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INTRACELLULAR SIGNALLING INVOLVED IN THE REGULATION OF ATRIAL NATRIURETIC FACTOR SECRETION

Astra I. Chang

This thesis is submitted in the fulfillment of the Master of Science program in Cellular and Molecular Medicine.

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INTRACELLULAR SIGNALLING INVOLVED IN THE REGULATION OF ATRIAL NATRIURETIC FACTOR SECRETION
Thesis for Master of Science in Cellular and Molecular Medicine

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List of Abbreviations

ANF	atrial natriuretic factor
BNP	brain natriuretic peptide
BSA	bovine serum albumin
cGMP	cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
DAG	diacylglycerol
DMSO	dimethyl sulphoxide
DNP	dendroaspis natriuretic peptide
EDTA	ethylenediaminetetraacetic acid
ET-18-OCH_3	edelfosine
EtOH	ethanol
KRBB	Kreb's Ringer bicarbonate buffer
IP_3	inositol 1,4,5-trisphosphate
IP_3R	inositol 1,4,5-trisphosphate receptor
MAPK	mitogen activated protein kinases
MLCK	myosin light chain kinase
PA	phosphatidic acid
PAM	peptidylglycine-α-amidating monooxygenase
PC	phosphatidyl choline
PIP_2	phosphatidyl inositol-4,5-bisphosphate (also PI-4,5-P)
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
RIA	radioimmunoassay
SR	sarcoplasmic reticulum
TCA	trichloroacetic acid

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Abstract

Atrial natriuretic factor (ANF) is a hormone that helps maintain fluid homeostasis and has many other physiological roles. Understanding of its regulation may have immense impacts in the treatment and understanding of cardiac diseases. Despite the recent and continuing unravelling of signalling cascades, the intracellular signalling governing ANF secretion from atrial cardiocytes remains mostly unknown. Following recent evidence of the involvement of G proteins in modulating ANF secretion, the role of G_q effector phospholipase C (PLC) and its proximal effectors was investigated in spontaneously beating rat atria. Phospholipase C and protein kinase C inhibitors dramatically increased basal secretion of ANF. Furthermore, although stretch is a potent stimulus for secretion, these inhibitor-mediated increases fell to baseline levels when the stretch of the atria was subsequently introduced. Inositol trisphosphate receptor inhibition did not appear to affect basal secretion but dose-dependently blocked stretch secretion coupling. These results reveal interesting novel phenomena and demonstrate key participation of the PLC cascade in the regulation of ANF secretion.

Acknowledgements

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1. Introduction

Intracellular signalling cascades are the means utilized by cells to communicate a message received from receptors on the extracellular surface to various regulatory targets within the cell, thus eliciting some action by the cell in response. Despite a recent explosion of information regarding proteins involved in vesicular transport and exocytosis (for reviews see Mayer, 2002 and Tooze *et al*, 2001), the intracellular signalling cascades underlying secretion have not been fully delineated and understanding of the stimulatory secretory pathways operating in different cells exists to varying degrees depending on the cell type. The intracellular signalling determining constitutive and stimulus-secretion coupling of the cardiac hormone, atrial natriuretic factor (ANF) is especially poorly understood with little known about the intracellular signalling that regulates its secretion. Atrial natriuretic factor is a hormone with wide-ranging physiological effects on the regulation of the body's water/salt balance, blood pressure, and cardiovascular growth (described further below). Following recent investigations that have revealed the intimate role of G proteins in modulating ANF secretion (Bensimon *et al*, 2004), the present study aims to delineate the signalling cascades regulating ANF secretion from the atria of the heart with particular emphasis on the $G\alpha_q$-phospholipase C cascade (described in Section 1.4 Signal Transduction and Second Messengers).

1.1 The heart and its endocrine function

Prior to 1981, the heart was known and accepted only as the vital muscular pump regulating blood flow and ensuring continuous circulation of blood throughout the body. However, observations that rats experienced strong natriuresis, the excessive loss of sodium in urine, and lower blood pressures following the infusion of atrial extracts (de Bold et al, 2001) provided evidence that the heart had another function – an endocrine function involving the synthesis and secretion of ANF and brain natriuretic peptide (BNP). The study of these peptides along with a third member of the family, C-type natriuretic peptide (CNP), makes up the field of cardiovascular endocrinology, involving the investigation of the heart as a hormone-secreting organ. ANF, BNP and CNP are explored further below. A fourth member, dendroaspis natriuretic peptide (DNP), originally isolated from the venom of the green mamba snake (Schweitz *et al*, 1992) has also been identified in human plasma and atrial myocardium (Lijnen *et al*, 2005; Schirger *et al*, 1999). Although this last member, DNP remains to be further studied it has recently been found to signal through cyclic guanosine monophosphate (cGMP) to induce apoptosis in H9c2 cells (a cardiac muscle cell line derived from rat heart) as measured by increased levels of pro-apoptotic factors such as Bax and cytochrome c while decreasing the anti-apoptotic factors inhibitor of apoptosis protein-1 and -2 (Ha *et al*, 2005). The amino acid structures of all four natriuretic peptides are depicted in Figure 1.

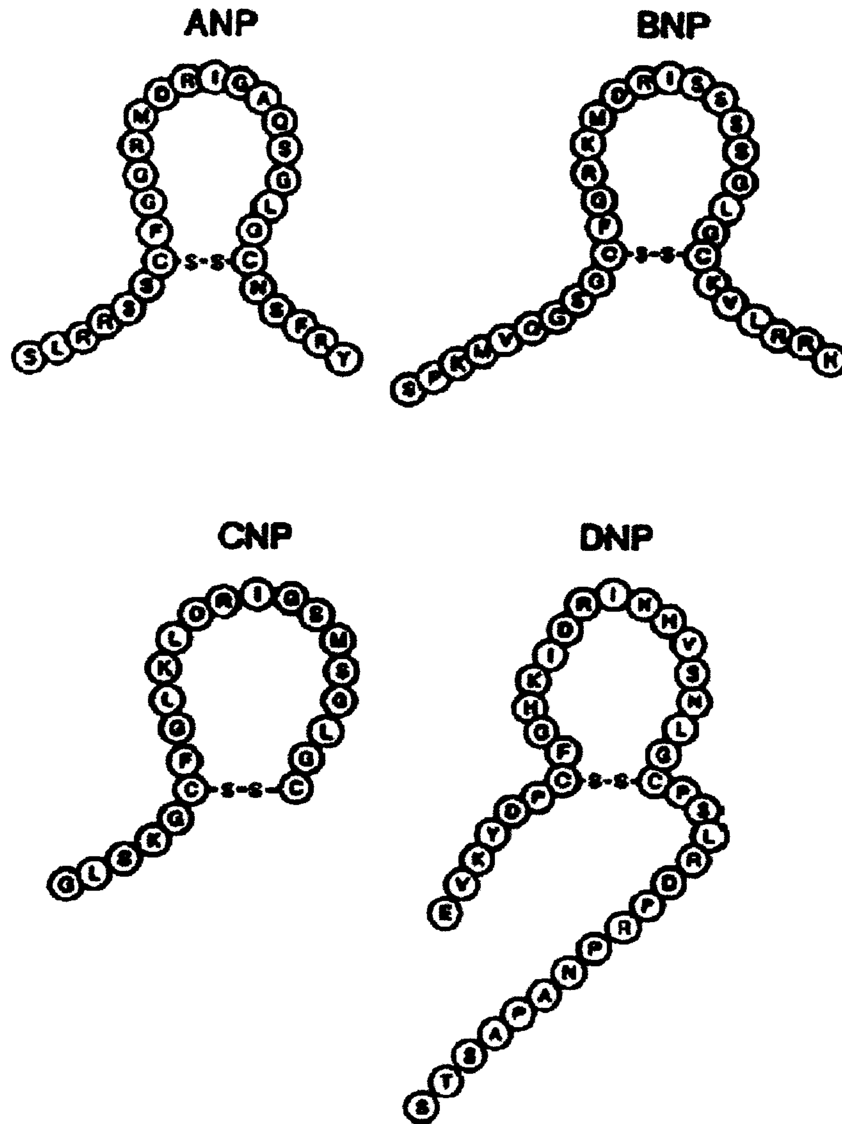


Figure 1. Amino acid structures of atrial natriuretic factor and the other natriuretic peptides

Amino acid structures of atrial, brain, C-type and Dendroaspis natriuretic peptides with similar 17-amino acid disulphide ring structures (Lijnen *et al*, 2005; Schirger *et al*, 1999).

Atrial natriuretic factor and brain natriuretic peptide have been studied in some detail and are currently exploited as markers of hypertrophy as well as morbidity and mortality in heart failure (Lijnen *et al*, 2005). Both ANF and BNP are predominantly produced in the atria of the heart, and are synthesized and secreted by cardiocytes. C-type natriuretic peptide has been less studied, but is slowly receiving more attention being recently identified as an endothelium-derived hyperpolarizing factor (Chauhan *et al*, 2003). Also a potent growth regulator, CNP demonstrates anti-atherogenic effects on vasculature suggesting the potentiality of CNP as a novel therapeutic target against inflammatory cardiovascular disorders (for a review see Ahluwalia and Hobbs, 2005). While CNP is found mostly in the central nervous system and vascular endothelial cells, it is also found in the heart, in both ventricular and atrial myocardium (Wei *et al*, 1993). All three natriuretic peptides are also secreted by cardiac fibroblasts (Lijnen *et al*, 2005).

Atrial natriuretic factor and brain natriuretic peptide are collectively stored in granules found in the atrial cardiocytes of the heart, known as specific atrial granules. However within the specific atrial granules ANF is stored in its unprocessed form and is proteolytically cleaved just prior to secretion, whereas BNP is stored in its processed form. Atrial natriuretic factor and BNP share many characteristics but are genetically distinct with their expression and secretion unique from each other under certain stimulatory conditions (Ogawa *et al*, 1999). Both natriuretic peptides are secreted in response to various stimuli including mechanical, endothelin-1, and neurohumoral/pharmacological α_1-adrenergic receptor agonists, however the dynamics of their secretion and expression in response to various secretogogues differ somewhat. For

example, stretch of the atrial wall is one of the most potent agonists of ANF secretion and atrial muscle stretch results in a rapid spike of ANF secretion which quickly decreases to approximately basal levels, even when the stretch-stimulus remains constant. A more sustained response is achieved by stimulation with other secretogogues such as endothelin-1 and the α_1-adrenergic agonist phenylephrine (Ogawa *et al*, 1999).

There are three different receptors for the natriuretic peptides: natriuretic peptide receptor-A, -B, and -C (NPR-A, NPR-B, and NPR-C, respectively). The first, NPR-A binds both ANF and BNP and signals through the cGMP second messenger cascade. C-type natriuretic peptide is the ligand for NPR-B through which it exerts its vasodilatory and growth inhibitory effects. Finally, NPR-C acts as a clearance receptor binding all three natriuretic peptides and removing them from circulation (Lijnen *et al*, 2005). After binding to their receptors and exerting their effects, the natriuretic peptides are hydrolytically degraded in lysosomes (Cohen *et al*, 1996).

1.2 Atrial natriuretic factor

All of the natriuretic peptides have significant diuretic, natriuretic and blood pressure-lowering properties, however under normal conditions the majority of circulating natriuretic hormone is atrial natriuretic factor (Vesely *et al*, 2002). Although there is some evidence of other circulating peptides derived from the unprocessed form of ANF (proANF), namely long-acting sodium stimulator (made up of proANF amino acids

1 to 30), vessel dilator (amino acids 31 to 67), and kaliuretic stimulator (amino acids 79 to 98) (Vesely, 2002; Vesely *et al*, 2002), it may be noted that NPR-A has selective affinity for ANF, the only cyclical peptide arising from proANF.

The early evolutionary nativity of ANF is evidenced by its appearance in an expansive variety of organisms ranging from simple vertebrates to humans. The natriuretic propeptide A (NPPA) gene, found on human chromosome 1p36 encodes ANF (Figure 2) and heterologues have been identified even in invertebrates and plants (Vesely, 2002; Gehring and Irving, 2003). In humans, ANF is synthesized in the endoplasmic reticulum as a 151-amino acid precursor protein, preproANF. Post-translational cleavage of the 25 N-terminal residues yields the storage form proANF, a 126 amino acid protein (Glembotski and Gibson, 1985). Secreted ANF (also sometimes referred to as $ANF_{(99-126)}$ or $ANF_{(1-28)}$) is a relatively small hormone, a polypeptide of only 28 amino acids having one tertiary structural element, a disulphide bond between the cysteine residues at positions 105 and 121 formed at the proANF stage. Thus the final secreted ANF hormone contains a 17-member ring which is essential for its biological activity (Figure 1; Iervasi et al, 1993).

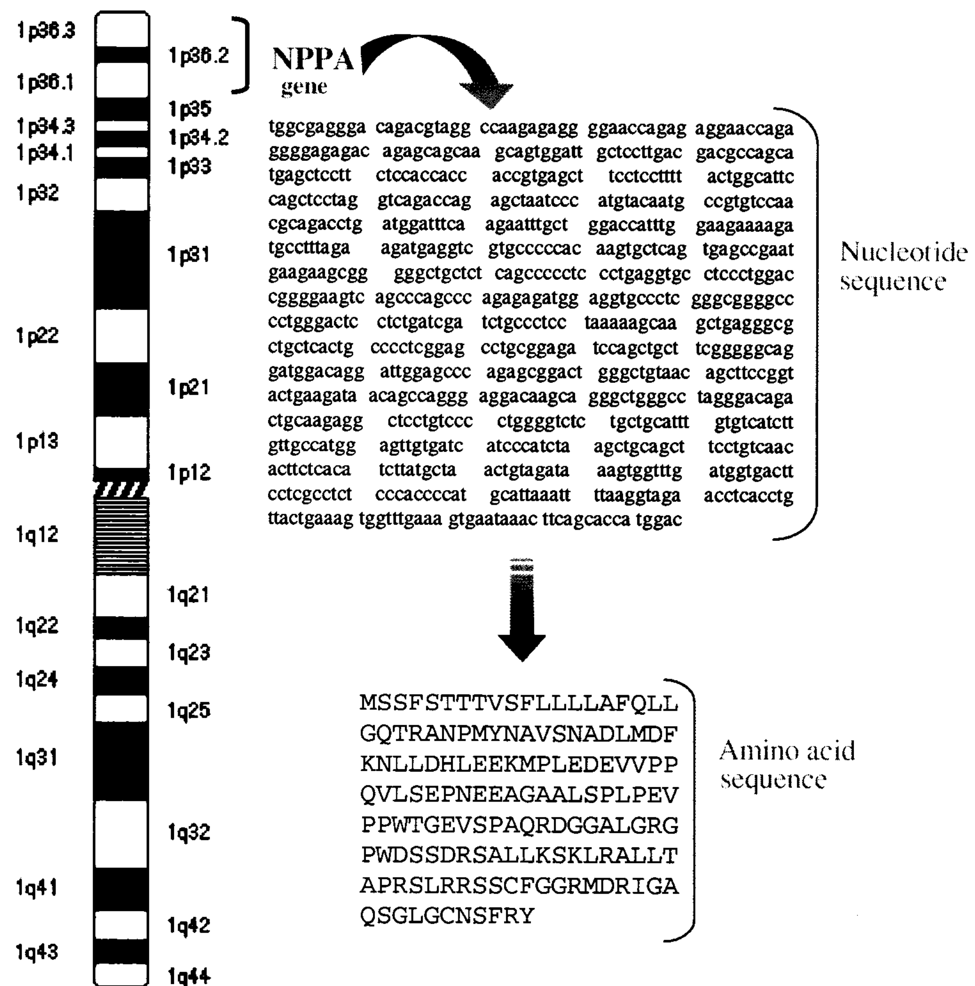


Figure 2. The natriuretic peptide precursor gene A

Shown is the natriuretic peptide precursor gene A (NPPA) on human chromosome 1 with its nucleotide and post-translational amino acid sequence. The NPPA gene encodes for prepro-atrial natriuretic factor (preproANF) which undergoes post-translational modifications to yield the biologically active hormone ANF. (Sequences from NCBI accession NM_006172.)

1.2.1 Functions and importance of atrial natriuretic factor

Atrial natriuretic factor can act as a neuromodulator, strongly participates in the moderation of blood pressure and growth, and has been utilized as a hypertrophic marker, but is best known for the actions implicit in its name. This cardiac hormone is a potent activator of natriuresis, and is thus intrinsically involved in the regulation of water and salt balance. As discussed above, ANF exerts its physiological actions through the guanylate cyclase receptor NPR-A, which is found in an expansive array of tissues including the kidney, brain, intestine, eye, testes, olfactory mucosa, adrenal glomeruli, thymus, spleen, lymph nodes, tonsils, macrophages, and vascular tissue (Kiemer and Vollmar 2001). Table 1 summarizes and references the effects of ANF in some of these tissues; nephritic, neural, and cardiovascular effects of ANF are described below.

In the kidneys, ANF causes an inhibition of proximal tubule Na^+ reabsorption and augments glomerular filtration by causing a relaxation of the glomerular mesangial cells thereby increasing the filtrative surface area. This results in heightened urinary volume and excretion of electrolytes (Tortora and Grabowski, 2000) and in these ways ANF effects its natriuretic and diuretic properties, resulting in a lowering of blood volume and pressure. At the same time, these actions are amplified by the ANF-antagonism of the renin-angiotensin-aldosterone system by decreasing renin release. The renin-angiotensin-aldosterone system is implicated in hypertension and it is suggested that ANF may play some important roles in the counteraction of this common pathological condition (Woodard *et al*, 2002).

Table 1. Functions and effects of atrial natriuretic factor on various tissues[§]

Tissue	Example of Function/Effect	References (abbreviated)
Intestine	- upregulates aquaporin 3 in colonic epithelia	Itoh et al. (2004) *Int J Mol Med* **14**:621-626
	- suppresses jejunal absorption	Gonzalez Bosc *et al.* (2000) *Peptides* **21**:875-887
Eye	- expressed in neural retinal, glial, vascular elements of retina	Rollin et al. (2004) *Mol Vision* **10**:15-22.
	- high vitreous levels with diabetes thus may have role in proliferative diabetic retinopathy	Rollin et al. (2004) *Mol Vision* **10**:450-457
Testes	- involved in early fetal testicular steroidogenesis	El-Gehani et al. (2001) *Biol Reprod* **65**:595-600.
Thymus	- site of ANF synthesis	Vollmar et al. (1990) *Peptides* **11**:33–37.
Spleen	- site of ANF synthesis	Throsby et al. (1991) *Endocrinology* **129**:991–1000.
Lymph nodes	- site of ANF synthesis	Vollmar and Schulz. (1990) *Comp Biochem Physiol A.* **96**:459–463.
Tonsils	- inhibits cell proliferation	Vollmar et al. (1996) *Endocrinology* **137**:1706–1713.
Macrophages	- increases phagocytosis and respiratory burst	Vollmar et al. (1997) *Eur J Pharmacol* **319**:279–285.
	- inhibits lipopolysaccharide-induced inducible nitric oxide synthase	Kiemer and Vollmar. (1997) *Endocrinology* **138**:4282–4290.
Vascular tissue	- causes vasodilation through guanylyl cyclase and cGMP	Katzung (2001) *Basic and Clinical Pharmacology* (McGraw-Hill) 302-303.
	- antagonizes increased vascular tone by endothelin	Agapitov and Haynes. (2002) *J Renin Angiotensin Aldosterone Syst* **3**:1-15.

[§] Please see text for descriptions of ANF effects on the renal, nervous, and cardiovascular systems.

In the brain, ANF decreases the release of corticotrophin while also affecting the blood pressure/volume regulatory regions, and can influence sympathetic activity (Raidoo *et al*, 1998). Corticotropin stimulates the release of glucocorticoids such as cortisol from the adrenal cortex, which influences glucose metabolism (Tortora and Grabowski, 2000). ANF may also affect metabolism by promoting lipolysis (reviewed in Dessi-Fulgheri *et al*, 2003; Kalra and Tigas, 2002). ANF has been detected in numerous hypothalamic centres where it is thought to help influence the osmolality and blood pressure regulatory effects of the peptide. Such hypothalamic areas include the neurons of the paraventricular nucleus, the median eminence, lamina terminalis, infundibular and ventromedial nuclei. Atrial natriuretic factor has also been found in ventricular ependymal lining, epithelial cells of the choroid plexus, and in neurons of the brain stem (Raidoo *et al*, 1998).

In the heart, ANF improves left ventricular performance in part by decreasing the preload to the heart and stimulates coronary vasodilatation (de Bold and Bruneau, 2000) and thereby protecting against such cardiac pathological conditions as hypertension and heart disease. This is explored further in 'Natriuretic peptides and heart disease' (section 1.3.1) below. Through the stimulation of guanylate cyclase, ANF is a potent vasodilator. Guanulate cyclase cleaves GTP to to generate cGMP which stimulate cGMP-dependent protein kinases (cGK). Type Iα cGK (cGKIα) bind actives myosin phosphatase to cause smooth muscle relaxation (Feil and Kemp-Harper, 2006).

1.2.2 Atrial granules and mechanisms of atrial natriuretic factor secretion

A basal level of ANF is continuously released to the circulation (~15-20ng/min from rat right atrium – my own experimental observations). This peptide hormone is stored in Golgi-packaged granules in the form of $proANF_{(1\text{-}126)}$ (same as proANF). These atrial secretory granules also contain many of the proteins characteristic of other endocrine and neuronal granules, such as carboxypeptidase, chromogranin A and B, cytochrome b_{561}, H^+-ATPase, G proteins, and peptidylglycine-α-amidating monooxygenase (PAM) (respectively Lynch *et al*, 1998; Steiner *et al*, 1990; Pruss and Shepard, 1987; Apps and Percy, 1987; Eipper and Mains, 1988; Bensimon *et al*, 2004; and O'Donnell *et al*, 2003). The trans-Golgi network acts to sort and target secretory proteins to granules, which are created by an out-pouching of the trans-Golgi that surrounds and enshrouds a dense core of secretory proteins. Subsequent modifications follow such as changes in size and composition of both the granular membrane and the encapsulated proteins. Thus these nascent granules can be termed 'immature'. It is thought that there are two pools of atrial granules, an immature and mature pool. The mature pool appears to be less acidic and lacking the clathrin coat observed on immature granules (Tooze *et al*, 2001; Mangat and de Bold, 1993).

