BRAIN TYPE NATRIURETIC PEPTIDE INCREASES L-TYPE Ca^{2+} CURRENT IN ATRIAL MYOCYTES BY ACTIVATING NATRIURETIC PEPTIDE RECEPTOR A

by

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "BRAIN TYPE NATRIURETIC PEPTIDE INCREASES L-TYPE Ca^{2+} CURRENT IN ATRIAL MYOCYTES BY ACTIVATING NATRIURETIC PEPTIDE RECEPTOR A" by Jeremy E. Springer in partial fulfilment of the requirements for the degree of Master of Science.

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Signature of Author

To my grandfather Ben Springer
who passed away on 5 January, 2011

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ABSTRACT

Natriuretic peptides are a group of hormones, including atrial-, brain-, and C-type-natriuretic peptides (ANP, BNP, CNP). BNP can bind to two NP receptors (NPRs) denoted NPR-A (activates guanylyl cyclase) and NPR-C (activates inhibitory G-proteins). This study investigated the electrophysiological effects of BNP on isolated mouse atrial myocytes. Current-clamp experiments show that BNP had no effect on action potential (AP) parameters in basal conditions; however, when pre-stimulated with the β-adrenergic receptor agonist isoproterenol (ISO), BNP prolonged AP duration. Voltage-clamp experiments demonstrate that BNP increased L-type calcium current ($I_{Ca,L}$) in the presence of ISO without altering cardiac potassium currents. The BNP effect on $I_{Ca,L}$ was blocked by A71915 (a selective NPR-A antagonist), maintained in myocytes lacking NPR-C receptors and blocked by the phosphodiesterase-3 (PDE-3) inhibitor milrinone. These data demonstrate that BNP prolongs AP duration and increases $I_{Ca,L}$ in atrial myocytes by activating NPR-A, increasing intracellular cGMP, and inhibiting PDE-3.

LIST OF ABBREVIATIONS USED

β-AR	β-adrenergic receptor
AC	adenylyl cyclase
ACE	angiotensin converting enzyme
AF	atrial fibrillation
ANP	atrial natriuretic peptide
AP	action potential
$APD_{50,70,90}$	action potential duration at 50, 70 and 90% repolarization
ASCEND-HF	Acute Study of Clinical Effectiveness of Nesiritide in Decompensated Heart Failure
ATP	adenosine triphosphate
BNP	brain type natriuretic peptide
BP	blood pressure
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
DAG	diacylglycerol
DD-H_2O	double distilled water
DMSO	dimethyl sulfoxide
EHNA	erythro-9-(2-Hydroxy-3-nonyl) adenine
FRET	Förster resonance energy transfer
fsANP	frame shift mutated ANP
FUSION I&II	Follow-UP Serial Infusions of Nesiritide I and II
GC	guanylyl cyclase

GC-A	guanylyl cyclase A
G_i	inhibitory G protein
G_{max}	maximum current conductance
G_s	stimulatory G protein
GTP	guanosine triphosphate
HF	heart failure
$I_{Ca,L}$	L-type calcium current
I_f	hyperpolarization activated current
I_{K1}	inwardly rectifying potassium current
I_K	potassium currents
I_{Kr}	rapidly activating outwardly rectifying potassium current
I_{Ks}	slow activating outwardly rectifying potassium current
I_{Na}	inward sodium currents
IP3	inositol triphosphate
ISO	isoproterenol
IV	current-voltage relationship
k	activation curve slope factor
KHD	kinase homology domain
KO	knock out
LBD	ligand binding domain
NO	nitric oxide
NP	natriuretic peptide
NPR	natriuretic peptide receptor

NPR-A	natriuretic peptide receptor - A
NPR-B	natriuretic peptide receptor - B
NPR-C	natriuretic peptide receptor - C
PDE	phosphodiesterase
PDE-1-5	phosphodiesterase type 1-5
p-GC	particulate – guanylyl cyclase
PKA	protein kinase –A
PKG	protein kinase – G
PLCβ	phospholipase –C
SAN	sinoatrial node
SC	stem cells
s-GC	soluble guanylyl cyclase
TRP-C	transient receptor potential-C channel
$V_{1/2(act)}$	voltage required for 50% channel activation
V_{max}	maximum rate of depolarization
WT	wild type

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CHAPTER 1: INTRODUCTION

1.1 Overview

The hormonal regulation of electrical activity in the heart is an increasingly important topic with respect to normal cardiac physiology and cardiac disease. Studying isolated cardiac myocytes has proven to be a valid method to study cardiac electrophysiology and with inference, whole heart cardiac function. Cardiac myocytes are electrically active cells that generate action potentials (APs). These APs propagate throughout the myocardium in a synchronous fashion leading to properly timed contractions in the atria and ventricles (52). There are five phases to the cardiac AP that are classified based on the specific ion channels responsible for each phase (Fig. 1) (36, 87). When the cell is at rest (phase 4), the main current that is responsible for maintaining the approximate -80 mV membrane potential is the inwardly rectifying potassium current (I_{K1}) (87). When the cell is stimulated, and the membrane potential reaches a threshold voltage (≈-55mV) to activate inward sodium currents (I_{Na}), depolarization of the membrane potential occurs (87), which is referred to as the upstroke or phase 0. Phase 1 and phase 2 are somewhat indistinguishable in mouse cardiac APs, however in humans and other mammals are characterized by a short plateau phase and beginning of repolarization. L-type Ca^{2+} channels ($I_{Ca,L}$), along with distinct potassium currents are mainly responsible for these phases. Finally, phase 3 is the repolarization, as a result of outward potassium currents, that restores the membrane potential to the resting potential (36).

This research focused primarily on the electrophysiological effects of B-type natriuretic peptide (BNP) on atrial myocytes. Atrial myocytes were utilized because our future goals include the investigation of the relationship between natriuretic peptides and atrial arrhythmias such as atrial fibrillation (AF). Current-clamp measurements were

initially performed on isolated mouse cardiac myocytes to assess the effect of BNP on atrial APs. Following this, the ionic mechanism responsible for the effects of BNP on atrial APs was determined using voltage-clamp experiments.

FIGURE 1:

Action potential waveform and underlying ionic currents in mouse atrial myocytes. Different AP phases are shown in red, and adjacent labels represent the underlying ionic mechanism responsible for that portion of the AP. Note: Due to the rapid nature of the mouse AP, phase 1 and 2 are difficult to distinguish (87).

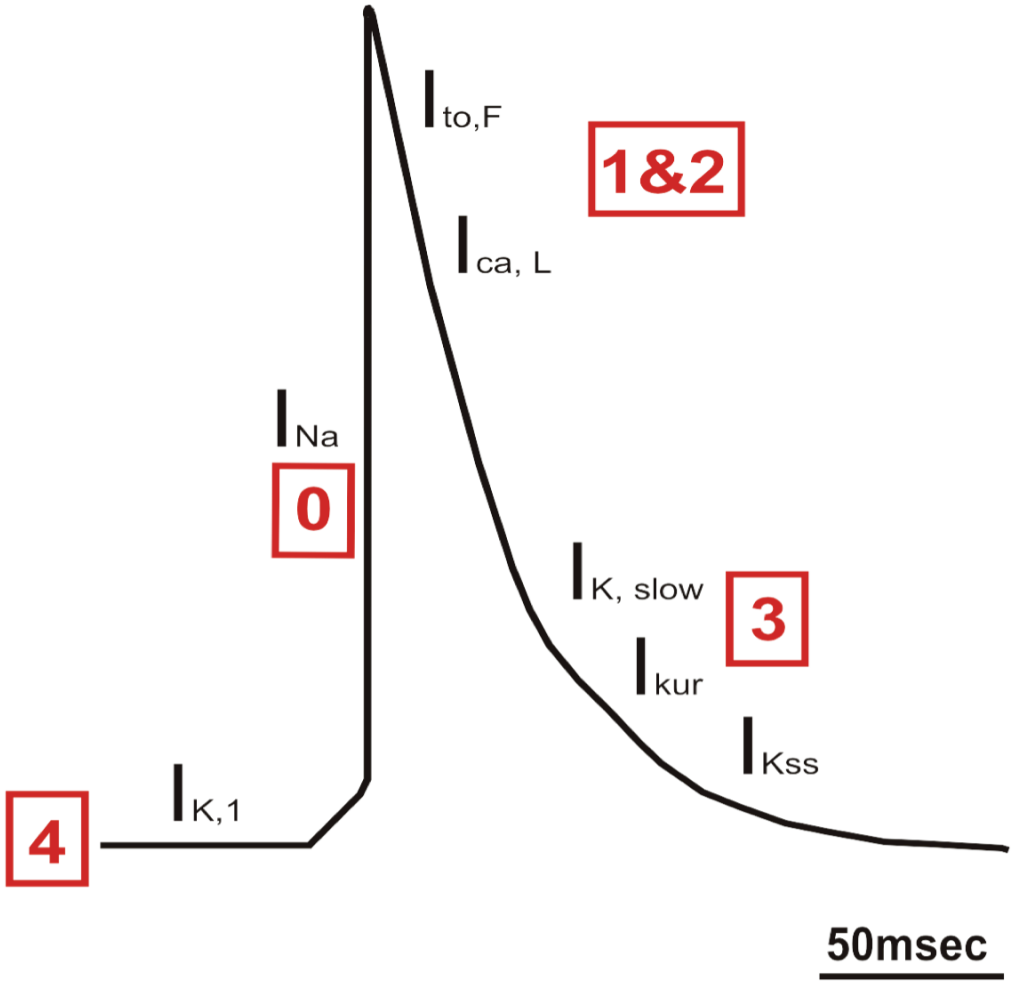


Figure 1.

1.2 Natriuretic Peptides

Natriuretic peptides (NP) are a family of hormones that are produced in various tissues in a wide range of species. They are generally characterized as hormones that elicit their physiological effects by regulating blood pressure (BP) and maintaining cardiovascular homeostasis through the induction of natriuresis and diuresis (59, 63). The natriuretic peptide family currently consists of numerous structurally similar peptides. Atrial natriuretic peptide (ANP) was originally discovered by de Bold and colleagues, when they performed a relatively simple yet ground-breaking experiment that demonstrated an immediate decrease in BP along with an increase in water and sodium excretion when rats were injected with homogenized atrial tissue (14). Shortly after the discovery of ANP, an alternate splice variant of pro-ANP was isolated and referred to as urodilatin (115). BNP was discovered shortly after, from porcine brains and was found to cause smooth muscle relaxation as well as to induce natriuresis and diuresis along with hypotension (130). C-type natriuretic peptide (CNP) was similarly discovered in porcine brains and also elicited potent smooth muscle relaxation (131). All NPs share the conserved sequence CFGXXXDRIXXXXGLGC where X is any amino acid. A 17-peptide disulfide linked ring structure, which enables biological activity, is formed between the cysteines positioned at the beginning and end of the above sequence (82). Two additional intestinal peptides are functionally similar to the cardiac natriuretic peptide family and are referred to as guanylin and uroguanylin. Both intestinal peptides are secreted in response to elevated NaCl ingestion and exert natriuretic and diuretic effects on the kidney through guanylyl cyclase (GC) activation and cyclic guanosine monophosphate (cGMP) mediated cellular events (32).

NP Synthesis and Processing

ANP is primarily synthesized and stored in secretory granules located in atrial myocytes; however it is also present in other tissues throughout the body at lower concentrations. ANP is originally synthesized as a preprohormone that is 151 amino acids in length. Prepro-ANP is cleaved by an unknown enzyme and is stored in atrial granules in the 126-amino acid proANP form (102). An increase in atrial wall stretch and the associated increase in transmural pressure due to increased intravascular volume is the principal trigger controlling acute release of ANP into the coronary sinus (27). Once ANP is released into the coronary sinus it is rapidly cleaved by the transmembrane cardiac serine protease called corin in order to form the biologically active 28-amino acid ANP (102, 147). Once in its biologically active form, ANP enters circulation and acts as an endocrine peptide exerting its effects on various target tissues throughout the body. Recently, it has been shown that ANP release is a cyclic adenosine monophosphate (cAMP) dependent process that relies on phosphorylation events through protein kinase-A (PKA) in isolated rabbit atrial myocytes (23). Normal circulating ANP concentration is approximately 10 fmol/ml and these levels are elevated 10-30-fold in patients suffering from congestive heart failure (22). The human ANP gene is located at the distal end of the short arm of chromosome 1, in band 1p36, and the mouse gene is located on chromosome 4 (146).

BNP is stored in atrial secretory granules along with ANP and is synthesized as a preprohormone of 134 amino acid residues, which is cleaved by an unknown enzyme into the proBNP form of 108 residues. Upon secretion into circulation the prohormone is presumably cleaved by the proprotein convertase enzyme furin, because the prohormone precursor contains a furin cleavable RXXR sequence at its processing site. Furthermore,

it has been shown that furin is the major processing enzyme for the pro-BNP in rat cardiomyocytes (114). Furin is a calcium dependent serine endoprotease (127) and produces a biologically inactive 76-residue amino terminal peptide and the biologically active 32-residue carboxyl-terminal peptide (130). BNP is synthesized and stored along with ANP in atrial granules. BNP is also synthesized through a constitutive secretory pathway in ventricular myocytes because of the lack of secretory granules. BNP is only produced in ventricular myocytes during cardiac wall stretch due to volume overload. It has been proposed that ventricular BNP synthesis is primarily regulated by the nuclear transcription factor; GATA 4 (135). In normal humans, circulating BNP concentrations are approximately 1fmol/ml, however in patients with congestive heart failure circulating levels increase 200-300-fold (84). Plasma BNP concentrations are currently utilized as an indicator and marker for acute decompensated congestive heart failure due to the enormous increases in circulating BNP during this disease (102). Similar to ANP, the human BNP gene is located on chromosome 1 in humans and is 8 kb upstream of the ANP gene. The human BNP gene contains 3 exons and 2 introns (127). BNP is located on chromosome 4 in mice (102).

Unlike ANP and BNP, CNP is highly expressed throughout the central nervous system (58) and in endothelial cells (133), rather than the heart. CNP is a vasodilator, as well as a regulator of vascular tone, and is released in response to vascular injury (132). There are two major endogenous forms of CNP, designated CNP-22 and CNP-53, both of which elicit similar physiological effects in different parts of the body (102, 148). Similar to BNP, pro-CNP (103 amino acids) is cleaved by furin to the biologically active CNP-53 (144). The enzyme that is responsible for cleaving CNP-53 to CNP-22 is currently

unknown (102). Unlike ANP and BNP, the human CNP gene is located on chromosome 2 (91), while the mouse CNP gene is located on chromosome 1 (91).

NP Metabolism, Clearance and Degradation

Natriuretic peptides are metabolized, cleared or degraded from the body shortly after their synthesis, which results in short biological half-lives. The three major mechanisms that contribute to the short half-life are: degradation through receptor binding, degradation by proteases and finally clearance of peptides into excretory body fluids (101). Renal excretion of NPs has previously been shown, however likely plays an insignificant role in the regulation of circulating NP concentrations and will not be discussed further (121).

The first mechanism of NP clearance involves the internalization of peptides upon natriuretic peptide receptor (NPR) binding. Based on the fact that all NPRs remove NPs from circulation upon binding, all NPRs can be considered clearance receptors. However, natriuretic peptide receptor - C (NPR-C) was originally referred to as a unique clearance receptor because of its lack of association with a GC enzyme, and the fact that NPR-C has the ability to preferentially bind biologically inactive NPs over their respective NPRs (73). It is speculated that upon ligand binding NPR-C internalizes NPs through a clathrin-dependent lysosomal hydrolysis mechanism, while simultaneously recycling the receptor back to the membrane (89). Although NPR-C was originally referred to as a clearance receptor, it is now known that NPR-C mediates numerous physiological functions, mediated by its interaction with a heterotrimeric inhibitory G protein, (G_i) (7, 110, 113) and is therefore inappropriately named a clearance receptor. The details surrounding NPR-C will be further explored in following sections.

The second mechanism responsible for NP clearance and degradation involves the activity of several extracellular proteases that preferentially degrade specific NPs in a species-specific manner. Numerous enzymes have been linked to NP degradation including neprilysin (a neutral endopeptidase), enkephalinase and CD10 for ANP and CNP, however less so for BNP. Neutral endopeptidase 24.11 is an endothelium derived circulating protease that is largely responsible for the hydrolysis of all the NPs by cleaving the hormone between Cys7 and Phe8 (28, 53, 124). Additionally, insulin-degrading enzyme has been found to cleave ANP, and meprin A may be involved in improving neprilysin mediated degradation of BNP (101).

1.3 Natriuretic Peptide Receptors

In the following sections, the three natriuretic peptide receptors will be briefly discussed, focusing mainly on natriuretic peptide receptor - A (NPR-A) in order to provide information on the receptor most relevant to this research.

Natriuretic Peptide Receptor – A/B

NPR-A and natriuretic peptide receptor - B (NPR-B) share many similarities and therefore will be discussed together in this section. NPR-A is widely dispersed throughout the body, and experiments measuring expression levels show high expression patterns in kidney, heart, adrenal and lung tissue (70, 88). NPR-B is highly expressed in brain, lung and kidney (143) and to a lesser extent the heart (88). The primary ligands for NPR-A are ANP and BNP. In terms of the concentration required for half-maximal response, NPR-A has a 1000-times higher affinity for ANP compared to BNP (71). Additionally, BNP is 10-fold less potent in activating NPR-A compared to ANP (71). The primary ligand for NPR-B is CNP, however both ANP and BNP have the ability to bind NPR-B with low affinity. The binding affinity for CNP to NPR-B is 50-500-fold

greater than the other NPs (57) Both NPR-A and -B are linked to a guanylyl cyclase enzyme (sometimes referred to as GC-A) and contain five homologous domains (Fig. 2) (71).

FIGURE 2:

Schematic representation of the natriuretic peptide receptors and their respective ligands. NPR-A,-B,-C: natriuretic peptide receptor type A, B, C, ANP; atrial natriuretic peptide, BNP; brain-type natriuretic peptide; CNP; C-type natriuretic peptide; DNP; *Dendroaspis* natriuretic peptide; cANF; selective natriuretic peptide type C receptor agonist, cGMP; cyclic guanosine monophosphate, G_i; inhibitory G-protein.

