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## Augmented potassium current is a shared phenotype for two genetic defects associated with familial atrial fibrillation

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### Abstract

**Background**—Mutations in multiple genes have been implicated in familial atrial fibrillation (AF), but the underlying mechanisms, and thus implications for therapy, remain ill-defined.

**Methods and Results**—Among 231 participants in the Vanderbilt AF Registry, we found a mutation in *KCNQ1* (encoding the  $\alpha$ -subunit of slow delayed rectifier potassium current [ $I_{Ks}$ ]) and separately a mutation in natriuretic peptide precursor A (*NPPA*) gene (encoding atrial natriuretic peptide, ANP), both segregating with early-onset lone AF in different kindreds. The functional effects of these mutations yielded strikingly similar  $I_{Ks}$  “gain of function.” In Chinese Hamster Ovary (CHO) cells, coexpression of mutant *KCNQ1* with its ancillary subunit *KCNE1* generated ~3-fold larger currents that activated much faster than wild-type (WT)- $I_{Ks}$ . Application of the WT *NPPA* peptide fragment produced similar changes in WT- $I_{Ks}$ , and these were exaggerated with the mutant *NPPA* S64R peptide fragment. Anantin, a competitive ANP receptor antagonist, completely inhibited the changes in  $I_{Ks}$  gating observed with *NPPA*-S64R. Computational simulations identified accelerated transitions into open states as the mechanism for variant  $I_{Ks}$  gating. Incorporating these  $I_{Ks}$  changes into computed human atrial action potentials (AP) resulted in 37% shortening (120 vs. 192 ms at 300 ms cycle length), reflecting loss of the phase II dome which is dependent on L-type calcium channel current.

**Conclusions**—We found striking functional similarities due to mutations in *KCNQ1* and *NPPA* genes which led to  $I_{Ks}$  “gain of function”, atrial AP shortening, and consequent altered calcium current as a common mechanism between diverse familial AF syndromes.

### Keywords

atrial fibrillation; ion channels; atrial natriuretic peptide; genetics; action potentials

### INTRODUCTION

Atrial fibrillation (AF) is the most common cardiac arrhythmia affecting ~2% of the US population and resulting in substantial morbidity and mortality [1-4]. While most AF is

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associated with other cardiac or systemic disorders, 10-30% of individuals with AF have no evidence of structural heart disease (so called idiopathic or 'lone' AF) [5,6]. There is now accumulating evidence that genetic factors play a role in the pathogenesis of lone AF [7,8]. Identification of specific electrophysiologic abnormalities in genetically-defined AF holds the promise of moving therapy from the current empiric approach to one that is mechanism-based.

The role of gene variants in the pathogenesis of AF has only recently begun to be appreciated [7,9]. *KCNQ1*, the first disease gene identified for familial AF [10], encodes the pore-forming  $\alpha$ -subunit of the potassium channel that conducts the slow component of the delayed rectifier potassium current ( $I_{Ks}$ ) in the atrium and ventricle. Functional analysis of the S140G mutant revealed a gain-of-function, which contrasts with the dominant negative or loss-of-function effects of *KCNQ1* mutations previously associated with the congenital long QT syndrome [11]. Similarly, small kindreds with AF and mutations in other potassium channel genes, including *KCNE2* [12], *KCNJ2* [13], and *KCNA5* [14], and in genes encoding sodium channel  $\alpha$ - and  $\beta$ -subunits have been reported [15,16].

To date, familial AF has been linked to mutations in potassium and sodium channels that are predicted to either shorten or lengthen the duration of the cardiac action potential (AP). However, a recent report has identified a novel molecular genetic basis for AF with the identification of a mutation in the natriuretic peptide precursor A gene (*NPPA*), which encodes atrial natriuretic peptide (ANP) [17]. This mutation segregated with familial AF, thereby uncovering an unexpected association between a defect in a circulating hormone and susceptibility to AF. Although the precise mechanism through which the mutant ANP leads to the development AF is not completely clear, a shortening of the atrial AP duration was demonstrated in an isolated rat heart model.