Constitutive (basal) secretion is thought to occur in a manner described as a 'passive vesicle-mediated pathway' (Ogawa *et al*, 1999) involving newly synthesized peptide-containing granules, whereas regulated ANF secretion is thought to be released from a storage pool in response to a stimulus. However, when protein synthesis is experimentally blocked (e.g. by cycloheximide) basal secretion of ANF remains

continuous, suggestive of a constitutive-like mechanism whereby vesicles are thought to bud off from mature secretory granules containing a dense core of hormone, in a manner similar to that observed in pancreatic cells (Ogawa *et al*, 1999; Arvan *et al*, 1991). Brain natriuretic peptide is often packaged in the same storage granules as ANF and can be released simultaneously from cardiocytes (Ogawa *et al*, 1999; Ruskoaho, 1992). However, co-regulation of ANF and BNP occurs in atrial cardiocytes and is not observed from cardiocytes in the ventricles of the heart (please see Biancotti and de Bold, 2000).

Adrenergic agonists such as phenylephrine and endothelin-1 are known secretogogues of cardiac ANF and are thought to act through the G_q signalling pathway (described below). Further to these and other neurohumoral agonists, the dominating physiological stimulus for ANF secretion is the mechanical distension of the atrial wall caused by an increase in blood volume flowing to the atria, known as stretch-secretion coupling (de Bold *et al*, 2001). Such mechanical distension (or stretch) of the atrial wall alone results in sudden increased secretion of ANF from the heart (Bensimon *et al*, 2004). Although the precise mechanisms of stretch stimulus sensing and mechano-transduction have not been delineated, the cytoskeleton is intuitively an important participant and has been shown as such (Knöll *et al*, 2002; Hamill and Marinac, 2001). For example, Knöll *et al* (2002) recently demonstrated the probability of the involvement of the Z-disc protein, muscle LIM protein, in cardiac myocyte stretch-sensing. When tissue or cells are stretched, the plasma membrane is altered and the actin cytoskeleton is reorganized affecting stretch-activated ion channels (e.g. Ca^{2+} and K^{+} ion channels) and the

scaffolding (including microtubules) that acts in part to coordinate the transport of secretory granules to the membrane for exocytosis (Webster, 2002; Larsen *et al*, 1993).

Upon stimulus, mature (and some immature) granules move to release sites and proANF is proteolytically cleaved to its physiologically active form, ANF_{99-126} (same as ANF) before being released to the circulation (Ogawa et al, 1999; Mangat and de Bold, 1993). The processing enzyme responsible for cleavage of proANF to the biologically active secreted hormone, ANF is currently not known. While evidence exists suggesting that the protease corin may play this processing role (Yan *et al* 2000, Wu *et al* 2002), a recent proteomic evaluation of the atrial secretory granule did not find corin to be present, although limitations of the study are acknowledged (O'Donnell *et al*, 2003). Nonetheless, peptidylglycine-α-amidating monooxygenase (PAM) was localized to the atrial granules in the study and the finding that PAM abundantly resides as a secretory vesicle membrane-associated protein presents a novel function for this enzyme as its enzymatic product of alpha-amidated peptides are not readily found within cardiac atrial tissue. Rather, PAM may act to help sequester and anchor ANF within the granule and/or may function in the processing of proANF to ANF (O'Donnell *et al*, 2003). Consequently, the possibility of enzymes other than corin acting for the purpose of processing the peptide hormone that have been found to be associated with the granule is verisimilar with likely candidates including PAM, carboxypeptidase E/H, neprilysin, and tumor necrosis factor-alpha converting enzyme (TACE) (Muth *et al*, 2004; O'Donnell *et al*, 2003).

1.3 Cardiac pathophysiology

1.3.1 Natriuretic peptides and heart disease

Under various conditions, there is a heightened necessity for the actions of ANF (described above) for example to help improve cardiac performance under conditions of hemodynamic overload (Colucci and Braunwald, 2005). Described in this section are some cardiomyopathic conditions in which natriuretic peptide levels are significantly increased in response to the heightened need. The identification of such conditions and the correlation with natriuretic peptide expression and/or secretion may provide useful diagnostic indices and possible therapeutic targets while helping to increase our understanding of intracellular signalling. For example, ANF and BNP plasma levels have been found to be augmented with aortic stenosis. Through this finding, the prognosis of patients with severe aortic stenosis is aided through the measurements of natriuretic peptides (Gerber *et al*, 2003).

Increases in ANF and BNP plasma levels and production are stimulated under hypertrophic conditions, following myocardial infarction, and under conditions of cardiac failure. Although hypertrophy is an adaptive response of the heart to meet increasing demands due to various stimuli, over an extended period of time, hypertrophy can become pathological with associated symptoms of decompensation, cardiomyopathy, arrhythmias, fibrotic disease, sudden death and heart failure (Levy *et al*, 1990). The development of pathological hypertrophy is marked by the induction of fetal gene

expression program which includes increased ANF expression not just in the atria but also in the ventricles. Similarly, it has been demonstrated that ANF gene expression increases in an apparent biphasic manner 1 to 2 days and 3 to 7 days following acute myocardial infarction. The increase in ANF gene expression occurs in the adjacent non-infarcted region, while increases in BNP expression following myocardial infarction occur in the periphery of the infarct as well as more remotely (Backlund *et al*, 2003, Lijnen *et al*, 2005). Under such conditions, ANF and BNP are thought to act as antifibrotic factors (Tamura *et al*, 2000).

Synthesis of CNP by the myocardium has been shown in chronic heart failure and levels of CNP are increased by 2 to 3 times under these conditions (Kalra *et al*, 2003; Wei *et al*, 1993). In congestive heart failure, increases in ANF plasma levels, up to 10-fold, correlate well with the severity of heart failure as well as with right and left atrial pressure (Lijnen *et al*, 2005). In compensated heart failure there is a mild to moderate reduction in renal perfusion and sodium excretion is amplified by augmented levels of ANF, resulting in a sodium/potassium ratio of greater than 1.0 in the urine. In decompensated heart failure, despite similarly augmented levels of ANF, the urinary sodium/potassium ratio becomes less than 1.0 due to a strong activation of the renin-angiotensin-aldosterone system by moderate to severe reductions in renal perfusion. This activation of the renin-angiotensin-aldosterone system prevails over the effects of ANF resulting in almost complete renal sodium reabsorption, which leads to excessive fluid volume in the venous system causing plethoric cardiac distension and muscle weakening, as well as possible fluid leakage into tissues such as the lungs causing pulmonary edema

and dyspnea (Colucci and Braunwald, 2005; Weber, 2001; Weber 1993). Many of these complications may be preventable if this prevalence of the renin-angiotensin-aldosterone system over the actions of ANF can be overcome, for example by further heightened ANF secretion. The present study shows that the $G\alpha_q$-phospholipase C signalling pathway is involved in the transduction leading to ANF secretion. This pathway has also been implicated in many of the cardiomyopathic conditions in which natriuretic peptides are increased. The involvement of $G\alpha_q$ and phospholipase C (PLC) as well as the proximal effectors inositol-1,4,5-trisphosphate (IP_3) and protein kinase C (PKC) in some cardiomyopathies are described in the following section.

1.3.2 $G\alpha_q$, phospholipase C, inositol 1,4,5-trisphosphate, protein kinase C, and heart disease

There are many different isoforms of heterotrimeric guanine nucleotide binding proteins (G proteins) (described below in 'Signal transduction and second messengers') however among these, the G_q appears to be the primary isoform to act as the mediator of cardiomyopathic signalling in cardiomyocytes. Implication of G_q in cardiomyopathies such as hypertrophy has resulted from numerous molecular studies in which cardiomyocyte overexpression of $G\alpha_q$ in both *in vitro* (cell culture) and *in vivo* (transgenic mice) systems is sufficient to cause hypertrophy and significantly increased susceptibility of cardiac failure when the heart is exposed to stress such as pressure overload (reviewed in Aoki and Izumo, 2001). Notably, many secretogogues of ANF, which is often used as a marker of pathological hypertrophy (for example Morris *et* al, 2004; Morabito *et al*, 2001) utilize G_q signal transduction.

Activation of G_q induces the phosphatidylinositol cycle, consisting of the breakdown of phosphatidylinositol 4,5-bisphosphate by phospholipase C to generate the second messengers inositol 1,4,5-trisphosphate and diacylglycerol/PKC (described below), which has been implicated in the mediation of many of the processes that are involved in such heart diseases as hypertrophy and atherogenesis. These processes include expression and activation of adhesion molecules, platelet aggregation, secretion of endothelium-derived factors, and mitogenic responses of vascular smooth muscle cells (Siddiqui *et al*, 2000). Thus, both PKC and IP_3 are implicated in the signal mediation of cardiomyopathies. For example, overexpression of PKCβ in transgenic mice results in cardiac hypertrophy leading to dysfunction (Bowman *et al*, 1997; Wakasaki *et al*, 1997).

Calcium (Ca^{2+})/calmodulin-dependent enzymes such as calmodulin kinases and calcineurin are suggested to participate in the mediation of hypertrophic signals and are activated by increases in free intracellular Ca^{2+} as induced by IP_3 binding to the IP_3 receptor (Figure 3). For example, GATA4 is a transcription factor thought to be involved in the gene regulation of cardiac hypertrophy. A cofactor of GATA4, NF-AT3 is dephosphorylated by calcineurin, which activates the cofactor to translocate to the nucleus where it acts as a transactivator for various genes. Among these genes are those involved in hypertrophy, as evidenced by a strong hypertrophic response following overexpression of NF-AT3 in transgenic animals (Aoki and Izumo, 2001). Such signalling, however, is not necessarily to be taken as being disadvantageous. Ibarra *et al* (2004) recently presented evidence that IP_3 plays key roles in the signal transduction of

insulin-like growth factor activities. It has been demonstrated in diverse models of myocardial ischemia and myocardial infarction that insulin-like growth factor, which actuates cardiac hypertrophy both *in vitro* and *in vivo*, also possesses protective and anti-apoptotic properties (Ibarra *et al*, 2004).

During the development of cardiac hypertrophy, protein kinase-mediated phosphorylation cascades of which protein kinase C is intimately involved, are essential in signal transduction between the plasma membrane and nucleus. In cardiocytes, activated PKC is thought to be a primary hypertrophic signal transducer stimulating specific signalling pathways that result in increased protein synthesis. Downstream of G-protein coupled receptor and PKC signalling is the activation of c-fos, c-src, Ras and the mitogen-activated protein (MAP) kinase cascade (Figure 3). As mentioned above, activation of the MAP kinase cascade leads to increased expression of a number of hypertrophic genes in cardiomyocytes. One action of MAP kinase is to phosphorylate $p90^{rsk}$ which in turn phosphorylates the ribosomal subunit protein S6, as well as serum response factors involved in regulating the transcription of c-fos, which in turn leads to specific gene expression. Although Ras is conventionally thought of as belonging to the tyrosine kinase pathway, considerable cross-talk between this and the G_q/PLC/PKC pathway exists such as observed in the activation of the EGF receptor, in increases in cardiomyocyte phosphotyrosine content, and in the activation of Ras. It has been demonstrated both *in vitro* and *in vivo* that activation of Ras alone is sufficient to arouse a significant hypertrophic response (Siddiqui *et al*, 2000; Aoki and Izumo, 2001).

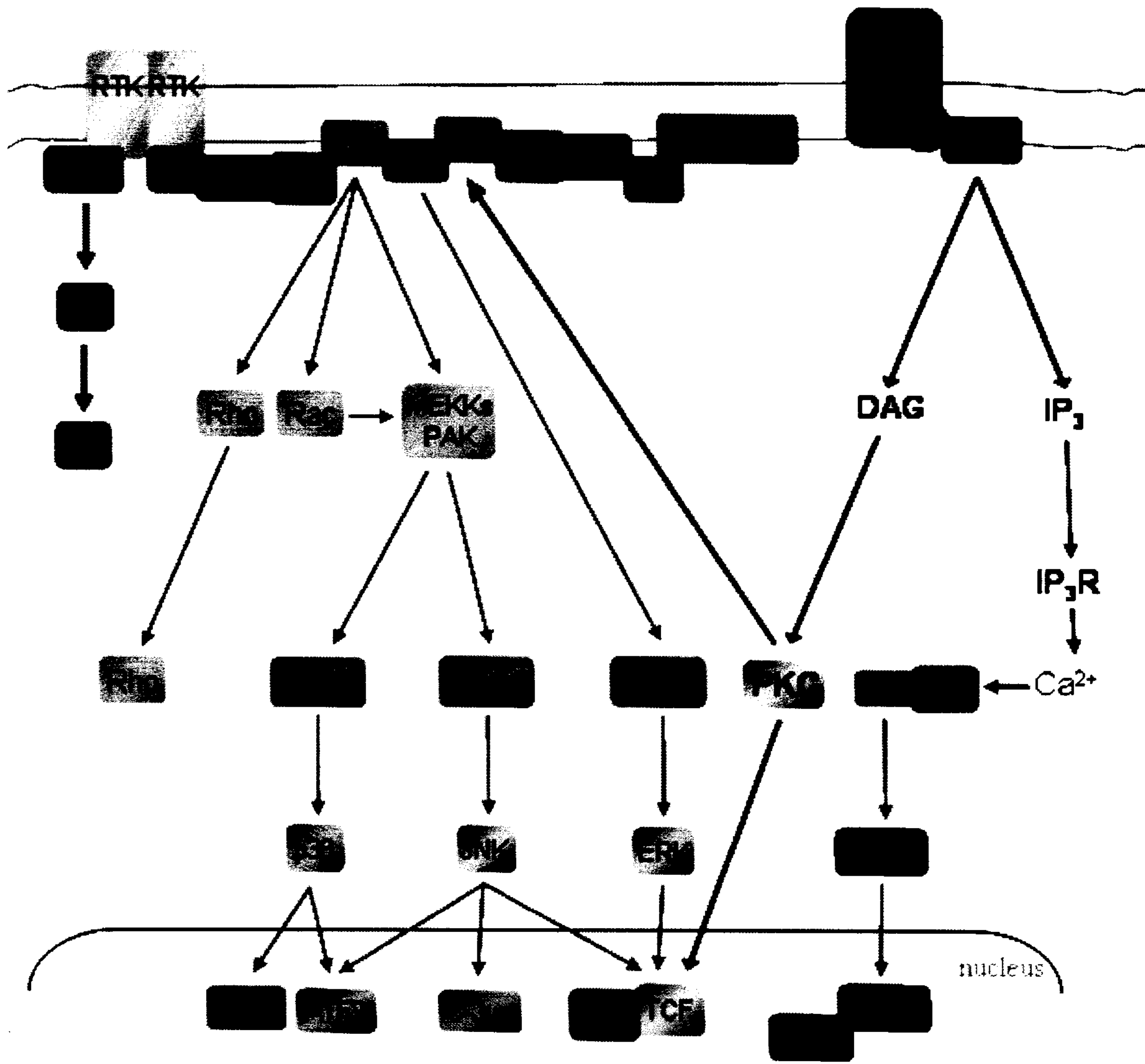


Figure 3. The G_q protein, protein kinase C and mitogen activatied kinase cascade

Shown are some of the intracellular signalling cascades that follow from G_q protein coupled receptor (GPCR) activation including the mitogen activated protein kinase (MAPK) cascade by way of protein kinase C activity.
(Based on Aoki and Izumo, 2001.)

1.4 Signal transduction and second messengers

1.4.1 G proteins

It has been demonstrated that many secretogogues of ANF from the atria of the heart utilize G proteins to trigger signal transduction leading to exocytosis of the hormone. G proteins were originally described by Nobel laureates Martin Rodbell and Alfred G. Gilman (Coles 1994) and, as Gilman reports, are named after their guanine nucleotide binding property. Many isoforms of G proteins have now been described and in addition to the now classical signalling pathways, novel anomalous G protein signalling pathways are slowly being unravelled (for a review see McCudden *et al*, 2005). G proteins exist as heterotrimers having α, β, and γ subunits, and are bound to the intracellular region of 7-transmembrane G-protein coupled receptors (GPCRs) or localized to intracellular membranes such as the Golgi stacks. In the inactive form of the heterotrimeric protein, guanine diphosphate (GDP) is bound to the α-subunit. Ligand binding to the GPCR triggers a series of events commencing with the exchange of GDP to GTP (guanosine triphosphate). This activates the heterotrimer and dissociation of the α-GTP from its β and γ subunits, which may or may not continue to exist as a complex (i.e. as $\beta\gamma$) follows. Any of the three subunits can activate or deactivate various specific downstream effectors. One such pathway, the phosphoinositide cycle, activated by the $G\alpha_q$ subunit is the focus of the present study and is discussed in the following section.

It has recently been shown that Mastoparan-7, an activator of $G_{i/o}$ proteins, results in increased ANF release and Pertussis toxin, an inhibitor of $G_{i/o}$ proteins, diminishes stretch-induced release. It is therefore thought that regulatory signalling under the stretch stimulus occurs, at least in part, through the $G\alpha_{i/o}$ isoforms (Bensimon *et al*, 2004). Other agonists are thought to act through the $G\alpha_q$ isoforms, whose cascade in other secretory cells has been well characterized (e.g. in neuronal cells). The present study investigates the role of the PLC cascade, which is traditionally known to follow $G\alpha_q$ activation, in cardiac ANF secretion.

1.4.2 Phospholipase C β

Agonists, such as drugs or other chemicals that combine with cellular receptors to produce a physiological response, for example endothelin-1 and other α_1-adrenergic and muscarinic agonists can cause the stimulation of the phosphoinositide cycle in the heart (van Heugten et al, 1996). Receptors for these agonists are G-protein coupled receptors, as stated above, resulting in the activation of G_q proteins. G_q (or other G proteins), in turn activate an essential enzyme in the phosphoinositide cycle, phospholipase C (Figure 4). There are 11 isoforms of PLC that have been identified in mammals. These 11 isoforms are divided into 4 classes - β, γ, δ, and ε - each of which have subtypes (Fukami, 2002) and are mediated by different factors and/or mechanisms (Table 2).

The isoform of phospholipase C that is activated by $G\alpha_q$ in the heart and other tissues is PLCβ of which there are 4 subtypes. In their proteomic investigation of atrial secretory granules, Muth *et al* (2004) identified the $PLC\beta_4$ isoform associated with the granular membrane. This particular isoform has been implicated as an effector in the bradykinin-induced inhibition of M currents in sympathetic ganglion neurons through the G protein coupled bradykinin receptor. M currents are potassium currents that are activated in the sub-threshold range for action potentials in neurons (Haley *et al*, 2000). The presence of $PLC\beta_4$ in association with atrial granules additionally highlights the possibility of a role for K^+ participation (or other ions) in the signalling regulation of ANF secretion. The calcium cation (Ca^{2+}) is another ion that is often enmeshed in cellular signalling transduction and its release from IP_3-sensitive sarcoplasmic reticulum stores is an ensuing event following phospholipase C activation, as illustrated in Figure 4 and discussed in the following section.

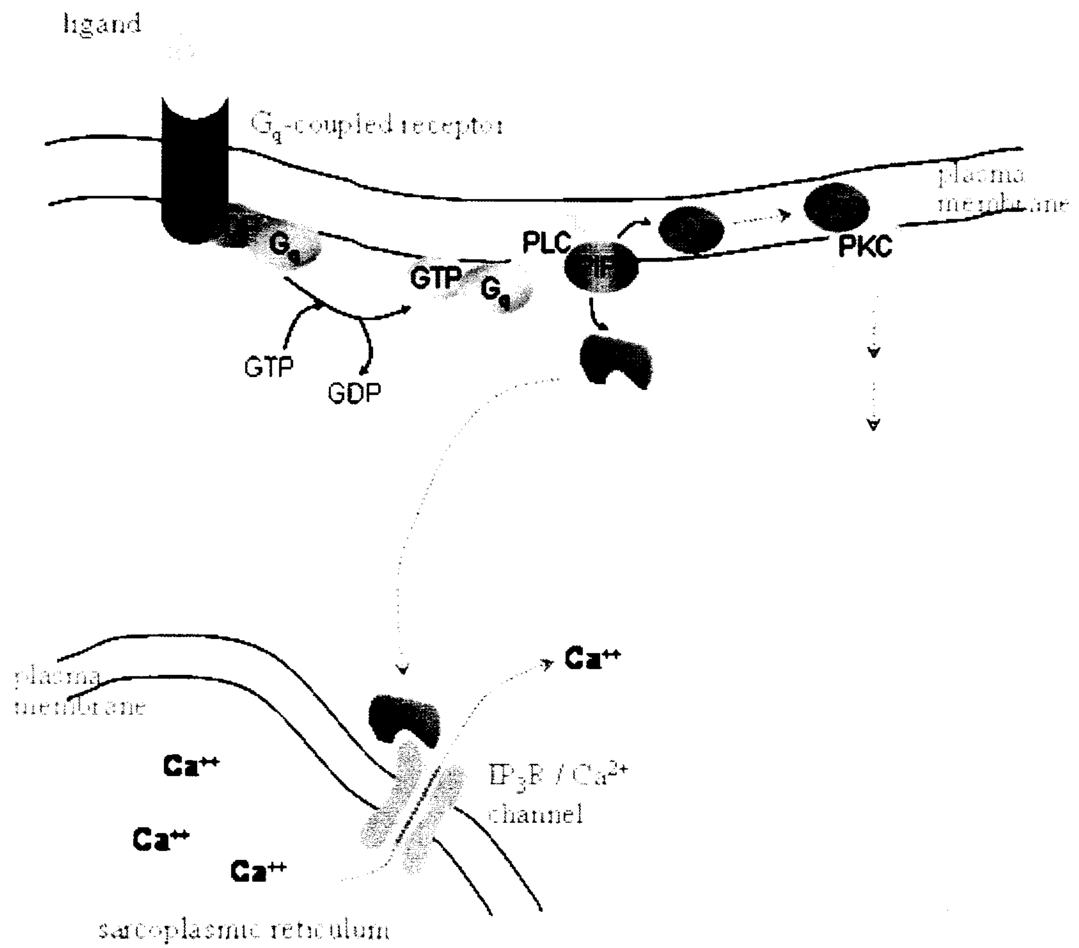


Figure 4. G_q activation of phospholipase C and the resultant proximal effects.

