Effects of Wildtype and Mutant Forms of Atrial Natriuretic Peptide on Atrial Electrophysiology and Arrhythmogenesis

Running title: Hua et al.; Effects of ANP and mutant ANP in the atria

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Abstract:

**Background** - Atrial natriuretic peptide (ANP) is a hormone with numerous beneficial cardiovascular effects. Recently, a mutation in the atrial natriuretic peptide (ANP) gene, which results in the generation of a mutant form of ANP (mANP), was identified and shown to cause atrial fibrillation (AF) in people. The mechanism(s) through which mANP causes AF are unknown. Our objective was to compare the effects of wildtype ANP and mANP on atrial electrophysiology in mice and humans.

**Methods and Results** - Action potentials (APs), L-type Ca\(^{2+}\) currents (I_{Ca,L}) and Na\(^{+}\) current (I_{Na}) were recorded in atrial myocytes from wildtype or natriuretic peptide receptor C knockout (NPR-C\(^{-/-}\)) mice. In mice ANP and mANP (10 – 100 nM) had opposing effects on atrial myocyte action potential (AP) morphology and I_{Ca,L}. ANP increased AP upstroke velocity (V_{max}), AP duration, and I_{Ca,L} similarly in wildtype and NPR-C\(^{-/-}\) myocytes. In contrast mANP decreased V_{max}, AP duration and I_{Ca,L} and these effects were completely absent in NPR-C\(^{-/-}\) myocytes. ANP and mANP also had opposing effects on I_{Ca,L} in human atrial myocytes. In contrast, neither ANP nor mANP had any effect on I_{Na} in mouse atrial myocytes. Optical mapping studies in mice demonstrate that ANP sped electrical conduction in the atria whereas mANP did the opposite and slowed atrial conduction. Atrial pacing in the presence of mANP induced arrhythmias in 62.5% of hearts while treatment with ANP completely prevented the occurrence of arrhythmias.

**Conclusions** - These findings provide mechanistic insight into how mANP causes AF and demonstrate that wildtype ANP is antiarrhythmic.

**Key words:** natriuretic peptide, action potential, calcium channel, atrial fibrillation
Introduction

Atrial fibrillation (AF) is a highly prevalent cardiac arrhythmia\(^1,2\) and is a major clinical problem due to the fact that current therapeutic approaches have significant limitations.\(^3\) AF, which is characterized by the very rapid and irregular activation of the atria, can occur in association with a number of cardiovascular disorders (i.e. heart failure, hypertension) or in the absence of structural disease (lone AF).\(^4,5\) Lone AF is known to occur in association with genetic mutations in, for example, ion channels and gap junction proteins.\(^6-9\)

Recently, a mutation in the \textit{NPPA} gene, which encodes the cardioprotective hormone atrial natriuretic peptide (ANP)\(^10\) was identified and linked to the occurrence of AF.\(^11\) This mutation is characterized by a 2 base pair deletion in exon 3, which causes a frameshift that abolishes the stop codon and extends the normal reading frame. This \textit{NPPA} mutation results in the production of a 40 amino acid mutant ANP (mANP) consisting of the normal 28 amino acid ANP with an abnormal 12 amino acid carboxyl terminal extension.

ANP is one member of a family of natriuretic peptides (NPs) that also includes B-type NP (BNP) and C-type NP (CNP).\(^10,12\) ANP is produced in atrial myocytes and is released into the circulation in response to atrial stretch. ANP is best known for its ability to regulate blood volume and blood pressure through effects in the kidneys (natriuresis, diuresis) and blood vessels (smooth muscle relaxation, endothelial permeability).\(^10\) We have recently demonstrated that NPs have potent effects on cardiac electrophysiology.\(^13-16\) For example, BNP and CNP can increase action potential (AP) duration in association with increases in L-type \textit{Ca}\(^{2+}\) current (\textit{I}_{\text{Ca,L}}) in atrial myocytes.\(^13\) We have also shown that wildtype NPs (BNP, CNP) can potently increase electrical conduction in the sinoatrial node and atrial myocardium.\(^17\)

Wildtype ANP is able to elicit its physiological effects by binding to two NP receptors
called NPR-A and NPR-C.\textsuperscript{10,18,19} NPR-A is a particulate guanylyl cyclase (GC) receptor. When bound to ANP, NPR-A increases GC activity and the production of cyclic GMP (cGMP), which can modulate several downstream signaling proteins including protein kinase G (PKG) and phosphodiesterases (PDEs).\textsuperscript{10,20} NPR-C is coupled to the activation of inhibitory G proteins (G\textsubscript{i}) that reduce adenylyl cyclase (AC) activity and intracellular cyclic AMP (cAMP) levels.\textsuperscript{21,22} NPR-C activates G\textsubscript{i} through a specific G\textsubscript{i}-activator domain located in the 37 amino acid intracellular portion of the receptor.\textsuperscript{23,24}

mANP was found to circulate at concentrations 5 – 10 times greater than wildtype ANP in patients affected by this mutation and it has been hypothesized that this leads to enhanced effects of ANP that could create an electrophysiological substrate for AF.\textsuperscript{11,25} However, the ionic and molecular mechanism(s) through which mANP causes AF in humans are currently unknown. Accordingly, the goal of this study was to compare the effects of wildtype ANP and mANP on atrial electrophysiology in mouse and human atrial tissues. Our findings demonstrate that ANP and mANP have opposing effects on atrial electrophysiology due to the activation of distinct signaling pathways. These findings may explain how mANP leads to AF and also provide new insight into the electrophysiological effects of wildtype ANP in the atria.

**Methods**

An expanded methods section is available in the Supplemental Material.

**Mice**

This study used wildtype and NPR-C knockout (NPR-C\textsuperscript{\textminus\textminus})\textsuperscript{14,26} mice between the ages of 10-15 weeks. All experimental procedures were approved by the Dalhousie University Committee for Laboratory Animals and followed the guidelines of the Canadian Council on Animal Care.
Human tissue samples and patient characteristics

Right atrial tissue samples were obtained from cardiac surgery patients who were in sinus rhythm and had no prior history of arrhythmia. Patient characteristics are listed in Supplemental Table 1. All patients gave informed written consent to participate in our study, which was approved by the Research Ethics Board of the Capital Health District Authority.

Peptides

This study used wildtype ANP and mANP, which were both obtained from Bachem (Torrance, CA). mANP (amino acid sequence: SLRRSCFGGRMDRIGAQSGGLCNSFRYRITAREDKQGWA)\textsuperscript{11,27} was synthesized and confirmed by mass spectrometry, amino acid analysis and HPLC by Bachem.

Patch-clamping of atrial myocytes from mice and humans

Right or left atrial myocytes were isolated from mice enzymatically as we have described previously.\textsuperscript{13,28} Human right atrial myocytes were isolated from tissue samples obtained from cardiac surgery patients using similar procedures to those in mice, with some specific modifications as outlined in the Supplemental Material. Mouse and human atrial myocytes were used to record action potentials (APs) using the perforated patch-clamp technique and/or $I_{\text{Ca,L}}$ and $\text{Na}^+$ current ($I_{\text{Na}}$) using the whole-cell patch-clamp technique. The solutions and experimental protocols for these experiments are available in the Supplemental Material.

Quantitative PCR

Quantitative gene expression in human right atrial samples was performed using approaches we have described previously.\textsuperscript{13,28} Intron spanning primers were designed for human NPR-A, NPR-B, NPR-C and GAPDH. Experimental protocols and primer sequences are provided in the Supplemental Material.
cAMP assay

cAMP was measured in isolated mouse right atrial myocytes using a HTRF cAMP Femto2 kit (Cisbio US) according to manufacturer’s instruction (see Supplemental Material).

High resolution optical mapping

Patterns of electrical conduction, atrial effective refractory period (ERP) and susceptibility to arrhythmias were studied in isolated atrial preparations using optical mapping techniques we have previously described.\textsuperscript{17,28,29} Optical mapping was done using the voltage sensitive dye di-4-ANEPPS (10 \( \mu \)M) and blebbistatin (10 \( \mu \)M) to suppress contractile activity. All analyses were performed using custom software. Details are provided in the Supplemental Material.

To measure ERP atrial preparations were given an 8 stimulus drive train at a cycle length of 90 ms followed by a single extra stimulus at progressively shorter cycle lengths. ERP was defined as the shortest coupling interval allowing for capture of the atrial preparation.

For arrhythmia studies we used S1-S2 pacing protocols in which the atria were paced at a fixed cycle length (90 ms) and then given 3-6 premature stimuli at progressively shorter cycle lengths (S2). The details for these experimental approaches are provided in the Supplemental Material.

Statistical analysis

All data are presented as means \( \pm \) standard deviation (SD). Data were analyzed using a Student’s \( t \)-test, one-way ANOVA with Tukey’s posthoc test, one-way repeated measures ANOVA with Tukey’s posthoc test or Fisher’s exact test as indicated in each figure legend. \( P<0.05 \) was considered significant.
Results

**ANP and mANP have opposing effects on atrial myocyte electrophysiology in mice**

Although circulating concentrations of mANP were found to be higher than wildtype ANP in individuals who have this NPPA mutation\(^1\)\(^1\) our goal was to determine if ANP and mANP have distinct effects on atrial electrophysiology independent of differences in dose. Therefore, we initially measured the effects of identical doses of wildtype ANP (100 nM) and mANP (100 nM) on AP morphology in mouse right atrial myocytes in basal conditions (Supplemental Fig. 1A). These measurements demonstrate that neither ANP nor mANP had any effects on mouse atrial AP properties in basal conditions (Supplemental Fig. 1B; Supplemental Tables 2 and 3). Since we have previously shown that \(I_{Ca,L}\) is a major target of NPs in the atria\(^1\),\(^3\)\(^,\)\(^1\)\(^5\), we also measured the effects of ANP and mANP (100 nM doses) on basal \(I_{Ca,L}\) in mouse right atrial myocytes (Supplemental Figs. 1C, 1F). In agreement with the absence of effects on AP morphology, ANP and mANP had no effects on mouse atrial \(I_{Ca,L}\) density (Supplemental Figs. 1D, 1G) or \(I_{Ca,L}\) activation kinetics (Supplemental Figs. 1E, 1H; Supplemental Tables 4 and 5) in basal conditions.

The absence of effects of ANP on AP morphology and \(I_{Ca,L}\) in basal conditions is consistent with our prior studies showing that the related peptides BNP and CNP only affect mouse atrial myocyte electrophysiology in the presence of the \(\beta\)-adrenergic receptor agonist isoproterenol (ISO).\(^1\)\(^3\) Accordingly, we next measured the effects of ANP and mANP on AP morphology in mouse atrial myocytes following application of ISO (10 nM). In these experiments ANP and mANP were each applied at doses of 10 and 100 nM in order to consider the dose dependence of each of these peptides.

The effects of ANP (10 and 100 nM) on mouse atrial AP morphology are illustrated in
Fig. 1. Representative recordings (Fig. 1A and 1D) as well as summary data illustrate that ISO increased ($P<0.05$) the AP maximum upstroke velocity ($V_{\text{max}}$; Figs. 1B and 1E) as well as AP duration at 50% (APD$_{50}$), 70% (APD$_{70}$) and 90% (APD$_{90}$) repolarization (Figs. 1C and 1F). Application of ANP at both doses further increased ($P<0.05$) $V_{\text{max}}$ (Figs. 1B and 1E; Supplemental Tables 6 and 7) and APD at all repolarization times (Figs. 1C and 1F; Supplemental Tables 6 and 7). There was no difference in the magnitude of the increases in $V_{\text{max}}$ ($P=0.841$), APD$_{50}$ ($P=1.000$), APD$_{70}$ ($P=0.697$) or APD$_{90}$ ($P=0.146$) elicited by ANP at doses of 10 and 100 nM (Fig 1G). The effects of ANP were fully reversible on washout.