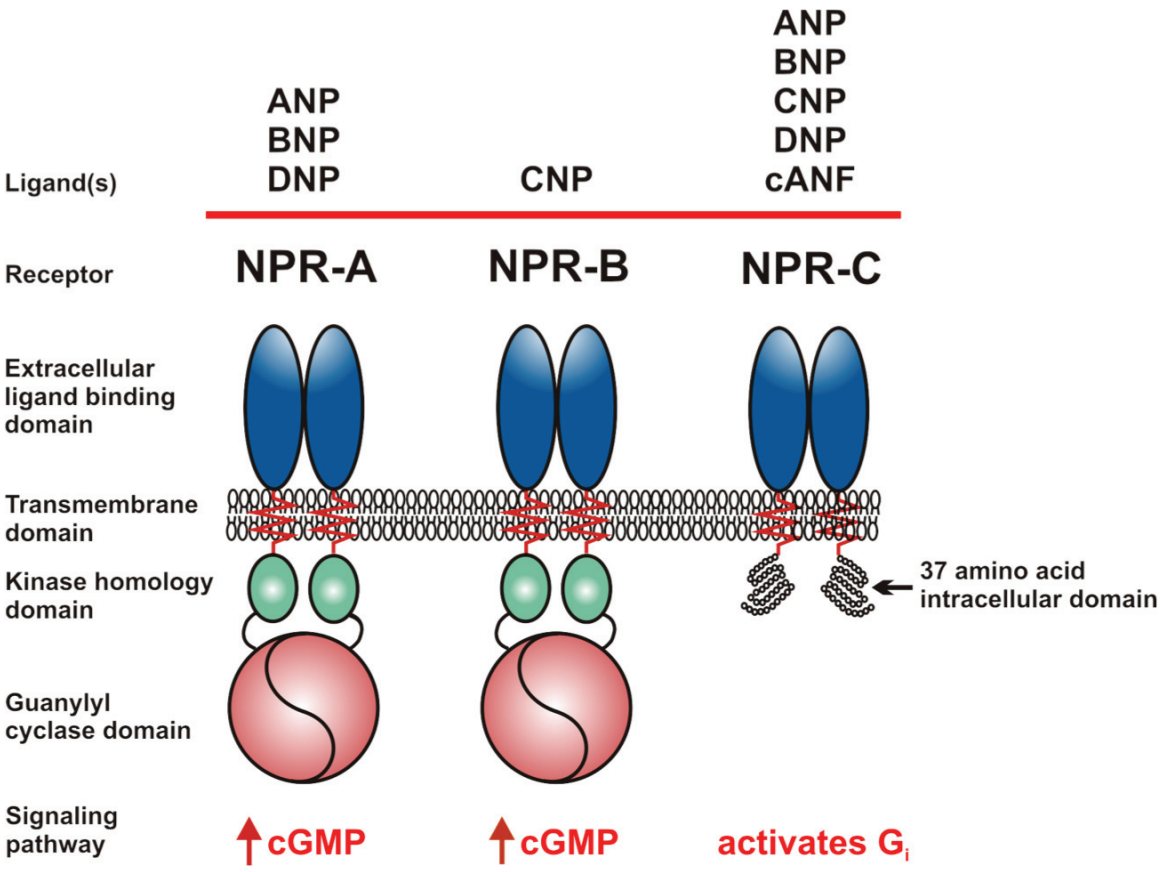

Ligand(s)
ANP
BNP
DNP
CNP
ANP
BNP
CNP
DNP
cANF
Receptor
NPR-A
NPR-B
NPR-C
Extracellular ligand binding domain
Transmembrane domain
Kinase homology domain
Guanylyl cyclase domain
37 amino acid intracellular domain
Signaling pathway
cGMP
cGMP
activates G_i

Figure 2.

Beginning with the amino terminus, the first segment of the NPR is called the ligand binding domain (LBD) and spans 450-amino acids. Following the LBD, is the hydrophobic region which spans the plasma membrane and consists of 21-amino acids. The next region, referred to, as the kinase homology domain (KHD) is poorly understood; however, it is thought to act as a repressor of the GC enzyme when the receptor is unbound to its respective ligand. Following the KHD is the 41-amino acid α-helix, which behaves similar to a hinge and connects the KHD to the catalytic portion of the receptor. The final region of the receptor is located at the C-terminus and contains the GC enzyme responsible for converting guanosine triphosphate (GTP) to cGMP (106). The crystal structure of the unliganded, glycosylated dimerized NPR-A has been solved at 2.0-Å resolution, and provided further structural evidence that NP binding to NPR-A is a chloride dependent process (139), which was previously experimentally shown by Misono (81).

Unlike most G-protein coupled receptors, which are phosphorylated after ligand binding (100), an event activating a series of intracellular events, NPR-A and -B are phosphorylated in the basal state, maintaining an inactive GC enzyme. Upon ligand binding, NPR-A and NPR-B undergo an adenosine triphosphate (ATP) dependent conformational change, which results in the removal of the inhibitory effect the KHD normally, has on GC (50). Once GC is no longer inhibited by the KHD, the enzyme facilitates the conversion of GTP to cGMP (105). It has been shown that ANP increases the intracellular level of cGMP in a concentration and time-dependent fashion (20, 117). Elevated levels of intracellular cGMP can then result in activation of protein kinase-G (PKG), which phosphorylates many biologically active targets such as phosphodiesterases (PDEs). Both NPR-A and NPR-B experience desensitization with

constant exposure to NPs, which is of interest when considering the therapeutic use of exogenous NPs. Numerous studies have shown that with chronic NP exposure NPRs are desensitized by ligand induced dephosphorylation events (33, 103, 104).

Natriuretic Peptide Receptor – C

NPR-C is distributed widely in several tissue and cell types including; the cardiac conduction system, platelets, vascular smooth muscle cells, glomeruli, collecting ducts, pituitary glands, adrenal glands the central nervous system and the eye (1-4, 12, 30, 65). NPR-C has an extracellular domain similar to NPR-A and NPR-B, a single transmembrane domain and a short cytoplasmic domain (Fig. 2) (100). The crystal structure for the non-liganded and liganded form has been resolved to 2.9-Å resolution (44). It has been shown in humans and rats that NPR-C can bind all NPs with the following affinity ANP>CNP>BNP (11, 133). NPR-C is not linked to a GC enzyme and therefore was originally referred to as a biologically silent receptor that was thought to clear and degrade NPs from the circulation without directly initiating signaling events (73). More recently, however, it was shown that the NPR-C cytoplasmic domain contains several G_i activator sequences, which bind and activate G_i regulatory proteins (97). Various studies have shown the physiological effects of NPR-C activation through its association with the adenylyl cyclase (AC)/cAMP signal transduction pathway (8, 112, 113). Specifically, the 37 amino acid cytoplasmic domain of NPR-C inhibits AC through a direct interaction with G_i in a GTP dependent manner (6). In addition to NPR-C's effects on G_i, it has been shown that NPR-C can also activate the β-isoform of phospholipase C (PLCβ) (97). This results in increased production of diacylglycerol (DAG) and inositol triphosphate (IP3), and increased concentrations of intracellular Ca^{2+} (1). Contrary to the initial clearance receptor hypothesis, NPR-C is responsible for

numerous physiological effects ranging from inhibiting leuteinizing hormone-stimulated progesterone secretion in Leydig tumor cells (5) to exerting anti-proliferative actions in neuroblastoma cell lines (62). NPR-C also mediates electrophysiological effects in cardiac myocytes. It has been shown that NPR-C activation by CNP in the presence of maximal concentrations of isoproterenol (ISO) shortens the AP duration and inhibits $I_{Ca,L}$ in frog atrial myocytes (112). CNP similarly reduces $I_{Ca,L}$ in mouse sinoatrial node (SAN) cells via activation of NPR-C (113).

In addition to being produced in cardiomyocytes, NPs are also secreted from cardiac fibroblasts and all three NPRs are expressed in these fibroblasts (46, 137). All three NPRs have been associated with eliciting antifibrotic and anti-proliferative effects on cardiac fibroblasts, and NPR-C specifically is known to regulate transient receptor potential – C channels (TRP-C) in cardiac fibroblasts through DAG activation (110). Cardiac myocytes were the main focus of this study and therefore fibroblasts were not considered and will not be mentioned in this context.

1.4 Natriuretic peptides, cAMP, cGMP and Phosphodiesterases

Activation of NPR-A and NPR-B by their respective NPs results in elevated levels of intracellular cGMP through activation of the linked particulate GC (p-GC) (102). An equally important secondary messenger, cAMP, is generated through AC and is stimulated by catecholamines such as epinephrine. The main downstream target for cGMP and cAMP are PKG and PKA respectively (153). It is relatively well known that these two pathways exert opposing effects on cardiac function, as a result of the opposing effects of their downstream phosphorylation targets; PKA and PKG (153). Interestingly, it has been established that cGMP exerts a negative effect on metabolic function in the whole heart and single cell preparation (85, 119, 120). PDEs are enzymes responsible for

the hydrolysis of cyclic nucleotides and are responsible for maintaining the highly regulated concentrations of cAMP and cGMP in a compartment specific manner, due to the their association with particular parts of the cytoplasm (34). There are five major families of PDEs with multiple isoforms expressed in each family (31). There are numerous PDE isoforms that are species and region specific, however in the case of the murine cardiac model, it has been stated that PDE-1-5 are important (72). Specifically, PDE-2 and PDE-3 are important with regards to the their involvement with NPRs and their downstream products (72). Regulation of PDE activity is complex and involves feedback loops and crosstalk between cAMP and cGMP (Fig. 3) (153). PDE-1 is activated by a Ca^{2+}calmodulin complex and hydrolyses cAMP (72). PDE-2 is activated by cGMP and hydrolyses both cAMP and cGMP (72). PDE-3 is inhibited by cGMP and activated by PKA and hydrolyses cAMP (72). PDE-4 is activated by PKA and hydrolyzes cAMP. Finally PDE-5 is activated by PKA and PKG, and is responsible for hydrolyzing cGMP (Fig. 3) (153). It is obvious based on this description that an extensive cross-talk relationship between cAMP and cGMP exists in cardiac myocytes. It also shows that while NPs traditionally elicit their effects through cGMP/PKG pathways, the involvement of other secondary messengers cannot be ruled out.

FIGURE 3:

Schematic diagram showing the relationship between natriuretic peptides, intracellular second messengers and phosphodiesterases. Red arrows indicate an activating sequence, black hashed lines indicate an inhibitory or degrading sequence. PDE; phosphodiesterase 1-5, NP/NPR-A/B; natriuretic peptide, natriuretic peptide receptor – A/B signaling cascade, PKG; protein kinase – G, PKA; protein kinase – A, cAMP; cyclic adenosine monophosphate, cGMP; cyclic guanosine monophosphate, Ca^{2+}/CaM; calcium/calmodulin complex.

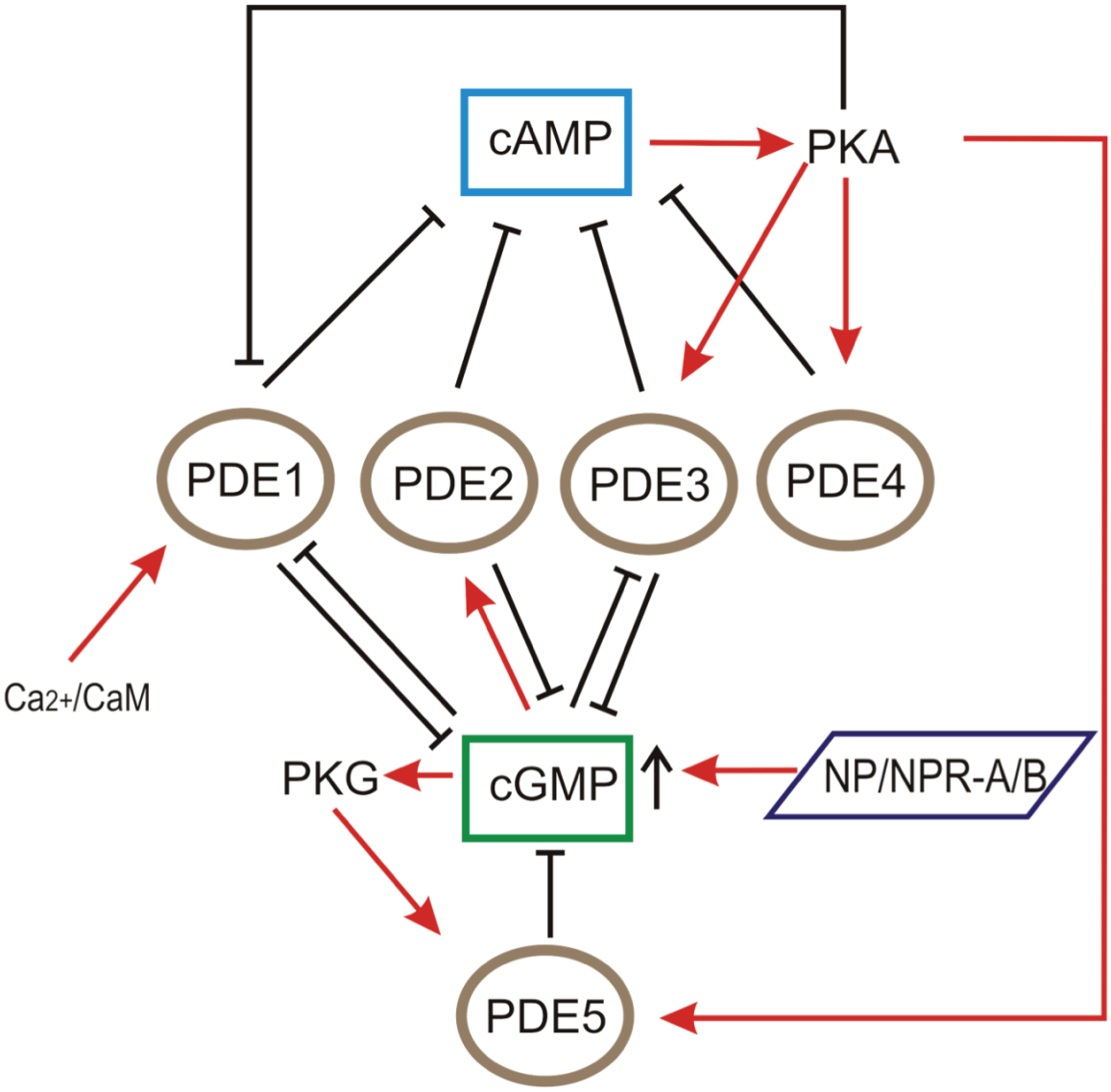


Figure 3.

1.5 Physiological effects of NPs

Natriuretic peptides are typically described as endogenous paracrine and autocrine hormones that primarily regulate blood volume and BP by acting on the cardiovascular and/or renal system, as well as the central nervous system (26, 59, 63). These peptides regulate the various systems by effecting downstream molecular targets through a complex interaction of peptide concentrations or receptor expression levels. This highly coordinated system is particularly evident when NP levels or NPR expression patterns are altered by genetic mutations and subsequent pathologies arise, which will be discussed further below (59). Due to the wealth of information available regarding the physiological effects of NPs in various organ systems and species, this thesis will specifically focus on the cardiac specific physiological effects of NPs mediated through NPR-A and NPR-C.

Effects on Blood Pressure

NPs exert their effects on BP by binding to NPRs and activating an intracellular cascade of second messengers that result in vasodilation and natriuresis, both of which are important in BP regulation. Oliver and colleagues showed clearly that the result of genetic NPR-A ablation in mice is elevated BP in both sexes (93). The hypotensive effects of NPs also results in part from a reduction in cardiac preload caused by the transport of intravascular fluid to the interstitial space, due to an increase in capillary permeability (142). Conversely, mice lacking a functional NPR-C protein exhibit lower systolic and diastolic BP when compared to wild type (WT) animals (76). There is currently a very limited understanding regarding the role of NPR-C in BP regulation, and no explanation why NPR-C ablation results in lower BP (76).

Blood volume is regulated by the renin-angiotensin-aldosterone system and by NPs. Normally, the liver secretes angiotensinogen, which is converted to angiotensin I by the kidney enzyme renin. Angiotensin I is then converted to angiotensin II by angiotensin converting enzyme (ACE), and acts on the adrenal cortex to release aldosterone. Aldosterone increases sodium reabsorption in the cortical collecting ducts thus decreasing sodium and water excretion. It has been shown that activation of NPR-A by ANP more so than BNP promotes natriuresis and diuresis by inhibiting the aldosterone response to angiotensin II by decreasing renin secretion (47, 102, 142). This decrease in renin secretion and subsequent decrease in BP does not occur when humans are injected with high dose ANP, presumably because of protective compensatory responses associated with large reductions in BP (102). Additionally, activation of the NPR-A signal cascade increases glomerular filtration rate by increasing the glomerular capillary pressure through dilation of the afferent arteriole, and constriction of the efferent arteriole (75).

Effects on the Heart

Isolated cardiomyocyte contractility is a good indicator of whole heart contractility, pump function and cardiac output, and therefore will be discussed in relation to NPs. Zhang and colleagues clearly showed that application of ANP, BNP and CNP at various concentrations (1 nM, 10 nM & 100 nM) to isolated rabbit ventricular myocytes caused a decrease in contractility in a concentration dependent manner (156). Using a PKG inhibitor (KT5823), they demonstrated that this decrease in contractility is a cGMP dependent process (156). Pierkes *et al.* (99) showed similar results using an isolated perfused mouse working heart preparation and an NPR-A knockout (KO) mouse to determine the receptor responsible for mediating the effects. They showed a biphasic effect with the application of CNP (100 nM); an immediate increase in contractility and

relaxation, followed by a slowly developing negative inotropic effect. Since these effects were present in the NPR-A KO mouse, they determined that their CNP effect was mediated by NPR-B and cGMP. Interestingly, Pierkes *et al.* (99) found conflicting results with Zhang *et al.* (156) who demonstrated that ANP had no effect on cardiac function. Using a slightly different model Brusq and colleagues showed that CNP (1μM) only exerts a cGMP mediated positive lusitropic effect in rat papillary muscles (17). Differences in peptide concentration, species and experimental protocol may explain the difference in published results. Additionally, the current confusion amongst groups may provide support to the hypothesis that NPs may elicit different effects depending on distinctive threshold levels of secondary messengers.