Table 2. Isoforms of phospholipase C[§]

Isoform		Regulation	Distribution	References
Class	Subtype			
β	1	$G\alpha_q$, PI(3)P	brain (cerebellum, hippocampus), adrenal gland, lungs, heart	Wang *et al.* (1999) PNAS 96:7843-7846 Razzini *et al.* (2000) JBC 275:14873-14881 Kim *et al.* (1997) Nature 389:290-293 Arthur *et al.* (2001) JBC 246:37341-37346
	2	$G\beta\gamma$, $G\alpha_q$	haematopoietic cells	Wang *et al.* (2000) JBC 275:7466-7469 Wang *et al.* (1999) PNAS 96:7843-7846
	3	$G\beta\gamma$, $G\alpha_q$	brain, liver, parotid gland platelets	Wang *et al.* (2000) JBC 275:7466-7469 Hwang *et al.* (2000) JBC 275:16632-16637
	4	$G\alpha_q$	brain (cerebellum, retina)	Kim *et al.* (1997) Nature 389:290-293
γ	1	tyrosine kinase, $PI(3,4,5)P_3$	brain (neurons), lungs, thymus	Rhee and Bae. (1997) JBC 272:15045-15048 Bae *et al.* (1998) JBC 273:4465-4469 Falasca *et al.* (1998) EMBO J 17:414-422
	2	tyrosine kinase, $PI(3,4,5)P_3$	lungs, thymus, spleen	Rhee and Bae. (1997) JBC 272:15045-15048
δ	1	Ca^{2+}, $G\alpha_h$, $PI(4,5)P_2$, small GTPase Ral, calmodulin	brain, heart, lungs, testes, skeletal muscle, spleen	Essen *et al.* (1996) Nature 380:595-602 Feng *et al.* (1996) JBC 271:16451-16454 Lomasney *et al.* (1996) JBC 271:25316-25326 Sidhu *et al.* (2005) JBC 280:21933-21941
	2	expression correlates with (but unknown if regulated by) proliferation cell marker PCNA	brain, stomach	Marchisio *et al.* (2004) Int J Immunopathol Pharmacol 17:381-388 Marchisio *et al.* (2001) Am J Pathol 159:803-808
	3	Ca^{2+}, cAMP, $PI(4,5)P_2$, phosphatidic acid	brain, heart, skeletal muscle	Lin *et al.* (2001) Biochem Biophys Res Commun 286:274-280
	4	Ca^{2+}, $PI(4,5)P_2$	brain, skeletal muscle	Fukami *et al.* (2000) Eur J Biochem 267:28-36
ε		Ras, $G\beta\gamma$, $G\alpha_{12}$	lungs, liver, heart, skeletal muscle	Song *et al.* (2001) JBC 276:2752-2757 Lopez *et al.* (2001) JBC 276:2758-2765 Wing *et al* (2001) JBC 276:48257-48261

[§] Please note this table is by no means exhaustive. (Based on Fukami, 2002.)

1.4.3 Inositol 1,4,5-trisphosphate and its receptor

In the heart, as in many other tissues, the two second messengers IP_3 and diacylglycerol (DAG) are generated via the cleavage of phosphatidyl inositol-4,5-bisphosphate (PIP_2), a plasma cell membrane component, by activated phospholipase C. Inositol-1,4,5-trisphosphate traverses the cytosol from the cell membrane to bind to IP_3 receptors (IP_3R) on the sarcoplasmic reticulum (SR). The IP_3R simultaneously acts as a transmembrane calcium channel whereby IP_3 binding to its receptor opens this calcium channel thus freeing SR-sequestered calcium cations (Ca^{2+}) to move via the electrochemical gradient into the intracellular space, resulting in an increase of free intracellular calcium concentrations ($[Ca^{2+}]_i$) (Figure 4).

There are three different isoforms of IP_3R, types I, II, and III (Huh *et al*, 2005) of which type II is found to be the predominant isoform expressed in rat atrial and ventricular myocytes, with atrial cardiomyocytes having an expression level approximately 7-fold higher than their ventricular counterparts. In atrial myocytes, immunostaining has revealed the distribution of IP_3R around the subsarcolemmal region in discontinuous loci, but not deeper within the myocytes compared to ryanodine receptors which are found perpendicular to the longitudinal axis of the myocyte alongside the sarcoplasmic reticulum as well as adjacent to the sarcolemma (Lipp *et al*, 2000).

In cardiocytes, depolarization causes the opening of voltage-operated calcium channels on the sarcolemma and the resultant activation of ryanodine receptors on the sarcoplasmic reticulum due to the increase in free intracellular Ca^{2+}. The activated

ryanodine receptors open transiently releasing sequestered Ca^{2+} into the cytosol in the form of localized "sparks", which culminate over the myocyte to lead to contraction (Berridge *et al*, 2003). Thus, excitation-contraction coupling has traditionally been thought to be modulated by the ryanodine receptor Ca^{2+} channels. However there is some speculation as to the possible involvement of IP_3R in excitation-contraction coupling. Co-immunostaining in the mammalian atrium has provided evidence that the ryanodine receptor and IP_3R overlap and that there is a modulation of the ryanodine receptor by IP_3R activity whereby IP_3-dependent Ca^{2+} release occurring in close proximity to the ryanodine receptor may solicit a positive inotropic effect by facilitating the Ca^{2+}-induced ryanodine receptor Ca^{2+} release (Mackenzie *et al*, 2004; Zima and Blatter, 2004). Under normal conditions this effect may be insignificant, but can become substantial in disease conditions such as in the development of end-stage heart failure when the ratio of the number of IP_3R to ryanodine receptors is dramatically skewed towards IP_3R (Go *et al*, 1995).

1.4.4 Protein kinase C

The effective transduction of cellular signals relies on specific controls to modulate components of the signalling transduction pathway. Most such components are proteins whose activities are tuned or altered through the binding of ligands or second messengers, or by covalent modifications that result in conformational changes. It is well established that protein phosphorylation is a key mechanism regulating enzyme activity and cellular signalling, and is the most observed form of covalent modification (Hug and Sarre, 1993). Protein phosphorylation is achieved through the action of kinases which

add negatively charged phosphate groups (PO_3^-) to serine, threonine, and tyrosine amino acid residues of regulatory proteins. Consequently, the chemical properties of these regulatory proteins are altered to allow for recognition, binding, activation, or deactivation (Gomperts *et al*, 2003). In fact, the amplification and transduction of most extracellular signals are achieved within the cell by protein kinase cascades (Egan and Weinberg, 1993).

Differential expression of various protein kinases in various tissues helps to control tissue- and cell-specific responses to numerous stimuli. One group of kinases, protein kinase C, whose original isoforms (α, β, γ) were discovered by Nishizuka and colleagues in rat brain (Inoue *et al*, 1977), phosphorylate various proteins at serine and threonine residues situated circa the arginine residues of the consensus sequence, RxxS/TxRx (Kemp and Pearson, 1990; Nishikawa *et al*, 1997). Protein kinase C is now known to be involved in a multitude of processes including growth, differentiation, development, aging, synaptic transmission, axonal regeneration, muscle contraction and relaxation, tumour promotion, and secretion (Liu, 1996) including, as evidenced in the present study, the regulation of ANF secretion.

Traditionally, the activation of PKC is attributed to DAG formed along with IP_3 from phosphoinositide hydrolysis triggered through G protein coupled receptors on the extracellular membrane (Gomperts *et al*, 2003). The intracellular Ca^{2+} released from endoplasmic reticulum stores by IP_3 may contribute to the full activation of PKC (Taskinen, 1999) however it is DAG that causes the association of PKC with the plasma

membrane resulting in the activation of the Ca^{2+}-sensitive enzyme (Nishizuka, 1986; von Harsdorf *et al*, 1989). Thus, DAG is known to stimulate membrane-bound, phospholipid- and calcium-dependent isoforms of protein kinase C (von Harsdorf *et al*, 1989). The sustained activation of these PKC isoforms requires both elevated levels of DAG and simultaneous high-frequency Ca^{2+} spikes (Gomperts *et al*, 2003).

The implication of PKC in vast numbers of cellular responses is emphasized in that most ligands, growth factors, hormones, and neurotransmitters result in the production of DAG (and release of intracellular Ca^{2+} stores) and subsequently lead to PKC activation (Gomperts *et al*, 2003) that results in various cellular responses which may also include a change in the expression and secretion of ANF. Experimental activation of PKC has been achieved using tumour promoting phorbol esters such as 12-O-tetradecanoyl-4β-phorbol-13-acetate (TPA), also known as 4β-phorbol-12β-myristate-13α-acetate (PMA). These pharmacological agents are structurally similar to DAG and are thought to bind to the same activation sites on PKC as DAG. Nonetheless, phorbol esters may have indirect effects, which can result in either the activation or inactivation PKCs. For example, the commonly used phorbol ester, TPA also activates the serum response element (SRE) (Buscher *et al*, 1988). SRE regulates the serum-mediated expression of c-*fos* (Treisman, 1985) and also binds the two transcription factors, serum response factor (SRF) and $p62^{TCF}$ (Elk) through which growth factors also effect their actions (Marais *et al*, 1993). Nonetheless, numerous experiments utilizing phorbol esters have been conducted in almost all cell types demonstrating the importance of PKC in its

participation in many cellular processes, however, interpretation of such studies including those involving ANF should be interpreted with caution.

Through molecular cloning techniques, 12 distinct isoforms of PKC have been identified in mammals (Parker *et al*, 1986; Coussens *et al*, 1986) having various substrates and activators. These 12 isoforms are grouped into three categories based on sequence similarity and modes of activation. The conventional PKCs (α, $\beta 1$, $\beta 2$, γ) referred to above, are all activated by phospholipid, DAG, and Ca^{2+}; the novel PKCs (δ, ε, η, θ) are activated by phospholipid and DAG but are not Ca^{2+} dependent; and the atypical PKCs (λ, ι, ζ, μ) are activated by phospholipids, but not by DAG or Ca^{2+}. Nor are the atypical PKCs activated by phorbol esters. In fact, both the novel and atypical PKCs can be directly activated, or their activation potentiated, by other lipid second messengers namely the 3-phosphorylated inositol lipids, phosphoinositol 3,4-bisphosphate [$PI(3,4)P_2$] and phosphoinositol 3,4,5-trisphosphate [$PI(3,4,5)P_3$], in the presence of phosphatidylserine (Gomperts *et al*, 2003). These are formed through the actions of phosphoinositide 3-kinases, as shown in Figure 5. Activity of PKCs can also be potentiated by arachidonic acid, lysophosphatidic acid and lysophosphatidylcholine, or inhibited by sphingolipids (e.g. sphingosine and lysosphingolipids). Additionally, of the known isoforms, PKCλ is unique in its activation by the protein lambda-PKC interaction protein (LPA).

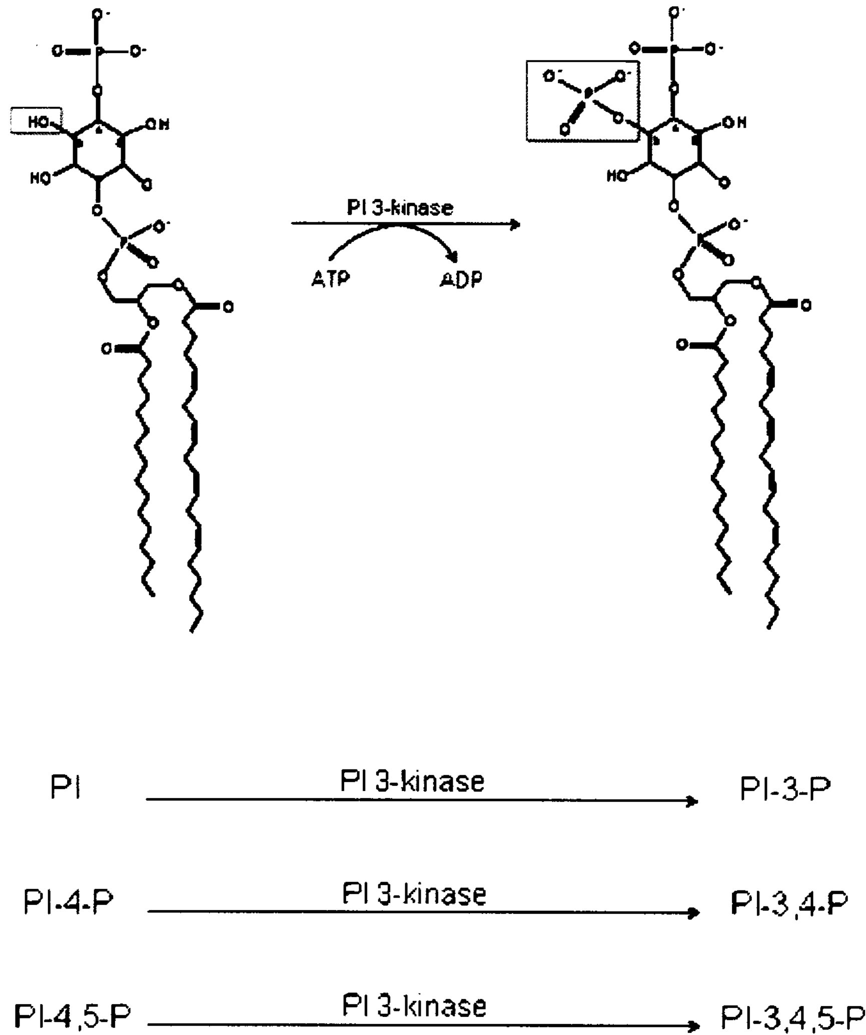


Figure 5. Generation of 3-phosphorylated lipids by phosphoinositol 3 kinase

The 3-phosphorylated inositol lipids, phosphoinositol 3,4-bisphosphate [PI(3,4)P_2] and phosphoinositol 3,4,5-trisphosphate [PI(3,4,5)P_3], are in part generated through the activity of phosphoinositide 3-kinases (PI3K) which phosphorylate the 3 OH-position of the inositol ring of the phosphatidylinositol lipids. (Based on Gomperts *et al*, 2003.)

All of the PKC isoforms identified to date share four conserved regions and five regions that show sequence variation among the different subtypes (Kohout and Rogers, 1993; Hayashi *et al*, 1999), illustrated in Figure 6a. The four conserved regions, designated C1-C4, share some similarities with other signalling proteins such as PKA, ERK, MEK1, CDK2 and CDK7, and Src family protein tyrosine kinases (Parker *et al*, 1986; Newton, 1995; Gomperts *et al*, 2003). The first two conserved regions, C1 and C2 are regulatory domains while the latter, C3 and C4 represent the catalytic domain of the kinase. A zinc finger motif of C1, made up of a cysteine-histidine rich motif that integrates two zinc atoms, is the site to which activators such as DAG bind. The C2 region binds negatively charged phospholipids that also act as activators (as described above). In the Ca^{2+}-dependent isoforms a Ca^{2+}-binding site, for Ca^{2+}-dependent lipid binding, is also found within the C2 regulatory domain. Between the regulatory domains (C1 and C2) and the catalytic domains (C3 and C4) is a hinge region, which confers an elbow-like conformation to the enzyme (depicted in Figure 6b). When the kinase is bound to a membrane, this hinge region is vulnerable to proteolytic cleavage (e.g. by trypsin) which can result in the detachment of the kinase domain (protein kinase m) and thus a constitutive activation of the kinase (Gomperts *et al*, 2003). In the inactive state, the catalytic domain of PKC binds to a pseudosubstrate domain, causing the hinge linking the C2 and C3 to bend (Figure 6b). These conserved and variable domains are important in identifying which isoforms are present and which of these may be activated or inactivated in regulatory processes such as those that are involved in signalling for ANF secretion.

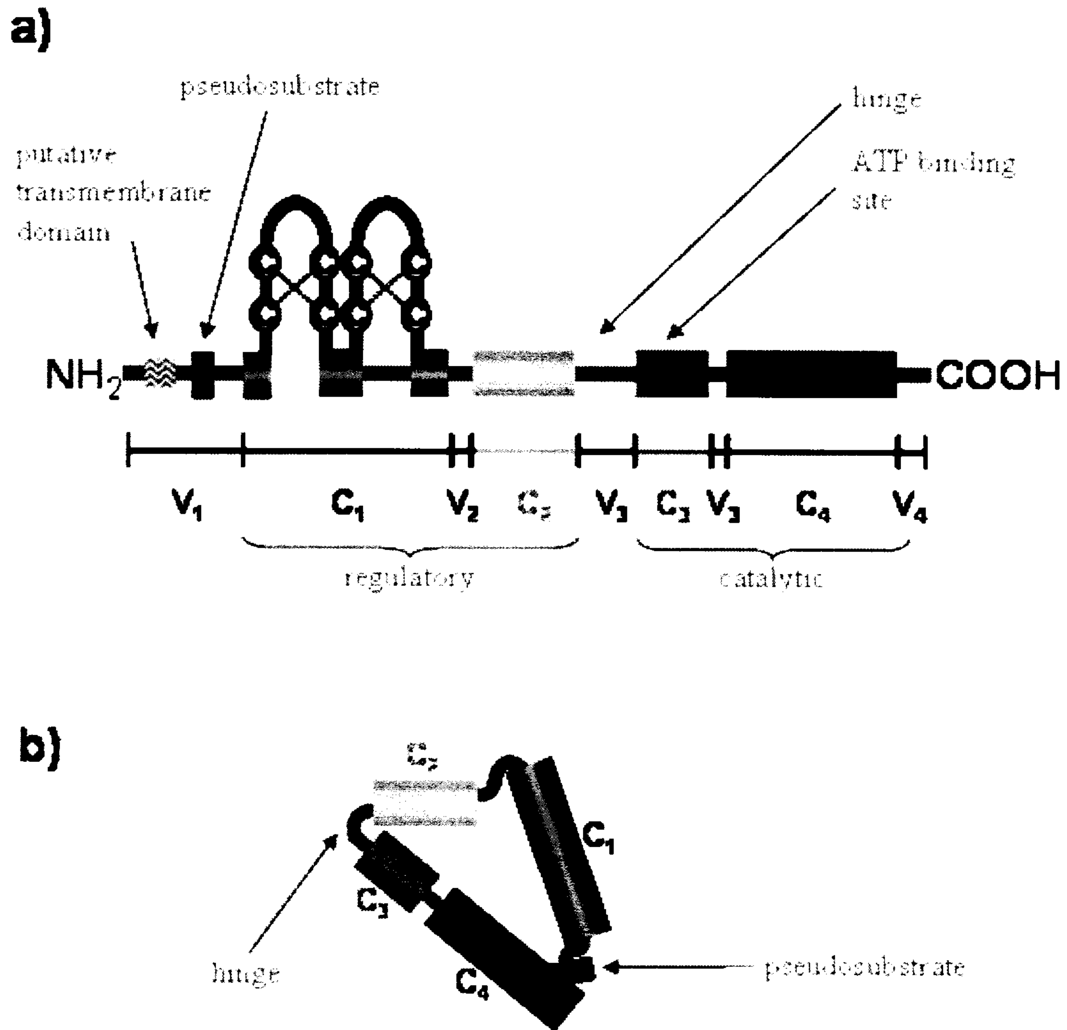


Figure 6. Structural domains of protein kinase C.

All of the PKC isoforms identified to date share four conserved regions - C1 and C2 are regulatory domains; C3 and C4 are the catalytic domains. Activators such as diacylglycerol bind at the zinc finger motif of C1 and other negatively charged phospholipid activators, as well as Ca^{2+}, bind within C2. A hinge (elbow-like) region is found between the regulatory and catalytic domains which is vulnerable to proteolytic cleavage when PKC is bound to the membrane. b) In the inactive sate, the catalytic domain binds to the pseudosubstrate domain causing the hinge between C2 and C3 to bend.

(Domains are not to scale. Based on Gomperts et al, 2003; Kohout and Rogers, 1993.)

It appears that nuclear targeting of both calcium-dependent and -independent isoforms, such as stimulated by phorbol esters or other mitogens, may have a more long-term activity and the nuclear effects of PKC activity present good examples of isoform-specificity. Such nuclear substrates of PKC include lamins, DNA topoisomerase II, CCAAT-enhancer binding protein, and myogenin. For example, in cell cycle regulation, G2 to M phase transition is promoted by PKCβII, which is selectively activated at the nuclear periphery during G2. The nuclear envelope polypeptide lamin B is phosphorylated by PKCβII at key sites involved in mitotic nuclear lamina disassembly and promotes the breakdown of the nuclear envelope during mitosis (Black, 2000; Goss *et al*, 1994). Thus PKC can promote cell growth, however again there are opposing effects depending on which isoforms are involved and such opposing effects have been demonstrated within the same cell type, which may also occur in atrial cardiocytes with differing effects on ANF secretion. An example of this is provided by studies done on the R6 rat embryo fibroblast line where overexpression of PKCβI enhanced growth and overexpression of PKCε promoted transformation, while similar overexpression of PKCα significantly inhibited both growth and transformation (for a review see Black, 2000).

Results from Northern and Western blot analyses show that PKC α, β1, β2, δ, ε, and ζ all appear to be ubiquitously expressed in the brain, however, PKCα and ε do not appear in the liver and PKCβ is not detected in renal mesangial cells. Similarly, PKCγ has thus far been found only in the central nervous system, and PKCθ is predominantly found in skeletal muscle and at lesser amounts in the lungs, spleen, skin, and brain. PKCη is strongly expressed in the skin and lungs but also in low amounts in the brain and

spleen (Hug and Sarre, 1993). Besides differential tissue/cell expression, the PKC isoforms can be differentially localized or compartmentalized within the cell with the aid of anchoring proteins. Although not all isoforms act on the same substrate and some activation/deactivation differences exist, there are not necessarily unique substrates for each and every isoform of the enzyme. Thus subcellular compartmentalization and unique protein complexes allow the different PKC isoforms to have specialized functions (Gomperts *et al*, 2003). While evidence exists to support such subcellular compartmentalization and protein complexes (e.g. Otte *et al*, 1991 and Goodnight *et al*, 1995) these have not yet been fully elucidated in all tissue types.