Surprisingly, mANP (10 and 100 nM), had the opposite effects to ANP on mouse atrial AP morphology. Representative recordings (Figs. 2A and 2D) and summary data illustrate that following application of ISO, mANP reversibly decreased ($P<0.05$) $V_{\text{max}}$ (Figs. 2B and 2E; Supplemental Tables 8 and 9) and APD at all repolarization times (Figs. 2C and 2F; Supplemental Tables 8 and 9). There was no difference in the magnitude of the decreases in $V_{\text{max}}$ ($P=0.112$), APD$_{50}$ ($P=0.289$), APD$_{70}$ ($P=0.525$) or APD$_{90}$ ($P=0.825$) elicited by mANP at doses of 10 and 100 nM (Fig. 2G).

The individuals affected by the $NPPA$ mutation being studied were all heterozygous and thus produce both wildtype ANP and mANP.$^{11}$ Accordingly, we next studied the effects of ANP and mANP in combination on mouse atrial myocyte AP morphology. In these studies we initially applied ISO (10 nM) and then simultaneously applied ANP and mANP (100 nM each). Representative AP recordings (Fig. 3A) and summary data (Figs. 3B and 3C) demonstrate that, in the presence of ISO, combined application of equimolar doses of ANP and mANP had no effect on $V_{\text{max}}$ ($P=0.070$), APD$_{50}$ ($P=0.387$), APD$_{70}$ ($P=0.127$) or APD$_{90}$ ($P=0.067$) although there was an overall trend toward a reduction in these AP parameters (Supplemental Table 10).
These findings suggest that when present together at equal doses the effects of ANP and mANP negate each other.

Next we measured the effects of ANP and mANP (100 nM each) on I_{Ca,L} in the presence of ISO (10 nM) in mouse right atrial myocytes (Fig. 4). Representative I_{Ca,L} recordings (Fig. 4A) and time course data (Fig. 4B) demonstrate that ISO increased atrial I_{Ca,L} and subsequent application of ANP further augmented I_{Ca,L}. These effects were reversible upon washout. The stimulatory effects of ISO and ANP are also evident in summary current-voltage (IV) relationships (Fig. 4C). The effects of ANP on atrial I_{Ca,L} were further studied by performing steady-state conductance analysis (Fig. 4D; Supplemental Table 11). These measurements demonstrate that ISO increased I_{Ca,L} maximum conductance (G_{max}) in association with a negative shift (P<0.05) in the V_{1/2} of channel activation (V_{1/2(act)}) from -10.2±0.3 mV to -16.3±1.1 mV. Application of ANP in the presence of ISO further increased (P<0.05) G_{max} and shifted (P<0.05) the V_{1/2(act)} to -18.6±1.1 mV.

Consistent with the AP data presented above, representative I_{Ca,L} recordings (Fig. 4E), time course data (Fig. 4F) and summary IV relationships (Fig. 4G) demonstrate that mANP (100 nM) had the opposite effects to ANP and decreased atrial I_{Ca,L} in the presence of ISO. Conductance analysis demonstrates that mANP decreased (P<0.05) I_{Ca,L} G_{max} and shifted (P<0.05) the V_{1/2(act)} to more positive membrane potentials (Fig. 4H; Supplemental Table 12).

Thus, as was the case for AP measurements, ANP and mANP have distinct and opposing effects on atrial I_{Ca,L}.

To assess the dose dependence of the effects of ANP and mANP on atrial I_{Ca,L} we measured the effects of each peptide at concentrations between 1-100 nM (in the presence of ISO) on peak right atrial I_{Ca,L} (Supplemental Fig. 2). These studies demonstrate that ANP and
mANP modulated ICa,L at each dose tested. There was no significant difference in the magnitude of the effects of ANP (P=0.30) and mANP (P=0.10) at doses of 10 and 100 nM although there was a trend towards larger effects at the higher dose. In contrast the effects of ANP and mANP at 1 nM doses were smaller (P<0.05) than those at the higher two doses. We also measured the effects of ANP and mANP (100 nM each) on ICa,L in mouse left atrial myocytes (Supplemental Fig. 3). These data illustrate that the effects of ANP and mANP on ICa,L are very similar in right and left atrial myocytes.

Because ANP and mANP had opposing effects on AP Vmax, which is importantly influenced by sodium currents (INa), we also measured the effects of ISO (10 nM) and each peptide (100 nM) on mouse atrial INa (Supplemental Fig. 4; Supplemental Tables 13 and 14). INa IV relationships and steady state conductance analysis demonstrate that neither ISO, ANP or mANP had any effects on atrial INa, suggesting that ICa,L is the key ion channel regulated by these peptides (see Discussion).

Role of NPR-A and NPR-C in mediating the effects of ANP and mANP on ICa,L
We have previously demonstrated that NPs (BNP, CNP) can modulate ICa,L via the GC-linked NPR-A/B receptors as well as NPR-C. Furthermore, ANP and mANP have each been shown to bind NPR-A and NPR-C. To determine the mechanism for the opposing effects of ANP and mANP on atrial ICa,L we measured the effects of these two peptides (100 nM doses) on ICa,L (in the presence of ISO; 10 nM) in right atrial myocytes from NPR-C−/− mice (Fig. 5). These data demonstrate that ANP (Figs. 5A-C) increased ISO stimulated ICa,L and that the magnitude of the effect of ANP was very similar to that observed in wildtype mice (compare to Fig. 4).

Strikingly, the inhibitory effects of mANP on ICa,L (in the presence of ISO) were completely absent in atrial myocytes from NPR-C−/− mice. The data in Figs. 5D-F demonstrate
that ISO increased \( I_{\text{Ca,L}} \) as expected, but that mANP had no effects on \( I_{\text{Ca,L}} \) density. Thus, the ability of ANP to increase \( I_{\text{Ca,L}} \) is maintained in NPR-C\(^{-/-}\) atrial myocytes, whereas the inhibitory effect of mANP on \( I_{\text{Ca,L}} \) is absent mice lacking NPR-C receptors. This suggests that ANP and mANP are signaling via separate pathways.

Because the stimulatory effect of ANP on atrial \( I_{\text{Ca,L}} \) was maintained in NPR-C\(^{-/-}\) atrial myocytes we hypothesized that this effect must be mediated by NPR-A, the only other receptor that ANP binds to physiologically.\(^{10}\) This hypothesis was tested using the NPR-A antagonist A71915.\(^{30}\) We have previously demonstrated that A71915 has no direct effects on atrial \( I_{\text{Ca,L}} \); therefore, we applied A71915 (500 nM) concomitantly with ISO (10 nM) for 5 min followed by application of ANP (100 nM) in mouse right atrial myocytes. These data (Supplemental Fig. 5) demonstrate that A71915 completely blocked the ability of ANP to increase atrial \( I_{\text{Ca,L}} \).

**ANP and mANP have opposing effects on cAMP production in mouse atrial myocytes.**

The data described above demonstrate that ANP increases \( I_{\text{Ca,L}} \) via NPR-A while mANP decreases \( I_{\text{Ca,L}} \) via NPR-C. We have previously demonstrated that NPR-A mediated increases in \( I_{\text{Ca,L}} \) involve the inhibition of phosphodiesterase 3 (PDE3) by cGMP\(^{13}\), which would increase cAMP by preventing its hydrolysis. Conversely, NPR-C activation is known to reduce cAMP levels via the activation of Gi proteins.\(^{23,31}\) Accordingly, we hypothesized that the effects of ANP would be associated with increased cAMP levels while the effects of mANP would be associated with decreased cAMP production. This was tested using a cAMP assay in isolated mouse right atrial myocytes. In these experiments (Supplemental Fig. 6), the effects of ANP (100 nM) and mANP (100 nM) on intracellular cAMP levels were measured in basal conditions and in the presence of ISO (10 nM).

Consistent with an absence of effects on mouse atrial myocyte electrophysiology (AP
morphology, $I_{Ca,L}$) in basal conditions neither ANP ($P=0.463$) nor mANP ($P=0.445$) had any effect on cAMP concentrations in the absence of ISO. In contrast, in the presence of ISO, ANP and mANP had opposing effects on intracellular cAMP concentrations. Specifically, ISO increased ($P<0.05$) cAMP as expected. Application of ANP in the presence of ISO resulted in a further increase in cAMP ($P<0.05$) whereas application of mANP in the presence of ISO resulted in lower cAMP concentrations ($P<0.05$). Thus, the opposing effects of ANP and mANP on atrial myocyte electrophysiology (in the presence of ISO) occur in association with opposing effects of these two peptides on intracellular cAMP concentrations.

**Effects of ANP and mutant ANP on human right atrial myocyte $I_{Ca,L}$**

The next set of experiments sought to translate our findings in mouse atrial myocytes to the human heart. Using quantitative PCR, we assessed the mRNA expression pattern of all three NPRs in human right atrial samples obtained from patients undergoing cardiac surgery (Supplemental Fig. 7). These measurements demonstrate that all three NPRs are expressed in human right atrium and that NPR-C is more highly expressed ($P<0.05$) than NPR-A and NPR-B. We also isolated atrial myocytes from human right atrial appendage samples and measured the effects of identical doses of ANP (100 nM) and mANP (100 nM) on human right atrial $I_{Ca,L}$ in baseline conditions and in the presence of ISO (10 nM). Human $I_{Ca,L}$ was recorded using the same voltage clamp protocols used in our mouse recordings. Supplemental Fig. 8 illustrates a representative example of a family of $I_{Ca,L}$ recordings at membrane potentials between -60 mV and +60 mV along with the human $I_{Ca,L}$ IV relationship, which was very similar to that measured in mouse atrial myocytes (Supplemental Fig. 1; Fig. 4).

Representative $I_{Ca,L}$ recordings (Fig. 6A) and summary data (Fig. 6B) illustrate that ANP reversibly increased basal $I_{Ca,L}$ in human atrial myocytes. The magnitude of this effect was
comparable to that seen in mice. Notably; however, this stimulatory effect of ANP on human atrial \( I_{\text{Ca,L}} \) was observed without the requirement for pre-stimulation with ISO. Next, we measured the effects of mANP on basal \( I_{\text{Ca,L}} \) in human right atrial myocytes. Representative recordings (Fig. 6C) and summary data (Fig. 6D) demonstrate that, as in the mouse, mANP had no effect on \( I_{\text{Ca,L}} \) density in human atrial myocytes in the absence of ISO. Finally, we measured the effects of ANP and mANP on human right atrial \( I_{\text{Ca,L}} \) in the presence of ISO (10 nM; Figs. 6E-H). Consistent with our findings in mouse myocytes, ANP increased \((P<0.05)\) ISO-stimulated \( I_{\text{Ca,L}} \) while mANP inhibited \((P<0.05)\) ISO-stimulated \( I_{\text{Ca,L}} \) in human atrial myocytes. These effects of ANP and mANP were fully reversible following washout.

Our findings in human right atrial myocytes demonstrate that, similar to the mouse, the main effect of ANP is to increase \( I_{\text{Ca,L}} \) while mANP decreases \( I_{\text{Ca,L}} \); however, a notable difference was that ANP was able to stimulate \( I_{\text{Ca,L}} \) in basal conditions and in the presence of ISO in human atrial myocytes, whereas in mice ANP only increased \( I_{\text{Ca,L}} \) in the presence of ISO. We have previously shown that BNP and CNP (NPs closely related to ANP) also increase mouse atrial \( I_{\text{Ca,L}} \) in the presence of ISO, but not in basal conditions, and that this is because mouse atria do not have constitutive PDE3 activity, which is necessary for the stimulatory effects of these NPs on atrial \( I_{\text{Ca,L}} \).\(^{13,32}\)

Based on this, we hypothesized that human atrial myocytes must have constitutive PDE3 activity, which would explain the ability of ANP to increase human atrial \( I_{\text{Ca,L}} \) in basal conditions. To test this hypothesis we measured the effects of the PDE3 inhibitor milrinone (10 µM) on basal \( I_{\text{Ca,L}} \) in human right atrial myocytes (Supplemental Fig. 9). These data illustrate that milrinone potently, and reversibly, increased human atrial \( I_{\text{Ca,L}} \) confirming that, unlike the mouse\(^{32}\), human atrial myocytes display constitutive PDE3 activity.
ANP and mANP have opposing effects on electrical conduction in the mouse atria

Alterations in electrical conduction within the atria can contribute importantly to susceptibility to AF\(^4,33\); therefore, we used high resolution optical mapping to determine the effects of ANP and mANP on activation patterns in the mouse atria. These studies were performed in an isolated atrial preparation\(^17,34,35\) (Supplemental Fig. 10) and used an intermediate dose of ANP (50 nM) and mANP (50 nM). Representative activation maps are presented in control conditions, after application of ISO (10 nM) and following subsequent application of ANP or mANP in the presence of ISO (Fig. 7A). These activation maps demonstrate that the first electrical breakthrough occurs in the right atrial posterior wall, which corresponds to the location of the sinoatrial node\(^17\), and that conduction propagates from this site into the right and left atrial appendages. As expected, ISO sped conduction time throughout the atrial preparation (note fewer isochrones with more space between successive isochrones in the presence of ISO).