Heart rate and SAN AP firing rate is another key determinant of cardiac output, and is regulated by NPs. It has been shown that CNP acting through NPR-B has a positive chronotropic effect in dogs, by increasing the slope of the SAN AP diastolic depolarization phase and decreasing the AP duration at 75 and 90% repolarization (10). Interestingly, Rose and colleagues have shown that CNP acting through NPR-C, has a negative chronotropic effect in the presence of isoproterenol in Langendorff-perfused mouse hearts, due to the inhibition of $I_{Ca,L.}$ in isolated SAN myocytes (113). Herring *et al.* (43) demonstrated that CNP and BNP induce bradycardia as a result of presynaptic acetycholine release facilitating vagal neuro-transmission in isolated guinea pig atrial preparations. They suggest that the increase in cGMP caused by the NP, antagonizes PDE-3, resulting in elevated levels of cAMP and PKA dependent phosphorylation of presynaptic N-type calcium channels (43).

While NPs are more commonly associated with their effects on blood volume and BP, a few studies have recently shown that NPs alter the electrophysiological properties

of cardiac myocytes. Rose and colleagues showed that CNP (10 nM) shortened the AP duration in bullfrog atrial myocytes prestimulated with the β-adrenergic receptor (β-AR) agonist isoproterenol by activating NPR-C. The ionic mechanism responsible for the AP shortening was an inhibition of $I_{Ca,L}$, which is the predominant Ca^{2+} current in atrial myocytes (112). More recently it has been shown that BNP (1 μM) increased AP duration, yet paradoxically decreased $I_{Ca,L}$ amplitude in rat ventricular myocytes. The same study showed that BNP caused a marked reduction in ventricular myocyte shortening and calcium transient amplitudes (123). Another group recently studied the effects of ANP (20 nM) on various developmental stages of murine embryonic stem cells (SC). In their preparation, ANP decreased the frequency of APs in early developmental SCs and whole embryo hearts by inhibiting $I_{Ca,L}$ peak amplitude. This inhibition of current was abolished in the presence of erythro-9-(2-Hydroxy-3-nonyl) adenine (EHNA), indicating the involvement of PDE-2 and the relationship between elevated levels of cGMP and depressed levels of cAMP by PDE-2 (80).

In summary, the above studies illustrate that the effects of NPs in the heart are likely complex and may involve multiple receptor subtypes and signaling pathways. Furthermore, the above studies provide support that additional work is needed to determine which NP effects are most important, and which conditions are required to elicit the effects.

Other Non-Cardiac Effects of BNP

NPs are mainly known for their role in regulating aspects of the cardiovascular system, however novel research has shown that NP/NPR pathways are also involved in other physiological systems. Zhang and colleagues recently demonstrated that BNP and NPR-A are expressed in dorsal root ganglion neurons and that this system is upregulated

following peripheral tissue inflammation (154). Furthermore, BNP inhibits the excitability of these neurons in the presence of glutamate by activating the NPR-A/PKG pathway acting on calcium activated potassium channels. This research provided another potential therapeutic target for pain therapy (154).

All three NPs and their respective receptors are expressed in pulmonary tissue. NPs bind the p-GC linked receptors in airway smooth muscle cells, causing elevated levels of intracellular cGMP, which results in relaxation, and inhibition of proliferation through PKG phosphorylation (37).

Lipolysis is the process of triglyceride breakdown into free fatty acids for the use in metabolism through beta-oxidation. Recent evidence is beginning to show the role NPs play in lipolysis and obesity related metabolism. NPR-A mediated ANP activation causes a cGMP-dependent, PDE-3 independent increase in lipolysis in human adipocytes (118). Further research regarding the involvement of NPs and obesity has shown that NP induced lipolysis is unrelated to obesity in young men (35).

NPs play a significant role in post-partum long bone growth, which is evident in transgenic NPR-B or -C mutant mice showing severe skeletal abnormalities. There is numerous data demonstrating with transgenic and pharmacological experiments that CNP, acting through the NPR-B/PKGII pathway causes a differentiation of hypertrophic chondrocytes resulting in long bone growth (102). An interesting study has even shown the relationship between chondrocyte specific NPR-B gene mutations and the autosomal recessive disease, acromesomelic dysplasia, type Maroteaux (short stature, shortened limbs) (9).

1.6 Pathologies Associated with NPs/NPRs

There are numerous mouse models that exist with genetic mutations in NP and NPR genes that were either genetically engineered or arose spontaneously (59). To highlight the importance of the NP/NPR system, this section will briefly highlight the various phenotypes that arise when the NP system is disturbed.

NPs play a critical role in a variety of homeostatic processes such as BP, and severe phenotypes arise when NPs are genetically overexpressed or deleted *in vivo*. ANP is a critical component for the maintenance of BP. Mice overexpressing ANP experience arterial hypotension (128), and mice that have an ANP deletion accordingly are hypertensive (51). Interestingly, a recently identified family containing a hereditary mutation in ANP, experience high circulating peptide concentrations of a mutant ANP, which causes cardiac electrical disturbances, mainly characterized by shortened APs and sustained AF (45). This study provides further evidence that NPs play a role in cardiac electrophysiology, including the atrial myocardium. Although ANP and BNP bind the same NPR, mutations in BNP mainly result in cardiac structural abnormalities. BNP deletion results in cardiac fibrosis (134) and BNP overexpression results in increased plasma cGMP concentration and arterial hypotension (90), as well as skeletal overgrowth (129). There is currently limited understanding on the consequences of CNP peptide alterations, however one study showed that mice with targeted disruption of CNP experience severe dwarfism and early death as a result of impaired endochondral ossification (21).

NPR expression patterns are also critical in the maintenance of homeostasis; therefore, when they are genetically altered, the phenotypes that arise are similar to the phenotypes that occur in animals with altered NP expression levels. Several studies have

shown that when NPR-A is genetically disrupted throughout the entire animal, mice demonstrate hypertension, cardiac hypertrophy and sudden cardiac death (68, 69, 93). Conversely when NPR-A is globally overexpressed, mice experience hypotension, and protection from dietary salt intake (94), and in cardiomyocytes specific overexpression, experience a decrease in myocyte size (56). When NPR-B is globally deleted, animals demonstrate dwarfism, accumulation of white adipose tissue, epilepsy and infertility (59). Finally, when NPR-C is deleted, mice experience an usual protection against heat-stable enterotoxins, demonstrating NPR-Cs potential protective effect against pathogens (116). Additionally, mice with a dysfunctional NPR-C, show elongated slender bodies that have arachnodactyly (a condition that presents with abnormally long fingers in relation to the size of the hand), thoracic kyphosis and tail kinks (49). Finally, NPR-C mutant mice generally exhibit lower systolic and diastolic BP (76).

1.7 Purpose and Hypothesis

There is currently a very limited understanding of how NPs elicit their electrophysiological effects in the heart. Additionally, the signaling mechanisms involved in NP effects are even less understood. It is well known electrophysiologically and biochemically that NPR-C is linked to G_i and upon activation of this receptor, AC activity is inhibited and downstream targets of cAMP can be modulated (7, 109, 112, 113). It is also well known that NPR-A/B activation increase intracellular cGMP, resulting in an increase in all cGMP downstream targets (102). Although, cAMP and cGMP are often described as having opposing effects on cardiac function and myocyte electrophysiology (153) this is likely an overly simplistic description due to the involvement of PDEs and the crosstalk between cAMP and cGMP pathways (Fig. 3). Since BNP has the ability to bind both NPR-A and NPR-C, two conflicting pathways

may potentially be activated and may interact. Based on previous studies investigating the electrophysiological effects of NPs in the heart (112, 113) this research set out to further investigate the electrophysiological effects of BNP in murine atrial myocytes, and further elucidate the involvement of both the NPR-C and NPR-A pathways. It was hypothesized that BNP would decrease the AP duration, by activating NPR-A or NPR-C by inhibiting $I_{Ca,L}$ similar to the results obtained for CNP in bullfrog atrial myocytes and mouse SAN cells (112, 113).

CHAPTER 2: MATERIALS AND METHODS

2.1 Experimental Animals

C57BL/6 Mice

All mice utilized in these experiments were male, between 8 and 10 weeks of age and weighed between 25-30 g. Male mice were utilized as opposed to female mice to eliminate unknown variables associated with the estrous cycle. C57BL/6 mice (Charles River) were used to study the effects of BNP in a WT background. Animals were housed 5 to a cage in the Carleton animal facility at Dalhousie University and were subjected to a 12-hour dark/light photoperiod cycle. Animals were fed standard rodent chow, which was provided *ad libitum*. Room temperature ranged between 21- 24° C. Dalhousie University Council for Animal Care approved all experimental protocols in this study.

NPR-C Mutants

For experiments examining the role of natriuretic peptide receptor C, an NPR-C mutant mouse was used (strain: B6;C-*Npr3*^{lgj}/J; Jackson Laboratories). This NPR-C mutant was characterized after a mutation arose spontaneously in a C57BL/6 inbred strain that led to obvious phenotypic skeletal abnormalities (49). The B6;C-*Npr3*^{lgj}/J strain contains an in-frame 36 base pair deletion between position 195 and 232 on chromosome 15 and expresses a dysfunctional protein lacking 12 amino acids in the extracellular domain of the NPR-C protein (49). We bred our own NPR-C mutant colony, which was backcrossed into a C57BL/6 strain for more than 6 generations. All NPR-C mutant mice were male and between the ages of 8-10 weeks. NPR-C mutant mice were subjected to identical living conditions as the WT mice.

2.2 Single cell isolation.

Cells from the right atrial appendage were isolated from the hearts of adult male C57BL/6 mice or NPR-C mutant mice using techniques previously described (74, 111, 113). Briefly, adult mice were anesthetized with isoflurane (Baxter Laboratories). The heart was excised and placed in Tyrode's solution consisting of (in mM) 140 NaCl, 5.4 KCl, 1.2 KH_2PO_4, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 5.5 glucose, and 5 HEPES, with pH adjusted to 7.4 with NaOH, at 35°C. The atrial appendage was removed and cut into strips that measured approximately 2x6 mm. Strips were removed from the first Tyrode's solution and were transferred to a "low-Ca^{2+} -Mg^{2+} -free" solution containing (in mM) 140 NaCl, 5.4 KCl, 1.2 KH_2PO_4, 0.2 $CaCl_2$, 50 taurine, 18.5 glucose, 5 HEPES, and 1 mg/ml BSA, with pH adjusted to 6.9 with NaOH. Atrial strips were washed in low-Ca^{2+} -Mg^{2+} -free solution by successive transfers between three separate round-bottom tubes. Following the washing procedure atrial strips were transferred into 5 ml of low-Ca^{2+}- Mg^{2+} -free solution containing 3.8 mg of collagenase (type II, Worthington; 280 U/mg), 63.1 µl of elastase (Worthington; 5.23 µ/mgP), and 65.2 µl of 1 mg/100 µl protease solution from streptomyces griseus (type XIV, Sigma-Aldrich; 4.3 U/mg) for 30-32 min in order for sufficient enzymatic digestion to occur. The tissue was then transferred to 2.5 ml of modified Kraft-Brühe (KB) buffer solution containing (in mM): 100 K-glutamate, 10 K -aspartate, 25 KCl, 10 KH_2PO_4, 2 $MgSO_4$, 20 taurine, 5 creatine, 0.5 EGTA, 20 glucose, and 5 HEPES, and 1% BSA, with pH adjusted to 7.2 with KOH, for three successive washing steps where tissue was transferred into three separate tubes also containing modified KB solution. The tissue strips were placed in a final round bottom tube containing 2.5 ml of modified KB solution and left to rest for 5 min before strips were mechanically triturated for 7.5 min at 35°C with a wide-bore (3mm diameter) pipette.

The wide-bore pipette was custom made by fire polishing the end of a standard glass Pasteur pipette to ensure a smooth surface. This procedure yielded a sufficient number of atrial myocytes with clearly definable striations and proper morphology. Approximately 5 ml of the KB solution was added to the tube containing the tissue and was placed at room temperature (21°C) for one hour before electrophysiological experiments were performed. During the experiment, approximately 0.5 ml aliquots were transferred to the recording chamber situated on the stage of an inverted microscope (Nikon TE-300). Throughout the experiment the bath solution was superfused through the recording chamber (1.5-2 ml/min) at room temperature (21°- 23°C).

2.3 Solutions and Pharmacology

2.3a Experimental drugs

BNP-32 (rat; Bachem) was obtained as a trifluroacetate salt and was dissolved in double distilled water (DD-H_20) to produce a 10 µM stock solution, which was stored at -80°C in 250 µl aliquots until time of use, at which point 250 µl aliquots were dissolved in 25 ml of external bath solution to yield the desired final experimental concentration of 100 nM. This solution was superfused through the recording chamber (1.5-2 ml/min) at room temperature (21°- 23°C).

Isoproterenol hydrochloride (Sigma-Aldrich) was utilized as a nonselective β-AR agonist. An initial 1 mM stock was prepared and subsequently diluted to produce a final stock concentration of 10 µM. The final stock was prepared fresh every hour and 25 µl of this stock solution was added to 25 ml of external bath solution to achieve a final experimental concentration of 10 nM.

Milrinone (97% pure powder) (Sigma-Aldrich) was utilized as a PDE-3 specific inhibitor at 10 µM (54). An initial 1 mM stock was prepared in dimethyl sulfoxide

(DMSO) and stored in 25 µl aliquots at -80°C. To achieve a final experimental concentration of 10 µM, the 25 µl aliquots were dissolved in 25 ml of external bath solution.

A71915 (Bachem) is a well-characterized NPR-A specific antagonist with a IC_{50} of 0.65 nM (24). A71915 was utilized in experimentation to determine which NP receptor mediates BNPs electrophysiological effects. A71915 was dissolved in DD-H_2O and separated into 100 µl aliquots. The aliquots were dissolved in 25 ml of external bath solution for a final experimental concentration of 500 nM.

2.3b Action Potential/Potassium Current Solutions

AP external recording solution consisted of (in mM) 140 NaCl, 5.4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 5.5 Glucose, and buffered to a pH of 7.4 with NaOH. Internal pipette solution consisted of (in mM) 140 KCl, 5 NaCl, 2 $CaCl_2$, 5 EGTA, 4 K-ATP, 1 $MgCl_2$, 10 HEPES, 6.6 Na-phosphocreatine, 0.3 Na-GTP, and buffered to a pH of 7.2 with KOH. The internal solution was filtered through a 0.22 micron filter prior to use. Amphotericin-B was used to achieve the perforated patch clamp configuration. Amphotericin-B was dissolved in DMSO to make a final stock concentration of 20 mg/ml. Next, 10 µl of amphotericin stock solution was added to 0.5 ml of the AP internal solution to create a final concentration of 200 µg/ml of amphotericin-B. All solutions were vigorously vortexed prior to use, and were re-made every hour throughout the experiment in order to ensure fresh amphotericin-B was being used.

2.3c Calcium current solutions

The external calcium current recording solution consisted of (in mM) 140 CsCl, 5.4 TEA-Cl, 3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 1 Glucose, and buffered to a pH of 7.4 with CsOH. The internal calcium current recording solution consisted of (in mM) 135 CsCl, 5

NaCl, 0.2 $CaCl_2$, 5 EGTA, 4 Mg-ATP, 1 $MgCl_2$, 10 HEPES, 6.6 Na-phosphocreatine, 0.3 Na-GTP, and buffered to a pH of 7.2 with CsOH. The internal solution was filtered through a 0.22 micron filter prior to use.

2.4 Electrophysiological Protocols

Single atrial myocytes were patch clamped using the perforated patch clamp technique for AP recordings and the whole cell configuration technique for Ca^{2+} and K^+ current recordings (38). Micropipettes were pulled from borosilicate glass (with filament, 1.5 mm OD, 0.86 mm ID; Sutter Instrument, Novato, CA) using a Flaming/Brown pipette puller (model P-97, Sutter Instrument). The resistance of these pipettes was 4-7 MΩ when filled with recording solution.

Microelectrodes were positioned with a piezoelectric micromanipulator (Burleigh® Instruments, Burleigh TS-5000-150) mounted on the stage of an inverted microscope (Nikon Eclipse TE300). Seal resistances were 1–5 GΩ. After perforation of the membrane using the perforated patch clamped technique a series resistances of 15-30 MΩ was typically achieved. When the membrane was ruptured using manual negative pressure for the whole cell configuration the series resistances was typically 5–15 MΩ.

Agar bridges were used to provide an electrical connection between the bath solution and ground electrode while minimizing the transfer of ions or solute from the electrical environment. Agar bridges were made by filling standard surgical grade silicon tubing with 3 M KCl/agarose containing solution, which were eventually cut into 1.5cm lengths for electrophysiological work. 3 M KCl/agarose solution was made by dissolving 35 mg of agarose into 3 M KCl which was brought to a boil. After boiling, the solution was injected into silicon tubing and allowed to cool and solidify.

APs were elicited from atrial myocytes by applying a 20 ms depolarizing stimulus (0.03-0.1 nA) every 5 seconds. Using this protocol, APs were recorded over 15 minute periods, during which the experiment took place and drugs were applied.

Time course $I_{Ca,L}$ experiments were recorded by first applying a depolarizing voltage-clamp step from a -80 mV holding potential to -60 mV for 200 ms. Immediately after this prepulse, a 250 ms voltage-clamp step was applied from -60 mV to 0 mV. Current-voltage (IV) relations for $I_{Ca,L}$ were generated by applying a series of 250 ms steps in 10 mV increments from -80 mV to +60 mV. The peak inward current (at 0 mV) and I-V relations were plotted. All measurements of $I_{Ca,L}$ were made in Na-free bath solution to eliminate voltage-gated sodium currents. $I_{Ca,L}$ kinetic analysis was performed by calculating chord conductance from the IV curve using the equation:

$$G = \frac{I}{Vm - Vrev} x1000$$

where G represents the maximum conductance, at the current measured, I, applied membrane potential, V_m, and V_{rev} is the reversal potential. Peak conductance density was fit to the multi-component Boltzmann function to produce a sigmoidal curve in order to calculate maximum conductance (G_{max}), $V_{1/2(act)}$, and k (slope factor) from the equation:

$$G(V_m) = G_{\max} x \sum_{i=1}^{n} A_i / (1 + \exp((V_m - V_{1/2,i}) / k_i))$$

where G_{max} is the maximum conductance, and $A_i, V_{1/2i}, k_i$ represent the amplitude, membrane voltage for 50% activation, and slope factor for the ith component. $\sum_{i=1}^{n} A_i$ represents the sum over the number of component, n.