In the heart (rabbit), eleven PKC isoforms (α, β1, β2, γ, δ, ε, ζ, η, ι, λ and μ) have been identified and experimentally investigated (Carson and Korzick, 2003; Ping *et al*, 1997). PKC is present in both the membrane and cytosolic fractions in atrial and ventricular myocytes (Kuo *et al*, 1984; Yuan and Sen, 1986) and appears to have similar properties in the heart as those reported for other tissues (Kuo *et al*, 1984). Interestingly, however, PKC activity appears to be higher in the atria than in the ventricles (Wrenn *et al*, 1988). While investigations have sought to identify isoforms present in the whole heart or ventricular cardiomyocytes, the particular isoforms of PKC present specifically in the atria as well as their activities, have not been defined. Limited studies on animal models have implicated PKCs α, βII, γ, ε, δ, and ζ as being activated through cardiac $G\alpha_q$-mediated signalling, however Kilts *et al* (2005) suggest PKCε and δ as the main isozymes involved in such signalling in human atria, attributing the difference to varying activation profiles of the isozymes by a given $G\alpha_q$-coupled receptor among animal

species. Which particular PKC isozymes are directly involved in G-protein signalling may have implications in the regulation of ANF secretion from the atria, thus it will be both important and interesting to more fully delineate this profile.

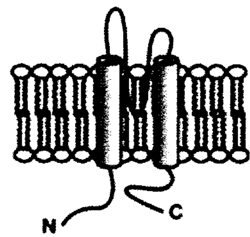
A temperature dependence of ANF secretion has been demonstrated (Bilder *et al*, 1986; Agnoletti *et al*, 1990; Page *et al* 1990), supporting the participation of such energy-dependent reactions as those carried out by protein kinase C (and others such as PKA and Ca^{2+}/calmodulin kinases) (Ruskoaho, 1992). Phorbol esters have been experimentally utilized manifesting in an increase in ANF secretion and suggesting the participation of PKC. However, the non-PKC specific effects of phorbol esters described above introduce some uncertainty as to whether the effects on ANF secretion are due to direct or indirect actions of the phorbol esters. For example, these compounds may result in the tissue or cells becoming ischemic. Ischemia of the myocardium has been found *in vivo* to strongly stimulate the secretion of ANF and *in vitro* experiments suggest that myocardial ischemia directly affects ANF secretion. Furthermore, *in vivo* and *in vitro* stimulation of ANF secretion has been demonstrated as a result of hypoxia (Ruskoaho, 1992), and hypoxia may also be a consequence of phorbol esters. Nonetheless, although the actions of phorbol esters on transcription may be indirect, it is thought that they are PKC-mediated. Protein kinase C activity in the heart such as induced with endothelin-1, phenylephrine, or PMA, has been shown to lead to the phosphorylation of p38 and p42/44 MAP kinases. These MAP kinases have been demonstrated to cause the induction of ventricular ANF gene expression (Morabito *et al*, 2001). Thus, while there is evidence supporting the participation of PKC in ANF secretion, whether PKC

increases or decreases secretion from cardiac atria and which isoforms cause such increases or decreases, has not been definitively elucidated.

1.4.5 G protein-activated inwardly rectifying K^+ channels

In addition to activating various second messenger cascades, G proteins may also directly interact with ion channels. Localization of G protein-activated inwardly rectifying K^+ channels (GIRK or Kir3.x) has been demonstrated in the atria and sinoatrial node of the heart (Ji *et al*, 1998). Like other inward rectifiers, the topology of GIRK consists of two membrane spanning repeats and a pore loop, as depicted in Figure 7a (Tomaselli and Roden, 2005). GIRK come together as tetramers to make up receptor-regulated ion channels such as the atrial M_2 muscarinic receptor-regulated K^+ channel (K_{ACh}, Figure 7b) which acts as the primary mediator of parasympathetic chronotropic and dromotropic effects (Tomaselli and Roden, 2005). K_{ACh} is a heterotetromeric channel consisting of homologous subunits made up by GIRK1 (Kir3.1) and GIRK4 (Kir3.4 also known as cardiac inward rectifier, CIR).

a)



INWARD RECTIFIERS (Kir)

Clone	Current
Kir2.x	I_{K1}
Kir3.1	I_{KACh}
Kir3.4	
Kir6.2+Sur1	I_{KATP}

b)

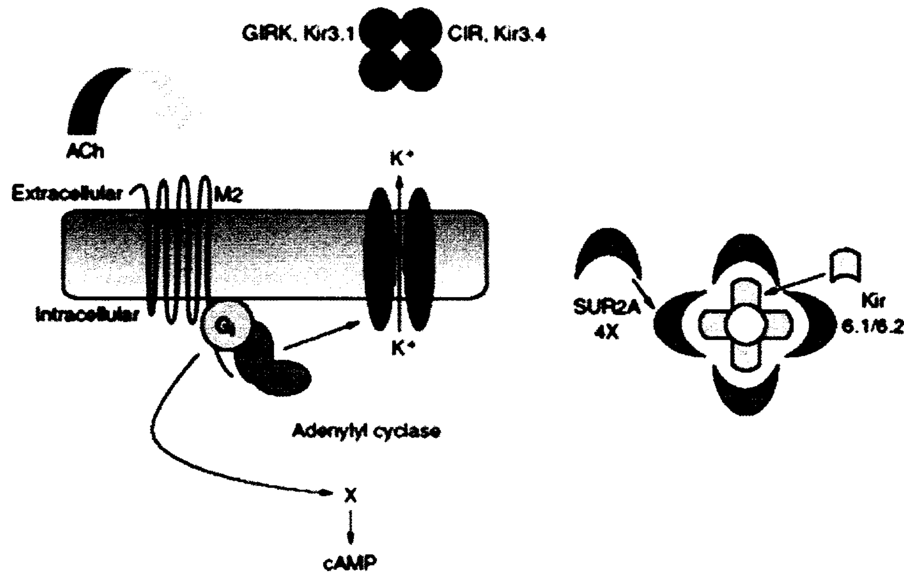


Figure 7. The potassium ion inward rectifying channel

a) Inward rectifiers such as GIRK consist of two membrane spanning repeats and a pore loop. b) GIRK come together as tetramers to make up receptor-regulated ion channels. An example is K_{ACh}, a heterotetromeric channel consisting of homologous subunits made up by GIRK1 (Kir3.1) and GIRK4 (Kir3.4 also known as cardiac inward rectifier, CIR).
(From Tomaselli and Roden, 2005 – Figures 1-7 and 1-11.)

M_2 muscarinic receptor activation leads to the subsequent activation of G_i proteins which other than being known to inhibit adenylate cyclase involved in modulating L-type Ca^{2+} channels, also stimulate the opening of K_{ACh} through the direct binding of $G\beta\gamma$ to the channel, thus decreasing heart rate through a hyperpolarization of cardiocytes (Tomaselli and Roden, 2005; Nikolov and Ivanova-Nikolova, 2004). Inward rectifiers of the heart have various specialized functions responding to neurohormones as well as metabolic and mechanical stress (such as muscle stretch). It is therefore not surprising that although GIRK is activated by $G\beta\gamma$, recent investigations show that associated with GIRK are also a number of signalling molecules including cytoplasmic ATP, $G\alpha_i$ (not just $\beta\gamma$, Clancy *et al*, 2005), and PIP_2 (Sui *et al*, 1998) with the involvement of PKC (Zhang *et al*, 2004), all which contribute to modulating K_{ACh}. Correspondingly, implications for GIRK in the present study should not be disregarded.

1.5 Isolated Perfusion Preparation

Investigations using explanted organs/tissues are able to maintain relatively normal physiological conditions as compared for example with cell culture studies, while removing influences of other control/organ systems normally present in the whole animal. This is especially important in the heart as various hormones, factors, and other nervous system controls may influence the responses of the heart to various experimental treatments, thus skewing the experimental data and the understanding of cardiac function itself.

The present study utilizes a unique *ex vivo* tissue perfusion system described in detail in *Materials and Methods* below, however the inaugural isolated heart perfusion setup is probably that of the Langendorff system. Although some similar preparations and modifications have been developed, many studies involving perfusion of the isolated heart or cardiac tissue still involve the Langendorff system, and since its first experimental use in 1895 by Oscar Langendorff, this preparation is of one the most widely used models for cardiovascular studies (Sutherland *et al*, 2003). The Langendorff system involves the insertion of a cannula into the ascending aorta and retrograde perfusion through the aorta towards the heart (Figure 8a). A polymesh support can be used for the heart to rest upon Chinchoy *et al*, 2000. The perfusate is allowed to flow through the coronary system exiting the coronary sinus in the right atrium. In the whole heart preparation, according to the Frank-Starling principle, the force of contraction is proportional to the pressure inside the ventricles. Thus to obtain a more physiological contraction, the ventricular pressure and thus force of contraction is often artificially increased for example, through the insertion of inflated balloons into the ventricles. Prior to this, the heart is often placed into cold media (~4°C) immediately following explantation from the animal, from which it is subsequently cannulated and slowly brought back up to a more physiological temperature (Sutherland *et al*, 2003; Chinchoy *et al*, 2000; Dehnert, 1988).

While the Langendorff setup is mostly used in whole heart studies, other preparations exist for studies involving isolated atria or ventricles. One such atrial preparation involves the insertion of a cannula accommodating three smaller catheters,

through the atrio-ventricular aperture. The cannula is secured with ligatures shown in Figure 8b (Cao *et al*, 2003; Cho *et al*, 1988). Although some obvious differences exist due to the use of atria versus whole heart, the isolated atrial perfusion preparation utilized in the present study strives to maintain the normal physiological morphology and orientation of the organ (Figure 8c). For example, myocardial "stunning" or postischemic dysfunction is a prolonged significant decrease in contractility following ischemia that persists even without any irreversible damage and after reperfusion (Bolli, 1990; Gao *et al*, 1996). In contrast to the commonly utilized perfusion preparations described above, that of the present study does not involve cooling of the heart, which may result in such "stunning" injuries of the organ and subsequent effects on normal functioning (Sutherland *et al*, 2003). No artificial devices (e.g. balloons) are inserted into the atrial chamber and the organ is neither hanging nor resting on a support. The arrangement of the cannulae allows the organ to be suspended without resting on any material and without the atrial tissue itself being punctured or tied in any way, thus avoiding any possibility of injury to the tissue.

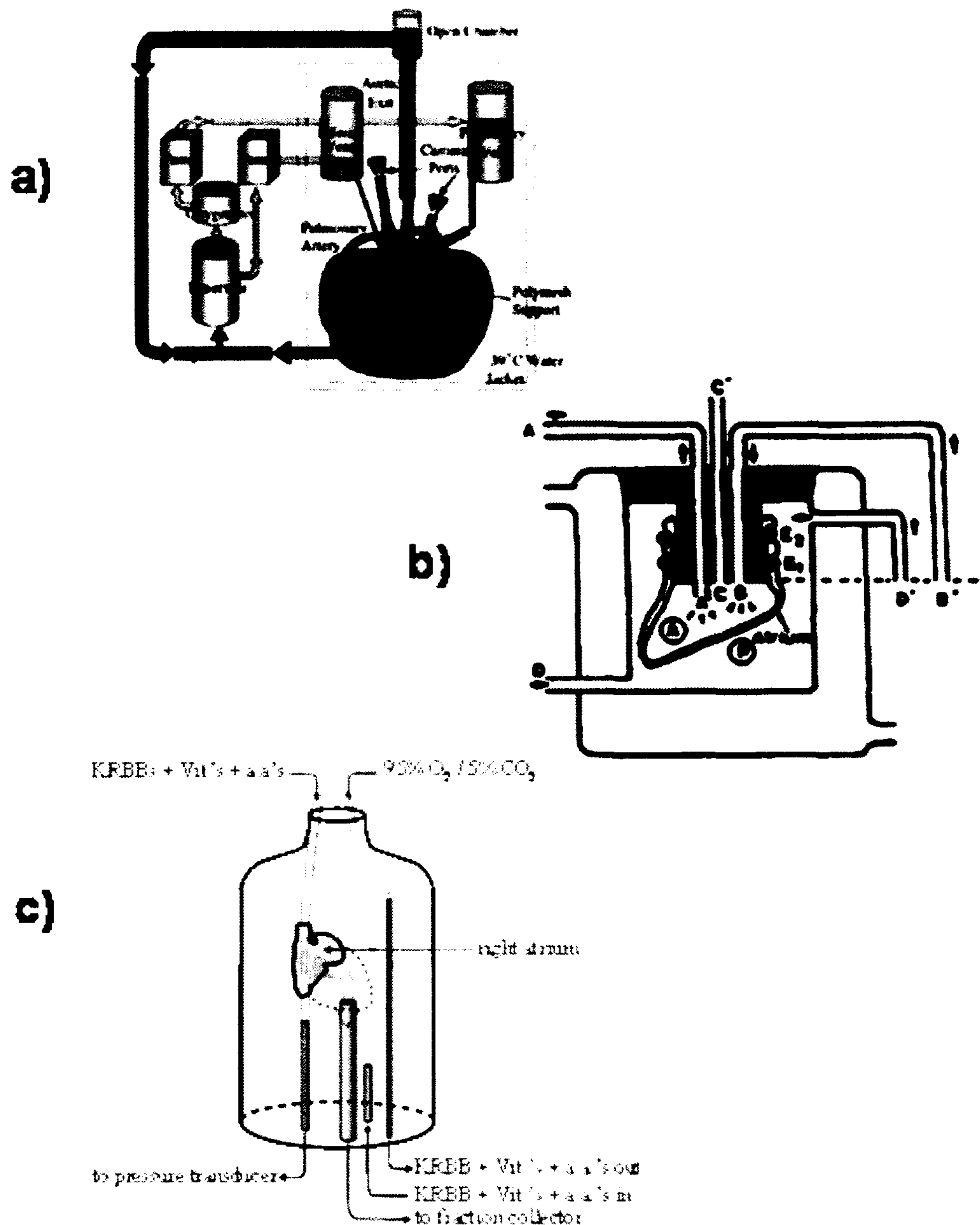


Figure 8. Various perfusion set-ups.

a) Langendorff preparation – The heart rests upon an optional polymesh support. The perfusate is allowed to flow through the coronary system exiting the coronary sinus in the right atrium. Force of contraction can be artificially increased for example through the insertion of inflated balloons to the ventricles. b) Isolated atrial preparation involving the insertion of a cannula accommodating three smaller catheters through the atrio-ventricular aperture. The cannula is secured with ligatures. c) Isolated atrial preparation used in the present study. This preparation maintains the normal morphology and orientation of the organ. Dashed lines show where the ventricle would be. [from a) Chinchoy et al, 2000 b) Cho et al, 1988 c) based on Bensimon, 2002].

1.6 Pharmacological agents

Described below are the various agents experimentally utilized in the current investigation and the characteristics which allowed them to be used to manipulate phospholipase C and its proximal effectors, to test their participation in regulating secretion of ANF from cardiac atria.

1.6.1 Phospholipase C inhibitors

1.6.1.a Edelfosine

Edelfosine, or 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OMe) is a membrane-active alkyl-lysophospholipid that is clinically administered as an anti-cancer agent. Edelfosine achieves its antineoplastic effects in part through preventing neovascularization (Vogler *et al*, 1998). The growth of tumours, like all tissues, depends on such angiogenesis for oxygen and nutritional supply as well as for metabolite removal. In preventing such vascularization, edelfosine may also help to prevent metastasis of such neoplasms. Edelfosine has additionally been shown to result in the down regulation of endothelial-cell adhesion molecules (Volger *et al*, 1998), increased presence of transferrin receptors and affinity of transferrin for the receptor in breast cancer cells (reviewed in Arthur and Bittman, 1998), as well as the activation of Fas/CD95 and the capping of membrane lipid rafts both through which it promotes apoptosis of tumor cells, while absolving normal cells (Gajate and Mollinedo, 2001).

Alkyl-lysophospholipids, of which edelfosine is one of the most potent, have been demonstrated to modulate protein kinase C signalling, consistent with an upstream effect on phospholipase C, which edelfosine acts to inhibit. Alkyl-lysophospholipids may also inhibit phospholipase D, phosphatidylcholine biosynthesis and the Na^+/K^+/ATPase. Notwithstanding the above effects, edelfosine potently acts to inhibit phosphoinositide-specific phospholipases C (PI-PLC) and is suggested to do so by blocking association of $G\alpha_{q/11}$ with PLC (Gajate and Mollinedo, 2002; Strassheim *et al*, 2000). Edelfosine has been utilized experimentally for such purposes in a variety of different systems (some examples are Kuo *et al*, 1997; Strassheim *et al*, 2000).

1.6.1.b U73122

1-[6-[17β-3-methoxyestra-1,3,4(10)-trien-17-yl]amino]-hexyl]1H-pyrrole-2,5-dione, commonly and commercially and known as U73122, is a selective inhibitor of PI-PLC and therefore inhibits the generation of DAG and IP_3 or other inositol phosphate products through receptor mediated actions of PI-PLC (Smith *et al*, 1990; Bleasdale and Fisher, 1993). It was found that U73122 does not affect the activity of phospholipase A_2 at the doses used for the present study, nor does it have effect on the levels of cAMP (Bleasdale and Fisher, 1993; Smith *et al*, 1990). The U73122 compound is an aminosteriod that was developed in response to the increasing understanding that PI-PLC play important roles in many cellular pathologies in addition to their importance in normal cell physiology and that the control of these enzymes has diverse therapeutic potential (Bleasdale, 1992). The inhibitory actions of U73122 on PLC have been extensively utilized in a variety of investigations involving many different cell types and

signal transduction systems (some recent examples are Lucas *et al* 2003, Herwald *et al* 2004, Zeng *et al* 2003).

Although the precise mechanism of U73122 has not been fully detailed, it is thought that this compound acts as an uncoupler of G_q proteins with PLC, thus disabling the signalling ability of the G protein to activate the phospholipase enzyme. Some of the structural requirements for PI-PLC inhibition include the maleimide group of U73122, which is crucial to its inhibitory actions. Similarly, the 3-methoxy group and the A ring appear to be important for PLC-inhibition since removal of the 3-methoxy group and/or saturation of the A ring results in a lowered potency of the agent (Bleasdale and Fisher, 1993).

1.6.1.c U73343

By substituting the maleic anhydride (maleimide) group of U73122 with succinimide, the inactive analogue, 1-[6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]-hexyl]-2,5-pyrrolidine-dione, commonly known as U73343, is formed. This inactive analogue has proven useful as a control compound in most studies employing U73122 (examples include: Munakata *et al*, 2002; Bleasdale *et al*, 1990; Smith *et al*, 1990) and has been similarly used in the present investigations.

1.6.2 Inositol 1,4,5-trisphosphate receptor inhibitor

1.6.2.a 2-APB

Inositol 1,4,5-trisphosphate-induced Ca^{2+} release from the sarcoplasmic reticulum is inhibited by 2-aminoethoxydiphenyl borate (2-APB). Maruyama and colleagues (1997) introduced 2-APB as the first membrane-penetrable modulator of IP_3R and it has since been utilized to investigate the physiological role of the IP_3R in various cells including cardiocytes (recent examples include Mackenzie *et al*, 2004 and Zima *et al*, 2004). 2-APB does not prevent IP_3 binding to the IP_3 receptor but is thought to interact directly with the IP_3R to prevent opening of the channel and subsequent Ca^{2+} release. Evidence of direct interaction of 2-APB with IP_3R without the interference in IP_3 binding is based in part on observations that 2-APB does not cause an increase in the level of IP_3. Additionally, 2-APB does not appear to interact with the ryanodine receptor or to affect Ca^{2+} release from the ryanodine-sensitive Ca^{2+} store (Maruyama *et al*, 1997).

1.6.3 Protein Kinase C Inhibitors

The inappropriate activity of protein kinase C has been implicated in numerous pathophysiological disorders including (but not limited to) developmental and proliferative disorders such as cancer, diabetes, inflammatory diseases, central nervous system disorders, neovascularization, and certain cardiac injuries (see respectively, Aleksandrovski, 2003; Nadra *et al*, 2005; Battaini, 2001; Kondo *et al*, 2005; Armstrong, 2004). Such widespread implication has made this enzyme a relevant therapeutic target that is widely being exploited (for recent reviews see: Hofmann, 2004 and Shen, 2003).

The following inhibitors of PKC, Calphostin C and Gö6976, were chosen due to their consistency of action and high specificity for PKC over other kinases such as PKA and MLCK compared to other traditionally utilized inhibitors such as chelerythrine chloride, ebselen, GF109603X (bisindolmaleimide I), and staurosporine. For example, some doubt has been raised as to whether chelerythrine actually possesses inhibitory activity against PKC (Lee *et al*, 1998); and although staurosporine is a potent inhibitor of PKC, it also acts on S6 kinase, phosphorylase kinase, PKA, and src kinase (Martiny-Baron *et al*, 1993).

1.6.3.a Calphostin C

Calphostin C is a perylenequinone and a secondary metabolite of the fungus *Cladosporium cladosporioides*, having a characteristic deep red colour. Perylenequinones are produced by various fungal plant pathogens and members of this family have been shown to be phototoxic to various plant, animal, and bacterial cells. Although also dependent on light, calphostin C is a specific inhibitor of PKC at non-cytotoxic concentrations. It interacts with the regulatory region of PKC, competing at the binding site of diacylglycerol and phorbol esters to exert its inhibitory effects (Martiny-Baron, 1993; Bruns *et al*, 1991). Thus, calphostin C is believed to inhibit the diacylglycerol-dependent isoforms namely α, $\beta1$, $\beta2$, γ, δ, ε, η, and θ. Some recent cardiac studies utilizing calphostin C as a specific PKC inhibitor include those by Weber *et al* (2005) and Tsai *et al* (2004).