Application of ANP in the presence of ISO further sped conduction whereas mANP had the opposite effect and slowed electrical conduction time across the atrial preparation.

To further quantify the effects of ANP and mANP (in the presence of ISO) on electrical conduction we measured local conduction velocity (CV) in the right and left atrial appendages using a previously described approach\(^17\) (see methods). Initially, CV was measured in atrial preparations in sinus rhythm where the cycle length was free to change. Summary data demonstrate that, as expected, ISO decreased (\(P<0.05\)) cycle length, which corresponds to an increase in heart rate. ANP further shortened (\(P<0.05\)) the ISO stimulated cycle length (Fig. 7B). In contrast, mANP tended to increase cycle length, although this did not reach statistical significance (\(P=0.09\); Fig. 7B).

Analysis of atrial CV (in sinus rhythm) demonstrates that ISO increased (\(P<0.05\)) CV in
the right and left atria and that subsequent application of ANP in the presence of ISO further increased \( P<0.05 \) right atrial and left atrial CVs (Fig. 7C). These changes in CV are consistent with the effects of ISO and ANP on atrial action potential \( V_{\text{max}} \) (Fig. 1), which is a key determinant of CV in the heart.\(^{36}\) Once again, mANP had the opposite effects to ANP and decreased \( P<0.05 \) CV in the right and left atria (Fig. 7D). This is in agreement with the finding that mANP also decreased \( V_{\text{max}} \) (Fig. 2) in isolated atrial myocytes. To account for the possibility of rate dependent effects, we also measured right and left atrial CV in atrial preparations paced at a fixed cycle length of 90 ms (Figs. 7E and 7F). These data demonstrate very similar effects of ANP and mANP on atrial CV to those observed in sinus rhythm.

Our optical mapping studies also enabled us to assess changes in AP duration in the intact atrial preparation by measuring optical APs (OAPs) (Supplemental Fig. 11). OAPs were measured in the right atrial myocardium following application of ISO (10 nM) and then ANP or mANP (50 nM each). ISO increased \( P<0.05 \) APD\(_{70} \) in atrial preparations and, consistent with our studies in isolated myocytes, subsequent application of ANP further increased \( P<0.05 \) APD\(_{70} \) while mANP decreased ISO-stimulated APD\(_{70} \).

Since changes in AP duration are known to affect atrial ERP we used programmed stimulation to measure the effects of ISO, ANP and mANP on ERP in isolated atrial preparations (Supplemental Fig. 11). Consistent with increases in AP duration in isolated myocytes and intact atrial preparations, ISO (10 nM) increased \( P<0.05 \) atrial ERP. Once again, ANP (50 nM) and mANP (50 nM) had opposite effects on refractoriness whereby ANP increased \( P<0.05 \) atrial ERP and mANP decreased \( P<0.05 \) atrial ERP.

**Effects of ANP and mANP on arrhythmogenesis**

Finally, we used high resolution optical mapping in conjunction with atrial pacing/programmed
stimulation to study the effects of ANP and mANP on inducibility of arrhythmias in mouse atrial preparations. These pacing studies were performed in atrial preparations in the presence of ISO (10 nM) and either ANP (50 nM) or mANP (50 nM) in order to mimic the conditions in which the two peptides elicited opposing effects on atrial conduction and ERP. Figs 8A and 8B illustrate activation maps before and after atrial pacing in the presence of ISO and ANP. These images demonstrate that the heart was in sinus rhythm and activation patterns were normal (i.e. conduction initiated in the right atrial posterior wall and then propagated uniformly throughout the atrial preparation) before and after pacing.

Fig. 8C illustrates a representative activation map in the presence of ISO and mANP before pacing. This map demonstrates a normal conduction pattern although, consistent with the data in Fig. 7, conduction was slowed in the presence of mANP compared to ANP. Strikingly, atrial pacing in the presence of mANP resulted in the induction of atrial arrhythmias (Fig. 8D). Specifically, delivery of premature stimuli resulted in the occurrence of multiple points of simultaneous activation (red color) including ectopic foci in the right atrium near the pectinate muscles. These ectopic foci were associated with a re-entrant pattern of conduction (white arrow in Fig. 8D) in the right atrium. In this example, we also observed conduction to the left atrium via an activation point in the right atrial posterior wall that was similar to the normal initial activation point before pacing (black arrow in Fig. 8D); however, conduction to the right atrial appendage from the normal excitation point was blocked. In other instances, we observed similar re-entrant patterns of conduction in the right atrium with no propagation to the left atrium (not shown). Summary data (Fig. 8E) demonstrate that in the presence of ISO and ANP all atrial preparations remained in normal sinus rhythm and no arrhythmias were induced following atrial pacing in these conditions. In contrast, we observed arrhythmias in 62.5% of hearts (5/8 hearts;
P<0.05) following atrial pacing in the presence of ISO and mANP. In all cases these arrhythmias were characterized by disorganized activation patterns, re-entrant conduction patterns and/or multiple ectopic foci similar to those shown in Fig. 8. Thus, mANP treated hearts were highly susceptible to induced arrhythmias while hearts treated with ANP were protected.

Discussion

In this study we have used mouse and human tissues to compare the effects of wildtype ANP and a mutant form of ANP (mANP) that has been associated with AF in the human population. Previous work that measured monophasic APs in rat hearts demonstrated that mANP could decrease AP duration.11 Furthermore, the human patients affected by this mutation had circulating levels of mANP that were 5 – 10 times greater than wildtype ANP.11 Subsequent biochemical studies showed that mANP was much more resistant to proteolytic degradation, which likely explains the elevated circulating levels of this mutant peptide compared to normal ANP.27 These findings led to the hypothesis that mANP functions similarly to ANP, but with enhanced effects due to the profound elevation in plasma concentrations of the peptide and that this could predispose these patients to AF. While the high circulating levels of mANP may contribute to its pro-arrhythmic actions, the specific electrophysiological effects of mANP and the mechanism(s) through which it elicits its effects have not been previously studied.

We studied the effects of ANP and mANP using identical doses (1-100 nM) in order to determine if mANP has unique effects on atrial electrophysiology independently of differences in plasma concentration. Surprisingly, we found that ANP and mANP had distinct and opposing effects on atrial electrophysiology (including V_{max}, AP duration, I_{Ca,L}, CV and ERP). The stimulatory effects of ANP and the inhibitory effects of mANP on I_{Ca,L} were found to be very similar in right and left atrial myocytes from mice. When ANP and mANP were applied
concomitantly at identical doses we observed no effect on atrial electrophysiology indicating that the peptides negated each other’s effects. Our mechanistic experiments using NPR-C-/- mice and an NPR-A antagonist conclusively demonstrate that ANP affects atrial electrophysiology via the NPR-A receptor while mANP signals via NPR-C.

These novel findings strongly suggest that mANP may be pro-arrhythmic not only because it circulates at a higher concentration that ANP, but also because it elicits electrophysiological effects that are distinct from ANP (i.e. mANP is not simply enhancing the effects of ANP in a concentration dependent manner). Because the circulating concentration of mANP was so much higher than ANP in individuals affected by this mutation it is possible that the effects of mANP may be more robust than those of ANP and that the effects of mANP may dominate in vivo. We also found that the expression of NPR-C, which mediates the effects of mANP, is much higher in human atria than NPR-A, which may further enhance the effects of mANP over ANP.

Many of the effects of NPs on the heart are dose dependent. Our electrophysiological studies demonstrate that the effects of ANP and mANP on atrial electrophysiology are comparable at doses from 10-100 nM and smaller, but still significant, at doses as low as 1 nM. While some of these doses of ANP are higher than typical circulating levels it is possible that mANP, because of its enhanced resistance to proteolytic degradation, could achieve low nanomolar concentrations in the circulation, particularly in conditions where atrial stretch is enhanced and NP production is increased. Furthermore, it is important to note that because ANP (and mANP) are produced in atrial myocytes the local concentrations of these peptides in the atria are likely to be much higher than circulating concentrations. All of these factors suggest that the effects of ANP and mANP we have described at low nanomolar
concentrations are physiologically relevant.

Interestingly, although we observed clear effects of ISO, ANP and mANP on $V_{\text{max}}$ and atrial CV, we found no direct effects of any of these compounds on atrial $I_{\text{Na}}$. This was surprising since $I_{\text{Na}}$ is the main determinant of $V_{\text{max}}$, which in turn contributes importantly to CV.36 Furthermore, we showed that the electrophysiological effects of ANP and mANP are associated with changes in cAMP production. Several studies have shown that cAMP and PKA can directly modulate voltage gated sodium channels37-39, although not all studies agree on this.40,41 The lack of direct effects of ANP and mANP on atrial $I_{\text{Na}}$ in our study suggests that ANP and mANP are modulating $V_{\text{max}}$ and atrial CV via their robust effects on $I_{\text{Ca,L}}$. Consistent with this hypothesis, changes in calcium are known to have potent effects on $V_{\text{max}}$42 and calcium is known to regulate sodium channels.43,45 Thus ANP and mANP could modulate $I_{\text{Na}}$ secondary to changes in $I_{\text{Ca,L}}$. In our $I_{\text{Na}}$ recordings, $I_{\text{Ca,L}}$ is blocked and Ca$^{2+}$ transients are suppressed, which could explain why we did not observe any effects of ANP/mANP on $I_{\text{Na}}$ in our experimental conditions.

It is also possible that changes in $I_{\text{Ca,L}}$ could directly contribute to changes in $V_{\text{max}}$ and CV as $I_{\text{Ca,L}}$ is known to have effects on electrical conduction in some conditions.36 Although these effects are smaller than those exerted by $I_{\text{Na}}$, they may be important in the atrial myocardium due to the expression of CaV1.3 L-type Ca$^{2+}$ channels in this region of the heart.46,47 CaV1.3 mediated $I_{\text{Ca,L}}$ activates at more negative membrane potentials than CaV1.246, which could result in a greater contribution by $I_{\text{Ca,L}}$ to the AP upstroke in the atrial myocardium. Our $I_{\text{Ca,L}}$ recordings were done using a voltage clamp protocol that measures total $I_{\text{Ca,L}}$ (CaV1.2 and CaV1.3 dependent). The left shifts in $I_{\text{Ca,L}} V_{1/2(\text{act})}$ elicited by ISO and ANP could result in a greater influence of $I_{\text{Ca,L}}$ on $V_{\text{max}}$ in atrial myocytes and contribute to the increases in $V_{\text{max}}$ we observed. In contrast, the right shift in $I_{\text{Ca,L}} V_{1/2(\text{act})}$ elicited by mANP could reduce $V_{\text{max}}$. CaV1.2
and Ca\textsubscript{v}1.3 are both known to be regulated by cAMP and PKA\textsuperscript{48-50}; therefore, we anticipate that both channels are being similarly modulated by ISO, ANP and mANP; however, we have not studied each channel separately in the present study.