There are numerous potassium channels that contribute to specific phases of the atrial AP and which are responsible for repolarization in the mouse atrial AP (Fig. 1) (16). These will be discussed in further detail in the discussion. In this study, potassium

currents (I_K) were measured by applying a series of 500 ms steps in 10 mV increments from -100 mV to +70 mV from a holding potential of -80 mV (66). These recordings permit analysis of several specific K^+ current components including the inward rectifier K^+ current (I_{K1}) and two separate regions of outward repolarizing I_K (41, 66, 98). In all cases the current amplitude was measured relative to the zero current (refers to zero net membrane current) (122). The first region of repolarizing K^+ current analyzed was the peak current amplitude that is dependent on the fast transient outward K^+ current of $I_{to,F}$ (16). The second region is the steady state K^+ current amplitude measured at the end of the 500 ms steps that is generated by several delayed rectifier K^+ currents including $I_{K,slow}$, I_{kur}, I_{Kss} (16). IV relationships were plotted for both peak and stead state I_K as indicated in Figure 17. In some cells peak and steady state I_K were measured using a single 500 ms voltage clamp step to +70 mV from a holding potential of -80 mV (66). These data are presented as summary bar graphs in Figure 17.

2.5 Statistical Analysis

Summary data are presented as means ± SEM. L-type Ca^{2+} current-voltage and kinetic data was analyzed using a paired T-test. One-way analysis of variance was used in all other cases, which was followed by student Newman Keuls post-hoc analysis when appropriate. Data was analyzed with Sigma Stat 2.0 statistical analysis software (Jandel Scientific) to identify significant differences. In all cases $P < 0.05$ was considered significant.

CHAPTER 3: RESULTS

Effects of BNP on atrial myocyte action potentials

The first series of experiments in this study investigated the effects of BNP (100 nM) on AP parameters in isolated mouse atrial myocytes (Fig. 4). Action potential duration at 50, and 90% repolarization times (APD_{50} and APD_{90}, respectively), AP peak amplitude and maximum rate of depolarization (V_{max}) were analyzed (Fig. 5). Application of BNP in basal conditions had no significant effect on any AP parameters measured (n=15). Specifically, APD_{50} was 7.4 ± 1.1 ms in control conditions and 7.3 ± 1.14 ms in the presence of BNP (P=0.234). APD_{90} was 36.32 ± 7.72 ms in control conditions and 35.7 ± 7.7 ms in the presence of BNP (P=0.073). AP peak amplitude was 137.1 ± 4.0 mV in control conditions and 136.83 ± 4.14 mV in the presence of BNP (P=0.305). Finally, V_{max} measured 157.5 ± 5.8 V/s in control conditions and 158.4 ± 4.14 V/s in the presence of BNP (P=0.291).

Previous studies have suggested that NP effects may be pronounced in the presence of β-AR activation (97, 112); therefore, the effects of BNP (100 nM) on atrial myocyte AP firing were further studied in the presence of a submaximal concentration of the β-AR agonist, isoproterenol (ISO; 10 nM), which stimulates AC activity and increases intracellular cAMP levels (77) (Fig. 6 and 7). As expected, application of ISO prolonged APD_{50}, APD_{70} and APD_{90} (P<0.05; n=12). Application of BNP in the presence of ISO further prolonged APD_{50} and APD_{70} (P<0.05; n=12). Application of BNP caused a similar trend with APD_{90}, however showed no significant effect (P=0.056; n=12). APD_{50} was 13.6 ± 0.83 ms in control conditions, 25.2 ± 1.8 ms in the presence of ISO and 31.2 ± 2.2 ms in the presence of ISO+BNP. APD_{70} was 23.7 ± 0.73 ms in control

conditions, 43.3 ± 2.0 ms in the presence of ISO and 51.3 ± 2.6 ms in the presence of ISO + BNP. APD_{90} was 46.8 ± 2.0 ms in control conditions, 67.0 ± 3.3 ms in the presence of ISO and 74.42 ± 3.9 ms in the presence of ISO + BNP. AP peak amplitude and V_{max} were not different (*P*=0.908, *P*=0.897 respectively) between treatment groups (Fig. 7). Together these data demonstrate that BNP has no effect on atrial AP firing properties in basal conditions; however, it increases AP duration following stimulation of the β-ARs.

FIGURE 4:

Effect of B-type natriuretic peptide on action potentials in acutely isolated mouse right atrial myocytes in basal conditions. Representative action potentials (AP) in control conditions and following the application of BNP (100 nM) are shown. These examples demonstrate that the acute application of BNP does not significantly change the AP duration compared to control.

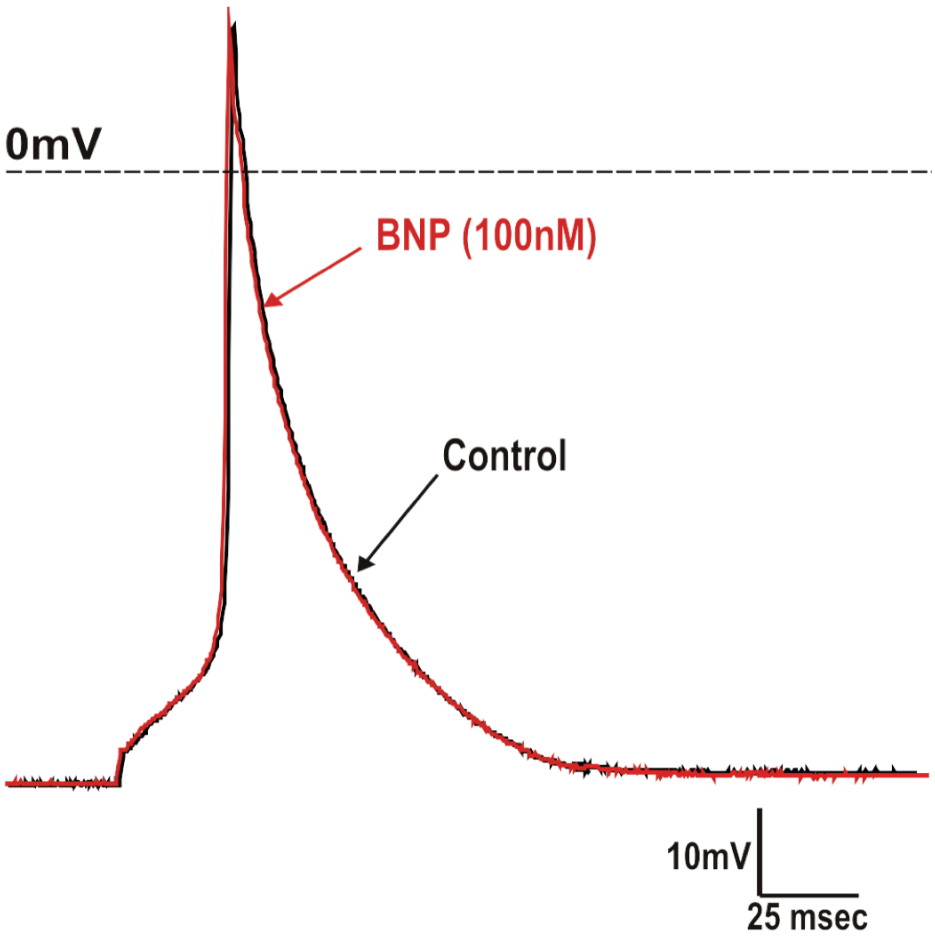


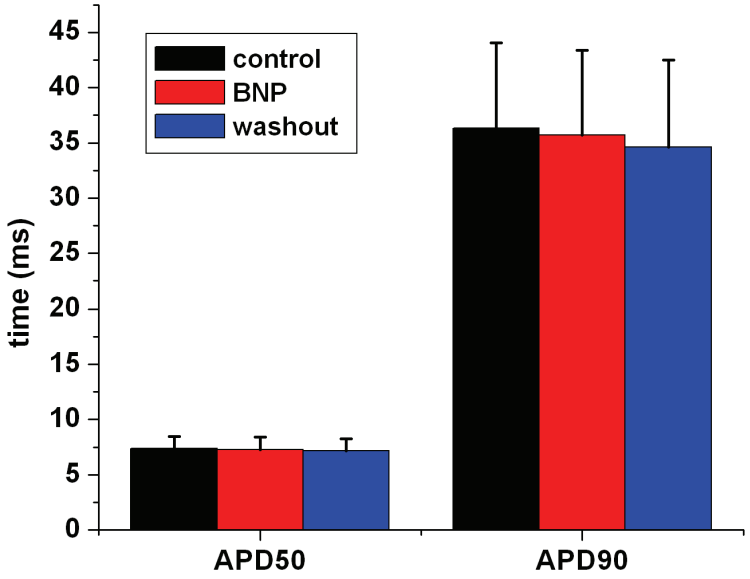
Figure 4.

FIGURE 5:

Summary data illustrating the effects of B-type natriuretic peptide on action potential (AP) parameters in acutely isolated mouse right atrial myocytes in basal conditions. A and B: Application of BNP (100 nM) had no significant effect on AP duration at 50% (APD_{50}) and 90% (APD_{90}) repolarization, AP peak amplitude (peak amp) or maximum rate of depolarization (V_{max}). Data are means ± SEM; *n*=15.

A.

B.


control
BNP
washout
time (ms)
45
40
35
30
25
20
15
10
5
0
APD50
APD90

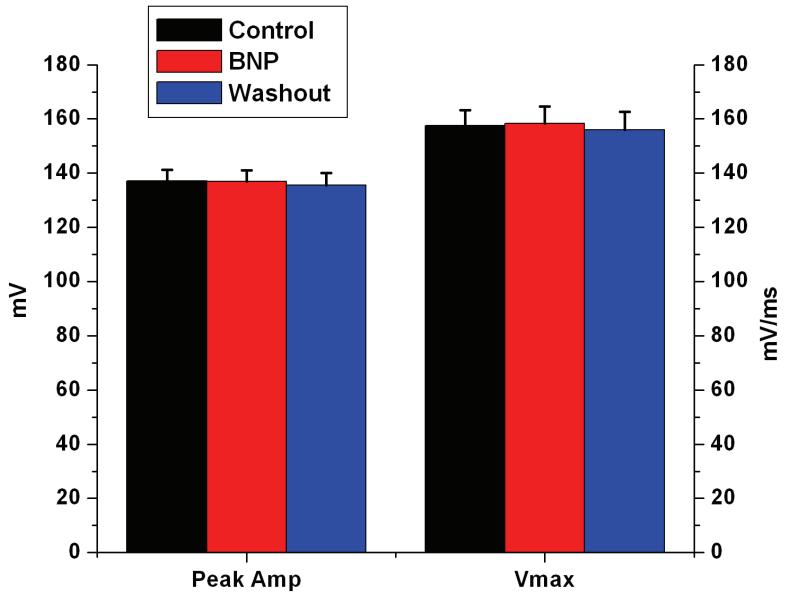

Control
BNP
Washout
mV
mV/ms
180
160
140
120
100
80
60
40
20
0
Peak Amp
Vmax

Figure 5.

FIGURE 6:

Effects of B-type natriuretic peptide on action potentials in mouse right atrial myocytes in the presence of isoproterenol (ISO). Representative action potentials (AP) in control conditions, in the presence of ISO (10 nM) and in ISO + BNP (100 nM) are shown. This example demonstrates the increase in AP duration when ISO is applied and a further increase when BNP is applied in the presence of ISO.

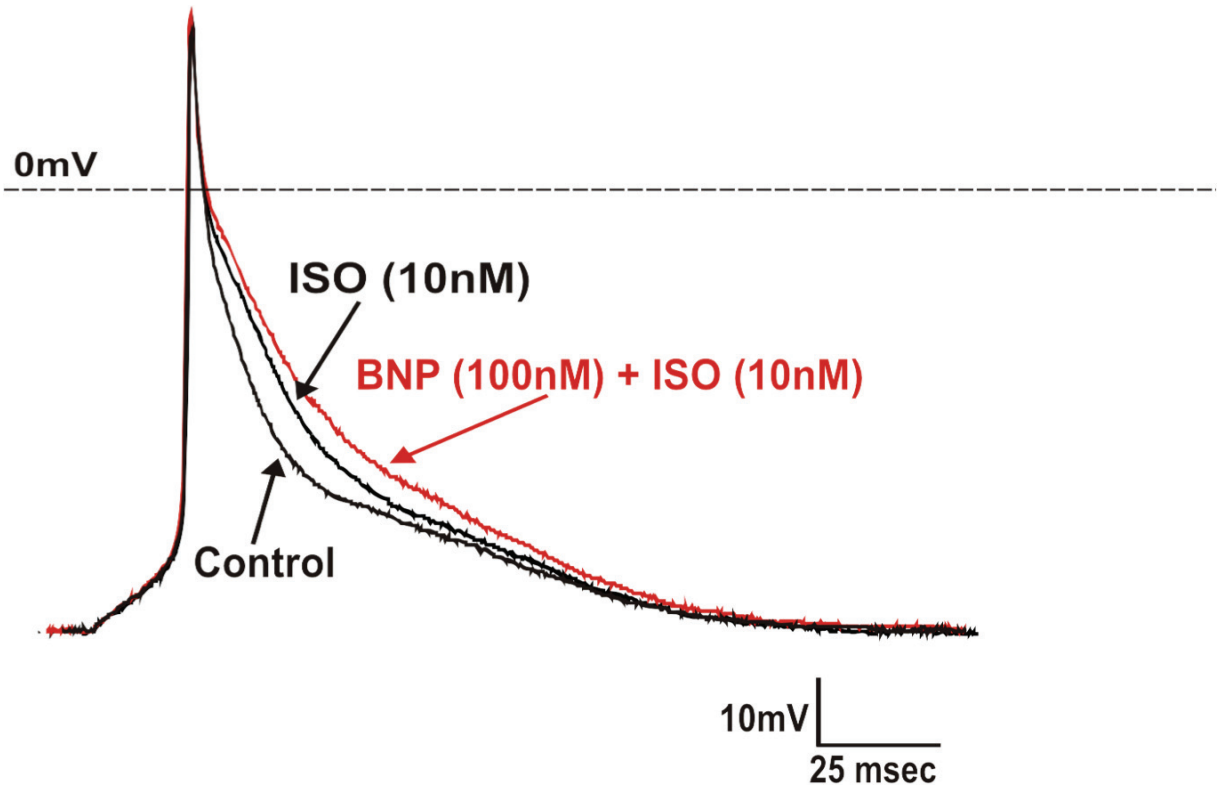


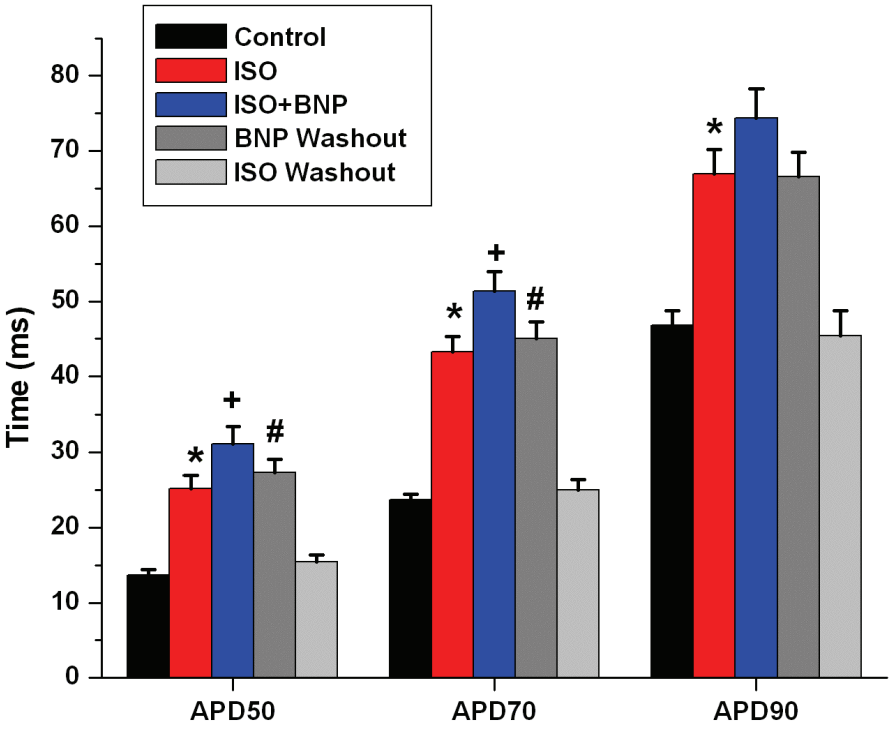
Figure 6.

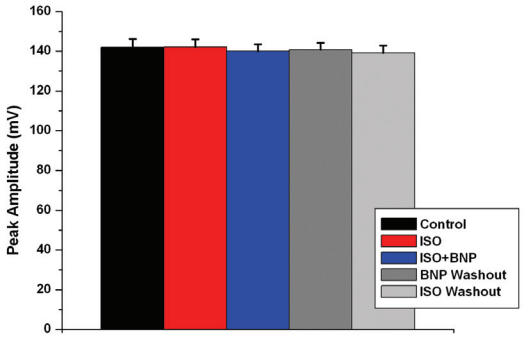
FIGURE 7:

Summary data illustrating the effects of B-type natriuretic peptide on action potential (AP) parameters in acutely isolated mouse right atrial myocytes following β-adrenergic receptor stimulation with isoproterenol (ISO; 10 nM). A: Application of BNP (100 nM) significantly increased AP duration at 50% (APD_{50}) and 70% (APD_{70}) repolarization. B and C: BNP had no significant effect on AP peak amplitude (peak amp) or maximum rate of depolarization (V_{max}). Data are means ± SEM; n=12; *P<0.05 vs. control, + P<0.05 vs. ISO, # P<0.05 vs. ISO + BNP.

A.

C.


Control
ISO
ISO+BNP
BNP Washout
ISO Washout
Time (ms)
0
10
20
30
40
50
60
70
80
APD50
APD70
APD90
*
+
#


Peak Amplitude (mV)
0
20
40
60
80
100
120
140
160
Control
ISO
ISO+BNP
BNP Washout
ISO Washout

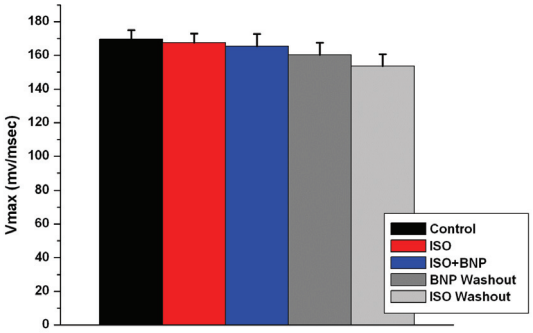

Vmax (mv/msec)
0
20
40
60
80
100
120
140
160
180
Control
ISO
ISO+BNP
BNP Washout
ISO Washout

Figure 7.