1.6.3.b Gö 6976

Indolocarbazoles are derivatives of staurosporine that were synthesized to have enhanced selectivity for PKC while maintaining high potency. Gö6976 is one of the most efficacious, selective, and consistent indolocarbazoles utilized for the experimental inhibition of protein kinase C (Pi and Walker, 2000; Martiny-Baron *et al*, 1993). Gö6976 is highly selective for the Ca^{2+}-dependent isoforms of PKC as inhibition of these isoforms by Gö6976 at nanomolar ranges has been demonstrated while significantly higher concentrations (micromolar ranges) have little or no effect on Ca^{2+}-independent isoforms. Thus Gö6976 is an inhibitor of the conventional PKC isoforms α, β1, β2, and γ; the additional isoforms (δ, ε, η, θ) inhibited by calphostin C as well as the atypical isoforms (λ, ι, ζ, μ) would therefore not be affected. Interestingly, the mechanism of action of Gö6976 is not through the Ca^{2+}-binding domain of PKC, but appears to be at the nucleotide binding site through the competitive interference of ATP-binding (Martiny-Baron *et al*, 1993). The utilization of Gö6976 for the inhibition of PKC isoforms is gaining popularity with some recent cardiac studies including those by Schreckenberg *et al* (2004) and Xie *et al* (2004).

1.7 Purpose and hypothesis

Under many cardiovascular-related pathophysiological states (e.g. hypertension, heart disease), there is a natural upregulation of secreted ANF, clinically observed by increased plasma levels. While initially ANF helps maintain cardiac performance, its

effects become inadequate with disease progression (see "Functions and importance of ANF" and "Natriuretic peptides and heart disease" above). Infusion of exogenous ANF has been observed to improve cardiac health (see Schmitt *et al*, 2004 for a review), thus the inadequacy of the actions of ANF may be due to a relative lack of the circulating hormone. Through the understanding of the signalling mechanisms regulating the secretion of ANF, novel therapeutic targets may be identified that may be utilized to increase endogenous ANF secretion and plasma levels in the treatment of such pathophysiological states.

Recent evidence has established that G proteins are intimately involved in the regulation of ANF secretion from heart atria. Pertussis toxin which causes ADP-ribosylation of $G\alpha_{i/o}$ disrupts stretch-secretion coupling (Bensimon *et al*, 2004), constitutive activation of $G\alpha_s$ with cholera toxin also diminishes stretch-secretion coupling (unpublished observations) and agonists of $G\alpha_q$, such as phenylephrine, are known to stimulate ANF secretion (Ogawa *et al*, 1999). Based on preliminary results suggesting substantial increases in ANF secretion with inhibition of the $G\alpha_q$ effector phospholipase C, it is hypothesized that PLC and its cascade may play key roles in the signal transduction governing ANF secretion. This investigation takes a closer look at PLC and its proximal signalling cascade in the regulation of ANF secretion.

2. Materials and Methods

2.1 Animals

Male Sprague-Dawley rats were obtained from Charles River Laboratories each weighing 225 to 275g and maintained with standard rat chow and water *ad libitum* at the Animal Care facilities of the University of Ottawa Heart Institute until reaching experimental body weights of 300 to 375g (approximately 5-7 days).

2.2 Solutions and pharmacological agents

The perfusion medium used was a Krebs' Ringer bicarbonate buffer (KRBB) solution consisting of 78mM NaCl, 4.7mM KCl, 2.54mM $CaCl_2 \cdot H_2O$, 1.36mM $NaH_2PO_4 \cdot H_2O$, 1.16mM $MgCl_2 \cdot H_2O$, 25.0mM $NaHCO_3$, 20mM *N*-2-hydroxyethylpirazine-*N*'-2-ethanesulfornic acid (HEPES), 2.0mM sodium glutamate, 4.0mM sodium fumarate, 2.0mM sodium lactate, 11.6mM glucose, 2.16mU/mL zinc insulin (Humulin R, Eli Lilly, Minneapolis MN), amino acids and vitamins as in Modified Eagle's Medium (MEM, Gibco-Invitrogen), 0.04g biotin (Sigma), 0.001% (wt/vol) bovine serum albumin (BSA), 0.01% (wt/vol) gelatine, 100U/mL penicillin G (Sigma-Aldrich Chemicals), and 0.25µg/mL fungizone (Gibco-Invitrogen). The solution

was brought to pH 7.4 by addition of NaOH, filtered at 0.45μm or finer and stored at 4°C for no more than 48 hours.

All pharmacological agents were purchased from EMD Biosciences, Calbiochem and prepared just prior to administration. Edelfosine was reconstituted in ethanol (5mg ET-18-OCH_3 in 1mL EtOH) and diluted to experimental concentrations with non-oxygenated KRBB. U73122 and U73343 were reconstituted in dimethyl sulphoxide (DMSO, Sigma) and further diluted to experimental concentrations by addition to Krebs-Ringer bicarbonate buffer (KRBB). 2-APB was dissolved and diluted to required concentrations in slightly warmed KRBB (RT to 30°C). Calphostin C (100μg) and Gö6976 (500μg) were reconstituted in 100μL and 1000μL DMSO, respectively, and diluted to experimental concentrations with KRBB.

Radioimmunoassay buffer consisted of 0.1M potassium phosphate (pH 7.4, VWR International, Mississauga ON), 0.9% NaCl, 0.01% sodium azide, 0.1% triton-X, and 0.1% heat-treated BSA.

The extraction solution for inositol 1,4,5-trisphosphate consisted of 5% (wt/vol) trichloroacetic acid (TCA), 5mM EDTA, 10mM LiCl, 0.321% (wt/vol) adenosine triphosphate (ATP). A 10% (wt/vol) $NaHCO_3$, 10% (wt/vol) NaOH solution was used for neutralization.

2.3 Isolated atrial perfusion preparation

The atrial preparation used for these experiments has been previously described (Bruneau *et al*, 1997) and is illustrated in Figure 8c. Briefly, following decapitation the heart was removed and placed in oxygenated Krebs-Ringer bicarbonate buffer solution (KRBB). A cannula was inserted from the inferior vena cava through the right atrium and out through the superior vena cava such that a small opening in the cannula faced the interior of the right atrial chamber as shown. Following ligation of the coronary sinus, the left atrium, aorta, and pulmonary vein as well as most of the ventricles were dissected away, thus isolating the right atrium. A heat-curved lipped cannula (PE-10) was inserted through the tricuspid valve. The atrial preparation was then placed in a tissue bath, and was continuously bathed by circulating KRBB in which was bubbled 95% carbon dioxide / 5% oxygen gas. Flow of KRBB through the atrium was via the superior vena cava, through the right atrial chamber, and out through the tricuspid valve. The final perfusion rate through the atrium was maintained at 3mL/min under all conditions. The perfusate was collected to a fraction collector (Retriever II, Isco Inc., NB), 15mL for 5-minute time intervals. An aliquot of these collections was taken every 20 minutes for radioimmunoassay, immediately frozen and stored at –20°C.

Intra-atrial pressure was recorded on a Narcobiosystems Narcotrace physiograph via connection to a pressure transducer. Basal atrial pressure was set to 0.5mmHg. For stretch experiments, intra-atrial pressure is increased to 8mmHg by raising the tip of the outflow cannula in relation to the level of the atrium and pressure transducer. For all

perfusion experiments, an equilibration time of one hour (represented -60' to 0') was allowed prior to the addition of pharmacological agents. All pharmacological agents were administered to the perfusion medium via a syringe pump at a rate of 0.33mL/min. Flow of the perfusion medium was adjusted to maintain the final atrial flow rate of 3mL/min. At the end of each perfusion, the tissue was quickly taken down from the perfusion apparatus and the cannulae were removed. Atria were dissected out, flash frozen in liquid N_2 and stored at $^-80^oC$ for future assays.

2.4 Atrial natriuretic factor radioimmunoassay

Standard competitive radioimmunoassay (RIA, Figure 9) was performed to quantify ANF (2-day RIA) and BNP (3-day RIA). Radioimmunoassay buffer solution was used for all dilutions. ANF antibodies were raised in rabbits against rat ANF_{99-126}. Radioactive ANF was prepared by iodination at the Cardiovascular Endocrinology Laboratory. Radioactive BNP was purchased from Peninsula Laboratories. All reactions were carried out in polystyrene tubes (12 × 75mm) on ice and away from direct sunlight. Standards were calculated using ANF_{99-126} (Peninsula Laboratories) at concentrations of 31.25, 62.50, 125.0, 250.0, 500.0, and 1000 pg/mL. Quality controls were included in each assay, having been previously prepared at known ANF_{99-126} concentrations of 125 pg/mL (Low QC) or 250 pg/mL (Medium QC). To increase pellet size, 6.25% polyethylene glycol (PEG) in milliQ H_2O was added to each tube immediately prior to centrifugation.

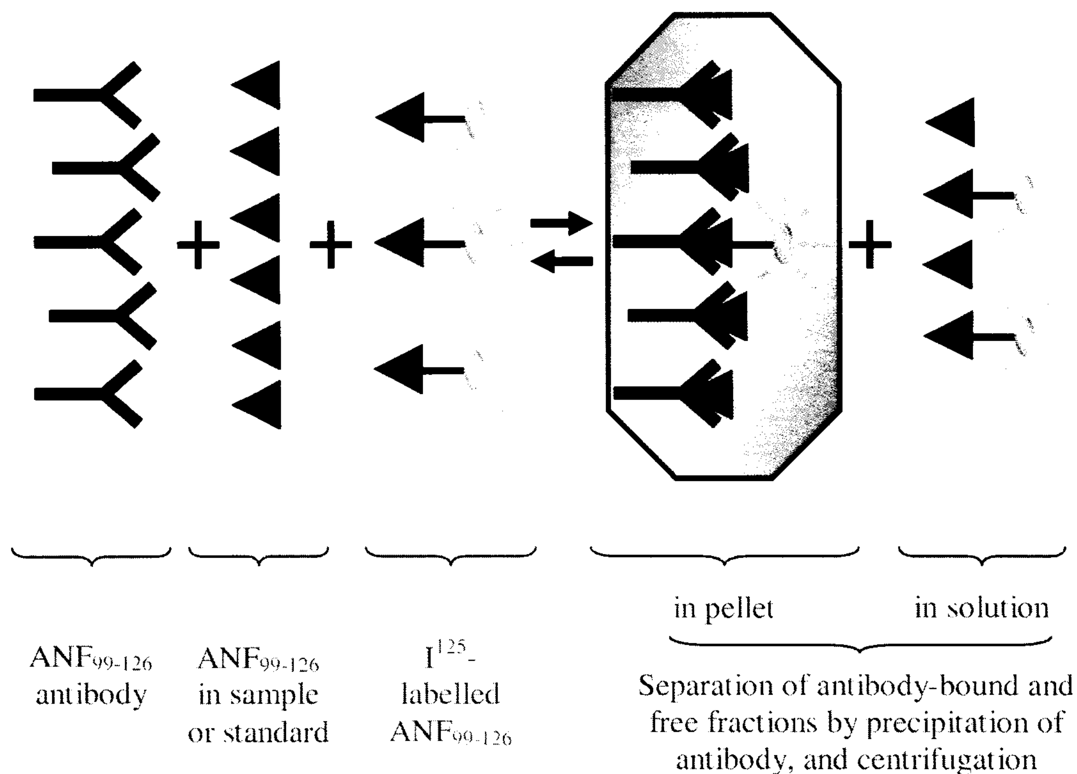


Figure 9. Radioimmunoassay for atrial natriuretic factor

Radioimmunoassay (RIA) is described in terms of competition between unlabelled antigen (ANF_{99-126}) in the sample or standard its radio-labelled counterpart (I^{125}-ANF) for binding to a limited quantity of specific antibody. The amount of unlabelled ANF_{99-126} present in the sample limits the amount of I^{125}-ANF that can bind to the antibody. Thus the amount of unlabelled species is inversely proportional to the amount of radioactivity associated with the antigen-antibody complex. (Based on Bensimon, 2002.)

2.5 Inositol 1,4,5-trisphosphate extraction and quantification

Cryopreserved perfused atrial tissue was washed into a polycarbonate tube with extraction solution and blended with a polytron homogenizer (3×10s with 15s chilling on ice in between). The acidified homogenate was centrifuged at 2000×g for 15' at 4°C and the supernatant was washed 3× with ~10 × volume water-saturated diethyl ether. The final aqueous phase was collected and neutralized to pH 7.5 using a Universal Indicator (Fluka/Riedel de Haën, Germany). Inositol 1,4,5-trisphosphate was measured using a commercial radioreceptor-binding kit (D-*myo*-Inositol 1,4,5-triphosphate [^{3}H] Biotrak Assay System TRK1000, Amersham Biosciences), according to manufacturer's instruction. Standards were prepared at concentrations of 0.19, 0.38, 0.76, 1.5 3.1, 6.2, 12.5, and 25 ρmol/tube. Radioactive signal from [^{3}H] labelled IP_3 was detected on a beta-scintillation counter.

2.6 Statistical analysis

All values (including Figures) are reported as mean ± standard error of the mean, with n representing the number of repetitions of independent experiments under the indicated treatments. Probability was set at 95% or higher for significance ($p \leq 0.05$) as evaluated by unpaired Student's *t*-test, with 2-tailed distribution.

3. Results

3.1 Role of phospholipase C signalling in atrial natriuretic factor secretion

3.1.1 Effect of phospholipase C inhibition on basal atrial natriuretic factor secretion

Isolated spontaneously beating atria were perfused with either edelfosine or U73122 for a period of 240'. Both edelfosine- and U73122-treatment (5μM each) resulted in dramatic augmentations of ANF secretion ($p < 0.01$) shown in Figures 10a and b. Increases were detected after 20 and 40 minutes of treatment initiation (U73122 and edelfosine, respectively) and were sustained until the end of the experimental period. In contrast, the inactive analogue U73343 (5μM) did not have a significant effect on secretion (Figure 10c) illustrating that inhibition of PLC is the cause of the observed enhanced levels of ANF secretion. Dose response curves (0.5μM, 5μM, and 20μM) were obtained for U73122 (data not shown) and U73343. The higher concentration of U73343 (20μM) showed some increase in ANF secretion therefore the more medial concentration of 5μM for both the inhibitor and inactive analogue was chosen.

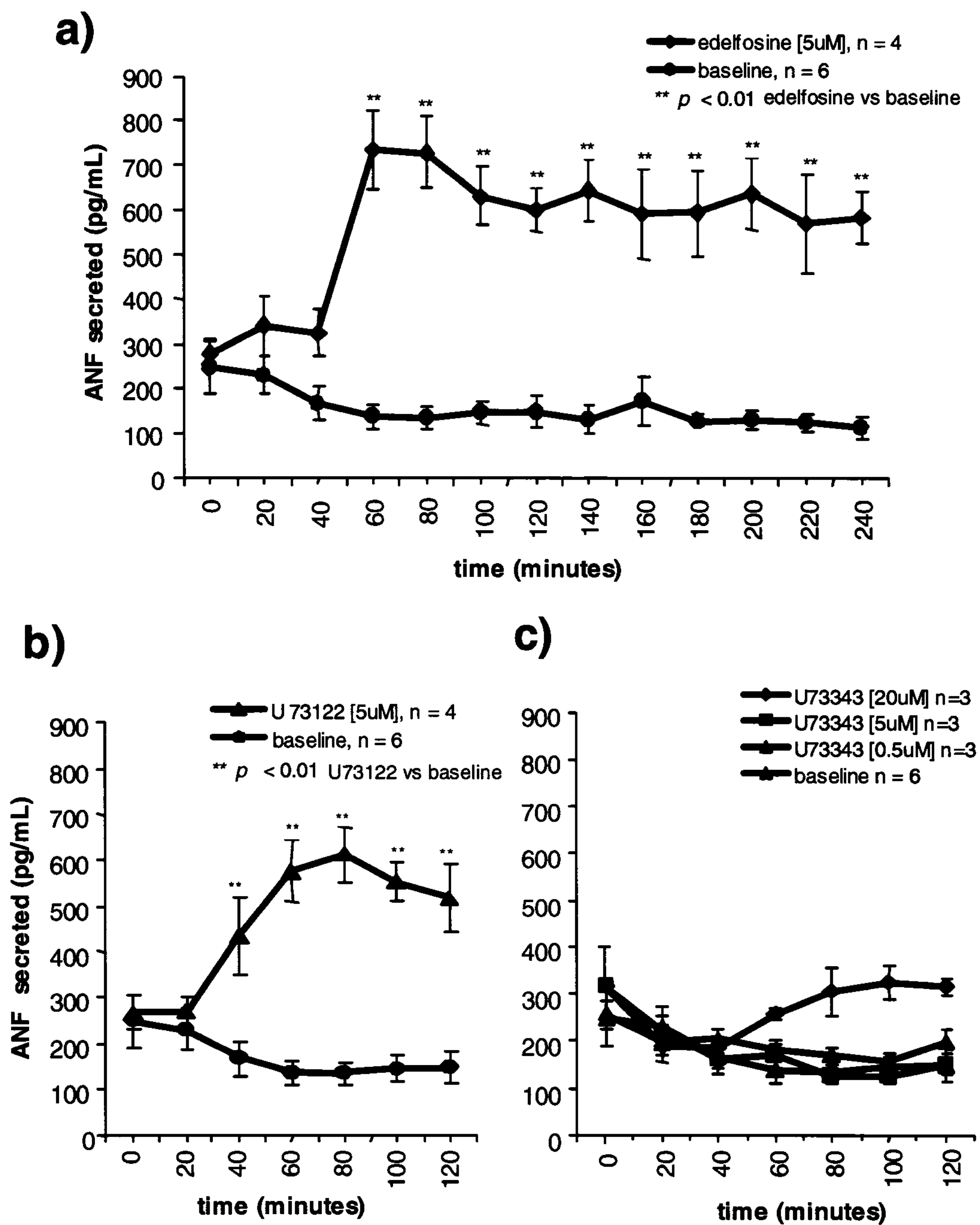


Figure 10. Effects of phospholipase C inhibitors on atrial natriuretic factor secretion

Spontaneously beating atria were perfused for 120' or 240' following a 60' equilibration period. a) Edelfosine [5uM] b) U73122 [5uM] or c) U73343 were administered from 0' to the end of the experimental period. Edelfosine and U73122 but not U73343 caused dramatic increases in ANF secretion.

3.1.2 Effect of phospholipase C inhibition on stretch-secretion coupling of atrial natriuretic factor secretion

Stretch-stimulated secretion results in a characteristic acute peak in ANF release (significance $p < 0.01$), as shown in Figure 11. In experiments where isolated perfused atria underwent either edelfosine- or U73122-treatments (5μM each) this characteristic peak was not observed. In contrast, the increased level of ANF secretion occurring as a result of treatments with the PLC-inhibitors fell contiguously with an additional stretch stimulus induced by increasing intra-atrial pressure from 0.5mmHg to 8mmHg (Figure 12a and b). ANF secretion in these experiments was at baseline levels beginning at sampling points 20 and 40 minutes following atrial-stretch induction.

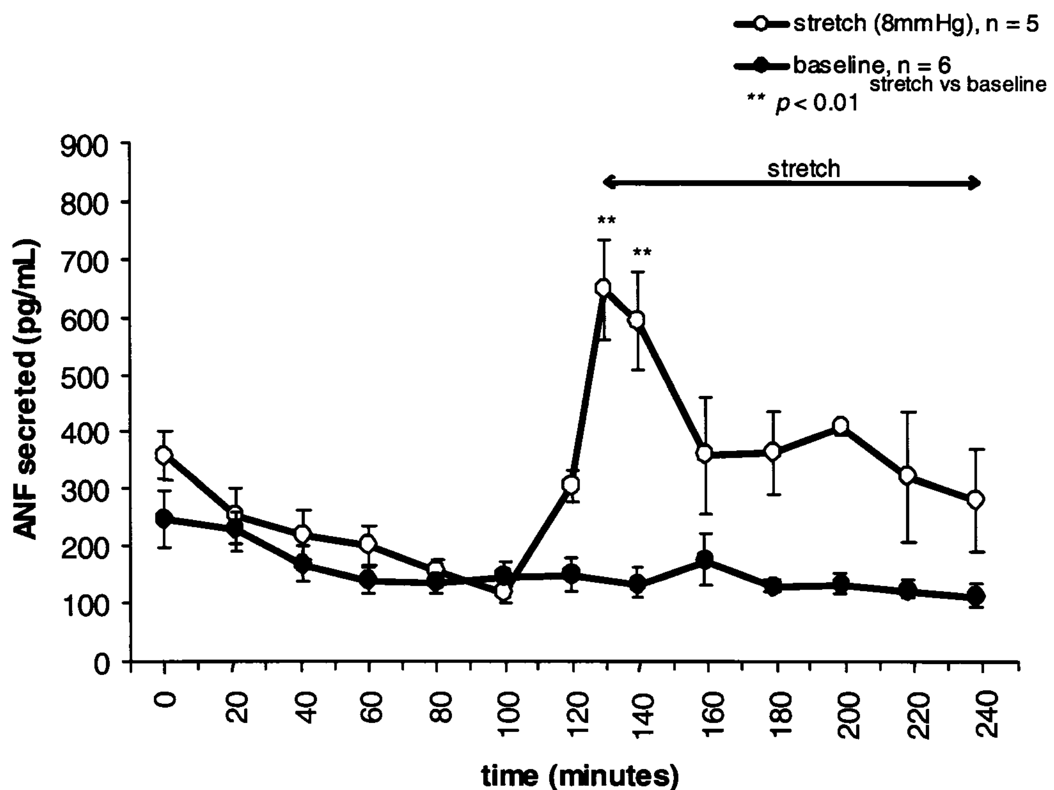


Figure 11. Stretch-stimulated atrial natriuretic factor secretion

Spontaneously beating isolated atria were perfused for 240' following a 60' equilibration period. At 120' a stretch-stimulus was added by increasing intra-atrial pressure from the baseline value of 0.5mmHg to 8mmHg, and maintained for the remaining duration of the experiment. An acute peak in secretion was observed, characteristic of ANF stretch-secretion coupling.