Electrical re-entry is thought to be a major factor in AF with the wavelength of re-entry being determined by the product of the atrial effective refractory period and atrial CV\textsuperscript{4,33,51,52}. Reductions in wavelength that favour the occurrence of AF are commonly associated with changes in ion channel function or regulation in atrial myocytes\textsuperscript{53-55}. Our novel studies demonstrate that mANP decreases AP duration (which corresponds to a reduction in atrial refractory period) and $V_{\text{max}}$ (which is consistent with the slowing of atrial CV). In agreement with these findings, direct assessment of refractoriness in intact atrial preparations illustrates that mANP shortened atrial ERP. These effects on CV and ERP would be expected to decrease the wavelength of re-entry and could explain how mANP leads to the occurrence of AF. In contrast, and consistent with our prior studies of the effects of BNP and CNP on atrial electrophysiology\textsuperscript{13,14,17}, our experiments show that wildtype ANP increases AP duration and $V_{\text{max}}$, speeds electrical conduction in both the right and left atria, and increases atrial ERP. These effects would increase the wavelength of re-entry and would be expected to decrease the likelihood of AF, suggesting that wildtype ANP could be antiarrhythmic. Consistent with these concepts, we found that mANP greatly increased the susceptibility to arrhythmias during pacing studies in atrial preparations. These arrhythmias were characterized by re-entrant conduction patterns, ectopic foci of activation and conduction block, all of which are associated with atrial flutter and atrial fibrillation\textsuperscript{56-58}. In agreement with a key role for $I_{\text{Ca,L}}$ in the occurrence of atrial arrhythmias in the presence of mANP, reductions in both Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 expression or function are known to profoundly increase susceptibility to AF in animal models and
humans.47,58-61

The present study indicates that changes in cAMP are central to the electrophysiological effects of wildtype NPs, as well as mANP, in the heart. Effects mediated by the NPR-A receptor involve a cGMP mediated inhibition of PDE3, which would lead to elevated cAMP levels while effects mediated by NPR-C involve the inhibition of adenylyl cyclase (AC) and reductions in cAMP. Importantly, our present study shows that the electrophysiological effects of ANP and mANP were only evident in the mouse in the presence of ISO. This is in agreement with the observations that neither ANP nor mANP had any effects on cAMP production in the absence of ISO in mouse atrial myocytes and further supports the conclusion that the electrophysiological effects of ANP and mANP are associated with changes in cAMP signaling. Changes in cAMP concentration are known to affect \(I_{Ca,L}\) via changes in phosphorylation of L-type Ca\(^{2+}\) channels by protein kinase A (PKA), which shifts the voltage dependence of activation and increases the channel open probability.62 Our biophysical analyses showing that the effects of both ANP and mANP on atrial \(I_{Ca,L}\) are associated with shifts in the \(V_{1/2}\) of channel activation strongly suggest that these peptides causes cAMP dependent changes in PKA phosphorylation of L-type Ca\(^{2+}\) channels.

Our study demonstrates that the distinct effects of ANP and mANP on \(I_{Ca,L}\) are also present in human atrial myocytes with some minor differences compared to the mouse. Specifically, in mice, ANP only increased atrial \(I_{Ca,L}\) in the presence of ISO whereas in humans ANP potently increased atrial \(I_{Ca,L}\) in basal conditions and in the presence of ISO. This is because the stimulatory effect of NPs on atrial \(I_{Ca,L}\) (mediated by NPR-A) occurs via the inhibition of PDE3.13 We have previously shown that mouse atrial myocytes lack constitutive PDE3 activity, which is why NPs, including ANP, have no effect on basal atrial
electrophysiology in mice. In contrast, our present study shows that human atrial myocytes have robust constitutive PDE3 activity and this explains why ANP can modulate human ICa,L in basal conditions. Our observation that human atrial myocytes have constitutive PDE3 activity is consistent with prior studies showing similar results. The inhibitory effects of mANP on atrial ICa,L were very similar in mice and humans and were only observed in the presence of ISO. This is highly consistent with our prior studies demonstrating that NPR-C mediated effects of NPs on cardiac electrophysiology are most prominent in the setting of β-adrenergic receptor activation. All of our human ICa,L measurements were performed in right atrial myocytes. Based on our data showing that the effects of ANP and mANP are similar in right and left atrial myocytes in mice we hypothesize this is the case in the human heart as well; however, we have not directly measured the effects of NPs in human left atrial myocytes.

The similarities between the electrophysiological effects of ANP and mANP in mice and humans (particularly in the presence of ISO) strongly suggests a mechanism for how mANP causes AF in humans. Nevertheless, extrapolation of our mouse arrhythmogenesis studies to humans must be done with some caution as our results do demonstrate some differences in PDE3 activity and how ICa,L is regulated by NPs in mice and humans. Clearly, additional studies in humans are warranted.

While our study provides novel insight into how mANP could create a substrate for atrial fibrillation our experiments also demonstrate that wildtype ANP is protective against atrial arrhythmias, possibly in association with its AP lengthening and CV speeding effects. These effects of ANP would be expected to increase the wavelength of re-entry and decrease the likelihood of AF being initiated. The anti-arrhythmic effects of wildtype ANP have not been well studied; however, there is evidence that ANP can reduce the incidence of post-operative AF
during cardiothoracic surgeries. To better appreciate the role of wildtype ANP in human AF it will be important to study the electrophysiological effects of ANP in atrial myocytes from human patients with a history of AF. AF is associated with electrical remodeling, including alterations in Ca\(^{2+}\) channel expression, which may impact the effects of ANP on I\(_{\text{Ca,L}}\) in this setting. For example, it has recently been shown that the effects of nitric oxide on atrial I\(_{\text{Ca,L}}\) are attenuated and distinct, while the responsiveness to ISO is preserved, in atrial myocytes isolated from patients with chronic AF. It is presently unknown how chronic AF impacts the electrophysiological effects of ANP.

In conclusion we have studied the effects of ANP and mANP on atrial electrophysiology in mice and humans. Our study provides new insight into the unique electrophysiological effects of mANP and suggests that this mutation promotes AF by decreasing AP V\(_{\text{max}}\), shortening atrial AP duration, decreasing I\(_{\text{Ca,L}}\) and slowing atrial CV. In contrast, wildtype ANP has the opposite effects, which would be expected to decrease the susceptibility to AF by increasing the wavelength of re-entry, although additional studies are required to fully assess the impact of differences between mouse and human atrial electrophysiology. These findings advance our understanding of the electrical effects of wildtype and mutated NPs in the heart and suggest that the NP system could be a target for treating or preventing AF.

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**Conflict of Interest Disclosures:** None.
References:


**Figure Legends:**

**Figure 1:** Effects of ANP on mouse atrial action potential morphology in the presence of isoproterenol. ISO was applied at a dose of 10 nM and two doses of ANP (10 and 100 nM) were studied. (A and D) Representative right atrial APs in control conditions, in the presence of ISO (10 nM) and after application of ANP (10 or 100 nM) in the presence of ISO. Dashed lines are at 0 mV. (B and E) Summary of the effects of ISO and ANP on right atrial AP upstroke velocity ($V_{max}$). (C and F) Summary of the effects of ISO and ANP on right atrial AP duration at 50% ($APD_{50}$), 70% ($APD_{70}$) and 90% ($APD_{90}$) repolarization. *$P<0.05$ vs control, †$P<0.05$ vs. ISO by
one-way repeated measures ANOVA with Tukey’s posthoc test; \(n=5\) myocytes for the 10 nM dose of ANP; \(n=6\) myocytes for the 100 nM dose of ANP. (G) Summary of the percent increase in right atrial AP parameters elicited by 10 nM and 100 nM doses of ANP (in the presence of ISO). There was no significant difference in the magnitude of the effects of ANP at these two doses (data analyzed by Student’s \(t\)-test). In this and all subsequent figures summary data are presented as means ± SD. Refer to Supplemental Tables 6 and 7 for analysis of additional AP parameters.

Figure 2: Effects of mANP on mouse atrial action potential morphology in the presence of isoproterenol. ISO was applied at a dose of 10 nM and two doses of mANP (10 and 100 nM) were studied. (A and D) Representative right atrial APs in control conditions, in the presence of ISO (10 nM) and after application of mANP (10 or 100 nM) in the presence of ISO. Dashed lines are at 0 mV. (B and E) Summary of the effects of ISO and mANP on right atrial \(V_{\text{max}}\). (C and F) Summary of the effects of ISO and mANP on right atrial \(\text{APD}_{50}\), \(\text{APD}_{10}\) and \(\text{APD}_{90}\). \(^*P<0.05\) vs control, \(^P<0.05\) vs. ISO by one-way repeated measures ANOVA with Tukey’s posthoc test; \(n=7\) myocytes for the 10 nM dose of mANP; \(n=9\) myocytes for the 100 nM dose of mANP. (G) Summary of the percent decrease in right atrial AP parameters elicited by 10 nM and 100 nM doses of mANP (in the presence of ISO). There was no significant difference in the magnitude of the effects of mANP at these two doses (data analyzed by Student’s \(t\)-test). Refer to Supplemental Tables 8 and 9 for analysis of additional AP parameters.

Figure 3: Effects of combined application of ANP and mANP on mouse atrial action potential morphology in the presence of isoproterenol. (A and D) Representative right atrial APs in
control conditions, in the presence of ISO (10 nM) and after application of ANP and mANP together (100 nM each) in the presence of ISO. Dashed lines are at 0 mV. (B) Summary of the effects of ISO and ANP + mANP on right atrial V_{max}. (C) Summary of the effects of ISO and ANP + mANP on right atrial APD_{50}, APD_{70} and APD_{90}. *P<0.05 vs control; combined application of ANP and mANP had no significant effect on AP parameters; data analyzed by one-way repeated measures ANOVA with Tukey’s posthoc test; n=7 myocytes. Refer to Supplemental Table 10 for analysis of additional AP parameters.

Figure 4: Effects of ANP and mANP on mouse right atrial I_{Ca,L} in the presence of isoproterenol. (A) Representative right atrial I_{Ca,L} recordings in control conditions, in the presence of ISO (10 nM) and after application of ANP (100 nM) in the presence of ISO. (B) Time course of the effects of ISO and ANP on I_{Ca,L}. (C) Right atrial I_{Ca,L} IV relationships in control conditions, in the presence of ISO and after application of ANP in the presence of ISO. *P<0.05 vs control; +P<0.05 vs. ISO by one way repeated measures ANOVA with Tukey’s posthoc test; n=8 myocytes. (D) Summary I_{Ca,L} conductance density plots demonstrating the effects of ISO and ANP on I_{Ca,L} activation kinetics. Refer to Supplemental Table 11 for analysis of I_{Ca,L} activation kinetics. (E) Representative right atrial I_{Ca,L} recordings in control conditions, in the presence of ISO (10 nM) and after application of mANP (100 nM) in the presence of ISO. (F) Time course of the effects of ISO and mANP on I_{Ca,L}. (G) Right atrial I_{Ca,L} IV relationships in control conditions, in the presence of ISO and after application of mANP in the presence of ISO. *P<0.05 vs. control; 7P<0.05 vs. ISO by one-way repeated measures ANOVA with Tukey’s posthoc test; n=6 myocytes. (H) Summary I_{Ca,L} conductance density plots demonstrating the
effects of ISO and ANP on I_{Ca,L} activation kinetics. Refer to Supplemental Table 12 for analysis of I_{Ca,L} activation kinetics.

**Figure 5:** The effects of mANP on atrial I_{Ca,L} are absent in myocytes from NPR-C^{-/-} mice. (A) Representative I_{Ca,L} recordings (measured at 0 mV) illustrating the effects of ISO (10 nM) and ANP (100 nM) on right atrial myocytes from NPR-C^{-/-} mice. (B) Time course of the effects of ISO and ANP on I_{Ca,L} in NPR-C^{-/-} myocytes. (C) Summary of the effects of ISO and ANP on peak I_{Ca,L} in NPR-C^{-/-} right atrial myocytes. *P<0.05 vs. control; †P<0.05 vs. ISO by one-way repeated measures ANOVA with Tukey’s post-hoc test; n=6 myocytes. (D) Representative I_{Ca,L} recordings (at 0 mV) illustrating the effects of ISO (10 nM) and mANP (100 nM) in right atrial myocytes from NPR-C^{-/-} mice. (E) Time course of the effects of ISO and mANP on I_{Ca,L} in NPR-C^{-/-} myocytes. (F) Summary of the effects of ISO and mANP on peak I_{Ca,L} in NPR-C^{-/-} right atrial myocytes. *P<0.05 vs. control by one-way repeated measures ANOVA with Tukey’s post-hoc test; mANP had no effect on I_{Ca,L} amplitude in NPR-C^{-/-} atrial myocytes (P=0.989); n=9 myocytes.