Effect of BNP on $I_{Ca,L}$ in atrial myocytes

Voltage clamp experiments on isolated atrial myocytes were performed to investigate the underlying ionic mechanism(s) responsible for the BNP effect on APs. Since the effects of BNP (in the presence of ISO) were most prominent in early repolarization, initial experiments focused on $I_{Ca,L}$, a current that contributes prominently to this region of the AP (95, 112). Figure 8A illustrates sample $I_{Ca,L}$ measurements during a voltage clamp step to 0 mV (holding potential was -60 mV) in control conditions, following application of ISO (10 nM) and following application of BNP (100 nM) in the presence of ISO. These recordings, along with the time course data illustrated in figure 8B, demonstrate that ISO increased peak $I_{Ca,L}$ over the course of approximately 5 min. BNP further increased $I_{Ca,L}$ over the course of an additional 5 min. These effects were fully reversible when the drugs were washed off.

To quantify the effects of ISO and BNP on $I_{Ca,L}$ a summary current voltage (IV) relationship was plotted (n= 15; Fig. 9). Application of ISO caused a significant increase (186%) in the mean $I_{Ca,L}$ density at the peak of the IV relationship compared to control (P<0.05). Application of ISO also caused a left shift in the calcium current IV curve as expected (157). Application of BNP in the presence of ISO, caused a further increase in current in the voltage range of -20 mV to +30 mV and a 21% increase in peak $I_{Ca,L}$ current at 0 mV (P<0.05).

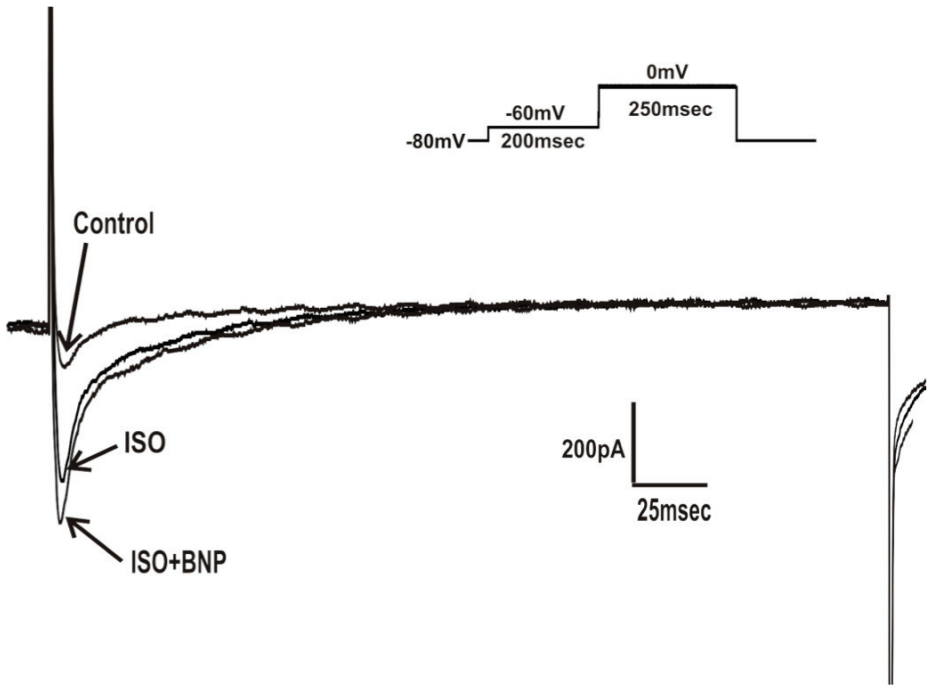
To further quantify the differences in $I_{Ca,L}$ between groups, maximum conductance (G_{max}), the voltage required for 50% channel activation ($V_{1/2(act)}$) and activation curve slope factor (k) were measured (n=15; Fig. 10) from Boltzman fits of chord conductance data. Analysis of maximum conductance revealed that ISO (10 nM) significantly elevated G_{max} from 101.56 ± 5.0 in control conditions to 200.13 ± 17.0

(pS/pF). G_{max} was further increased to 239.07 ± 16.0 (pS/pF) in the presence of ISO and BNP (P<0.05). $V_{1/2\ (act)}$ was significantly shifted from -8.26 ± 0.91 mV in control conditions to -13.85 ± 1.24 mV in the presence of ISO (10 nM), and -14.63 ± 1.4 mV in the presence of ISO and BNP (P<0.05). There were no significant differences in slope factor (k) between groups (control: 7.17 ± 0.23, ISO: 7.28 ± 0.48, ISO + BNP: 7.34 ± 0.37 [Table 1]).

FIGURE 8:

Effect of B-type natriuretic peptide on peak $I_{Ca,L}$ in acutely isolated mouse right atrial myocytes in the presence of isoproterenol (ISO). A: Representative $I_{Ca,L}$ recordings showing the effect of ISO (10 nM) and BNP (100 nM) + ISO (10 nM). B: Representative time course experiment elicited by a 0 mV depolarizing step from a -60 mV holding potential, demonstrating the change in peak $I_{Ca,L}$ with the application of each compounds over time (min).

A.


0mV
250msec
-60mV
200msec
-80mV
Control
ISO
ISO+BNP
200pA
25msec

B.

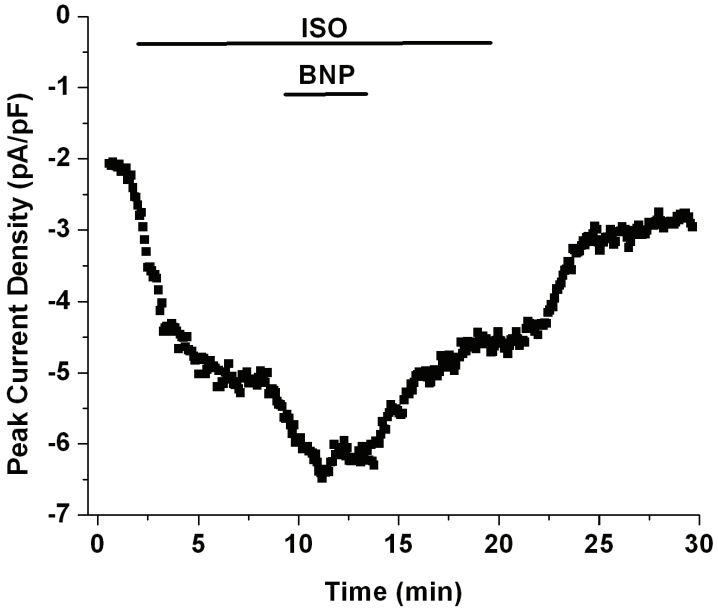

ISO
BNP
Peak Current Density (pA/pF)
0
-1
-2
-3
-4
-5
-6
-7
0
5
10
15
20
25
30
Time (min)

Figure 8.

FIGURE 9:

Summary current-voltage relationship showing the effect of B-type natriuretic peptide on peak $I_{Ca,L}$ density in acutely isolated mouse right atrial myocytes in the presence of isoproterenol. $I_{Ca,L}$ was significantly increased in the presence of isoproterenol (ISO; 10 nM) and was further increased when BNP (100 nM) was combined with ISO. Data are means ± SEM; n=15; *P<0.05 vs. Control, + P<0.05 vs. ISO.

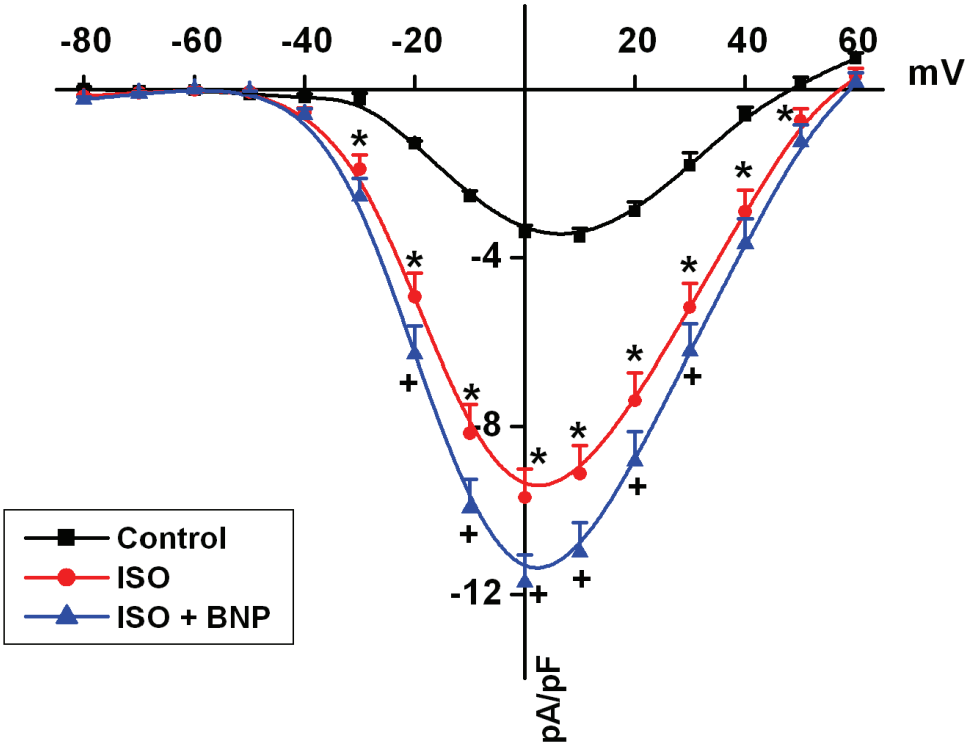


Figure 9.

FIGURE 10:

Summary activation curves showing the effect of B-type natriuretic peptide on $I_{Ca,L}$ activation kinetics in acutely isolated mouse right atrial myocytes in the presence of isoproterenol. $I_{Ca,L}$ G_{max} was significantly increased in the presence of isoproterenol (ISO; 10 nM), and further increased when BNP (100 nM) was combined with ISO. $V_{1/2\ (act)}$ was increased in the presence of ISO compared to control. $V_{1/2\ (act)}$ appeared to increase when BNP was applied in the presence of ISO, however did not reach significance. k (slope factor) was unchanged in the presence of ISO or BNP. Data are means ± SEM; *n*=15; * *P*<0.05 vs. control, + *P*<0.05 vs. ISO. Refer to Table 1 for a summary of conductance parameters in each condition.

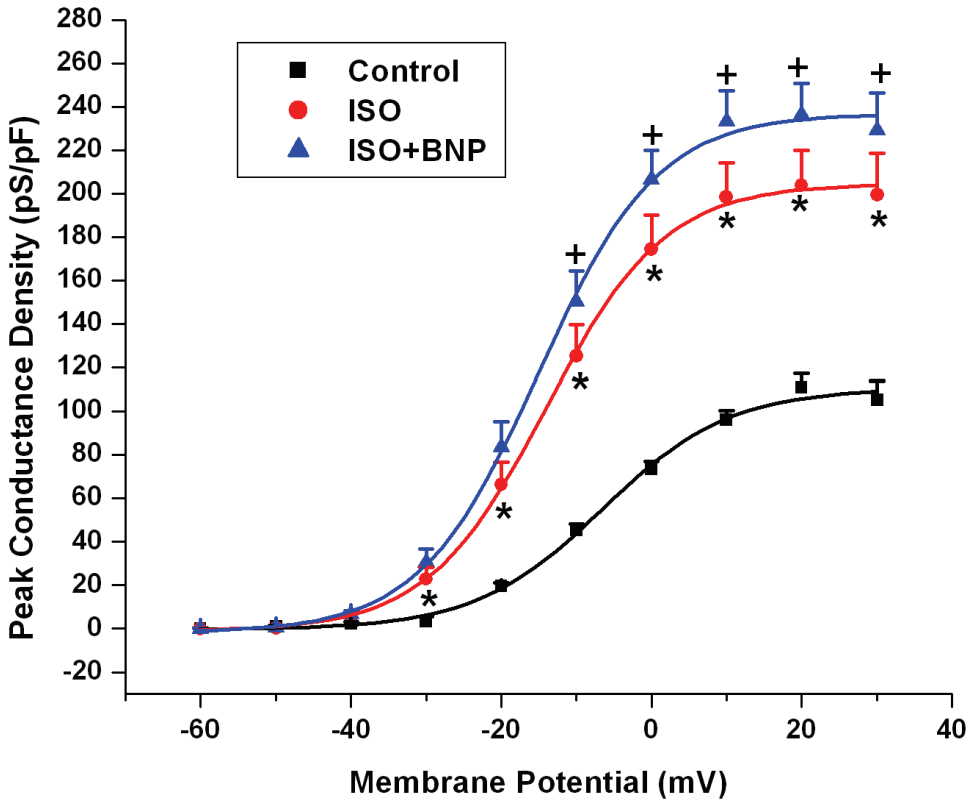

Control
ISO
ISO+BNP
Peak Conductance Density (pS/pF)
Membrane Potential (mV)
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
-20
-60
-40
-20
0
20
40
*
+

Figure 10.

TABLE 1:

Effect of B-type natriuretic peptide and isoproterenol on $I_{Ca,L}$ kinetics in acutely isolated mouse atrial myocytes.

Kinetic Parameter	Control	ISO (10 nM)	ISO+BNP (100 nM)
G_{max} (pS/pF)	101.56 ± 5.0	200.13 ± 17.0*	239.07 ± 16.0†
$V_{1/2\ (act)}$ (mV)	-8.26 ± 0.91	-13.85 ± 1.24*	-14.63 ± 1.4
k (mV)	7.17 ± 0.23	7.28 ± 0.48	7.34 ± 0.37

Values are means ± SEM. *n*=15. ISO; isoproterenol, BNP; B-type natriuretic peptide. * *P*<0.05 vs control; † *P*<0.05 vs ISO.

Effect of BNP on $I_{Ca,L}$ in NPR-C mutant atrial myocytes

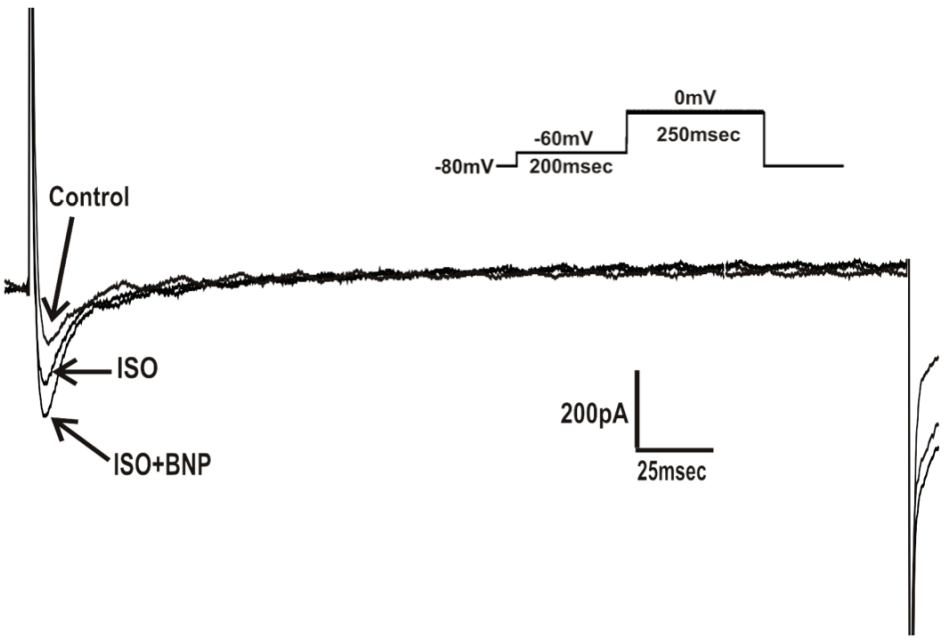
Measurements of $I_{Ca,L}$ in isolated atrial myocytes from NPR-C mutant mice lacking functional NPR-C receptors were performed to investigate whether NPR-C is involved in mediating the effects of BNP (n=8). Figure 11A illustrates sample $I_{Ca,L}$ measurements during a voltage clamp step to 0 mV (holding potential was -60 mV) in control conditions, following application of ISO (10 nM), and upon application of BNP (100 nM) in the presence of ISO. These recordings, along with the time course data illustrated in figure 11B demonstrate that ISO increased peak $I_{Ca,L}$ over the course of approximately 5 min. BNP further increased $I_{Ca,L}$ over the course of an additional 5 min. These effects were fully reversible when the drugs were washed off.

Summary data (Fig. 12) demonstrate that application of ISO caused a 123% increase in peak $I_{Ca,L}$ density compared to control (P<0.05; n= 8). Application of BNP in the presence of ISO caused a further 34% increase in $I_{Ca,L}$ (P<0.05 vs. ISO). These data demonstrate that the ability of BNP to increase $I_{Ca,L}$ is fully maintained in NPR-C mutant mice indicating that the effects of BNP on atrial $I_{Ca,L}$ are not primarily dependent on NPR-C.

FIGURE 11:

Effect of B-type natriuretic peptide on $I_{Ca,L}$ in acutely isolated mouse right atrial myocytes from NPR-C mutant mice lacking functional NPR-C receptors. A: $I_{Ca,L}$ recordings showing the effect of isoproterenol (ISO; 10 nM) and BNP (100 nM) + ISO (10 nM). B: Representative time course experiment elicited by a 0 mV depolarizing step from a -60 mV holding potential, demonstrating the change in peak $I_{Ca,L}$ with the application of each compound over time. This experiment demonstrates that BNP does not primarily elicit its effects through NPR-C.

A.


0mV
250msec
-60mV
200msec
-80mV
Control
ISO
ISO+BNP
200pA
25msec

B.