The three coding exons of *NPPA* transcribe a 151 amino acid peptide called preproANP (Figure 1) [18,20]. This peptide then undergoes dual modification involving a signal peptidase and the enzyme corin to produce ANP, the mature 28 amino acid carboxy terminal end and a 98 amino acid N-terminus [21,22]. The N-terminal peptide undergoes further degradation to produce a 16 amino acid peptide called long acting natriuretic peptide (LANP), a vessel dilator, and kaliuretic hormone [23,24]. These additional peptides generated from preproANP have been shown to have biological activity similar to that of the mature ANP [23,25].

Here we report genetic analyses of *KCNQ1* and *NPPA* in a large cohort of individuals with lone AF or AF associated with heart disease, as well as in population controls. We identified mutations in both genes that cosegregated with AF in separate kindreds. *In vitro* studies demonstrated strikingly similar gain-of-function defects associated with the mutant forms. Computational simulations of atrial APs incorporating wild-type (WT) or mutant  $I_{Ks}$  demonstrated a mutation-dependent change in AP configuration - AP shortening due to loss of the AP "dome" with premature stimuli - known to increase AF susceptibility [26,27].

## Materials and Methods

### Study subjects

Subjects prospectively enrolled between November 2002 and October 2005 in the Vanderbilt AF Registry, which comprises clinical and genetic databases, were studied. Individuals enrolled in the Registry were greater than 18 years old with an ECG-confirmed diagnosis of AF. Subjects were excluded if AF was diagnosed in the setting of recent cardiac surgery or if they were unable to give informed consent or report for follow-up. The study protocol was approved by the Institutional Review Board of Vanderbilt University, and participants were enrolled following informed written consent. Controls were a cohort of anonymous population

controls obtained from the Coriell repository, representing four different ethnic groups (Caucasian, African-American, Hispanic, Asian, n = 47 for each group).

Probands and their relatives were clinically classified by a consistently applied set of definitions. For the purposes of our study, AF was defined as replacement of sinus P waves by rapid oscillations or fibrillatory waves that varied in size, shape, and timing and were associated with an irregular ventricular response when atrioventricular conduction was intact. Documentation of AF on an ECG, rhythm strip, event recorder, or Holter monitor recording was necessary. Lone AF was defined as AF occurring in individuals less than 65 years of age without hypertension or overt structural heart disease as determined by clinical examination, ECG and echocardiography. An echocardiogram was obtained on all patients at time of enrollment into the registry.

Paroxysmal AF was defined as AF lasting more than 30 seconds that terminated spontaneously. Persistent AF was defined as AF lasting more than seven days and requiring either pharmacologic therapy or electrical cardioversion for termination. AF that was refractory to cardioversion or that was allowed to continue was classified as permanent.

Familial AF was defined as the presence of lone AF in one or more first-degree relatives of the index case. Family history information was initially obtained from the medical record and was supplemented by a questionnaire detailing past medical history, family history, and clinical symptoms. For individuals with a positive family history, a more detailed pedigree was generated by history and review of medical records of relatives.

### Mutation screening

Whole blood was collected for genomic DNA extraction and analysis from all participating subjects. The coding and flanking intronic regions of *KCNQ1* and *NPPA* were amplified by PCR using primers designed to obtain fragments of appropriate size (see online supplement for details). PCR-amplified DNA fragments were analyzed using the Reveal® Discovery System (based on temperature gradient capillary electrophoresis) to identify aberrant conformers, which were then directly sequenced. Plasma ANP levels were measured in participating subjects using the Phoenix radioimmunoassay (Phoenix Pharmaceuticals, Inc., Burlingame, CA).