**Figure 6:** Effects of ANP and mutant ANP on I_{Ca,L} in human right atrial myocytes in baseline conditions and in the presence of isoproterenol. (A) Representative I_{Ca,L} recordings (measured at 0 mV) in control conditions, after application of ANP (100 nM) and following ANP washout. (B) Summary data illustrating the effects of ANP on human atrial I_{Ca,L} in basal conditions. *P<0.05 vs. control by one-way repeated measures ANOVA with Tukey’s posthoc test; n=6 myocytes from 3 patient samples. (C) Representative I_{Ca,L} recordings in control conditions and after application of mANP (100 nM). (D) Summary data illustrating that mANP had no effect
(P=0.936) on human atrial I_{Ca,L} in basal conditions. Data analyzed by one-way repeated measures ANOVA; n=8 myocytes from 4 patient samples. (E) Representative I_{Ca,L} recordings in control conditions, in the presence of ISO (10 nM), after application of ANP (100 nM) in the presence of ISO, and following ANP washout. (F) Summary data illustrating the effects of ANP on human atrial I_{Ca,L} in the presence of ISO. *P<0.05 vs. control; +P<0.05 vs. ISO by one-way repeated measures ANOVA with Tukey’s posthoc test; n=6 myocytes from 4 patient samples. (G) Representative I_{Ca,L} recordings in control conditions, in the presence of ISO (10 nM), after application of mANP (100 nM) in the presence of ISO, and following mANP washout. (H) Summary data illustrating the effects of mANP on human atrial I_{Ca,L} in the presence of ISO. *P<0.05 vs. control; †P<0.05 vs. ISO by one-way repeated measures ANOVA with Tukey’s posthoc test; n=7 myocytes from 4 patient samples.

**Figure 7:** ANP and mANP have opposing effects on electrical conduction in the atria in mice. (A) Representative color maps showing activation patterns in atrial preparations (see Supplemental Fig. 10) in control conditions, in the presence of ISO (10 nM) and following application of ANP (50 nM; top row) or mANP (50 nM; bottom row). Scale bars are 2 mm. The right atrial appendage is on the left side of the image and the red color indicates the earliest activation time in the right atrial posterior wall. Time interval between isochrones is 1 ms. (B) Summary of the effects of ANP and mANP on cycle length in atrial preparations in sinus rhythm. (C and D) Summary data illustrating the effects of ANP and mANP on right and left atrial conduction velocities in atrial preparations in sinus rhythm (n=5 hearts for each condition). (E and F) Summary data illustrating the effects of ANP and mANP on right and left atrial conduction velocities in atrial preparations paced a fixed cycle length of 90 ms. *P<0.05 vs.
control; \(^*P<0.05\) vs ISO by one-way repeated measures ANOVA with Tukey’s posthoc test; \(n=8\) hearts for each condition.

**Figure 8:** Effects of ANP and mANP on susceptibility to arrhythmias during atrial pacing. These experiments were performed in atrial preparations in the presence of ISO (10 nM) and either ANP (50 nM) or mANP (50 nM). Arrhythmias were induced using S1-S2 protocols in which atrial preparations were paced a fixed cycle length (90 ms) and then given a series of premature extra stimuli (see Methods). (A and B) Representative color maps from before and after atrial pacing in the presence of ISO and ANP. These activation maps show that after pacing, atrial preparations treated with ANP remained in sinus rhythm (B) with activation patterns very similar to before pacing (A). (C and D) Representative color maps from before and after atrial pacing in the presence of ISO and mANP. In these examples, the atrial preparation is in normal sinus rhythm before atrial pacing has occurred (C). After pacing in the presence of mANP (D) electrical conduction became arrhythmic. Note the different time scales in these activation maps. Time interval between isochrones is 1 ms in all maps, scale bars are 2 mm. (E) Summary of the susceptibility to arrhythmias in atrial preparations treated with ANP or mANP (both in the presence of ISO). Numbers in parentheses indicate the number of hearts that showed arrhythmias; \(*P<0.05\) vs ANP by Fisher’s exact test.
Effects of Wildtype and Mutant Forms of Atrial Natriuretic Peptide on Atrial Electrophysiology and Arrhythmogenesis
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SUPPLEMENTAL MATERIAL

Methods

Animals

This study utilized male littermate wildtype (NPR-C\textsuperscript{+/+}) and NPR-C knockout (NPR-C\textsuperscript{-/-}) mice between the ages of 10-15 weeks. NPR-C\textsuperscript{-/-} mice were initially obtained from the Jackson Laboratory (strain B6;C-Npr3\textsuperscript{lgj}/J) and backcrossed into the C57Bl/6 line for more than 15 generations. This mouse contains a 36 base pair deletion that results in a truncated, non-functional NPR-C protein.\textsuperscript{1}

Isolation of mouse and human atrial myocytes

The procedures for isolating mouse atrial myocytes have been described previously\textsuperscript{2,3} and were as follows. Mice were administered a 0.2 ml intraperitoneal injection of heparin (1000 IU/ml) to prevent blood clotting. Following this, mice were anesthetized by isoflurane inhalation and then sacrificed by cervical dislocation. The heart was excised into Tyrode's solution (35°C) consisting of (in mM) 140 NaCl, 5.4 KCl, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 1.0 MgCl\textsubscript{2}, 1.8 CaCl\textsubscript{2}, 5.55 glucose, and 5 HEPES, with pH adjusted to 7.4 with NaOH. The right or left atrial appendage was dissected from the heart and cut into strips, which were transferred and rinsed in a ‘low Ca\textsuperscript{2+}, Mg\textsuperscript{2+} free’ solution containing (in mM) 140 NaCl, 5.4 KCl, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 0.2 CaCl\textsubscript{2}, 50 taurine, 18.5 glucose, 5 HEPES and 1 mg/ml bovine serum albumin (BSA), with pH adjusted to 6.9 with NaOH. Atrial tissue was digested in 5 ml of ‘low Ca\textsuperscript{2+}, Mg\textsuperscript{2+} free’ solution containing collagenase (type II, Worthington Biochemical Corporation), elastase (Worthington Biochemical Corporation) and protease (type XIV, Sigma Chemical Company) for 30 min. Then the tissue was transferred to 5 ml of modified KB solution containing (in mM) 100 potassium glutamate,
10 potassium aspartate, 25 KCl, 10 KH₂PO₄, 2 MgSO₄, 20 taurine, 5 creatine, 0.5 EGTA, 20 glucose, 5 HEPES, and 0.1% BSA, with pH adjusted to 7.2 with KOH. The tissue was mechanically agitated using a wide-bore pipette. This procedure yielded individual right or left atrial myocytes that were stored in KB solution until experimental use within 6 hours of isolation. Mouse atrial myocytes had membrane capacitances in the range of 40 – 65 pF.

Similar procedures were used to isolate human right atrial myocytes from tissue samples obtained from cardiac surgery patients (Supplemental Table 1) with some modifications. The tissue samples were removed from patients at the time of right atrial cannulation during cardiac surgery, placed in physiological saline on ice and transported immediately to the laboratory for enzymatic digestion. Strips of human right atrial appendage were digested in the same enzyme solutions described above; however, we typically had to digest the tissue for 45-60 min. Human tissue samples were also triturated twice with a wide bore pipette – once at 30 min of digestion and again at the end of digestion. These modifications yielded individual human right atrial myocytes that were stored in KB solution until experimental use within 6 hours of isolation. Human atrial myocytes had membrane capacitances in the range of 60 – 120 pF. For human myocyte studies we ensured that at least 3 different patient samples were used for each experimental condition and/or treatment group. We also designed our study so that ANP and mANP were each measured in cells from the same patient sample. These measures were taken to account for the possibility of patient to patient variability.

**Solutions and electrophysiological protocols**

Stimulated action potentials (APs) were recorded using the perforated patch-clamp technique. To record APs the recording chamber was superfused with a normal Tyrode’s
solution (22 – 23°C) containing (in mM) 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, and 5 glucose, with pH adjusted to 7.4 with NaOH. The pipette filling solution contained (in mM) 135 KCl, 0.1 CaCl₂, 1 MgCl₂, 5 NaCl, 10 EGTA, 4 Mg-ATP, 6.6 Na-phosphocreatine, 0.3 Na-GTP and 10 HEPES, with pH adjusted to 7.2 with KOH. Amphotericin B (200 µg/ml) was added to this pipette solution to record APs with the perforated patch clamp technique.

For recording I_{Ca,L} atrial myocytes were superfused with a modified Tyrode’s solution (22 – 23°C) containing the following (in mM) 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, and 5 glucose with pH adjusted to 7.4 with NaOH. The pipette solution for I_{Ca,L} contained (in mM) 135 CsCl, 0.1 CaCl₂, 1 MgCl₂, 5 NaCl, 10 EGTA, 4 Mg-ATP, 6.6 Na-phosphocreatine, 0.3 Na-GTP and 10 HEPES, with pH adjusted to 7.2 with CsOH. To block voltage gated Na⁺ currents (I_{Na}) when recording I_{Ca,L} cells were treated with lidocaine (0.3 mM). This approach was used in order to record atrial I_{Ca,L} from a holding potential of -60 mV due to the expression of CaV1.2 and CaV1.3 in atrial myocytes.²,³,⁵,⁶ Thus, our voltage clamp protocols enable us to record total I_{Ca,L}, which could include contributions from CaV1.2 as well as CaV1.3.

For recording I_{Na} atrial myocytes were superfused with a modified Tyrode’s solution (22 – 23°C) containing the following (in mM): 130 CsCl, 5 NaCl, 5.4 TEACl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, 5.5 glucose, 5.5 (pH 7.4, adjusted with CsOH). Nitrendipine (10 µM) was added to the superfusate to block I_{Ca,L}. The pipette solution for I_{Na} contained (in mM): 120 CsCl, 5 NaCl, 1 MgCl₂, 0.2 CaCl₂, 10 HEPES, 5 MgATP, 0.3 Na-GTP, 5 BAPTA (pH 7.2, adjusted with CsOH). I_{Na} was recorded using 50 ms voltage clamp steps between -100 and 10 mV from a holding potential of -120 mV.

Micropipettes were pulled from borosilicate glass (with filament, 1.5 mm OD, 0.75 mm ID, Sutter Instrument Company) using a Flaming/Brown pipette puller (model p-87, Sutter
Instrument Company). The resistance of these pipettes was 4 – 8 MΩ when filled with recording solution. Micropipettes were positioned with a micromanipulator (Burleigh PCS-5000 system) mounted on the stage of an inverted microscope (Olympus IX71). Seal resistance was 2 – 15 GΩ. Rupturing the sarcolemma in the patch for voltage clamp experiments resulted in access resistances of 5 – 15 MΩ. Series resistance compensation averaged 80 – 85% using an Axopatch 200B amplifier (Molecular Devices). For perforated patch clamp experiments access resistance was monitored for the development of capacitative transients upon sealing to the cell membrane with Amphotericin B in the pipette. Typically, access resistance became less than 30 MΩ within 5 min of sealing onto the cell, which was sufficient for recording stimulated APs in current clamp mode. Data were digitized using a Digidata 1440 and pCLAMP 10 software (Molecular Devices) and stored on computer for analysis.

$I_{Ca,L}$ and $I_{Na}$ activation kinetics were determined by calculating chord conductance ($G$) with the equation $G=I/(V_m-E_{rev})$, where $V_m$ represents the depolarizing voltages and $E_{rev}$ is the reversal potential estimated from the current-voltage relationships of $I_{Ca,L}$ or $I_{Na}$. Maximum conductance ($G_{max}$) and $V_{1/2}$ of activation for $I_{Ca,L}$ and $I_{Na}$ were determined using the following function: $G=[(V_m-V_{rev})][G_{max}]-1/[1+exp((V_m-V_{1/2})/k)]+1]$. 