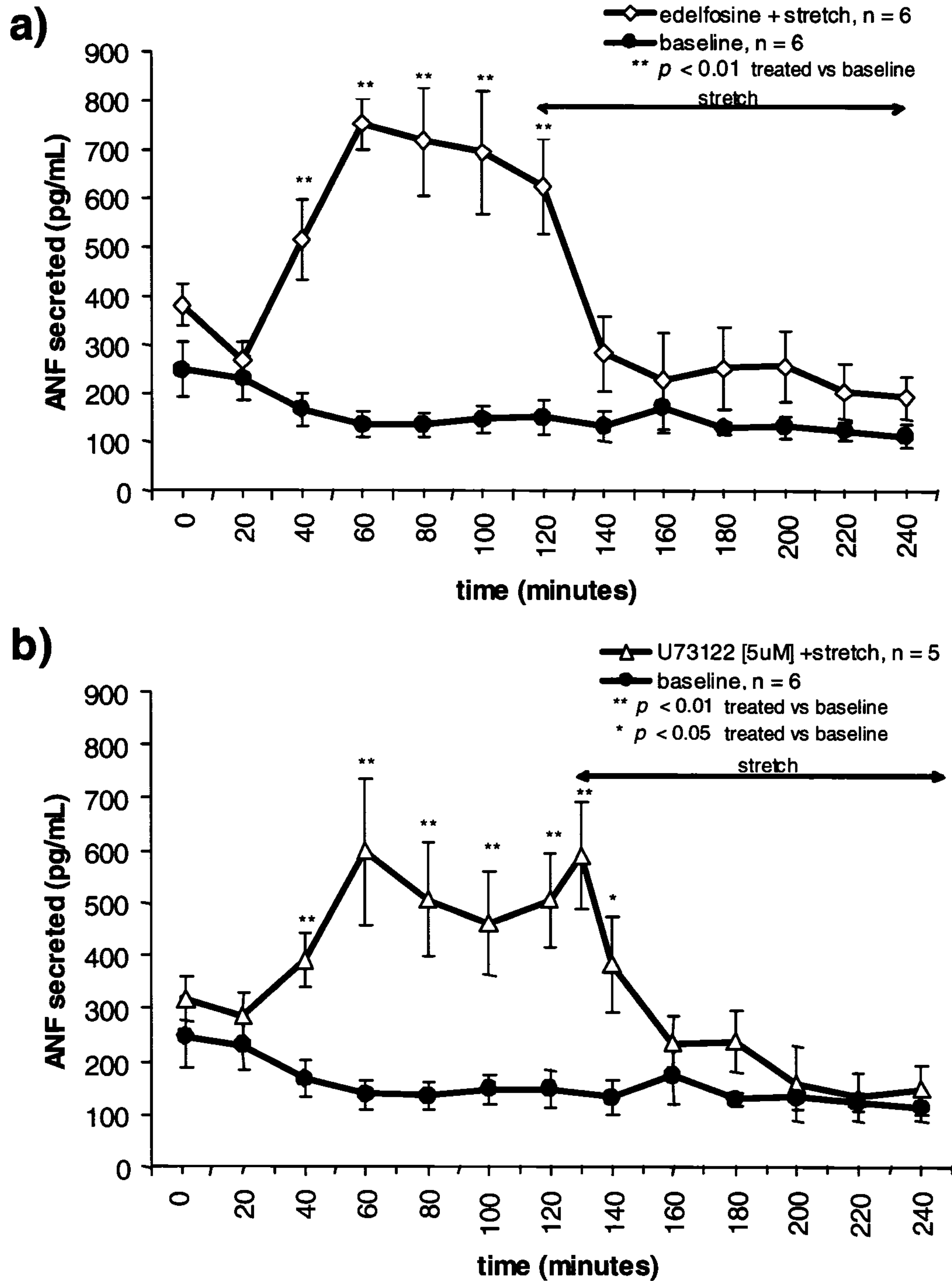


Figure 12. Effect of stretch on phospholipase C inhibition-mediated increases in atrial natriuretic factor secretion.

Spontaneously beating atria were perfused for 240' following a 60' equilibration period. a) Edelfosine [5uM] or b) U73122 [5uM] were administered from 0' to 240'. Stretch was induced at 120' through to 240' by increasing intra-atrial pressure to 8mmHg, from baseline pressure of 0.5mmHg.

3.2 Role of inositol 1,4,5-trisphosphate signalling on atrial natriuretic factor secretion

3.2.1 Effect of inositol 1,4,5-trisphosphate receptor inhibition on atrial natriuretic factor secretion

To test which arm of the PLC signalling cascade is responsible for the striking sustained increase in ANF secretion, the IP_3 receptor was inhibited by 2-APB (50μM). No significant effects were observed on basal secretion (Figure 13), however 2-APB treatment (50μM) did appear to significantly attenuate stretch-secretion coupling such that the characteristic spike in secretion fell below significance levels (Figure 14). To test whether stretch-secretion coupling could be completely blocked by IP_3R inhibition, the same experiments were repeated with the higher concentration of 2-APB (100μM) which resulted in a slightly, but not significantly, greater attenuation of the stretch response than that obtained with 50μM 2-APB (Figure 14).

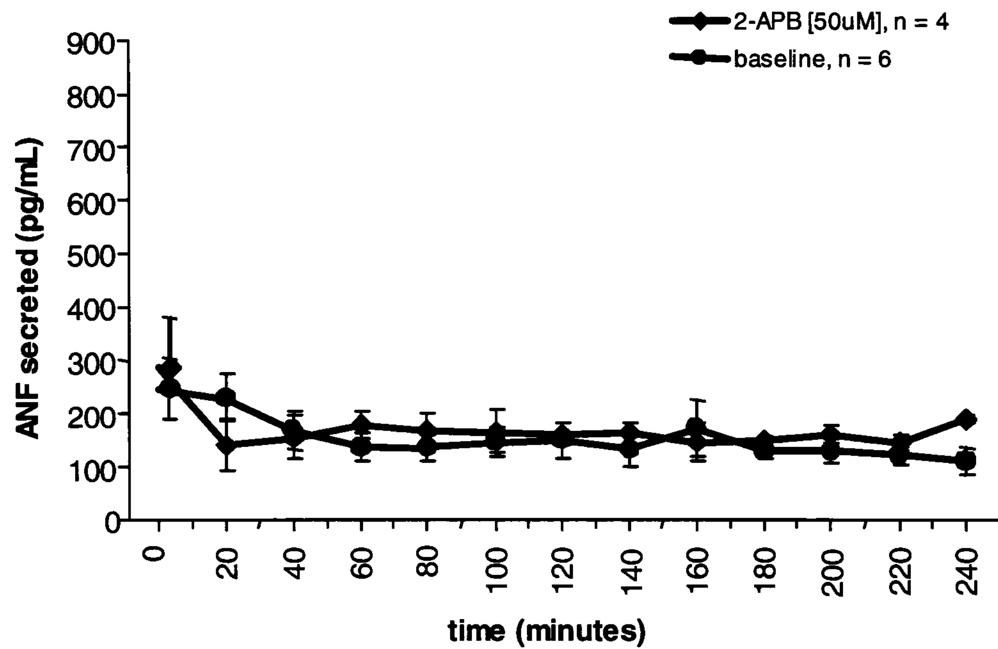


Figure 13. Effect of inositol trisphosphate receptor inhibition on atrial natriuretic factor secretion.

Spontaneously beating isolated atria were perfused for 240' following a 60' equilibration period. 2-APB [50μM] was administered from 0' to 240'. No significant effects were observed on ANF secretion.

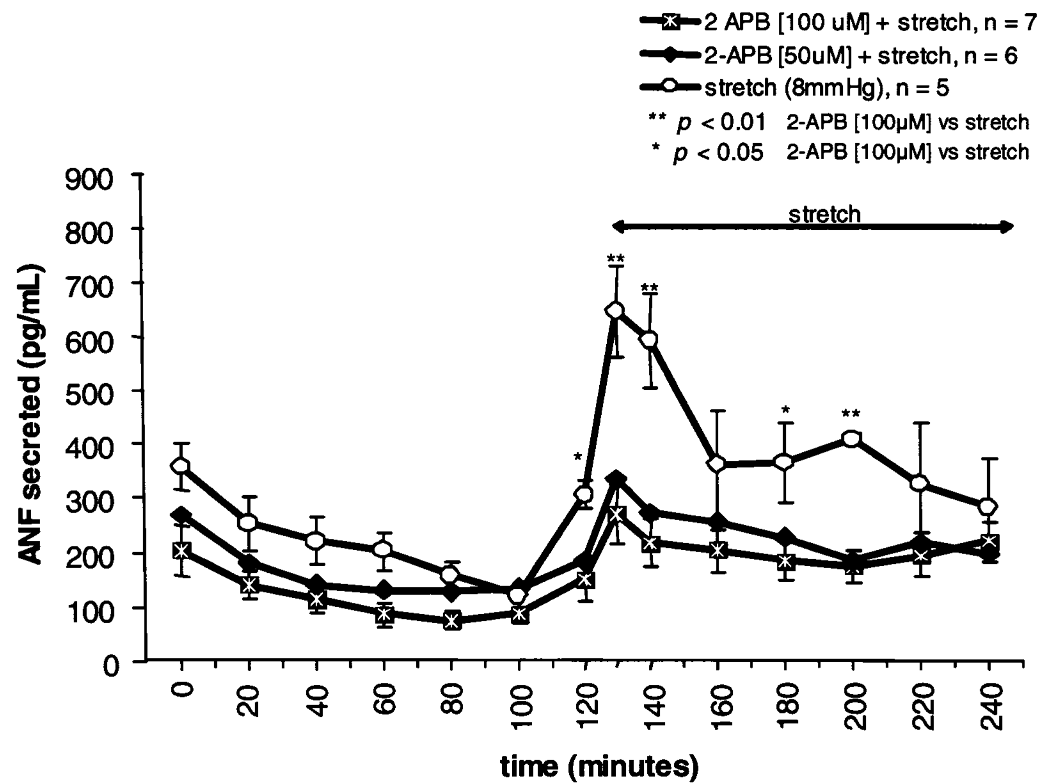


Figure 14. Effect of inositol trisphosphate receptor inhibition and stretch on atrial natriuretic factor secretion.

Spontaneously beating isolated atria were perfused for 240' following a 60' equilibration period. 2-APB [50μM or 100μM] was administered from 0' to 240'. A stretch-stimulus was induced at 120' through to 240' added by increasing intra-atrial pressure from the baseline value of 0.5mmHg to 8mmHg. 2-APB appeared to dose-dependently block ANF stretch-secretion coupling.

3.2.2 *Effect of combined phospholipase C and inositol 1,4,5-trisphosphate receptor inhibition on basal and stimulated atrial natriuretic factor secretion*

A combined administration of the PLC inhibitor edelfosine (5μM) and the IP_3R inhibitor 2-APB (50μM) did not result in any significant changes in ANF secretion from perfused isolated atria compared to baseline values (Figure 15). Similarly, as shown in Figure 16, when a stretch-stimulus was added by increasing intra-atrial pressure to 10mmHg at 120' no significant changes were observed, demonstrating a blockade of the stretch-response similar to that observed with administration of 2-APB alone (Figure 14).

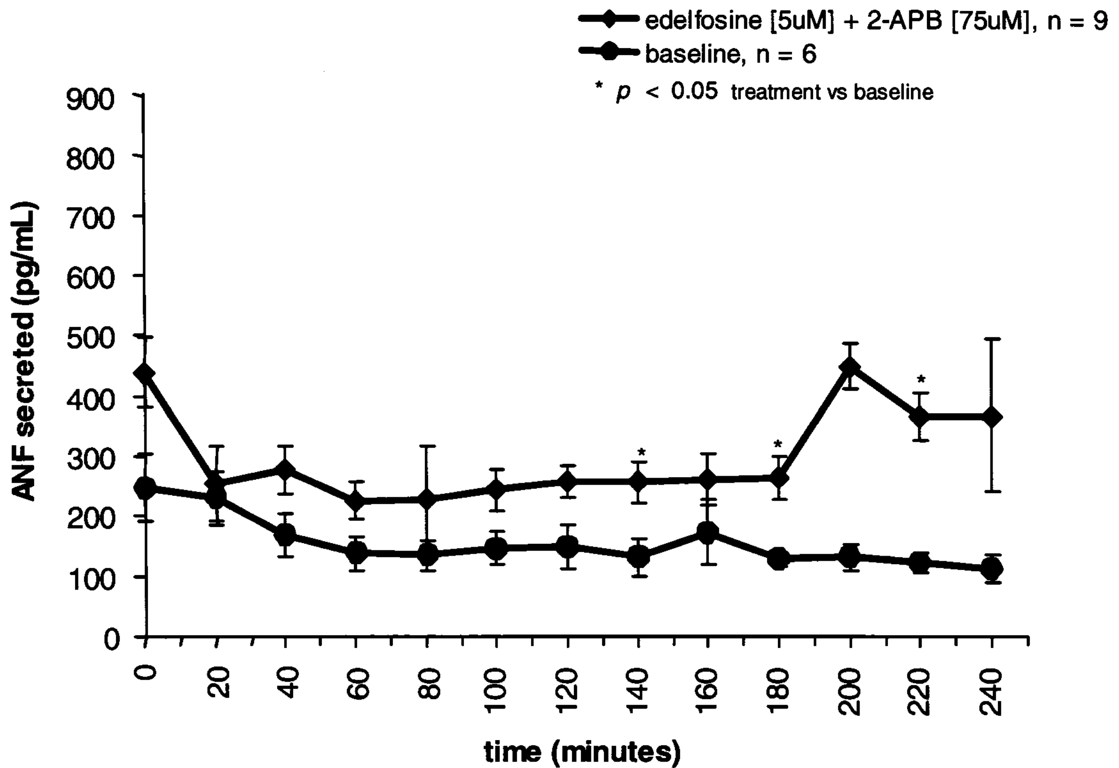


Figure 15. Effect of combined phospholipase C and inositol trisphosphate receptor inhibition on atrial natriuretic factor secretion.

Spontaneously beating isolated atria were perfused for 240’ following a 60’ equilibration period. Edelfosine [5μM] and 2-APB [75μM] combined, were administered from 0’ to 240’. A small but insignificant increase in secretion was observed compared to baseline.

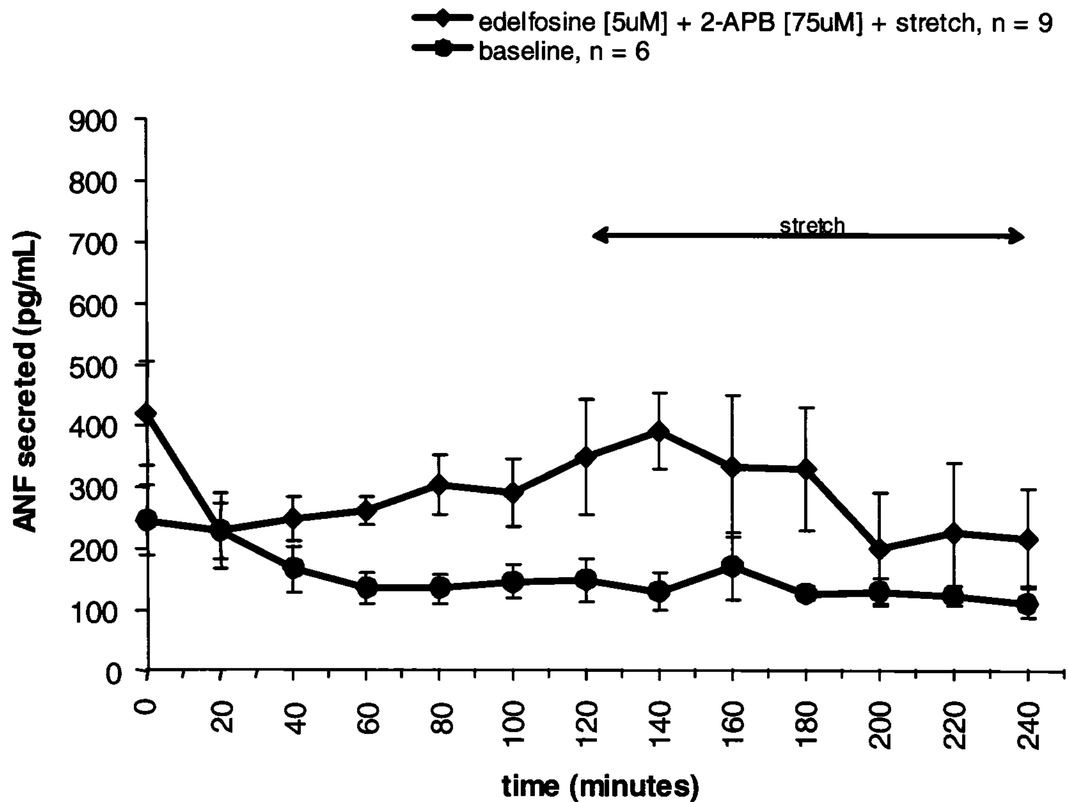


Figure 16. Effect of combined phospholipase C and inositol trisphosphate receptor inhibition, and stretch on atrial natriuretic factor secretion.

Spontaneously beating isolated atria were perfused for 240' following a 60' equilibration period. Edelfosine [5μM] and 2-APB [75μM] combined, were administered from 0' to 240'. Stretch was induced at 120' through to 240' by increasing intra-atrial pressure from the baseline value of 0.5mmHg to 8mmHg. No statistically significant changes in ANF secretion were observed.

3.3 Effect of protein kinase C signalling on atrial natriuretic factor secretion

3.3.1 Effect of protein kinase C inhibition on basal atrial natriuretic factor secretion

ANF secretion from isolated perfused atria was increased in response to treatments with either Calphostin C (200nM) or Gö6976 (1μM), shown in Figure 17a and b, respectively. Effects on secretion by treatment with either of the PKC inhibitors were not as sizeable and had an apparently slower onset, with increases in secretion observed after 40 minutes, as compared to treatment with the PLC inhibitors edelfosine and U73122, but were similarly sustained at the heightened level for the duration of the experimental period. Despite some variation between experiments, statistical evaluation shows that there is high significance between basal and treated secretion from both Calphostin C- and Gö6976-treated atria ($p < 0.01$).

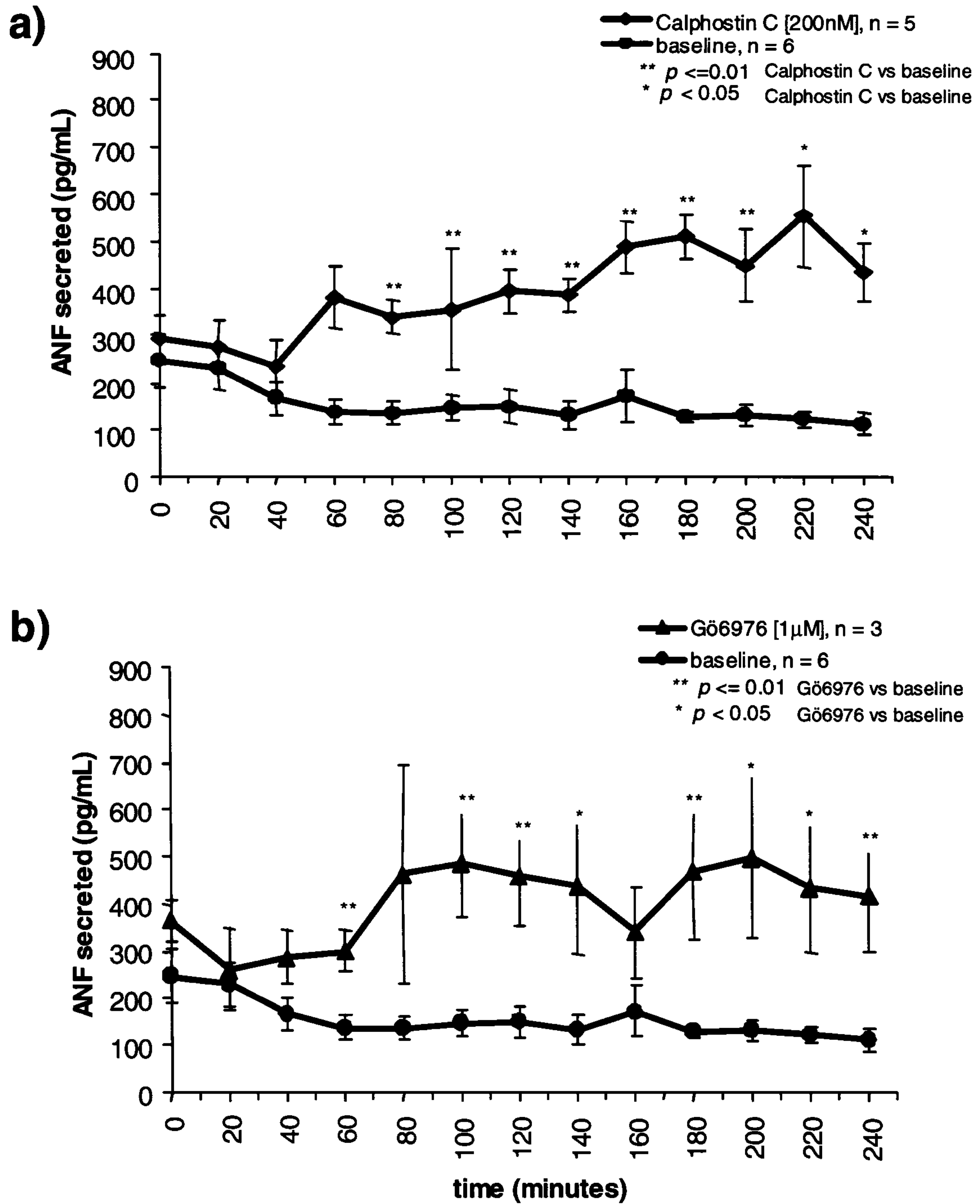


Figure 17. Effect of protein kinase C inhibition on atrial natriuretic factor secretion. Spontaneously beating atria were perfused for 240' following a 60' equilibration period. a) Calphostin C [200nM] or b) Gö6076 [1uM] were administered from 0' to 240'. Both PKC inhibitors caused significant increases in ANF secretion.