### Electrophysiology, FuGENE6-mediated channel expression and cell transfection

cDNAs for WT human *KCNQ1* and *KCNE1* were provided by Drs. Michael Sanguinetti (University of Utah) and Mark Keating (Novartis Institute for Biomedical Research, Cambridge, MA). CHO cells were used for transient transfections. Variant *KCNQ1* was engineered by site-directed mutagenesis using standard techniques [28], and plasmid sequences were verified by resequencing prior to use. To study  $I_{Ks}$ , 2  $\mu$ g WT or mutant *KCNQ1* plasmid was co-transfected with *KCNE1* in a bicistronic vector (*KCNE1*-IRES-eGFP, 2  $\mu$ g) into CHO cells with 12  $\mu$ l of FuGENE6 (Roche) in 0.5 ml serum-free medium for 30 min, after which standard medium was restored for 48 hours in culture. The cells were removed from the dish by brief trypsinization and stored in standard medium for experiments within the next 12 hours.

### Whole-cell voltage clamp

Cells showing green fluorescence were chosen for study. Voltage-clamp studies were performed using methods previously reported [29]. To obtain current-voltage (I-V) relations for  $I_{Ks}$ , cells were held at -80 mV. Activating currents were elicited with 5-sec depolarizing pulses from -60 to +100 mV in 20 mV steps, and tail currents were recorded upon return to -40 mV. Pulses were delivered every 30 sec. Current densities (in pA/pF) were obtained after normalization to cell surface area. I-V relationships were analyzed by fitting the Boltzmann

equation to the data:  $I = I_{\max} / \{1 + \exp[(V_t - V_{0.5})/k]\}$ , where  $I_{\max}$  is the maximal current,  $V_t$  is the test potential,  $V_{0.5}$  is the membrane potential at which 50% of the channels are activated, and  $k$  is a slope factor. Time constants for activation and deactivation were obtained by fitting mono-exponential functions to the current data using CLAMPFIT software.

The effects of WT *NPPA* peptide (and mutant *NPPA* S64R peptide fragment on  $I_{Ks}$  were assessed by adding peptide (100 nM) to the superfusate for 30 min pretreatment prior to recording  $I_{Ks}$ . The dose responses of  $I_{Ks}$  to WT and to mutant S64R were assessed by repeating the experiments with peptide concentrations of each ranging from 0.1 to 100 nM. To further address the role of receptor-mediated initiation of the cGMP-dependent ANP pathway in modifying  $I_{Ks}$  by WT and mutant S64R peptide, experiments were performed with the addition of two other membrane-permeable agents to the ANP pretreatment: the competitive ANP receptor antagonist anantin (500 nM) and the cGMP analog 8'-Br-cGMP (200  $\mu$ M).

### Solutions and drugs

To record  $I_{Ks}$ , the internal pipette filling solution contained (in mM): KCl 200, K<sub>4</sub>BAPTA 5, K<sub>2</sub>ATP 5, MgCl<sub>2</sub> 1 and HEPES 10. The solution was adjusted to pH 7.2 with KOH, yielding a final  $[K^+]_i$  of ~235 mM, as we have described previously [29,30]. The external solution was normal Tyrode's, containing (in mM) NaCl 130, KCl 4, CaCl<sub>2</sub> 1.8, MgCl<sub>2</sub> 1, HEPES 10, and glucose 10, and was adjusted to pH 7.35 with NaOH. Experiments were conducted at 22-23° C. WT *NPPA* peptide fragment (AA sequence: CQVLSEPNEEAG) and 8'-Br-cGMP were purchased from Sigma-Aldrich Co. (St Louis, MO). The mutant *NPPA* S64R peptide fragment (AA sequence: CQVLREPNEEAG) was commercially synthesized by Peptide 2.0 Co. (Chantilly, VA). Anantin was obtained from Bachem Americas Inc. (Torrance, CA).