**High resolution optical mapping**

To study patterns of electrical conduction in the mouse atria we used high resolution optical mapping in atrial preparations as we and others have done previously. To isolate our atrial preparation mice were administered a 0.2 ml intraperitoneal injection of heparin (1000 IU/ml) to prevent blood clotting and were then anesthetized by isoflurane inhalation and cervically dislocated. Hearts were excised into Krebs solution (35°C) containing (in mM): 118
NaCl, 4.7 KCl, 1.2 KH₂PO₄, 12.2 MgSO₄, 1 CaCl₂, 25 NaHCO₃, 11 glucose and bubbled with 95% O₂/5% CO₂ in order to maintain a pH of 7.4. The atria were dissected away from the ventricles and pinned in a dish with the epicardial surface facing upwards (towards the imaging equipment). The superior and inferior vena cavae were cut open so that the crista terminalis could be visualized and the preparation could be pinned out flat (Supplemental Fig. 10).

The atrial preparation was superfused continuously with Krebs solution (35°C) bubbled with 95% O₂/5% CO₂ and allowed to equilibrate for at least 30 min. During this time the preparation was treated with the voltage sensitive dye di-4-ANEPPS (10 µM) for ~15 min and blebbistatin (10 µM) was added to the superfusate to suppress contractile activity. Blebbistatin was present throughout the duration of the experiments in order prevent motion artifacts during optical mapping. Some experiments were performed in sinus rhythm so that the cycle length (i.e. beating rate) of the atrial preparation was free to change, while in other studies we used a pacing electrode to pace atrial preparations at a fixed cycle length in order to study electrical conduction independently of changes in cycle length. The pacing electrode was placed near the opening of the superior vena cava.

Di-4-ANEPPS loaded atrial preparations were illuminated with light at a wavelength of 520 – 570 nM using an EXFO X-cite fluorescent light source (Lumen Dynamics). Emitted light (590 – 640 nM) was captured using a high speed EMCCD camera (Evolve 128, Photometrics). Data were captured from an optical field of view of 8 x 8 mm² at a frame rate of ~1000 frames/s using Metamorph software (Molecular Devices). The spatial resolution was 67 x 67 µm for each pixel. Magnification was constant in all experiments and no pixel binning was used.

All optical data were analyzed using custom software written in Matlab. Pseudocolor electrical activation maps were generated from measurements of activation time at individual
pixels as defined by assessment of $\text{dF/dt}_{\text{max}}$. In all cases background fluorescence was subtracted. Local conduction velocity (CV) was quantified specifically in the right atrial myocardium (within the RAA) and the left atrial myocardium (within the LAA) using an established approach previously described. \cite{7,8,12} Briefly, activation times at each pixel from a 7 x 7 pixel array were determined and fit to a plane using the least squares fit method. The direction on this plane that is increasing the fastest represents the direction that is perpendicular to the wavefront of electrical propagation and the maximum slope represents the inverse of the speed of conduction in that direction. With a spatial resolution of 67 x 67 μM per pixel, the area of the 7 x 7 pixel array was 469 x 469 μM. Thus, using this method, we computed maximum local CV vectors in the atrial region of interest.

Optical APs (OAPs) were assessed by measuring changes in fluorescence as a function of time at individual pixels in the atrial preparation as we have previously described. \cite{7,13} OAPs were used to quantify changes in AP duration in the intact atrial preparation.

To measure the effects of ISO, ANP and mANP on atrial effective refractory period (ERP) we used programmed stimulation protocols in which the atrial preparation was paced at a cycle length of 90 ms for 8 beats and then given an extra stimulus at progressively shorter cycle lengths (1 ms increments). \cite{13} ERP was defined as the shortest coupling interval allowing for capture of the atrial preparation.

**Arrhythmia studies**

Susceptibility to atrial arrhythmias was studied using high resolution optical mapping in conjunction with atrial pacing. To induce arrhythmias we used S1-S2 pacing protocols in which we paced the atria at a cycle length of 90 ms (S1) and then delivered 3-6 premature extra beats at
shorter cycle lengths (50 or 25 ms). In all arrhythmia studies the atrial preparations were in normal sinus rhythm and displayed normal patterns of electrical activation and conduction before atrial pacing took place.

**Quantitative PCR**

A portion of our human right atrial samples was retained for molecular biology studies. Quantitative gene expression in human right atrial tissue was performed using methods similar to those we have previously described in mice. Intron spanning primers were designed for human Npr1, Npr2 and Npr3 (genes corresponding to NPR-A, NPR-B and NPR-C) as well as GAPDH, which was used a reference gene. Following synthesis (Sigma Genosys) primers were reconstituted in nuclease free water at a concentration of 100 nM and frozen at -20°C. Primer sequences were as follows:

**Npr1:**
Forward 5’-TCACGCACGCTACAAACA-3’
Reverse 5’-GAGAGAGAGAGAGAGAGAGAAAGG-3’
Amplification product: 106 base pairs

**Npr2:**
Forward 5’-GACGACCCATCCTGTGATAAA-3’
Reverse 5’-GGAAGCTGGAAACACCAAAC-3’
Amplification product: 97 base pairs

**Npr3:**
Forward 5’-GTGGGTAGGTGTGGAGATAAG-3’
Reverse 5’-CTACTGGGTGCAAAGCAGATA-3’
Amplification product: 77 base pairs

GAPDH:
Forward 5’-ATGACATCAAGAAGGTGGTG-3’
Reverse 5’-CATACCAGGAAATGAGCTTG-3’
Amplification product: 177 base pairs

RNA was extracted in PureZOL™ RNA isolation reagent according to kit instructions (Aurum total RNA fatty and fibrous tissue kit, BioRad). Tissue was eluted in 30-40 µl of elution buffer from the spin column. RNA concentrations were determined using a Qubit fluorometer (Invitrogen) and first strand synthesis reactions were performed using the iScript cDNA synthesis kit (BioRad) according to kit instructions with 0.5 µg RNA template. A260/280 readings were also performed to evaluate the purity of RNA extractions prior to first strand synthesis. Lack of genomic DNA contamination was verified by reverse transcription (RT)-PCR using a no RT control.

RT-qPCR using BRYT green dye was used to assess gene expression. Following RNA extraction cDNA was synthesized and 20 µl BRYT reactions were performed with 1 µl cDNA template. Reactions were carried out using a CFX96 Real-Time PCR Detection System (BioRad). Amplification conditions were as follows: 95°C for 2 min to activate Taq polymerase, 35 cycles of denaturation at 95°C for 30 sec, annealing using a gradient from 53-61°C for 30 sec and extension at 72°C or 1 min 30 sec. Melt curve analysis was performed from 65-95°C every 0.5°C increments. Single amplicons with appropriate melting temperatures and sizes were
detected. Data were expressed in the form $2^{-\Delta CT} \times 100$ vs. GAPDH for all tissue samples. $C_T$ values $> 32$ were eliminated due to lack of reproducibility.\textsuperscript{14} Primers were used at a concentration of 10 nM.

**cAMP assay**

Mouse right atrial myocytes were isolated as described above and centrifuged (2000 rpm) for 5 minutes. The supernatant was removed and the pellet was resuspended in normal Tyrode’s solution. A hemocytometer was used to determine myocyte density. Cells were either untreated (control) or treated with the following: ANP alone, mANP alone, ISO, ISO + ANP or ISO + mANP. Cells were incubated with these compounds for 15 min at 4°C. Intracellular cAMP concentrations were then determined using a HTRF cAMP Femto2 kit (Cisbio US) according to the manufacturer’s instructions.
Supplemental Figure 1: Effects of ANP and mANP on mouse atrial action potential morphology and L-type Ca\(^{2+}\) current in basal conditions. A, Representative right atrial APs in control conditions and after application of ANP (100 nM) or mANP (100 nM). B, Summary of the effects of ANP and mANP on right atrial AP duration at 50% (APD\(_{50}\)), 70% (APD\(_{70}\)) and 90% (APD\(_{90}\)) repolarization. ANP and mANP had no effect on atrial AP duration. Data analyzed by paired Student’s t-test; n=6 myocytes for ANP and 5 myocytes for mANP. Refer to Supplemental Tables 2 and 3 for additional AP parameters. C, Representative right atrial I\(_{Ca,L}\) recordings in control conditions and after application of ANP (100 nM). D, Right atrial I\(_{Ca,L}\) IV relationships in control conditions and after application of ANP. E, Summary I\(_{Ca,L}\) conductance density plots demonstrating the effects of ANP on I\(_{Ca,L}\) activation kinetics. F,
Representative right atrial $I_{Ca,L}$ recordings in control conditions and after application of mANP (100 nM). 