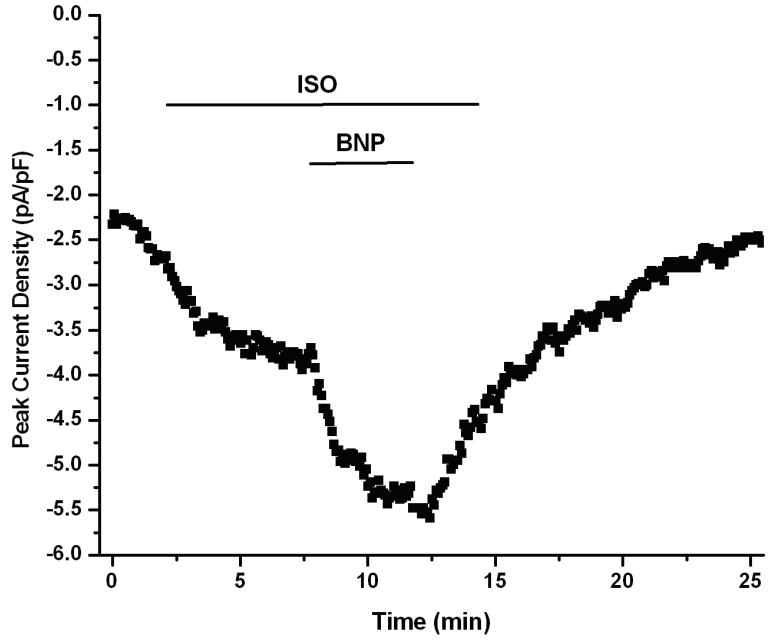

ISO
BNP
Peak Current Density (pA/pF)
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
-4.5
-5.0
-5.5
-6.0
0
5
10
15
20
25
Time (min)

Figure 11.

FIGURE 12:

Summary data illustrating the effects of B-type natriuretic peptide on peak current density at 0 mV in acutely isolated mouse right atrial myocytes from NPR-C mutant mice following β-adrenergic receptor stimulation with isoproterenol (ISO; 10 nM). A: Application of BNP (100 nM) significantly increased peak $I_{Ca,L}$ in the presence of isoproterenol (10 nM). This experiment demonstrates that BNP does not primarily elicit its effects through NPR-C. Data are means ± SEM; n=8; * P<0.05 vs. control, + P<0.05 vs. ISO.

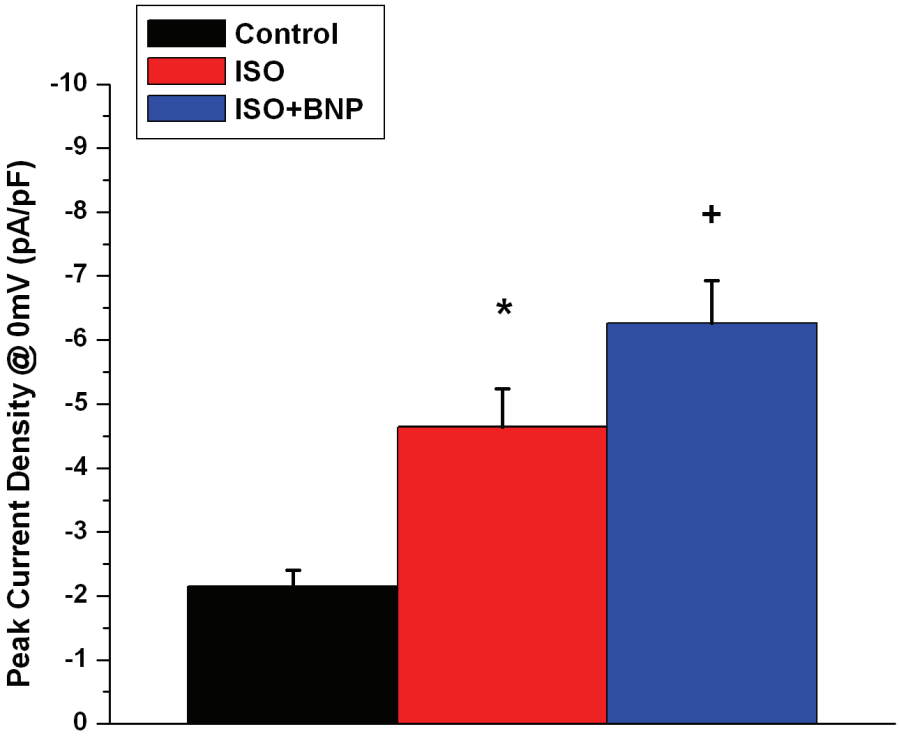

Control
ISO
ISO+BNP
Peak Current Density @ 0mV (pA/pF)
-10
-9
-8
-7
-6
-5
-4
-3
-2
-1
0
*
+

Figure 12.

Effect of BNP on $I_{Ca,L}$ in atrial myocytes in the presence of NPR-A block

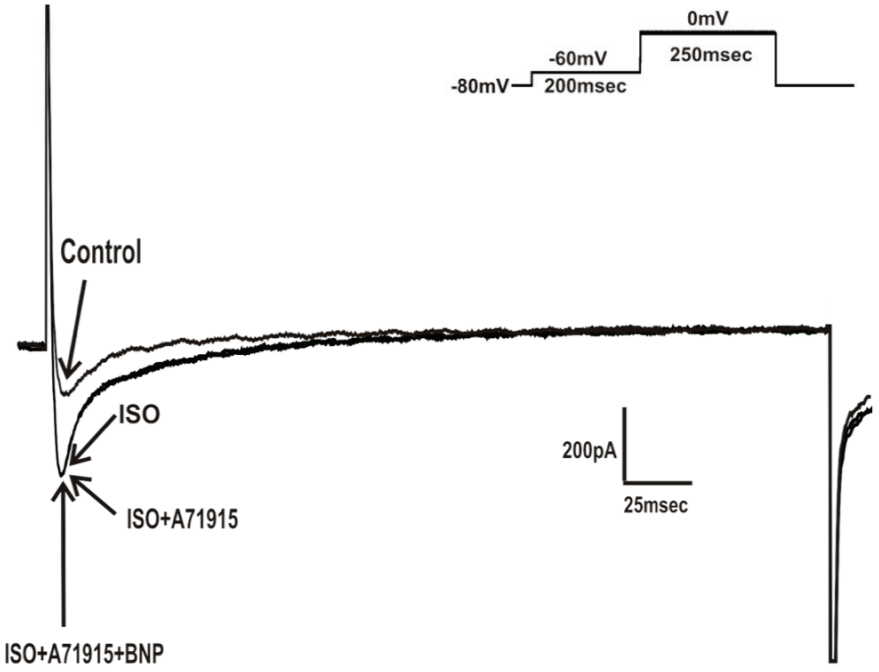
Measurements of $I_{Ca,L}$ in isolated atrial myocytes were performed using the previously characterized NPR-A antagonist A71915 (500 nM) (24) to investigate whether NPR-A is involved in mediating the effects of BNP (*n*=6). Figure 13A illustrates sample $I_{Ca,L}$ measurements during a voltage clamp step to 0 mV (holding potential was -60 mV) in control conditions, following application of ISO (10 nM), in ISO and A71915 (500 nM) and following application of BNP (100 nM) in the presence of ISO and A71915. These recordings, along with the time course data illustrated in figure 13B demonstrate that ISO increased peak $I_{Ca,L}$ over the course of approximately 5 min. A71915 had no effect on $I_{Ca,L}$; however in the presence of this NPR-A antagonist, the effect of BNP on $I_{Ca,L}$ was completely blocked. These effects were fully reversible when the drugs were washed off.

Summary data (Fig. 14) illustrates that application of ISO increased peak $I_{Ca,L}$ density by 156% compared to control ($P<0.05$; $n= 6$). Additionally, the effect of BNP was completely absent in the presence of the NPR-A antagonist A71915 ($P=0.225$).

FIGURE 13:

Effect of B-type natriuretic peptide on $I_{Ca,L}$ in acutely isolated mouse right atrial myocytes in the presence of isoproterenol (ISO) and the NPR-A antagonist A71915. A: Representative $I_{Ca,L}$ recording showing the effect of ISO (10 nM), ISO + A71915 (500 nM) and ISO + A71915 + BNP (100 nM). B: Representative time course experiment elicited by a 0 mV depolarizing step from a -60 mV holding potential, demonstrating the change in peak $I_{Ca,L}$ with the application of each compound over time. This experiment demonstrates that BNP elicits its effects through NPR-A.

A.


0mV
250msec
-60mV
200msec
-80mV
Control
ISO
ISO+A71915
ISO+A71915+BNP
200pA
25msec

B.

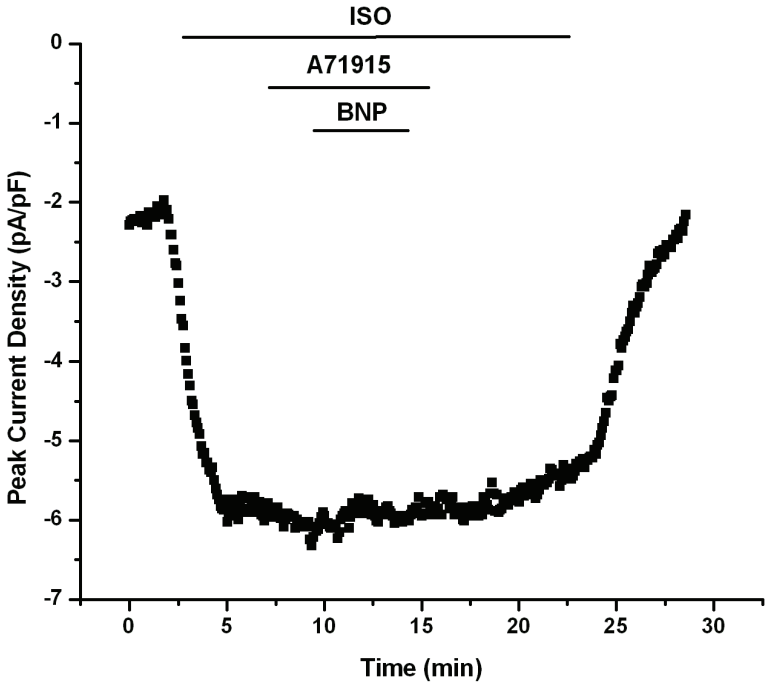

ISO
A71915
BNP
Peak Current Density (pA/pF)
0
-1
-2
-3
-4
-5
-6
-7
0
5
10
15
20
25
30
Time (min)

Figure 13.

FIGURE 14:

Summary data illustrating the effects of B-type natriuretic peptide on peak $I_{Ca,L}$ density at 0 mV in acutely isolated mouse right atrial myocytes in the presence of isoproterenol (ISO) and A71915. A: Application of ISO (10 nM) significantly increased peak current density (pA/pF), however BNP in the presence of A71915 and ISO had no significant effect. This experiment demonstrates that BNP elicits its effects through NPR-A. Data are means ± SEM; n=6; * P<0.05 vs. control.

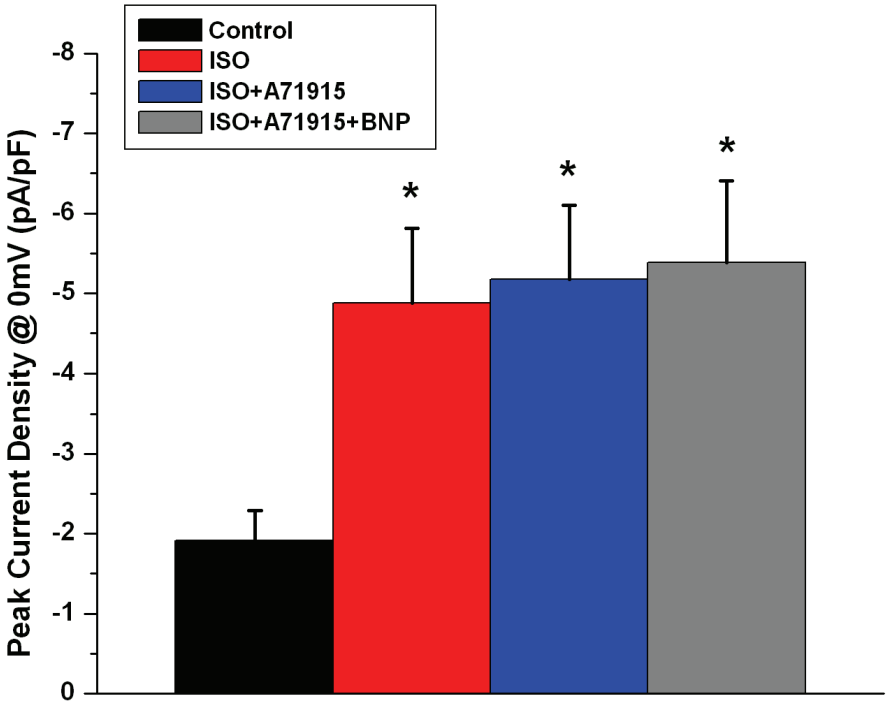

Control
ISO
ISO+A71915
ISO+A71915+BNP
Peak Current Density @ 0mV (pA/pF)
-8
-7
-6
-5
-4
-3
-2
-1
0
*
*
*

Figure 14.

Effect of BNP on $I_{Ca,L}$ in atrial myocytes in the presence of the PDE-3 antagonist milrinone

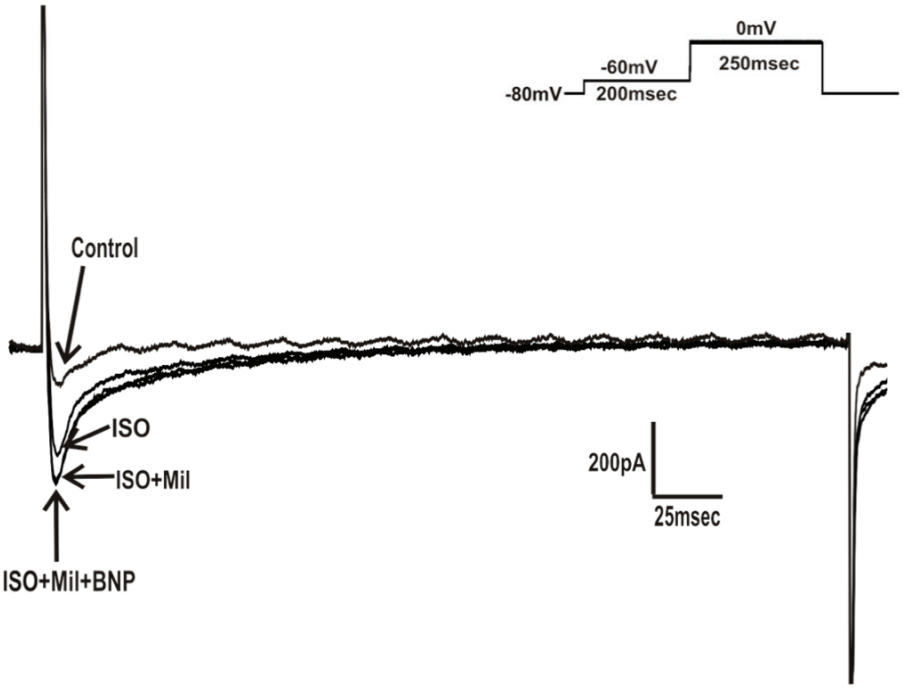
Thus far, these data presented demonstrate that BNP increases $I_{Ca,L}$ by binding to NPR-A which is known to elevate intracellular levels of cGMP (102). Traditionally, cGMP is known to decrease $I_{Ca,L}$ activity (19, 125, 136), however PDE-3 is a potential candidate molecule that could mediate an increase in $I_{Ca,L}$ (72). Measurements of $I_{Ca,L}$ in isolated atrial myocytes with the PDE-3 selective antagonist; milrinone (10 μM) (54) were performed to investigate whether BNP can still increase $I_{Ca,L}$ activity in the presence of a PDE-3 inhibitor. Figure 15A illustrates sample $I_{Ca,L}$ measurements during a voltage clamp step to 0 mV (holding potential was -60 mV) in control conditions, following application of ISO (10 nM), in ISO and milrinone (10 μM) and following application of BNP (100 nM) in the presence of ISO and milrinone. These recordings, along with the time course data illustrated in figure 15B demonstrate that ISO increased peak $I_{Ca,L}$ over the course of approximately 5 min. Milrinone caused a further increase in $I_{Ca,L}$, and, furthermore, the ability of BNP to enhance $I_{Ca,L}$ was completely absent in the presence of milrinone. These effects were fully reversible when the drugs were washed off.

Summary data (Fig. 16) demonstrate that application of ISO increased peak $I_{Ca,L}$ density by 179% compared to control ($P<0.05$; $n=7$), and application of milrinone further increased $I_{Ca,L}$ by 26.4% ($P<0.05$). BNP applied in the presence of milrinone had no further effect on peak $I_{Ca,L}$ density ($P=0.06$).

FIGURE 15:

Effect of B-type natriuretic peptide on $I_{Ca,L}$ in acutely isolated mouse right atrial myocytes in the presence of isoproterenol (ISO) and the PDE-3 inhibitor; milrinone. A: Representative $I_{Ca,L}$ recording showing the effect of ISO (10 nM), ISO + milrinone (10 μM) and ISO + milrinone + BNP (100 nM). B: Representative time course experiment elicited by a 0mV depolarizing step from a -60mV holding potential, demonstrating the change in peak $I_{Ca,L}$ with the application of each compound over time. This experiment demonstrates that BNP no longer increases $I_{Ca,L}$ activity in the presence of milrinone.

A.


0mV
250msec
-60mV
200msec
-80mV
Control
ISO
ISO+Mil
ISO+Mil+BNP
200pA
25msec

B.

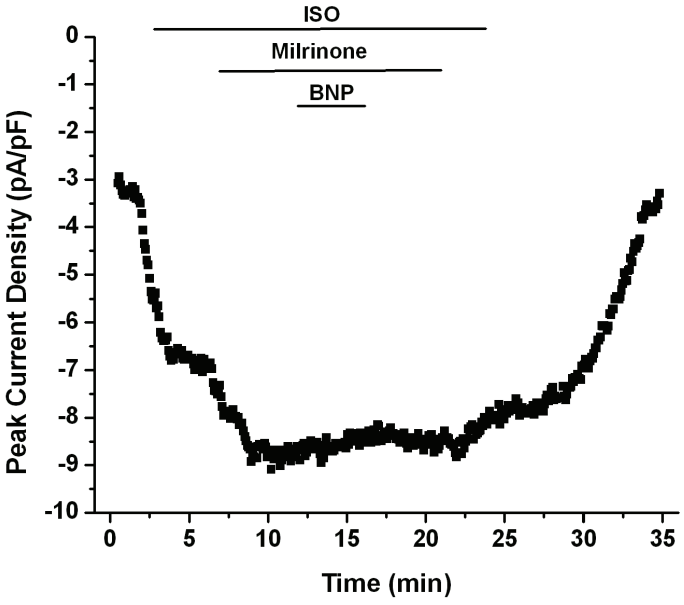

ISO
Milrinone
BNP
Peak Current Density (pA/pF)
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
-10
0
5
10
15
20
25
30
35
Time (min)

Figure 15.