3.3.2 Effect of protein kinase C inhibition on stretch-stimulated atrial natriuretic factor secretion

In a manner reminiscent of observed PLC-inhibitor treatment, the addition of a stretch-stimulus by way of acutely increasing intra-atrial pressure from 0.5mmHg to 8mmHg (sustained for the remainder of the experimental period) resulted in a diminishment of secretion from heightened levels to near baseline levels in the Gö6976-treated atria (Figure 18b). The drop in secretion with Calphostin C treatment was not as pronounced compared to that observed in Gö6976-treated atria, with levels remaining statistically augmented above baseline levels. Interestingly, while no increase in secretion was observed with Gö6976 with stretch-stimulus, there does appear to be a statistically significant acute increase in secretion above already augmented levels in response to atrial stretch with Calphostin C treatment ($p < 0.01$; Figure 18a). This further increase was a short-lived peak, characteristic of observed stretch-secretion coupling described above. Following this peak, the rate of secretion fell non-significantly below levels that had been observed just prior to the initiation of the stretch-stimulus.

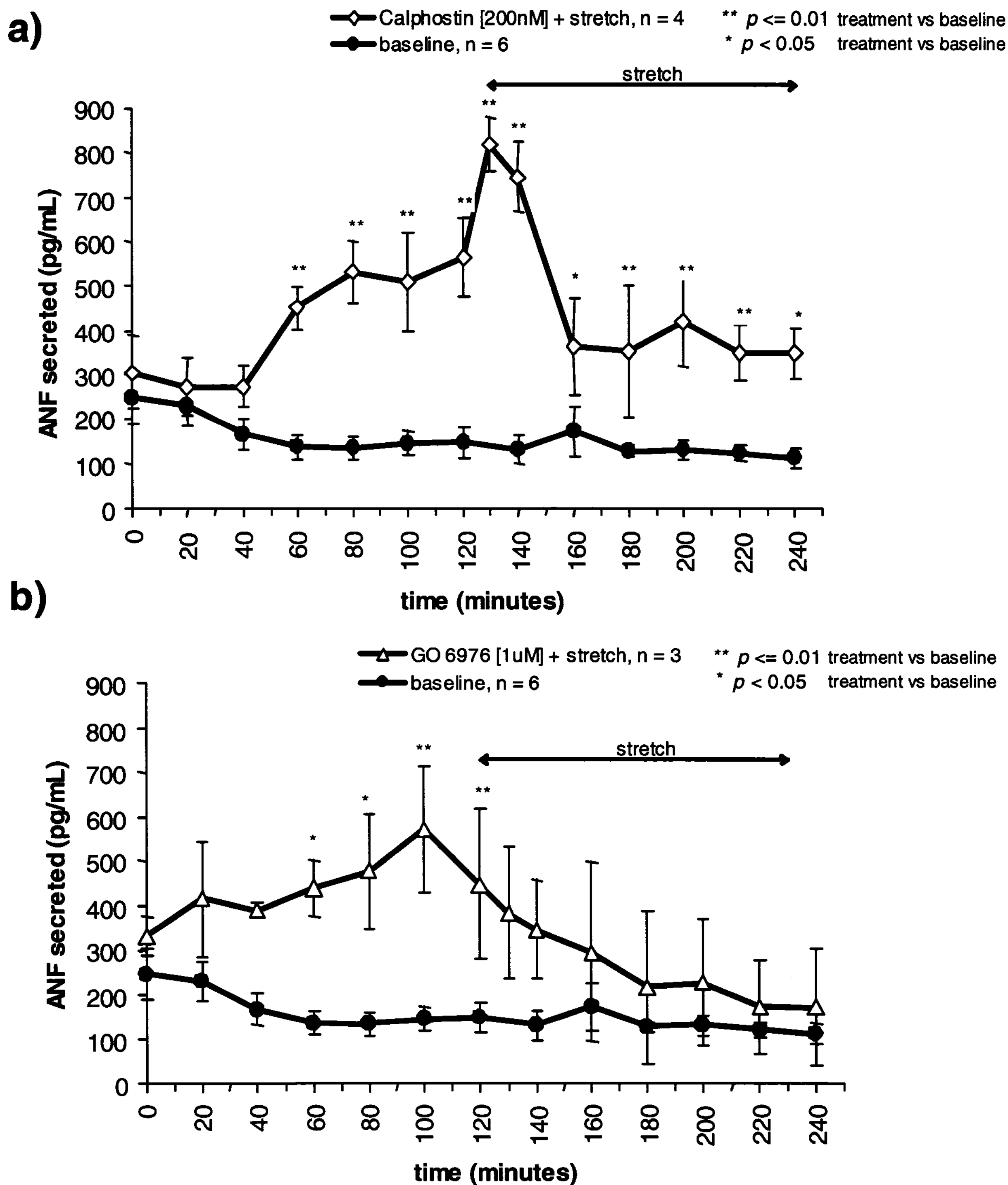


Figure 18. Effect of stretch on protein kinase C inhibitor-mediated increases in ANF secretion.

Spontaneously beating atria were perfused for 240' following a 60' equilibration period. a) Calphostin C [200nM] or b) Gö6076 [1uM] were administered from 0' to 240' and a stretch stimulus was added at 120' through to 240'.

3.4 Quantification of inositol 1,4,5-trisphosphate levels in isolated perfused atria

Relative IP_3 quantities measured in perfused atrial tissue are depicted in Figure 19. IP_3 levels in baseline atria were quantified giving a value of approximately 1.40pmol/tube containing an aliquot taken from extracts obtained from individual perfused atria. Extracts from perfused atria having undergone stretch-stimulation by increased intra-atrial pressure demonstrated significantly amassed quantities of IP_3, approximately 5-fold higher than that in baseline atria ($p < 0.01$). IP_3 levels in U73122- and U73343-treated atria were similar to baseline levels, as were levels in 2-APB treated atria. Consistent with PLC inhibition, U73122 blocked the stretch-induced increase in IP_3 formation, with levels again similar to baseline levels and significantly below that in stretch-stimulated atria ($p < 0.05$). Levels of IP_3 measured in atrial tissue that had been perfused with 2-APB and had undergone stretch-stimulation, were also significantly below levels measured in stretch-stimulated atria ($p < 0.05$).

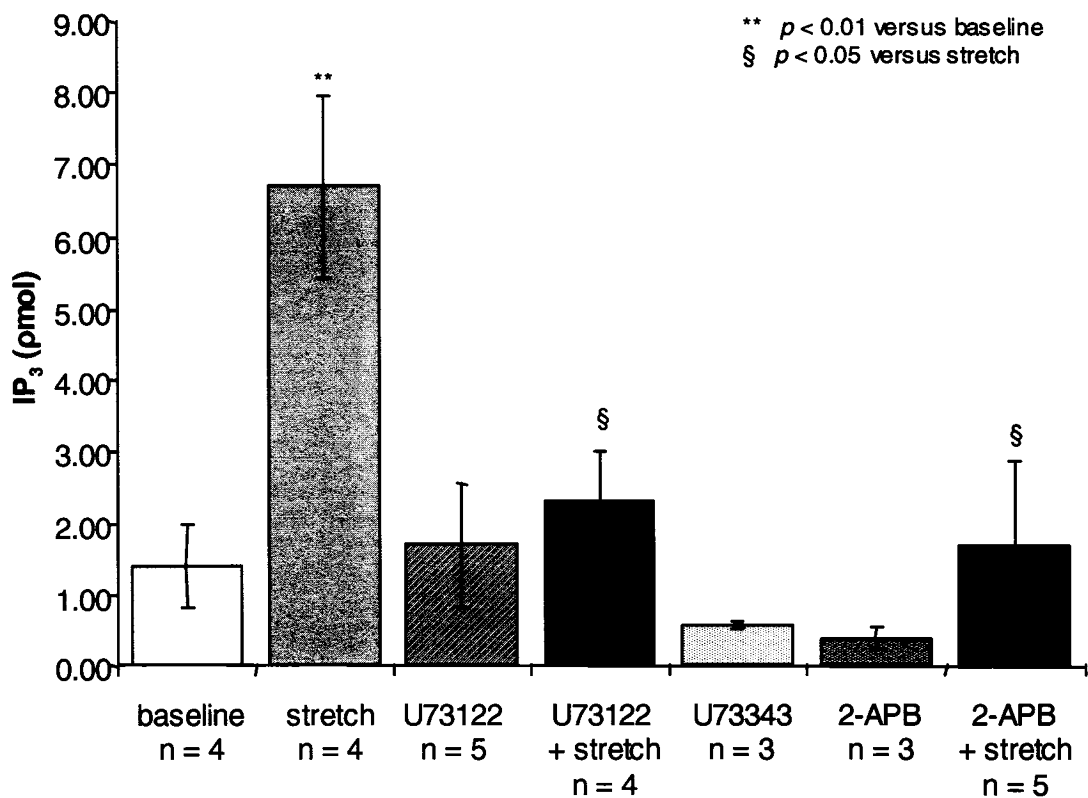


Figure 19. Inositol 1,4,5-trisphosphate levels in perfused atrial tissue.

IP_3 levels were measured in perfused atrial tissue that had undergone various treatments and which were cryopreserved following perfusion until the assay. IP_3 was quantified using the TRK1000 kit (Amersham Biosciences). In the abscissa, n = total number of tissue samples for that treatment. (Note: 2-APB and 2-APB + stretch were measured in a separate assay without samples of other treatment conditions and therefore cannot be conclusively compared with the other treatments.)

4. Discussion

The present investigation introduces exciting new aspects of the intracellular signalling cascades governing ANF secretion from the atria of the heart. There is strong evidence supporting an important involvement of phospholipase C and the proximal effectors in the cascade that follows its activity. Both phospholipase C and protein kinase C are demonstrated to be involved in the modulation of basal and stretch-stimulated ANF secretion, and inositol 1,4,5-trisphosphate along with its receptor IP_3R are shown to have involvement in stretch-secretion coupling. These aspects are described herein and present important implications which thus provide several interesting facets that should be further explored.

4.1 Phospholipase C involvement in the regulation of atrial natriuretic factor secretion

The present study clearly demonstrates a negative modulation of ANF secretion through the activity of phospholipase C, in that inhibition of the enzyme greatly enhances secretion and sustains secretion at this augmented level. Edelfosine was the initial agent used to inhibit PLC and because of the remarkable effects observed on ANF secretion, a second PLC inhibiting agent, U73122 was utilized, as well as quantification of IP_3 levels (described in methods) to verify these results. Despite its mechanism of action not being precisely delineated, U73122 has been successfully

employed in numerous studies to specifically inhibit IP_3 and DAG production through an inactivation of PLC (Bleasdale and Fisher, 1993; Thompson *et al*, 1991). Here, consistent with PLC inhibition, IP_3 levels were similar to baseline values following treatment with U73122 even with stretch-stimulation which has been observed to increase IP_3 levels and inositol turnover (von Harsdorf *et al*, 1989). This is an especially intriguing phenomenon not only because it is the first demonstration of such an increase in ANF release, but also because PLC is known as the immediate effector of $G\alpha_q$ through which many secretogogues of ANF are thought to signal and thus inhibition of PLC would not be expected to result in an increase in secretion. Several testable mechanisms exist to explain this paradox, which may have implications for other signalling molecules (such as those involved in the PLD cascade) and ions such as K^+, discussed further below, and which add insight into the signalling cascades that regulate ANF secretion.

4.1.1 Implications for phospholipase D activity

The precise mechanism behind the PLC-inhibited enhancement of ANF remains to be elucidated through scientific investigation. One possible explanation that presents a testable theory for this phenomenon is that by inhibiting PLC, the substrate phosphatidylinositol 4,5-bisphosphate (PIP_2) is liberated to act in its other role as a cofactor to advance the activity of phospholipase D (PLD) (Figure 20). Two isoforms of PLD have been identified in cardiac tissue, PLD1 and PLD2. The substrate phosphatidylcholine is cleaved by PLD to generate choline and phosphatidic acid (PA).

Activation of PKC by DAG occurs downstream of PLC and, like PLC-inhibition, the inhibition of PKC shows a similarly enhanced and sustained level of ANF secretion, discussed below. In the PLD cascade, illustrated in Figure 20, phosphatidic acid can be enzymatically acted upon by PA phosphohydrolase to form diacylglycerol, which activates PKC (Kurz *et al*, 2004). Although it remains to be elucidated which isoforms are specifically involved in either cascade ensuing from PLC or PLD activation, PKC isoforms activated through PLC-generated DAG are likely to be the DAG- and Ca^{2+}-dependent (i.e. the typical) isoforms (α, β_I, β_{II}, and/or γ) since, through IP_3 binding to IP_3R, there is an accompanying release of free intracellular Ca^{2+} which may potentiate PKC activity (Nishizuka, 1992).

In the PLD cascade, because there is no immediate accompanying Ca^{2+} release, it can be speculated that such isoforms of PKC activated by DAG generated from phosphatidylcholine and phosphatidic acid through PLD and PA phosphohydrolase are likely to be different from those activated by DAG generated from PIP_2 by PLC. Although studies have not yet been conducted to generate evidence supporting such a notion, it can be hypothesized that those isoforms activated through PLD and PA phosphohydrolase-generated DAG are DAG-dependent and Ca^{2+}-independent (i.e. the atypical isoforms - δ, ε, θ, η, and/or μ).

4.2 Protein kinase C involvement in the regulation of atrial natriuretic factor secretion

Both PKC inhibitors caused significantly heightened ANF secretion. There appears to be a slightly delayed inception of increases in secretion observed with Calphostin C and Gö6976 compared to those observed with PLC inhibitors edelfosine and U73122. It is uncertain whether this delay is of great relevance, and the reasons for it are unknown.

The activation of PKC by phorbol esters has been shown to increase ANF secretion from whole hearts (Ruskoaho *et al*, 1991). As discussed in the introduction, phorbol esters are utilized for their PKC-activating properties, although other regulatory proteins may also be affected. Nonetheless, it has also been demonstrated that PKC negatively regulates PLC-β and subsequent phosphoinositide hydrolysis (Cunningham *et al*, 1999), probably by way of a negative feedback. Thus, strong direct activation of PKC by phorbol esters may quickly result in an inactivation of PLC and the observed elevation of secreted ANF levels (Ruskoaho *et al*, 1991) supports the augmentation of ANF secretion by direct inactivation of PLC demonstrated in the present study.

As discussed below, it appears that the signal transduction mediating stretch-stimulated secretion involves the IP_3R. On the other side of the PLC signalling cascade is activation of PKC by diacylglycerol. Data of the present study suggest that inhibition of PKC by Calphostin C does not appear to affect stretch-secretion coupling of ANF in

isolated perfused atria since the characteristic acute peak in secretion is observed over heightened levels of secretions. This is supported by an investigation by Ishida *et al* (1988) who demonstrated that PKC inhibition did not affect ANF secretion induced by atrial wall stretch. On the other hand, no further increase in ANF secretion with stretch-stimulation was observed from atria treated with Gö6976. Differences in which PKC isoforms are affected by each of the inhibitors verisimilarly account for the differential effects on ANF secretion observed with stretch-stimulation. Calphostin C inhibits α, β1, β2, γ, δ, ε, η, and θ; whereas Gö6976 inhibits only α, β1, β2, and γ, since δ, ε, η, and θ are Ca^{2+}-independent and may be activated through PLD (Figure 20) or other signalling mechanism. These results are suggestive of a negative modulation of stretch-secretion coupling by δ, ε, η, and/or θ. Under 'Future Directions' below, a detailed approach is proposed which utilizes molecular and immunocytochemical techniques that will reveal the specific PKC isoforms expressed in atria. With determination of the presence and localization of these isoforms within atrial myocytes, further investigation should be conducted that will elucidate the involvement of PLD and various PKC isoforms in ANF secretion as well as which PKC isoforms are activated downstream of either PLC or PLD.

4.3 Inositol 1,4,5-trisphosphate and its receptor involvement in atrial natriuretic factor secretion

IP_3 levels measured in the present study were not significantly different between non-treated and U73122-treated perfused atria. Phosphatidic acid has been observed to cause elevated IP_3 levels in adult cardiomyocytes (Kurz *et al*, 1993). If inhibition of PLC indeed causes auxiliary activation of PLD, increased generation of PA would follow and therefore also IP_3. Nevertheless the lack of an observed augmentation of IP_3 level under the condition of PLC inhibition may be on account of the rapid metabolism of PA by phosphatidic acid phosphohydrolase, as described above.

It has long been known that atrial stretch is a potent stimulus for ANF release (Figure 10, Bensimon *et al*, 2004). However, the exact mechanisms by which such mechanical stress causes this release is still to be determined. Some evidence shows that IP_3 generation may be stimulated by atrial wall distension (experimentally demonstrated via balloon dilatation) and that the cardiac response to increases in atrial pressure may therefore be mediated through the phosphatidylinositol pathway. IP_3 levels and turnover in the myocardium, especially the right atrium, were found to be increased after just one minute of dilatation, and reached a maximal level after 10 minutes (von Harsdorf *et al* 1989). Caution is taken in evaluating these results as it may be noted that experimental procedures used in the von Harsdorf study involved cessation of perfusion to the isolated heart along with occlusion of the tricuspid valve.

This may have resulted in the heart becoming ischemic, which has been demonstrated *in vivo* to be a potent stimulant for ANF secretion (Chenu *et al*, 1991; Ruskoaho, 1992) and is supported by experimental studies *in vitro* suggesting that myocardial ischemia upregulates ANF secretion (Ruskoaho, 1992). Nevertheless, the remarkable drop in secretion rate from PLC inhibition-induced augmented levels to near baseline levels when atrial stretch is induced may be due to a reactivation of inositol trisphosphate formation. Whether or not such IP_3-formation occurs through PLC is unknown, however in some cell types a pertussis toxin-sensitive activation of PLC has been demonstrated. Furthermore, activation of PLC by $G\beta\gamma$ subunits released from G_i heterotrimeric proteins (Noh *et al*, 1995; dissociation of heterotrimeric G proteins is described in Introduction) not only explain the pertussis-toxin sensitivity of PLC activation but may also offer an explanation for why inhibition of PLC leads to such a dramatic increase in ANF and why the addition of the stretch-stimulus, and hence activation of G_i, abates this increase.

Inositol 1,4,5-trisphosphate levels measured in isolated atria treated with the PLC inhibitor U73122 and stretch in the present study, while slightly higher than atria treated with U73122 alone, were also significantly below levels measured in isolated atria that had undergone stretch stimulation only. Such determination of IP_3 gives a good estimation of relative levels. Notwithstanding, another possibility exists to explicate the observations of the present study involving G proteins and the IP_3 receptor/Ca^{2+} channel on the sarcoplasmic reticulum (SR).

It is evinced in the present study that inhibition of the IP_3 receptor using 2-APB attenuates stretch-secretion coupling (Figure 14), adding support for a role of IP_3 in the transduction of a stretch stimulus. The Cardiovascular Endocrinology laboratory has shown that the G-protein G_i is intimately involved in the signal transduction for stretch-secretion coupling of ANF (Bensimon *et al*, 2004) and in 2003, Zeng and colleagues showed a direct interaction of $G\beta\gamma$ released by $G\alpha_i$ with IP_3R. Furthermore, they demonstrated a potent activation of IP_3R by $G\beta\gamma$ subunits (Zeng *et al*, 2003). From these results it is postulated that the stretch stimulus may be transduced through increased levels of Ca^{2+} released through the IP_3R/Ca^{2+} channel, whereas basal (constitutive) secretion may be independent of Ca^{2+} or may actually be negatively modulated by normal Ca^{2+} levels. This suggestion is strongly supported by the results observed in the present study with 2-APB inhibition of the stretch response. As noted in the introduction IP_3R appears to be upregulated under severe pathological conditions (see section on 'Signal transduction and second messengers). Although the effects of ANF appear inadequate under such conditions, secreted levels of the hormone are elevated, and thus this corroborates with the suggestion that $G\beta\gamma$ release by $G\alpha_i$ may be signalling through IP_3R to help achieve such clinically observed elevated hormone levels (reviewed in Lijnen *et al*, 2005).

Although quantification of intracellular Ca^{2+} levels in the live tissue was not conducted, the increased level of basal secretion with PLC inhibition suggests a relief of the negative Ca^{2+} modulation thus allowing secretion of ANF to increase. However, with stretch stimulation, increased IP_3 and/or the $G\beta\gamma$ subunits released from G_i protein

may cause the opening of IP_3R Ca^{2+} release channels thus increasing intracellular Ca^{2+} concentrations and resulting in the observed drop in the augmented secretion back to baseline levels. Basal secretion was unaffected by the IP_3R blocker, 2-APB (Figure 13) therefore signalling for constitutive secretion may not be via this side of the pathway.

Myocardial stretch is known to increase ANF secretion, which at first may appear to conflict with the above model. It is likely however, that intracellular Ca^{2+} levels must reach a certain threshold before stretch-stimulated secretion occurs. Alternatively or additionally, it is plausible that any increases in intracellular Ca^{2+} are localized or act locally before being retaken up by Ca^{2+}ATPases on the SR. The inhibition of PLC may have caused a lower level of intracellular Ca^{2+} increasing the differential needed to meet threshold levels for the stimulated secretion. Thus, the added stretch stimulus may have indeed increased Ca^{2+} levels but only back to normal levels resulting in secretion returning to baseline, but not enough to meet the threshold to cause stretch stimulated secretion. This suggested model holds well with the fact that Ca^{2+} signalling in the heart is constantly ongoing, for example in contraction of the myocardium. Thus, as long as the heart is functioning normally, Ca^{2+} levels, though fluctuating, remain within a certain range which prevents basal ANF secretion occurring at too high a rate. Dysfunction of the heart resulting in increased pre-load and causing atrial stretch, opens Ca^{2+} channels which aids in increasing contractile force. Such stretch also stimulates ANF secretion to help combat the increased load. This therefore presents a testable model for the exact roles of Ca^{2+} in constitutive versus stretch-stimulated ANF secretion.