G, Right atrial $I_{Ca,L}$ IV relationships in control conditions and after application of mANP. H, Summary $I_{Ca,L}$ conductance density plots demonstrating the effects of mANP on $I_{Ca,L}$ activation kinetics. Neither ANP ($n=7$ myocytes) or mANP ($n=5$ myocytes) had any effects on mouse right atrial $I_{Ca,L}$. Refer to Supplemental Tables 4 and 5 for analysis of $I_{Ca,L}$ activation kinetics.
Supplemental Figure 2: Dose dependence of the effects of ANP and mANP on mouse atrial $I_{Ca,L}$ in the presence of isoproterenol. (A) Summary of the effects of ISO (10 nM) and ANP (1, 10, 100 nM) on peak $I_{Ca,L}$ (measured at 0 mV) in mouse right atrial myocytes. *$P<0.05$ vs. control; +$P<0.05$ vs. ISO by one-way repeated measures ANOVA with Tukey’s posthoc test; $n=5$ myocytes for 1 nM dose, 5 myocytes for 10 nM dose and 8 myocytes for 100 nM dose of ANP. (B) Summary data demonstrating the percent increase in $I_{Ca,L}$ (in the presence of ISO) elicited by each dose of ANP. *$P<0.05$ vs. 1 nM dose by one-way ANOVA with Tukey’s posthoc test. (C) Summary of the effects of ISO (10 nM) and mANP (1, 10, 100 nM) on peak $I_{Ca,L}$ (measured at 0 mV) in mouse right atrial myocytes. *$P<0.05$ vs. control; +$P<0.05$ vs. ISO by one-way repeated measures ANOVA with Tukey’s posthoc test; $n=5$ myocytes for 1 nM dose, 5 myocytes for 10 nM dose and 6 myocytes for 100 nM dose of ANP. (D) Summary data demonstrating the percent decrease in $I_{Ca,L}$ (in the presence of ISO) elicited by each dose of mANP. *$P<0.05$ vs. 1 nM dose by one-way ANOVA with Tukey’s posthoc test.
Supplemental Figure 3: Effects of ANP and mANP on I_{Ca,L} in mouse left atrial myocytes. (A) Representative left atrial I_{Ca,L} recordings in control conditions, in the presence of ISO (10 nM), after application of ANP (100 nM) in the presence of ISO, and after ANP washout. (B) Summary of the effects of ISO and ANP on left atrial I_{Ca,L} in mice. *P<0.05 vs. control; *P<0.05 vs. ISO by one-way repeated measures ANOVA with Tukey’s posthoc test; n=6 myocytes. (C) Representative left atrial I_{Ca,L} recordings in control conditions, in the presence of ISO (10 nM), after application of mANP (100 nM) in the presence of ISO, and following mANP washout. (D) Summary of the effects of ISO and mANP on left atrial I_{Ca,L} in mice. *P<0.05 vs. control; P<0.05 vs. ISO by one-way repeated measures ANOVA with Tukey’s posthoc test; n=6 myocytes.
Supplemental Figure 4: Effects of ANP and mANP on mouse atrial I_{Na} in the presence of isoproterenol. (A) Representative right atrial I_{Na} recordings in control conditions, in the presence of ISO (10 nM) and after application of ANP (100 nM) in the presence of ISO. (B) Right atrial I_{Na} IV relationships in control conditions, in the presence of ISO and after the application of ANP in the presence of ISO. There was no significant effect of ISO or ANP on I_{Na} density (P=0.511 by one-way repeated measures ANOVA); n=5 myocytes. (C) Summary I_{Na} conductance density plots demonstrating the effects of ISO and ANP on I_{Na} activation kinetics. Refer to Supplemental Table 13 for analysis of I_{Na} activation kinetics. (D) Representative right atrial I_{Na} recordings in control conditions, in the presence of ISO (10 nM) and after application of
mANP (100 nM) in the presence of ISO. (E) Right atrial $I_{\text{Na}}$ IV relationships in control conditions, in the presence of ISO and after the application of mANP in the presence of ISO. There was no significant effect of ISO or mANP in $I_{\text{Na}}$ density ($P=0.717$ by one-way repeated measures ANOVA); $n=6$ myocytes. (F) Summary $I_{\text{Na}}$ conductance density plots demonstrating the effects of ISO and mANP on $I_{\text{Na}}$ activation kinetics. Refer to Supplemental Table 14 for analysis of $I_{\text{Na}}$ activation kinetics.
Supplemental Figure 5: The effects of ANP on atrial $I_{Ca,L}$ are blocked by the NPR-A antagonist A71915. (A) Representative $I_{Ca,L}$ recordings (at 0 mV) in control conditions, in the presence of ISO (10 nM) and A171915 (500 nM) and following application of ANP (100 nM) in the presence of ISO and A71915. (B) Time course of the effects of ISO, A71915 and ANP on $I_{Ca,L}$. (C) Summary of the effects of ISO, A71915 and ANP on peak $I_{Ca,L}$ in right atrial myocytes. *$P<0.05$ vs. control by one-way repeated measures ANOVA with Tukey’s posthoc text; ANP had no effect ($P=0.991$) on $I_{Ca,L}$ amplitude following NPR-A blockade; $n=5$ myocytes.
Supplemental Figure 6: ANP and mANP have opposing effects on intracellular cAMP production in right atrial myocytes. The effects of ANP (100 nM) and mANP (100 nM) on cAMP production were measured in basal conditions and after application of ISO (10 nM). *P<0.05 vs. control; †P<0.05 vs. ISO; ‡P<0.05 vs. ISO+ANP by one-way ANOVA with Tukey’s posthoc test; n=6 right atrial myocyte isolations.
Supplemental Figure 7: Quantitative mRNA expression of natriuretic peptide receptors in human right atrial myocardium. Expression is shown for Npr1, Npr2 and Npr3, which correspond to NPR-A, NPR-B and NPR-C respectively, relative to GAPDH. *P<0.05 vs. Npr1, +P<0.05 vs. Npr2 by one-way ANOVA with Tukey’s posthoc test; n=10 human right atrial samples.
Supplemental Figure 8: Properties of L-type Ca$_{2+}$ current in human right atrial myocytes.
(A) Representative example of a family of $I_{Ca,L}$ recordings between -60 mV and +60 mV (holding potential = -60 mV). (B) Human $I_{Ca,L}$ IV relationship; $n$=5 right atrial myocytes from 3 patient samples.
Supplemental Figure 9: Effects of the PDE3 inhibitor milrinone on basal $I_{\text{Ca,L}}$ in human right atrial myocytes. (A) Representative $I_{\text{Ca,L}}$ recordings (at 0 mV) in basal conditions, after application of milrinone (10 µM) and after washout of milrinone. (B) Time course of the effects of milrinone on human atrial $I_{\text{Ca,L}}$. (C) Summary of the effects of milrinone on human atrial $I_{\text{Ca,L}}$. *$P<0.05$ vs. control by one-way repeated measures ANOVA with Tukey’s posthoc test; $n=7$ myocytes from 4 patient samples.
Supplemental Figure 10: Atrial preparation used for high resolution optical mapping studies. The preparation is oriented with the right atrium on the left side of the image. RAA, right atrial appendage; SVC, opening of superior vena cava; IVC, opening of inferior vena cava; LAA, left atrial appendage; Ao, aorta. The crista terminalis is shown by the dashed line. Scale bar is 2 mm.
Supplemental Figure 11: Effects of ANP and mANP on action potential duration and effective refractory period in mouse atrial preparations. (A) Representative optical APs (OAPs) in control conditions, in the presence of ISO (10 nM) and after application of ANP (50 nM) in the presence of ISO. OAPs were measured in the right atrial appendage using high resolution optical mapping. (B) Summary of the effects of ISO and ANP on APD$_{70}$ in mouse right atrium. (C) Summary of the effects of ISO and ANP on atrial effective refractory period. For panels B and C *$P<$0.05 vs. control; $^+P<$0.05 vs. ISO by one-way repeated measures ANOVA; $n=5$ hearts. (D) Representative optical APs (OAPs) in control conditions, in the presence of ISO (10 nM) and after application of mANP (50 nM) in the presence of ISO. (E) Summary of the effects of ISO and mANP on APD$_{70}$ in mouse right atrium. (F) Summary of the effects of ISO and mANP on atrial effective refractory period. For panels E and F *$P<$0.05 vs. control; $^+P<$0.05 vs. ISO by one-way repeated measures ANOVA; $n=5$ hearts.
### Supplemental Table 1: Patient characteristics

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<td>Gender (M/F)</td>
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<td>Age (years)</td>
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<td>Weight (kg)</td>
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<td>BMI (kg/m²)</td>
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BMI, body mass index; CAD, coronary artery disease; ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker.

### Supplemental Table 2: Action potential parameters at baseline and after application of ANP (100 nM) in mouse right atrial myocytes

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<th>Control</th>
<th>ANP</th>
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<tbody>
<tr>
<td>RMP (mV)</td>
<td>-77.9 ± 0.7</td>
<td>-78.0 ± 0.7</td>
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<tr>
<td>V_max (V/s)</td>
<td>150.0 ± 17.0</td>
<td>149.3 ± 16.8</td>
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<tr>
<td>OS (mV)</td>
<td>60.3 ± 15.8</td>
<td>60.1 ± 16.3</td>
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<tr>
<td>APD₅₀ (ms)</td>
<td>12.4 ± 4.8</td>
<td>12.6 ± 4.6</td>
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<tr>
<td>APD₇₀ (ms)</td>
<td>24.6 ± 7.9</td>
<td>24.7 ± 7.7</td>
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<tr>
<td>APD₉₀ (ms)</td>
<td>55.8 ± 10.8</td>
<td>55.5 ± 10.3</td>
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</table>

RMP, resting membrane potential; V_max, AP upstroke velocity; OS, overshoot; APD₅₀, AP duration at 50% repolarization; APD₇₀, AP duration at 70% repolarization; APD₉₀, AP duration at 90% repolarization. ANP had no effects on these AP parameters. Data analyzed by paired Student’s t-test; n=6 myocytes.
Supplemental Table 3: Action potential parameters at baseline and after application of mANP (100 nM) in mouse right atrial myocytes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>mANP</th>
</tr>
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<td>RMP (mV)</td>
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<td>-77.8 ± 0.7</td>
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<tr>
<td>( V_{\text{max}} ) (V/s)</td>
<td>144.2 ± 11.7</td>
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<td>OS (mV)</td>
<td>60.8 ± 8.6</td>
<td>59.8 ± 7.7</td>
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<td>( \text{APD}_{50} ) (ms)</td>
<td>15.2 ± 1.8</td>
<td>15.6 ± 1.3</td>
</tr>
<tr>
<td>( \text{APD}_{70} ) (ms)</td>
<td>29.3 ± 2.4</td>
<td>29.6 ± 2.6</td>
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<tr>
<td>( \text{APD}_{90} ) (ms)</td>
<td>55.3 ± 5.9</td>
<td>56.3 ± 5.9</td>
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</table>

RMP, resting membrane potential; \( V_{\text{max}} \), AP upstroke velocity; OS, overshoot; \( \text{APD}_{50} \), AP duration at 50% repolarization; \( \text{APD}_{70} \), AP duration at 70% repolarization; \( \text{APD}_{90} \), AP duration at 90% repolarization. ANP had no effects on these AP parameters. Data analyzed by paired Student’s \( t \)-test; \( n=5 \) myocytes.

Supplemental Table 4: \( I_{\text{Ca,L}} \) conductance analysis in baseline conditions and following application of ANP in mouse right atrial myocytes

<table>
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<tr>
<th>Parameter</th>
<th>Control</th>
<th>ANP</th>
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<tr>
<td>( G_{\text{max}} ) (pS/pF)</td>
<td>90.7 ± 3.4</td>
<td>91.6 ± 3.1</td>
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<tr>
<td>( V_{1/2(\text{act})} ) (mV)</td>
<td>-8.9 ± 1.0</td>
<td>-9.1 ± 1.0</td>
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<tr>
<td>( k )</td>
<td>6.5 ± 1.0</td>
<td>6.4 ± 0.8</td>
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\( G_{\text{max}} \), maximum conductance; \( V_{1/2(\text{act})} \), voltage at which 50% of channels are activated; \( k \), slope factor. ANP had no effects on \( I_{\text{Ca,L}} \) activation kinetics. Data analyzed by Student’s \( t \)-test; \( n=7 \) myocytes.

Supplemental Table 5: \( I_{\text{Ca,L}} \) conductance analysis in baseline conditions and following application of mANP in mouse right atrial myocytes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>mANP</th>
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<tbody>
<tr>
<td>( G_{\text{max}} ) (pS/pF)</td>
<td>101.8 ± 6.4</td>
<td>112.0 ± 3.3</td>
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<tr>
<td>( V_{1/2(\text{act})} ) (mV)</td>
<td>-10.9 ± 2.0</td>
<td>-11.1 ± 0.9</td>
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<tr>
<td>( k )</td>
<td>7.9 ± 1.8</td>
<td>8.4 ± 0.9</td>
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\( G_{\text{max}} \), maximum conductance; \( V_{1/2(\text{act})} \), voltage at which 50% of channels are activated; \( k \), slope factor. ANP had no effects on \( I_{\text{Ca,L}} \) activation kinetics. Data analyzed by Student’s \( t \)-test; \( n=5 \) myocytes.
### Supplemental Table 6: Action potential parameters at baseline and after application of ISO (10 nM) and ANP (10 nM) in mouse right atrial myocytes

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<th>ANP washout</th>
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<td>RMP (mV)</td>
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<td>-80.3 ± 2.0</td>
<td>-80.8 ± 2.4</td>
<td>-80.4 ± 2.0</td>
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<tr>
<td>V_{max} (V/s)</td>
<td>139.5 ± 17.6</td>
<td>145.7 ± 18.0*</td>
<td>151.5 ± 18.5**</td>
<td>144.5 ± 20.7*</td>
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<td>OS (mv)</td>
<td>57.3 ± 5.1</td>
<td>62.6 ± 2.6</td>
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<td>63.2 ± 3.1</td>
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<td>APD_{50} (ms)</td>
<td>10.0 ± 4.4</td>
<td>16.5 ± 7.9*</td>
<td>20.7 ± 8.6*</td>
<td>17.5 ± 7.0*</td>
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<td>APD_{70} (ms)</td>
<td>19.7 ± 3.1</td>
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<td>42.5 ± 7.0**</td>
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<td>APD_{90} (ms)</td>
<td>46.1 ± 4.8</td>
<td>63.6 ± 3.7*</td>
<td>76.8 ± 7.3**</td>
<td>65.5 ± 3.5*</td>
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RMP, resting membrane potential; V_{max}, AP upstroke velocity; OS, overshoot; APD_{50}, AP duration at 50% repolarization; APD_{70}, AP duration at 70% repolarization; APD_{90}, AP duration at 90% repolarization. *P<0.05 vs control, **P<0.05 vs. ISO by one-way repeated measures ANOVA with Tukey’s posthoc test; n=5 myocytes.