FIGURE 16:

Summary data illustrating the effects of B-type natriuretic peptide on peak $I_{Ca,L}$ density at 0 mV in acutely isolated mouse right atrial myocytes in the presence of milrinone and isoproterenol (ISO). A: Application of ISO (10 nM) and milrinone (10 μM) significantly increased peak current density (pA/pF), however BNP (100 nM) in the presence of milrinone and ISO had no significant effect. This experiment demonstrates that BNP no longer increases $I_{Ca,L}$ activity in the presence of milrinone. Data are means ± SEM; n=7; * P<0.05 vs. control, + P<0.05 vs. ISO.

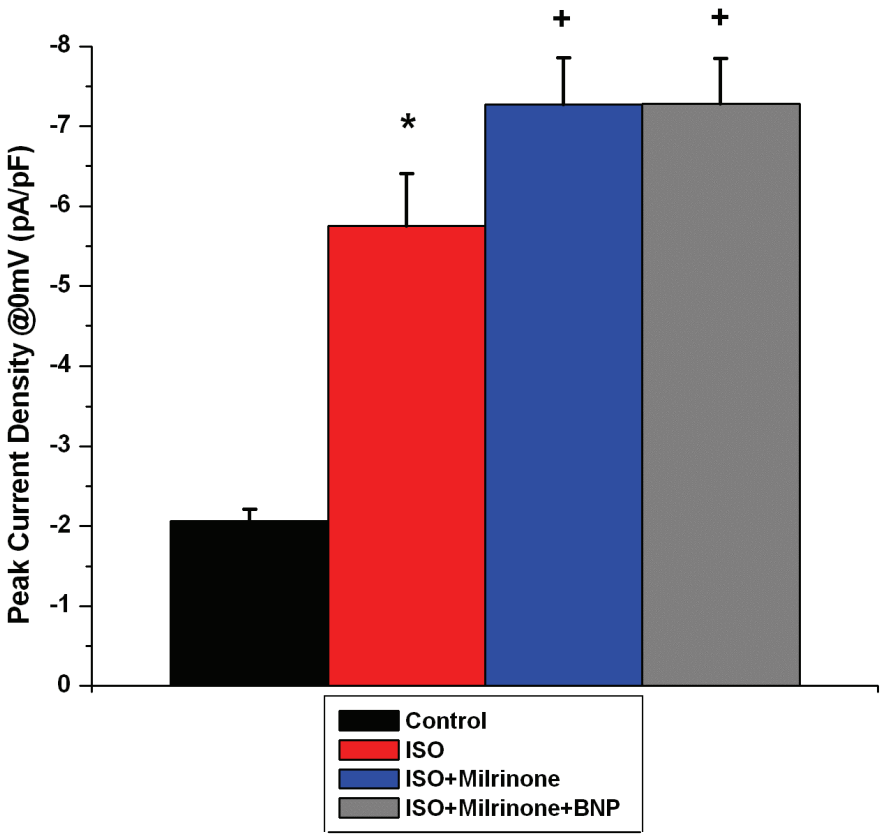

Peak Current Density @0mV (pA/pF)
-8
-7
-6
-5
-4
-3
-2
-1
0
*
+
+
Control
ISO
ISO+Milrinone
ISO+Milrinone+BNP

Figure 16.

Effect of BNP on I_K in atrial myocytes

To assess the selectivity of the BNP effect on $I_{Ca,L}$ described above, the effects of BNP on cardiac K^+ currents in isolated atrial myocytes were performed. Several K^+ currents (Fig. 1) contribute to the repolarization of the AP in mice (16, 87) and although it has been shown that mouse atrial myocytes do not express K^+ currents that are sensitive to cAMP and PKA phosphorylation (16) we wanted to determine whether the changes in AP duration elicited by BNP in the presence of ISO were partially dependent on K^+ currents. Figure 17A illustrates representative families of K^+ currents generated using the voltage clamp protocol shown in Figure 17A (inset) in control conditions, following application of ISO (10 nM) and in the presence of ISO + BNP (100 nM). As described in the Methods, IV relationships were generated for both peak and steady state I_K at the time points indicated by the open and filled circles in Figure 17A (16). Peak I_K amplitude (open circle) is mainly a measure of $I_{to,F}$ activity whereas the steady state I_K amplitude at the end of the 500 ms voltage clamp steps (filled circle) is a measure of the non-inactivating delayed rectifier K^+ currents including $I_{K,slow}$, I_{kur}, I_{Kss} (16, 41, 98). Application of ISO or BNP in the presence of ISO had no effect on the peak (P=0.254) and steady state (P=0.332) I_K IV relationships compared to control (Figure 17B; n=3 myocytes). The I_K IV curves also demonstrate that ISO and BNP had no effect on the inward rectifier K^+ current I_{K1}, which is evident at membrane potentials between -100 and -40 mV.

In some cells the effects of ISO and BNP on peak and steady state I_K were measured using a single voltage clamp step to +70 mV from a holding potential of -80 mV (Figure 17C, n=8 myocytes). These data further demonstrate that neither ISO or BNP

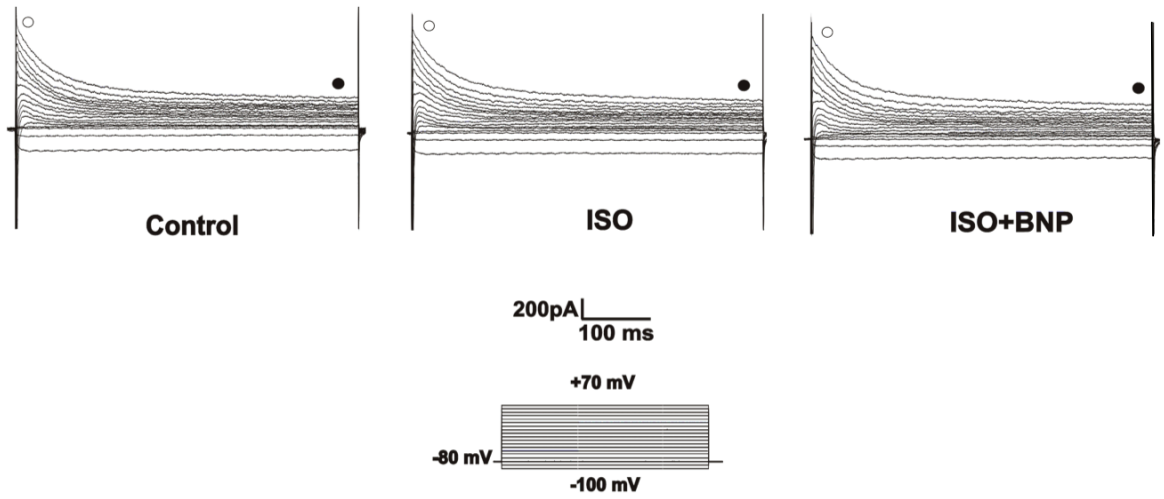
in the presence of ISO affected peak (P=0.268) or steady state (P=0.423) I_K in right atrial myocytes.

FIGURE 17:

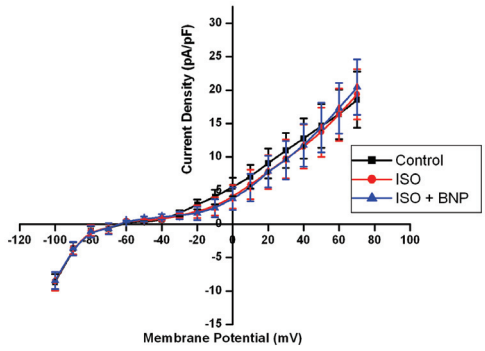
Effect of B-type natriuretic peptide and isoproterenol on K^+ currents in isolated mouse atrial myocytes. A. Representative families of potassium current (I_K) recordings elicited by a series of 10 mV depolarizing steps from -100 mV to +70 mV (holding potential was -80 mV) in control conditions, in the presence of isoproterenol (ISO; 10 nM), and after the addition of BNP (100 nM) in the presence of ISO. B. Summary IV relationships for peak (upper panel) and steady state (lower panel) K^+ current in control, ISO or BNP + ISO conditions. Peak I_K was measured at the time point indicated by the open circles in panel A. and steady state I_K was measured at the time point indicated by the filled circles in panel A. Data are means ± SEM; *n*=3 myocytes. C. Summary data illustrating the effects of ISO and BNP + ISO on peak and steady state I_K measured during a voltage clamp step to +30 mV from a holding potential of -80 mV. Application of ISO and BNP in the presence of ISO had no significant effect on peak or steady state I_K current compared to control. Data are means ± SEM; *n*=8 myocytes.

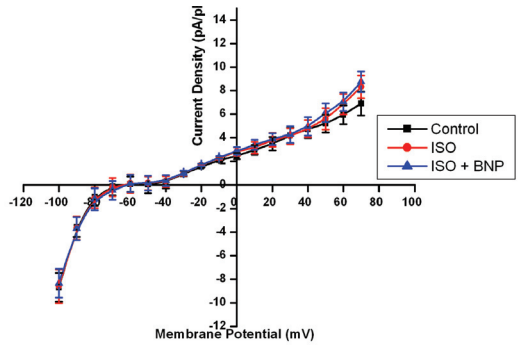
C.

A.



B.





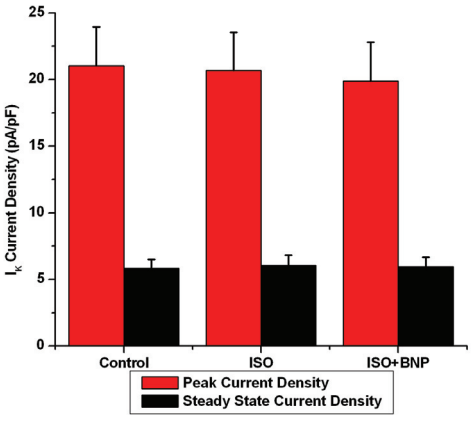


FIGURE 17.

CHAPTER 4: DISCUSSION

A small subset of the NP research field has begun to investigate the electrophysiological effects of NPs on the heart, however most of the published data have been obtained using ANP as an agonist, and the molecular mechanism(s) mediating these effects are still poorly understood. Therefore, the purpose of this study was to investigate the electrophysiological effects of BNP, to determine which NPR(s) mediates its effects and to study the intracellular signaling pathway(s) that are involved. We reject our hypothesis that stated that BNP would decrease the AP duration, by activating NPR-A and/or NPR-C and inhibiting $I_{Ca,L}$. The results obtained in this study provide the first electrophysiological evidence that BNP, at physiological concentrations, activates the p-GC linked NPR-A and can increase atrial myocyte AP duration by increasing $I_{Ca,L}$. The effects of BNP appear to be selective for $I_{Ca,L}$ in our conditions because BNP in the presence of ISO has no effect on I_{K1} or repolarizing K^+ currents. We have conclusively demonstrated that BNP activates NPR-A, because the BNP effect is completely abolished in the presence of the well-characterized NPR-A antagonist; A71915 (24). Using a transgenic NPR-C mutant mouse model, we have also demonstrated that NPR-C is not responsible for the increase in $I_{Ca,L}$ elicited by BNP. Furthermore, we have demonstrated that BNP is no longer able to increase $I_{Ca,L}$ when PDE-3 is inhibited (Fig. 18). To summarize, this study has shown that BNP activates NPR-A and increases $I_{Ca,L}$, however BNP can no longer increase $I_{Ca,L}$ in the presence of a PDE-3 inhibitor. NPR-A activation results in p-GC activation and elevated levels of intracellular cGMP (20, 102, 106). We hypothesize that BNP raises intracellular cGMP levels to a concentration that can sufficiently inhibit PDE-3, resulting in reduced hydrolysis of cAMP and increased activation of $I_{Ca,L}$ (Fig. 18).

Several studies have shown using molecular techniques that natriuretic peptides and their respective receptors are expressed in mammalian cardiac tissue (102). Specifically, BNP mRNA and protein, along with NPR-A and NPR-C are highly expressed in both atrial and ventricular tissue (92, 102, 105). Despite this expression pattern our data show that BNP primarily activates NPR-A in our experimental conditions (i.e. submaximal concentrations of ISO) because the ability of BNP to increase atrial $I_{Ca,L}$ was completely antagonized by the NPR-A antagonist A71915 and fully maintained in NPR-C mutant mice.

In order to assess the involvement of K^+ currents in the BNP effect on AP duration in the presence of ISO, inward rectifier and voltage gated K^+ currents were measured. In mouse atria, I_{K1} is primarily responsible for setting the resting membrane potential while several voltage gated potassium currents, including $I_{K,slow}$, I_{kur}, I_{Kss}, are responsible for AP repolarization in the mammalian myocardium (16). We have clearly demonstrated that BNP (in the presence of ISO) has no effect on I_{K1} or peak and steady state I_K, and therefore can confidently state that the AP duration was increased by BNP as a result of increasing $I_{Ca,L}$. The voltage gated K^+ channels that contribute to AP repolarization in particular vary depending on the cardiac region and/or species (16, 87). The two general classes of repolarizing K^+ currents are the transient outward potassium currents and the delayed rectifier potassium currents (87). It has been shown that the delayed rectifier K^+ currents in mouse atria are not cAMP sensitive (18, 150). Furthermore, it is well known that mouse atrial myocytes do not express the rapid and slow delayed outwardly rectifying potassium channels (I_{Kr} and I_{Ks}) which are known to be sensitive to cAMP and PKA phosphorylation (16, 18, 150). Our results demonstrate that neither ISO or BNP in the presence of ISO had any effect on repolarizing potassium

currents, and these data support the conclusion that mice do not express cAMP sensitive repolarizing potassium channels.

Interestingly, our results demonstrate that BNP has no effect under basal conditions, yet increases both AP duration and peak $I_{Ca,L}$ when prestimulated with the β-AR agonist; ISO. β-ARs are generally linked to a stimulatory G protein (G_s) and upon activation by catecholamines AC activity is increased resulting in the elevation of cAMP (145). This increase in cAMP results in elevated intracellular Ca^{2+} by stimulating $I_{Ca,L}$ and Ca^{2+} release from the sarcoplasmic reticulum. It has been previously suggested and shown that NP effects are more pronounced in the presence of elevated concentrations of cAMP (97, 112). Based on our results, this phenomenon could be explained by concluding that a threshold level of intracellular cAMP must exist in order for $I_{Ca,L}$ activity to be affected by NPs. It is possible that in order for the NP induced elevation in cGMP to have any noticeable effect on $I_{Ca,L}$ (through PDE-3) the cAMP pool must be elevated to a specific threshold concentration. An early study investigating the effects of cGMP on guinea-pig ventricular cells, clearly showed that under basal conditions, elevated intracellular cGMP had no effect on $I_{Ca,L}$, however when the cells were prestimulated with the β-AR agonist; isoproterenol at various concentrations (0.001-0.1 μM), cGMP (10 μM) caused a further significant stimulation in $I_{Ca,L}$ (96). The authors noted that the stimulatory effect on $I_{Ca,L}$ was dependent on the concentration of cGMP. Ono *et al.* (96) discuss the possibility that lower cGMP concentrations may result in a PKG mediated inhibitory effect on $I_{Ca,L}$, however at higher concentrations activation of cGMP sensitive PDEs (such as PDE-3) may supersede the inhibitory PKG effect, and therefore further elevate cAMP by reducing PDE dependent cAMP hydrolysis, resulting in $I_{Ca,L}$ stimulation.

The majority of the published literature investigating the electrophysiological effects of NPs and how they modulate $I_{Ca,L}$ involves ANP, however some studies have investigated BNP. Two independent groups have found in a rather contradictory manner that BNP increased AP duration in rat ventricular myocytes, yet decreased $I_{Ca,L}$ and cell shortening (29, 123). The paradoxical results were justified by stating that BNP reduces the Ca^{2+} transient amplitude which results in reduced Ca^{2+} - dependent inactivation of $I_{Ca,L}$, therefore leading to more Ca^{2+} entry through $I_{Ca,L}$, which would explain the increased AP duration (29, 123). Contrary to these studies, we have clearly shown that AP duration is increased as a result of increased $I_{Ca,L}$ activity. LeGrand and colleagues demonstrated that ANP (10 nM) decreased $I_{Ca,L}$ in human atrial trabeculae with a GTP containing pipette solution in both basal (37% reduction) and β-AR stimulated conditions (25% reduction). Interestingly, however they found that when GTP was removed from the internal solution ANP increased $I_{Ca,L}$ by 39% (61). Another group has shown in guinea pig ventricular myocytes that ANP has no effect on $I_{Ca,L}$ in basal conditions, yet when pre-stimulated with the broad-spectrum PDE inhibitor; IBMX or ISO, ANP decreased the current via a p-GC mediated pathway (48).

Potential Pathways Mediating the BNP effect

There are numerous downstream targets that could potentially be involved in the BNP signaling pathway (Fig. 3,18). BNP can activate both NPR-A and NPR-C and therefore can potentially simultaneously increase and decrease cAMP levels through different mechanisms (102). As a result, there are numerous second messengers that could potentially be modulated by BNP downstream of these two receptors and which could affect $I_{Ca,L}$. The following section will briefly discuss these receptors and signaling molecules.