When PLC inhibition was combined with IP_3 receptor inhibition the sustained increased levels of ANF secretion observed with PLC inhibition alone, were not apparent. It is not known whether the lack of increased secretion was due to signalling blockade or to chemical interference between edelfosine and 2-APB since they were both administered together from the same syringe and therefore may have had some interaction prior to reaching their respective targeting sites in the atria. To clarify this and to more accurately test whether there is involvement of IP_3 and/or its receptor, IP_3R, in the stretch-induced drop in secretion from the PLC-inhibitor mediated augmented levels, a modified experimental design is proposed in 'Future Directions' below. If the addition of the IP_3R-inhibitor does prevent this secretory drop, this would further corroborate the key role of IP_3/IP_3R in stretch-secretion coupling.

4.3.1 Secretory vesicles, chromogranins and the inositol 1,4,5-trisphosphate receptor

A direct interaction of the chromogranins of secretory granules with the IP_3R has recently been evidenced (Huh *et al*, 2005). Chromogranins are found in the secretory granules of numerous secretory cells including within the specific atrial granules containing ANF (Steiner *et al*, 1990). In addition to being major secretory granule proteins, chromogranins are also known as Ca^{2+} storage proteins and help to sequester an estimated 99.9% of the intra-granular Ca^{2+} found associated with most secretory granules. Furthermore, all three isoforms of IP_3R have been found to be present in the granules of secretory cells (Huh *et al*, 2005; Yoo *et al*, 2001) opening the likelihood of a similar presence in atrial secretory granules which would be consistent

with the recent atrial secretory granule-localization of inositol 1,4,5-triphosphate 3-kinase B (Muth *et al*, 2004). Huh and colleagues (2005) provide strong evidence of a direct correlation between the number of secretory granules present and the relative release of IP_3-stimulated free intracellular Ca^{2+}. Their study is suggestive of a molecular mechanism whereby chromogranins function as a major IP_3-sensitive intra-secretory granular Ca^{2+} store.

Taken altogether, this provides a possible explanation for the observed inhibition of stretch-stimulated ANF secretion with IP_3R inhibition. $G_{i/o}$ proteins have been shown to be intimately involved in stretch-secretion coupling (Bensimon *et al*, 2004) and as described above, there is also evidence that the $\beta\gamma$ subgroup dissociating from activated G_i directly binds to the IP_3R causing the channel to open (Zeng *et al*, 2003). It is therefore postulated that a signalling mechanism may be that stretch-activated $G_{\beta\gamma}$ interacts with IP_3R, located not as a sarcoplasmic reticulum transmembrane protein but as a transgranular protein and Ca^{2+} channel.

4.4 Implication of potassium ion channels in atrial natriuretic factor stretch-secretion coupling

The rapid increase in ANF secretion following stretch is highly suggestive of the involvement of ion channels (Biancotti, 2005), however much controversy and mystery continues to enshroud the role of certain ions in the modulation of ANF

secretion. For example the influence of calcium on secretion has long been debated with suggestions that intracellular Ca^{2+} may signal for increased ANF secretion (Schiebinger, 1989; Ruskoaho *et al*, 1985) while extracellular Ca^{2+} has been shown have a negative effect on ANF secretion (Kim *et al*, 2002; de Bold and de Bold, 1989). Despite the recent localization of $PLC\beta_4$ to atrial secretory granules and its role in the inhibition of M currents (see Introduction, section 1.4.2. Phospholipase C β), it appears to remain generally accepted for now that changes in ion currents may simply be part of the mechanism which maintains general tissue homeostasis in the midst of various signalling activities within the heart, although recent investigations involving G protein-activated inwardly rectifying (GIRK) and ATP-inhibited K^+ (K_{ATP}) channels may change this view.

4.4.1 Implications for G protein-activated inwardly rectifying K^+ channels

Mechanical stretch of the cell membrane has been demonstrated to cause the attenuation of K^+ currents flowing through G protein-activated inwardly rectifying K^+ channels (GIRK or Kir3.x) localized in the atria and sinoatrial node of the heart (Ji *et al*, 1998). Such mechanical stimulation causing distension of the atria wall is well known to concurrently increase inotropic and chronotropic cardiac contraction (Frank-Starling law of the heart), and ANF secretion (experimental observations; Bensimon *et al*, 2004). Thus GIRK provide a possible mechanistic culmination linking stretch-stimulation, signal transduction, and resulting alterations in contraction and/or ANF secretion. Phosphoinositol 4,5-bisphosphate directly interacts with the C terminus of

GIRK (Huang *et al*, 1998) and it has been suggested that underlying mechanisms regulating stretch-modulation of GIRK involves such PIP_2-channel interaction and protein kinase C (Zhang *et al*, 2004). The possible involvement of GIRK in the regulation of ANF secretion is supported in that the known secretogogues of ANF, endothelin-1 and α-adrenergic agonists, inhibit GIRK currents. Interestingly, this inhibition was blocked by transfection of phosphatidyl-4-phosphate 5-kinase, which promotes the synthesis of PIP_2 (Bender *et al*, 2002).

Although $G\alpha_i$ has been found to also directly interact with GIRK, whether it causes an inhibition or activation of K_{ACh} is unclear (Clancy *et al*, 2005), as is the precise role of GIRK in ANF secretion. It appears that if GIRK currents do indeed participate in the regulation of ANF secretion, that role may be a dual one. K_{ACh} currents are inhibited by PKC (Nikolov and Ivanova-Nikolova, 2004) and as described above are stimulated by PIP_2 binding (Zhang *et al*, 2004, Bender *et al*, 2002). Together with the data of the present study showing PLC- and PKC- inhibition resulting in increased ANF secretion, these studies suggest that K_{ACh} currents help to mediate the signalling regulating basal ANF secretion. On the other hand, atrial wall stretch, a potent ANF agonist, also inhibits K_{ACh} currents (Ji *et al*, 1998) as do endothelin-1 and α-adrenergic agonists that also act as ANF secretogogues, suggesting a negative role of K_{ACh} in modulating stimulated secretion.

4.4.2 *Implications for K_{ATP} ion channels*

It is generally accepted that endothelin-1 (ET-1) acts through a $G\alpha_q$ protein-coupled receptor. In support of this it has recently been demonstrated that ET-1 stimulates ANF secretion from isolated atria in a pertussis-toxin insensitive manner (Bensimon *et al*, 2004). Nonetheless, evidence also exists illustrating the inhibition of cardiac ATP-inhibited potassium (K_{ATP}) channels by ET-1 through pertussis toxin-sensitive G proteins (Watanuki *et al*, 1997; Hilgemann and Ball, 1996). Furthermore, the opening of K_{ATP} channels by pharmacological manipulations manifests an inhibition of stretch-stimulated ANF secretion from isolated whole hearts whereas K_{ATP} blockers increase stretch-stimulated secretion while basal secretion appears to be unaffected (Jiao *et al*, 2000; Xu *et al*, 1996). Conjointly, it has been suggested that there is a correlation between the magnitude of stretch-induced current through K_{ATP} channels and the inhibition of ANF secretion (Biancotti, 2005). Based on these investigations and the notion of extracellular Ca^{2+} as a negative modulator of ANF secretion, together with data from a recent investigation involving Kir6.2 knock out mice which subsequently have impaired K_{ATP} channel functioning, Biancotti (2005) intimates a conceivable signalling mechanism whereby atrial cardiomyocyte stretch stimulates both ANF secretion and concomitant K^+ current leading to negative modulation of intracellular Ca^{2+} therefore providing a putative feed-back mechanism (Biancotti, 2005; Saegusa *et al*, 2005).

A more sophisticated view additionally incorporates PIP_2 regulation of K_{ATP} and ties together $G_{i/o}$ and PLC signalling. G_i is so named for its inhibitory actions on adenylate cyclase which converts ATP to cAMP. Thus stretch activation of G_i would lead to higher intracellular levels of ATP. ATP increases the generation of phosphatidylinositol 4,5-bisphosphate from phosphatidylinositol to which K_{ATP} channels are evidenced to be sensitive (Hilgemann and Ball, 1996). The inhibition of PLC, as experimentally performed in the present study, would analogously have the consequence of increased PIP_2 and accordingly a similar inhibition of K_{ATP} with subsequent increases in ANF secretion. In conjunction, IP_3 is subsequently not formed due to PLC inaction. Therefore the negative feedback by intracellular Ca^{2+} is deficient and ANF secretion remains augmented. When the isolated atria is subsequently stretched, intracellular Ca^{2+} concentration is allowed to increase through $G_i\beta\gamma$ binding to IP_3R and/or the release of stretch-sensitive Ca^{2+} stores. This would re-establish the negative modulation of ANF and correlates with the observed drop in secretion back to baseline levels (Figure 12).

5. Conclusions

The present study demonstrates three compelling and very exciting phenomena – 1) the heightened and sustained secretion of ANF with PLC and PKC inhibition, 2) the dramatic declivity of secretion returning to baseline levels with the addition of atrial stretch to treatments with PLC or PKC inhibitors, and 3) the suppression of normal stretch-secretion coupling with IP_3R blockade. All of these strongly implicate the PLC-signalling cascade in the regulation of ANF secretion from the atria and suggest cross-talk between G_q and G_i signalling as well as between PLC and PLD signalling with strong implications for the participation of PKC isoforms and the involvement of K^+ currents through GIRK and/or K_{ATP}. Also suggested is the possible IP_3R localization to atrial secretory granules and direct signalling from receptor coupled G proteins to atrial secretory granules through IP_3R. Altogether a compendious illustration of the plausible intracellular signalling cascade(s) regulating ANF secretion is represented in Figure 20. Furthermore, the data presented herein introduce conceivable pharmacological targets to be studied in greater detail for the upregulation of endogenous ANF secretion in the treatment of heart diseases. What will be unravelled as further study is conducted is awaited with excitement as great advances both for cellular signalling and clinical implications are anticipated.

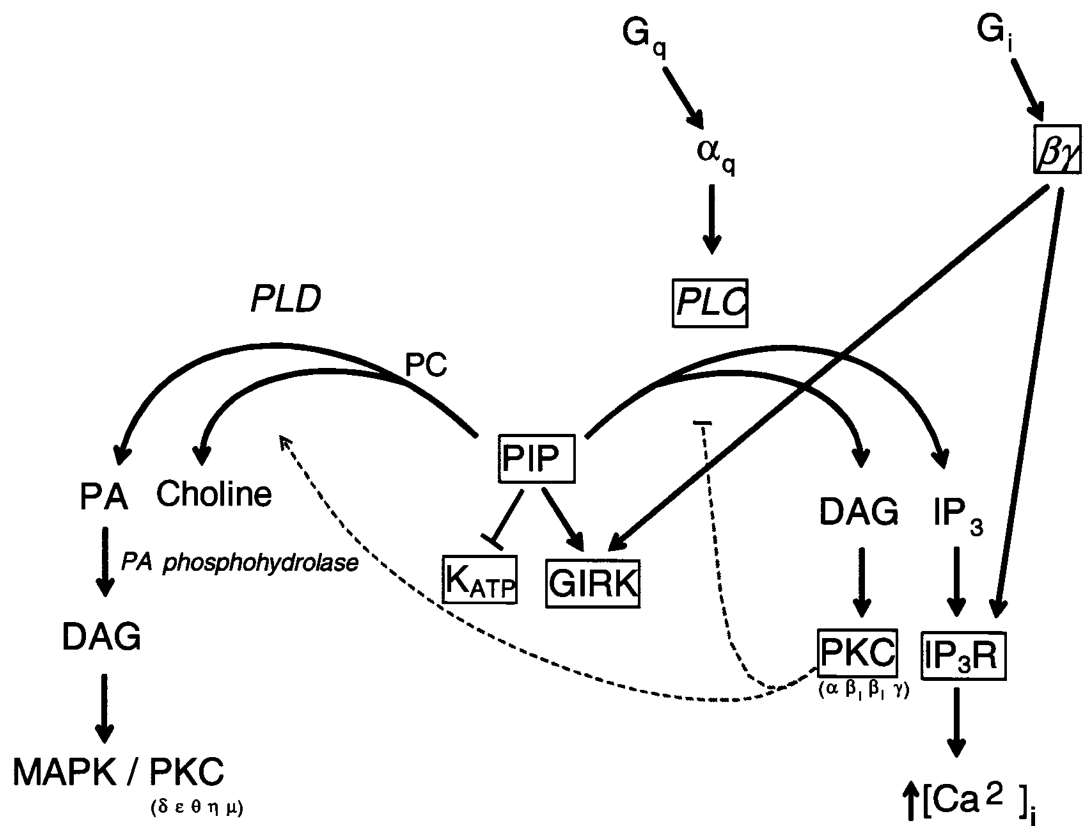


Figure 20. Proposed signalling mechanism mediating the regulation of ANF secretion.
Arrows represent an activation or generation. Blunted lines indicate inhibition. Respectively, green and red lines indicate what are thought to be positive or negative modulatory effects on ANF secretion.

6. Future Directions

In continuance of the present investigation and advancing the understanding of the intracellular signalling that regulates ANF secretion from the atria of the heart, herein are proposed further studies and recommended experimentations.

6.1 Investigating the combined effects of phospholipase C and inositol 1,4,5-trisphosphate receptor blockade on atrial natriuretic factor secretion

The current study involved a set of experiments whereby edelfosine and 2-APB were combined in a single syringe and thus administered together to spontaneously beating isolated atria. This was to test whether blocking the IP_3R would prevent the drop in secretion that occurs when a stretch-stimulus is added to atria being perfused with a PLC inhibitor. Instead of combining the pharmacological agents together in the same syringe prior to administration to the atria, it is proposed that the PLC inhibitor alone be administered until the heightened level of secretion is achieved (as in Figure 10). The IP_3R inhibitor can then be administered and allowed to act. Since this drug has been observed to act fairly quickly, intra-atrial pressure can be raised to stretch the atrial wall shortly thereafter. An example of the experimental timing is illustrated in Figure 21. By first allowing the PLC inhibitor-augmented level of ANF secretion to first be established, this experimental design should more accurately test whether IP_3 and/or the IP_3R will prevent the stretch-stimulated fall in secretion.

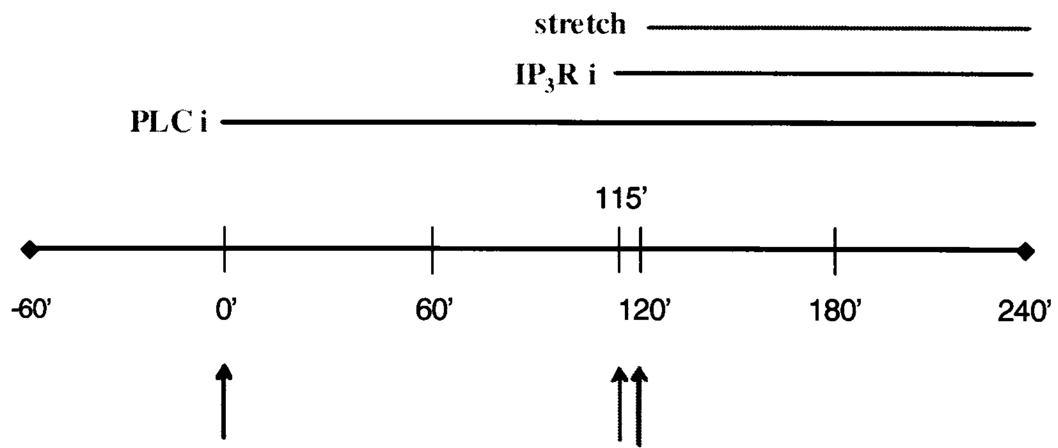


Figure 21. Experimental design for perfusion with combined phospholipase C and inositol trisphosphate inhibition.

Shown here is a proposed timeline for perfusion experiments to further test the role of IP_3/IP_3R in stretch-secretion coupling as well as to further investigate the phenomenon observed with PLC inhibition. (PLCi = PLC inhibitor; IP_3Ri = IP_3R inhibitor)

6.2 Characterization of atrial protein kinase C by degenerate/RACE PCR and sequencing

With 64 possible codons and only 20 amino acids, the genetic code is such that many different combinations of oligonucleotides can potentially code for a given set of amino acids. For example 64 possible sets of 18 oligonucleotides can code for the sequence Asn Phe Tyr Ala Trp Lys (example taken from Sambrook and Russell, 2001). In the organism however, there is usually only one combination present in the coding sequence, which may vary between organisms. To identify which combination makes up part of the organism's coding sequence, pools of oligonucleotides made of potential coding combinations can be synthesized and used as probes, or as PCR primers (degenerate primers) (Sambrook and Russell, 2001). In a study by Kohout and Rogers (1993) degenerate primers binding at the consensus cysteine-rich and ATP-binding sites of PKC were utilized. However, the authors were only able to identify some isoforms while citing sequence dissimilarities as the reason for not detecting other isoforms such as PKCζ for which the authors used specific primers.

Since the study by Kohout and Rogers (1993), 3 other isoforms have been discovered. Thus for the purposes of identifying consensus sequences among the 12 different described PKCs, which may also be useful for the discovery of new isoforms, different protein alignment software programs may be used.

It is recommended that the approach to identify atrial PKC isoforms be modified to include rapid amplification of cDNA ends (RACE) with which the unique C-terminal ends of individual PKC isoforms can be amplified and subsequently sequenced for identification. A similar approach has been widely utilized in numerous other studies involving rodents (e.g. Okamoto, 2004; Hanas *et al*, 2002) or other organisms (Talvinen and Nevalainen, 2002; Johjima *et al*, 2003; Murakami *et al*, 2003; Hugodot *et al*, 2004). The process of RACE adds an anchor sequence on to the end of the cDNA of interest. Subsequently, a universal primer complementary to this anchor sequence coupled with a specific primer is used in PCR reactions to amplify the unknown sequence of interest. However, here it is proposed that a degenerate primer be used with the universal primer in order to amplify multiple isoforms (Figure 22).

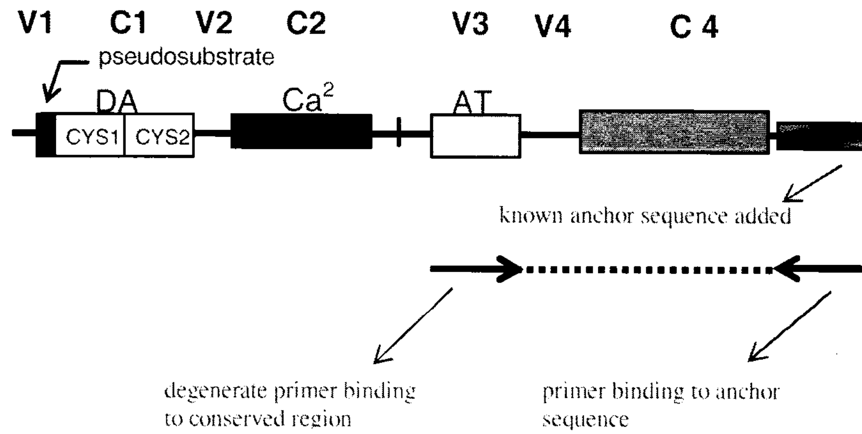


Figure 22. Combined RACE/PCR technique for characterization of PKC isoforms

The process of RACE adds an anchor sequence (purple block) on to the end of the cDNA of interest. A universal primer complementary to this anchor sequence is coupled with a degenerate primer in PCR reactions in order to amplify the unique C-terminal ends of multiple PKC isoforms which can be subsequently sequenced for identification.

6.3 Immunofluorescence localization of protein kinase C isoforms

In addition to PCR identification of PKC isoform expression described above, it will be pragmatic to localize the identified PKC isoforms to determine whether the kinases are found in cardiomyocytes or fibroblasts. An immunofluorescence protocol is proposed to achieve this, which will also serve to verify PCR results. Similarly immunofluorescence will localize not only those isoforms whose gene expression is PCR-diagnosed, but it may also be useful in the diligent testing for other isoforms.

6.4 Identification of inositol 1,4,5-trisphosphate receptor in atrial tissue

Although all three IP_3R are activated and respond in a similar manner, it would be of interest to identify which types are present in granule-containing atrial cardiocytes and which are similarly localized intra-granularly. Once the presence of IP_3R has been confirmed, further experimentation can be conducted to test for chromogranin A and/or B activation of these Ca^{2+}-channels as well as the whether or not there is direct interaction of $G_i\beta\gamma$ with sarcoplasmic and/or granular IP_3R. Elucidation of these interactions and what effects they have on stretch-secretion coupling of ANF will further the delineation of the signalling pathway that follows from muscle stretch to enhance ANF secretion.

6.5 Future therapeutic implications

The results of the present investigation are strongly suggestive of members of the PLC cascade as possible pharmacological targets to up-regulate endogenous atrial natriuretic factor secretion for the treatment of many cardiac maladies. For example a cardiac-specific PI-PLC inhibitor could act as both an inhibitor of angiotensin and a diuretic through the actions of ANF while circumventing the complication of the peptide hormone's very short circulating half-life since secretory augmentation can be sustained. Nonetheless it is recognized that caution should be taken with such interpretation and application, especially since the present study also demonstrates a dramatic drop in secretion of ANF when a stretch stimulus is added to PLC-inhibition. It has been demonstrated that while basal secretion from hypertrophied atrial tissue is increased, there is a quelling of both the response to endothelin and stretch-secretion coupling (Agnoletti *et al*, 1990). The increase in basal secretion is reminiscent of PLC- and PKC-inhibition while the attenuation of stimulus-induced secretion is somewhat reminiscent of IP_3R inhibition, which while conclusions can not be drawn with any certainty, gives at least some interesting targets for further investigation. It would also certainly be of interest to further investigate K^+ channel modulation.

7. References

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Thank you.