### Supplemental Table 7: Action potential parameters at baseline and after application of ISO (10 nM) and ANP (100 nM) in mouse right atrial myocytes

<table>
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<tr>
<th></th>
<th>Control</th>
<th>ISO</th>
<th>ISO + ANP</th>
<th>ANP washout</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP (mV)</td>
<td>-82.4 ± 0.9</td>
<td>-82.5 ± 1.7</td>
<td>-82.3 ± 1.9</td>
<td>-82.1 ± 1.9</td>
</tr>
<tr>
<td>V_{max} (V/s)</td>
<td>157.4 ± 12.7</td>
<td>173.9 ± 12.5*</td>
<td>183.2 ± 9.4**</td>
<td>173.6 ± 11.8*</td>
</tr>
<tr>
<td>OS (mv)</td>
<td>59.6 ± 3.8</td>
<td>64.9 ± 4.6*</td>
<td>68.0 ± 4.6*</td>
<td>64.8 ± 5.0*</td>
</tr>
<tr>
<td>APD_{50} (ms)</td>
<td>11.8 ± 4.6</td>
<td>15.5 ± 5.5*</td>
<td>19.5 ± 7.0**</td>
<td>15.6 ± 5.8*</td>
</tr>
<tr>
<td>APD_{70} (ms)</td>
<td>20.3 ± 8.2</td>
<td>27.6 ± 10.8*</td>
<td>36.7 ± 13.4**</td>
<td>27.9 ± 11.0*</td>
</tr>
<tr>
<td>APD_{90} (ms)</td>
<td>46.5 ± 10.8</td>
<td>58.3 ± 10.3*</td>
<td>71.5 ± 11.8**</td>
<td>58.7 ± 11.0*</td>
</tr>
</tbody>
</table>

RMP, resting membrane potential; V_{max}, AP upstroke velocity; OS, overshoot; APD_{50}, AP duration at 50% repolarization; APD_{70}, AP duration at 70% repolarization; APD_{90}, AP duration at 90% repolarization. *P<0.05 vs control, **P<0.05 vs. ISO by one-way repeated measures ANOVA with Tukey’s posthoc test; n=6 myocytes.

### Supplemental Table 8: Action potential parameters at baseline and after application of ISO (10 nM) and mANP (10 nM) in mouse right atrial myocytes

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ISO</th>
<th>ISO + mANP</th>
<th>mANP washout</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP (mV)</td>
<td>-80.0 ± 1.0</td>
<td>-80.2 ± 1.3</td>
<td>-80.3 ± 1.3</td>
<td>-80.4 ± 1.3</td>
</tr>
<tr>
<td>V_{max} (V/s)</td>
<td>130.4 ± 8.8</td>
<td>152.1 ± 11.7*</td>
<td>144.7 ± 9.6**</td>
<td>151.8 ± 12.5*</td>
</tr>
<tr>
<td>OS (mv)</td>
<td>56.5 ± 5.2</td>
<td>63.1 ± 6.0*</td>
<td>57.8 ± 7.3</td>
<td>62.7 ± 6.5*</td>
</tr>
<tr>
<td>APD_{50} (ms)</td>
<td>7.8 ± 3.4</td>
<td>13.6 ± 4.4*</td>
<td>11.4 ± 3.9*</td>
<td>13.7 ± 4.4*</td>
</tr>
<tr>
<td>APD_{70} (ms)</td>
<td>18.7 ± 5.5</td>
<td>28.7 ± 7.0*</td>
<td>23.5 ± 6.5**</td>
<td>27.5 ± 7.5*</td>
</tr>
<tr>
<td>APD_{90} (ms)</td>
<td>45.5 ± 5.7</td>
<td>58.0 ± 8.3*</td>
<td>51.4 ± 7.5**</td>
<td>56.2 ± 9.4*</td>
</tr>
</tbody>
</table>

RMP, resting membrane potential; V_{max}, AP upstroke velocity; OS, overshoot; APD_{50}, AP duration at 50% repolarization; APD_{70}, AP duration at 70% repolarization; APD_{90}, AP duration at 90% repolarization. *P<0.05 vs control, **P<0.05 vs. ISO by one-way repeated measures ANOVA with Tukey’s posthoc test; n=7 myocytes.
Supplemental Table 9: Action potential parameters at baseline and after application of ISO (10 nM) and mANP (100 nM) in mouse right atrial myocytes

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ISO</th>
<th>ISO + mANP</th>
<th>mANP washout</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP (mV)</td>
<td>-81.3 ± 1.5</td>
<td>-81.7 ± 1.5</td>
<td>-81.6 ± 0.9</td>
<td>-81.0 ± 2.1</td>
</tr>
<tr>
<td>V\text{max} (V/s)</td>
<td>146.4 ± 15.0</td>
<td>169.4 ± 15.6*</td>
<td>157.9 ± 19.5**</td>
<td>165.2 ± 16.5*</td>
</tr>
<tr>
<td>OS (mv)</td>
<td>63.0 ± 5.4</td>
<td>67.6 ± 5.1*</td>
<td>63.3 ± 4.5</td>
<td>65.6 ± 5.1</td>
</tr>
<tr>
<td>APD\text{50} (ms)</td>
<td>10.9 ± 3.3</td>
<td>15.0 ± 3.0*</td>
<td>12.1 ± 3.6**</td>
<td>15.9 ± 5.7*</td>
</tr>
<tr>
<td>APD\text{70} (ms)</td>
<td>22.0 ± 2.1</td>
<td>30.9 ± 4.5*</td>
<td>24.0 ± 3.9*</td>
<td>29.4 ± 6.9*</td>
</tr>
<tr>
<td>APD\text{90} (ms)</td>
<td>47.9 ± 6.0</td>
<td>60.8 ± 6.3*</td>
<td>54.1 ± 5.7**</td>
<td>59.1 ± 7.5*</td>
</tr>
</tbody>
</table>

RMP, resting membrane potential; V\text{max}, AP upstroke velocity; OS, overshoot; APD\text{50}, AP duration at 50% repolarization; APD\text{70}, AP duration at 70% repolarization; APD\text{90}, AP duration at 90% repolarization. *P<0.05 vs control, +P<0.05 vs. ISO by one-way repeated measures ANOVA with Tukey’s posthoc test; n=9 myocytes.

Supplemental Table 10: Action potential parameters at baseline and after application of ISO (10 nM) and ANP + mANP (100 nM each) in mouse right atrial myocytes

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ISO</th>
<th>ISO + ANP + mANP</th>
<th>ANP/mANP washout</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMP (mV)</td>
<td>-80.0 ± 2.3</td>
<td>-79.8 ± 1.8</td>
<td>-80.8 ± 2.1</td>
<td>-80.1 ± 1.8</td>
</tr>
<tr>
<td>V\text{max} (V/s)</td>
<td>133.9 ± 22.6</td>
<td>146.0 ± 21.6*</td>
<td>142.0 ± 23.4*</td>
<td>143.1 ± 25.2*</td>
</tr>
<tr>
<td>OS (mv)</td>
<td>59.5 ± 7.3</td>
<td>64.6 ± 7.8*</td>
<td>61.2 ± 9.6</td>
<td>62.4 ± 9.1</td>
</tr>
<tr>
<td>APD\text{50} (ms)</td>
<td>7.0 ± 3.1</td>
<td>12.1 ± 4.2*</td>
<td>11.3 ± 3.9*</td>
<td>12.1 ± 4.4*</td>
</tr>
<tr>
<td>APD\text{70} (ms)</td>
<td>18.4 ± 9.6</td>
<td>28.3 ± 12.2*</td>
<td>25.9 ± 12.0*</td>
<td>27.2 ± 11.4*</td>
</tr>
<tr>
<td>APD\text{90} (ms)</td>
<td>49.6 ± 15.3</td>
<td>62.2 ± 15.9*</td>
<td>58.1 ± 14.6*</td>
<td>61.2 ± 13.3*</td>
</tr>
</tbody>
</table>

RMP, resting membrane potential; V\text{max}, AP upstroke velocity; OS, overshoot; APD\text{50}, AP duration at 50% repolarization; APD\text{70}, AP duration at 70% repolarization; APD\text{90}, AP duration at 90% repolarization. *P<0.05 vs control, by one-way repeated measures ANOVA with Tukey’s posthoc test; n=7 myocytes.

Supplemental Table 11: I_{\text{Ca,L}} conductance analysis in baseline conditions and following application of ISO and ANP in mouse right atrial myocytes

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ISO</th>
<th>ISO + ANP</th>
</tr>
</thead>
<tbody>
<tr>
<td>G\text{max} (pS/pF)</td>
<td>79.3 ± 0.8</td>
<td>168.1 ± 6.4*</td>
<td>194.7 ± 6.7**</td>
</tr>
<tr>
<td>V\text{1/2(act)} (mV)</td>
<td>-10.2 ± 0.3</td>
<td>-16.3 ± 1.1*</td>
<td>-18.6 ± 1.1**</td>
</tr>
<tr>
<td>k</td>
<td>7.4 ± 0.3</td>
<td>6.6 ± 0.8</td>
<td>6.5 ± 0.8</td>
</tr>
</tbody>
</table>

G\text{max}, maximum conductance; V\text{1/2(act)}, voltage at which 50% of channels are activated; k, slope factor. *P<0.05 vs control, +P<0.05 vs. ISO by one-way repeated measures ANOVA with Tukey’s posthoc test; n=8 myocytes.
Supplemental Table 12: $I_{\text{Ca,L}}$ conductance analysis in baseline conditions and following application of ISO and mANP in mouse right atrial myocytes

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ISO</th>
<th>ISO + mANP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_{\text{max}}$ (pS/pF)</td>
<td>72.5 ± 3.1</td>
<td>178.2 ± 7.9*</td>
<td>152.9 ± 5.3**</td>
</tr>
<tr>
<td>$V_{1/2(\text{act})}$ (mV)</td>
<td>-12.0 ± 0.5</td>
<td>-20.6 ± 1.4*</td>
<td>-18.5 ± 1.0*</td>
</tr>
<tr>
<td>$k$</td>
<td>7.8 ± 1.0</td>
<td>5.7 ± 1.2</td>
<td>6.3 ± 1.0</td>
</tr>
</tbody>
</table>

$G_{\text{max}}$, maximum conductance; $V_{1/2(\text{act})}$, voltage at which 50% of channels are activated; $k$, slope factor. *$P<0.05$ vs control, **$P<0.05$ vs. ISO by one-way repeated measures ANOVA with Tukey’s posthoc test; $n=6$ myocytes.

Supplemental Table 13: $I_{\text{Na}}$ conductance analysis in baseline conditions and following application of ISO and ANP in mouse right atrial myocytes

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ISO</th>
<th>ISO + ANP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_{\text{max}}$ (pS/pF)</td>
<td>1167.3 ± 8.6</td>
<td>1240.7 ± 5.7</td>
<td>1219.3 ± 11.2</td>
</tr>
<tr>
<td>$V_{1/2(\text{act})}$ (mV)</td>
<td>-39.7 ± 0.2</td>
<td>-41.3 ± 0.2</td>
<td>-42.0 ± 0.4</td>
</tr>
<tr>
<td>$k$</td>
<td>6.7 ± 0.2</td>
<td>6.5 ± 0.2</td>
<td>6.6 ± 0.2</td>
</tr>
</tbody>
</table>

$G_{\text{max}}$, maximum conductance; $V_{1/2(\text{act})}$, voltage at which 50% of channels are activated; $k$, slope factor. There were no significant effects of ISO or ANP on $I_{\text{Na}}$ $G_{\text{max}}$ ($P=0.658$), $V_{1/2(\text{act})}$ ($P=0.235$) or $k$ ($P=0.67$). Data analyzed by one-way repeated measures ANOVA; $n=5$ myocytes.

Supplemental Table 14: $I_{\text{Na}}$ conductance analysis in baseline conditions and following application of ISO and mANP in mouse right atrial myocytes

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ISO</th>
<th>ISO + mANP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_{\text{max}}$ (pS/pF)</td>
<td>1679.2 ± 21.1</td>
<td>1752.8 ± 13.0</td>
<td>1709.9 ± 13.2</td>
</tr>
<tr>
<td>$V_{1/2(\text{act})}$ (mV)</td>
<td>-47.8 ± 0.5</td>
<td>-48.7 ± 0.2</td>
<td>-50.1 ± 0.2</td>
</tr>
<tr>
<td>$k$</td>
<td>6.4 ± 0.5</td>
<td>6.3 ± 0.2</td>
<td>6.7 ± 0.2</td>
</tr>
</tbody>
</table>

$G_{\text{max}}$, maximum conductance; $V_{1/2(\text{act})}$, voltage at which 50% of channels are activated; $k$, slope factor. There were no significant effects of ISO or ANP on $I_{\text{Na}}$ $G_{\text{max}}$ ($P=0.935$), $V_{1/2(\text{act})}$ ($P=0.193$) or $k$ ($P=0.955$). Data analyzed by one-way repeated measures ANOVA; $n=6$ myocytes.
References