BNP can elicit its effects through NPR-A and NPR-C activation (102). It is well known that NPR-A activation results in elevated cGMP levels (20, 102, 106), and NPR-C activation reduces cAMP levels through G_i (7, 112, 113). Our data clearly demonstrate that the increase in $I_{Ca,L}$ following BNP application is mediated by NPR-A because the BNP effect is still present in NPR-C mutant mice and antagonized by the NPR-A blocker A71915. Interestingly, BNP increased $I_{Ca,L}$ by 21% in WT mice and by 34% in the NPR-C mutant mouse. While these data were not significantly different, the trend of a larger increase in the NPR-C mutant group is interesting to note. It is possible that the smaller response observed in WT mice is caused by the simultaneous activation of both NPR-A and NPR-C. Activation of NPR-A results in elevated intracellular cGMP levels (102). Based on this research we propose that BNP causes an increase in $I_{Ca,L}$ in a PDE-3 dependent manner. Specifically, we hypothesize that activation of NPR-A by BNP will increase cGMP levels and inhibit the activity of PDE-3, which would result in an elevated cAMP pool (due to less cAMP hydrolysis) and a stimulatory effect on $I_{Ca,L}$. It is also well known that BNP can activate NPR-C, which can result in decreased cAMP levels through inhibition of AC by G_i (7, 102, 112, 113). Accordingly, it is possible that when the cell lacks a functional NPR-C receptor, BNP only activates NPR-A without the inhibitory effects of NPR-C, which could result in a larger cAMP pool and thus a larger increase in $I_{Ca,L}$. If this hypothesis is correct we would have expected a decrease in $I_{Ca,L}$ when NPR-A was pharmacologically antagonized by A71915 due to activation of NPR-C only; however our data demonstrates that when BNP is applied in the presence of A71915, there is no significant effect on $I_{Ca,L}$. To explain this we propose that A71915 may partially antagonize NPR-C, due to its structural similarities to ANP. In the characterization of NPR-A antagonists, Delporte and colleagues state that a criterion for

NPR antagonism is the presence of a disulfide bridge (24). Since NPR-C and NPR-A bind the same NPs that both contain a disulfide bridge, it is possible that the criterion for binding to NPR-A is similar to NPR-C and therefore theoretically A71915 could antagonize NPR-C as well. Extensive literature review revealed no research that investigated the antagonistic effects of A71915 on NPR-C. Additional work will be required to determine if both NPR-A and NPR-C are activated in these experimental conditions

It is well established that NPR-A is a p-GC linked receptor and that upon activation it increases intracellular cGMP (102). Most studies investigating the effects of NPs on cardiac $I_{Ca,L}$ suggest that cGMP modulates $I_{Ca,L}$ due to changes in PKG phosphorylation (79, 108, 152, 153, 155). Importantly, cGMP can stimulate PDE2 activity and inhibit PDE3 activity, in addition to alter PKG mediated phosphorylation events (72, 153). Indeed, the results obtained in this study demonstrate that BNP increases $I_{Ca,L}$ and that the BNP effect on $I_{Ca,L}$ no longer occurs in the presence of PDE-3 inhibition. This however, does not rule out the possibility that PKG (another downstream cGMP target) is also contributing to the effect we have observed. Importantly, it appears that the conditions in which elevated cGMP elicit effects on $I_{Ca,L}$ are critical and may lead to the preferential activation of one cGMP-dependent signaling pathway over others. This is likely related to the high level of complexity involved in cGMP signaling in the heart. An elevation in cGMP results in the activation of PKG which has numerous phosphorylation targets, including ion channels such as cardiac L-type Ca^{2+} channels. Furthermore, cGMP can also stimulate or inhibit various PDEs, producing either synergistic or antagonistic effects with cAMP. In a recent study investigating the compartmentalization of cAMP and cGMP using fluorescence resonance energy transfer

(FRET) techniques in rat ventricular myocytes, it was demonstrated that cGMP elevation through soluble - GC (s-GC) activation resulted in PDE-3 inhibition and elevation in cAMP while simultaneous activation of PDE-2 resulted in decreased cAMP levels in two separate intracellular compartments (126). Additionally, activation of p-GC generated increased cGMP levels, that specifically activated PDE-2, resulting in decreased levels of cAMP (126). Thus, cGMP can potentially modulate $I_{Ca,L}$ by regulating PKG, PDEs or both.

Further adding to this complexity is the fact that there is significant cross talk between cGMP, cAMP and PDEs. It is likely because of this complexity, numerous studies (using different models and experimental conditions) have failed to observe consistent effects of cGMP on $I_{Ca,L}$. For example, direct application of intracellular cGMP has been shown to inhibit (64, 140, 141) and stimulate (96, 141) $I_{Ca,L}$ in ventricular myocytes. cGMP dependent activation of PKG has been shown to stimulate $I_{Ca,L}$ in newborn and juvenile rabbit ventricular cells, however no effect is seen in adult cells (39, 60). Other groups have found conflicting results in adult guinea pig ventricular myocytes, showing cGMP dependent activation of PKG which has an inhibitory effect on $I_{Ca,L}$ (64, 96). The role of PDEs in mediating the effects of cGMP has received much attention and continues to develop as new PDEs are discovered and more selective PDE blockers are developed. An early study showed that activation of a cGMP-stimulated PDE (likely PDE-2) caused $I_{Ca,L}$ inhibition in frog ventricular myocytes, as a result of elevated cAMP hydrolysis (40). However, in two similar studies that agree with our results, it was more recently shown that intracellular perfusion of cGMP in human atrial myocytes resulted in elevated $I_{Ca,L}$ activity in basal conditions. Furthermore, it has been demonstrated that cGMP inhibition of PDE-3 appears to dominate over cGMP

stimulation of PDE-2, resulting in a net increase in cAMP concentration upon cGMP elevation and that modulation of $I_{Ca,L}$ results from PKA phosphorylation events (108, 138). In a similar experiment using nitric oxide (NO) to elevate cGMP through the s-GC enzyme, Kirstein and colleagues demonstrated that upon s-GC activation (using SIN-1; a well characterized NO analogue) elevated cGMP, inhibited PDE-3 resulting in decreased cAMP hydrolysis and increased $I_{Ca,L}$ activity (55). Qvigstad and colleagues demonstrated that CNP activated NPR-B, and increased intracellular cAMP levels in β-AR prestimulated rat ventricular cells through cGMP inhibition of PDE-3 (107). Our data utilized BNP to activate NPR-A and elevate cGMP levels but still demonstrates similar results.

It is generally agreed that the cGMP/PDE-3/cAMP signaling pathway stimulates $I_{Ca,L}$ by activating PKA which is known to exert stimulatory effects on $I_{Ca,L}$. To thoroughly investigate the effects of NPs and specific components that mediate $I_{Ca,L}$, the role of PKG and PKA should be considered. PKG is generally thought to have an inhibitory effect on $I_{Ca,L}$ (13, 64, 67, 78, 96, 136, 140), however some studies have shown the stimulatory effect of PKG on cardiac myocytes (39). The potential use of PKG inhibitors in future studies are discussed below.

Pathophysiological Significance

NPs and their receptors have been associated with normal physiological regulation of electrical activity as well as specific cardiac arrhythmias, such as AF. A recently identified family of northern European ancestry contained 11 members with a heterozygous frameshift mutation in the gene encoding ANP (fsANP), resulting in high circulating levels of fsANP (45). The mutation in the fsANP gene causes a two base pair deletion in exon 3, which removes a stop codon, causing 12 additional amino acids to

attach to the C terminus of the mature peptide (25). Dickey and colleagues have recently shown that fsANP circulates at higher concentrations because of reduced proteolytic degradation, as a result of the endogenous enzymes inability to effectively degrade the circulating peptide (25). The fsANP peptide also causes a shortening of the monophasic AP duration, providing a substrate for AF (45). AF is the most common cardiac arrhythmia and affects a very large percentage of elderly individuals (20% in patients > 85 years old) (42). AF is an arrhythmia characterized by electrical changes in the atria that have been linked to changes in ion channel expression and behaviour. AF greatly increases susceptibility to stroke due to blood stasis and clot formation in the atria and can also lead to reduced cardiac output by altering ventricular pump function (15, 86). The molecular changes that occur during AF result in an arrhythmia that is very difficult to treat (151). AF animal models have shown that sustained atrial tachycardia pacing results in reduced $I_{Ca,L}$ and transient outward current densities which results in decreased AP duration and a substrate for AF formation (149). Due to the current limited understanding of AF, the clear association between ion channel function and AF, and its significant implications on healthcare, substantial efforts to further improve our understanding of the molecular and hormonal aspects of the disease are being undertaken. Interestingly, our results demonstrate that BNP in the presence of ISO works in an opposing manner to fsANP previously discussed. Since BNP lengthens the AP duration by increasing $I_{Ca,L}$, it is possible that BNP may protect against the occurrence of AF. Additionally, it has been shown that other NPs, such as CNP and BNP exert electrophysiological effects and influence AP duration and $I_{Ca,L}$ (112, 113, 123). There is clearly a relationship between NPs and cardiac arrhythmias such as AF, suggesting an

opportunity to use NPs as a tool to further understand the molecular components of the disease as well as a therapeutic target.

Study Limitations

The preceding study contained experimental limitations that could be addressed in future experiments. This study only investigated the effects of BNP on $I_{Ca,L}$ and several K^+ currents, while many ion channels exist in mouse atrial myocytes and could be modulated by BNP. Additionally this study utilized only mouse atrial myocytes. While mice are a generally accepted model for the basic sciences and provide valuable information regarding human physiology, expression patterns of various intracellular components such as PDEs are different between species (72). Future experiments could address these issues by investigating the effects of BNP on all ionic currents in atrial myocytes obtained from other species.

We have shown that BNP has no effect in the presence of milrinone, however several questions arise from this result. Firstly, our experiments have not definitively shown that the intracellular cAMP pool has not been saturated in the presence of ISO and milrinone, therefore limiting the effect of BNP on $I_{Ca,L}$. In order to definitively prove our hypothesis that BNP increases AP duration and $I_{Ca,L}$ by inhibiting PDE-3 we would have to demonstrate that the intracellular cAMP pool has not been maximally saturated. To experimentally prove that the system hasn't been saturated, an identical experiment could be completed in the presence of another PDE inhibitor (rolipram for PDE-4) or a lower concentration of ISO. Assuming BNPs effect is not directly mediated by PDE-4, we would expect that BNP would still elicit an increase in $I_{Ca,L}$ in the presence of rolipram regardless of the elevated cAMP concentration. Similarly, we would expect that milrinone in the presence of a lower concentration of ISO (0.1nM) would still block the

BNP effect on $I_{Ca,L}$. Secondly, we have not ruled out the possibility that PDE-3 is involved in a parallel pathway to BNP and that both molecules target a similar downstream pool of cAMP to modulate $I_{Ca,L}$ activity. To definitively prove that BNPs effects on $I_{Ca,L}$ are directly mediated by PDE-3, the use of a cardiac specific PDE-3 KO animal model would be critical.

It is well documented that NPR-A activation results in increased intracellular cGMP levels (102). We presume that BNP stimulates $I_{Ca,L}$ through a cGMP/PDE-3/cAMP dependent pathway, however this particular study did not measure intracellular cGMP or cAMP concentrations. In order to directly assess the effects of BNP on intracellular cGMP and cAMP concentrations cGMP and cAMP assays could be completed (24, 83).

Future Direction

The purpose of this study was to investigate the electrophysiological effects of BNP in mouse right atrial myocytes. This study successfully determined that BNP activated NPR-A and increased $I_{Ca,L}$ in the presence of a β-AR agonist, however when PDE-3 was inhibited BNP no longer increased $I_{Ca,L}$ activity. While this appears to be the dominant pathway in the present experimental conditions there are numerous additional experiments to be completed that would provide more insight into this complex system, which will be discussed briefly in the following section.

This study showed that BNP increases $I_{Ca,L}$ activity through NPR-A activation, and that BNP no longer increased $I_{Ca,L}$ in the presence of a PDE-3 inhibitor. Since NPR-A activation results in increased intracellular cGMP levels (102) which can inhibit the ability for PDE-3 to hydrolyze cAMP (72), we propose that BNP increases $I_{Ca,L}$ activity by indirectly raising cAMP levels due to a reduction in cAMP hydrolysis by PDE3. NPs

have been shown to inhibit PDE3 activity in other studies (43). It is well known that cGMP and cAMP have opposing effects on cardiac function (153), and therefore to determine the exact role of both secondary messengers, future experiments would have to involve pharmacological inhibition of various second messenger downstream targets. To investigate the role of PKG, KT5823 (a commercially available potent PKG inhibitor) could be utilized. A similar experiment to determine the role of PKA could be performed using the PKA inhibitor; PKI (6-22) Amide. Based on these experiments, the contribution of each pathway could be separately determined providing evidence that multiple pathways may be eliciting opposing effects.

Since cGMP activates PDE-2 and inhibits PDE-3, it would be valuable to investigate BNPs effects in the presence of a PDE-2 inhibitor such as EHNA (79). PDE-2 hydrolyzes cAMP in the presence of cGMP (Fig. 3) and therefore when cGMP levels are elevated there is increased activation of PDE-2 and increased hydrolysis of cAMP (72). One may expect that $I_{Ca,L}$ activity may be larger when a PDE-2 inhibitor is combined with BNP, compared to when only BNP is present, because of a higher level of intracellular cAMP. The role of PDEs as mediators of cardiac function in both a normal and pathological state is a rapidly growing area of investigation. This study provides evidence that PDEs may play an important role in mediating NPs effects on cardiac electrophysiology, and that much more attention both clinically and experimentally is required to further understand this complex system.

FIGURE 18:

Schematic showing the downstream targets involved in NPR-A activation by BNP. Red lines indicate an inhibitory pathway, black arrows represent an activating pathway. PDE-2/3; phosphodiesterase 2 or 3, BNP/NPR-A/C; Brain-type natriuretic peptide, natriuretic peptide receptor – A/C, PKG; protein kinase – G, PKA; protein kinase – A, cAMP; cyclic adenosine monophosphate, cGMP; cyclic guanosine monophosphate, ATP; adenosine triphosphate, GTP; guanosine triphosphate, B-AR; β - adrenergic receptor, Gi; inhibitory G-protein, α, β, γ; accessory G-protein subunits- α, β, γ. The present study identified a dominant role of PDE-3 in mediating the effects of BNP on $I_{Ca,L}$. As indicated by the question marks (?), additional pathways may also be involved in these or other experimental condition.

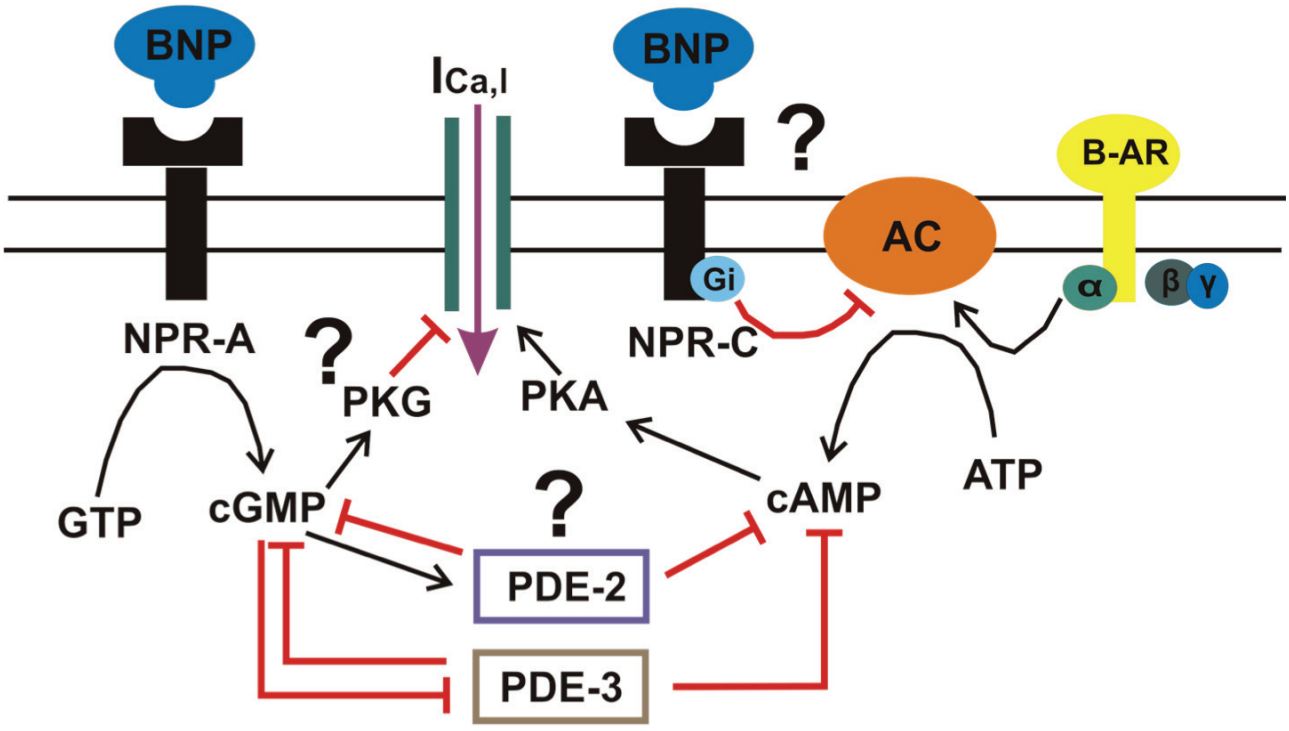

BNP
BNP
ICa,I
?
B-AR
AC
Gi
α
β
γ
NPR-A
?
PKG
PKA
NPR-C
GTP
cGMP
?
cAMP
ATP
PDE-2
PDE-3

Figure 18.

CHAPTER 5: CONCLUSIONS

In summary, our findings demonstrate that in mouse atrial myocytes, application of BNP at physiological concentrations increases AP duration by activating NPR-A and stimulating $I_{Ca,L}$. We have also shown that in the presence of PDE-3 inhibition, BNP can no longer accentuate $I_{Ca,L}$. It is well established that NPR-A activation results in increased intracellular cGMP levels (102). Since BNP can no longer stimulate $I_{Ca,L}$ in the presence of PDE-3 inhibition, we propose that BNP increases intracellular cGMP which inhibits PDE-3. Since PDE-3 is responsible for hydrolyzing cAMP (72), intracellular cAMP levels are predicted to be elevated in the presence of BNP, which could lead to the PKA dependent phosphorylation of $I_{Ca,L}$. Additionally, we only observed these effects when cells were prestimulated by the β-AR agonist, isoproterenol, and therefore conclude that the stimulatory effect of BNP on $I_{Ca,L}$ is highly dependent on the intracellular cAMP concentration.

It is clear that our current understanding of the cGMP/cAMP signaling pathways and their modulation by PDEs is limited. Various groups have shown opposing results in different species and conditions. Furthermore, it appears that these complex interacting pathways are highly dependent on concentrations of the various pathway components as well as the subcellular localization of these components. The system which mediates NPs effects on $I_{Ca,L}$ involves multiple interacting pathways that will not be solved in a single study but rather a combination of studies approaching the issue in different ways. The relationship between NPs and atrial arrhythmias such as AF are even less understood and lend further evidence to the complexity of the NP signaling pathways. This study provides further evidence that NPs do in fact elicit electrophysiological effects in the heart through a complex cross-talk interaction of intracellular second messengers. The

results obtained in this study will undoubtedly contribute to further understanding the mechanisms by which NPs act in the heart in normal physiological conditions as well as their relationship to cardiac diseases such as AF.

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