### Computational Simulations of $I_{Ks}$ and of the Human Atrial AP

Markov models of  $I_{Ks}$  were generated using the 17-state formulation described by Silva and Rudy [31] (Figure S-1 of the online data supplement). The current-voltage curves described above for WT *KCNQ1* cotransfected with *KCNE1* were used to generate the model of WT- $I_{Ks}$ . As noted below, both mutant *KCNQ1*-IAP54-56 and mutant *NPPA* S64R peptide fragment generated similar *in vitro* effects on  $I_{Ks}$  function. Accordingly, the current-voltage curves for *KCNQ1*-IAP54-56  $I_{Ks}$  were used to generate the "variant"  $I_{Ks}$  that here refers to both *KCNQ1*-IAP54-56  $I_{Ks}$  and S64R fragment  $I_{Ks}$ . The details of model generation for WT- $I_{Ks}$  and variant  $I_{Ks}$  are provided in Figures S-2, S-3 and Table S-1 of the online data supplement.

The Courtemanche-Ramirez-Nattel (CRN) simulation of human atrial APs was implemented [32]. The CRN  $I_{Ks}$  formulation was replaced with the Markov model of the human WT or variant  $I_{Ks}$  described above. Steady-state AP simulations were generated at pacing cycle length (CL) ranging from 300 to 4000 ms to compare the behavior of the simulation with implementation of WT and variant  $I_{Ks}$  (Figure S-4). Details of the CRN AP simulation are provided in the online data supplement (Table S-1). Electronic versions of the WT and variant  $I_{Ks}$  models as well as the CRN AP simulation are available upon request.

A simulation study was performed to evaluate the effects of premature atrial stimulation on morphology and duration of APs. Two premature atrial stimuli with a coupling interval of 400 ms were applied to the end of a 12-pulse train of 1000 ms pacing CL. APs as well as transmembrane current interactions were compared for WT and variant  $I_{Ks}$ .

### Statistical analysis

Data are expressed as mean  $\pm$  SEM. For comparisons among means of more than two groups, ANOVA was used, with *post hoc* pair-wise comparisons by Duncan's test if significant

differences among means were detected. If only two groups were being compared, Student's *t*-test was used. A *P*-value <0.05 was considered statistically significant.

## Results

### Mutation screening

During the 3-year enrollment period, 245 patients with AF were approached and 231 (94%) subjects agreed to participate in the study. This study cohort included 98 patients (42%) with lone AF and 133 with AF associated with heart disease. The majority of subjects were Caucasian (94%), and 5% were African-American. AF was diagnosed at a mean age of  $50 \pm 14$  years.

In the study cohort of 231 individuals with AF, screening for *KCNQ1* mutations in genomic DNA identified a unique sequence variant in a case of familial AF (Vanderbilt AF 313). In the proband, a 9-bp duplication was identified resulting in insertion of the amino acids isoleucine (I), alanine (A) and proline (P) at locations 54 to 56 (IAP54-56) in the N-terminus of the *KCNQ1* protein (Figure 2). The variant was also confirmed in three affected family members. The sequence change was not found in Caucasian, Chinese Han, or Asian population controls but was identified in 2.1% (2/94, the denominator refers to the number of chromosomes that were successfully screened and is therefore twice the number of African-American controls) of African-American individuals. As the controls were obtained from the anonymous Coriell repository, no clinical information about these individuals is available.

Genomic DNA sequencing of *NPPA* identified a novel missense mutation A190C (adenine to cytosine substitution at nucleotide 190) in exon 2 in a proband with familial AF (Vanderbilt AF 1111) (Figures 1 and 3). The mutation was also confirmed in two affected family members but was absent in unaffected family members. Furthermore, the mutation was not identified in Caucasian, Chinese Han, Asian, or African-American population controls. The mutation is also conserved across many species (dog, rat and mouse). Because of its location in the proANP peptide, subsequent post-translational modifications result in the arginine substitution being reflected not within ANP, but within the vessel dilator peptide (Figure 1). No other *KCNQ1* or *NPPA* non-synonymous variants were identified in these 231 subjects.

### Family phenotype evaluation

The *KCNQ1*-IAP54-56 proband in Vanderbilt AF 313 (III-4) presented with palpitations at age 38 but was not diagnosed with AF until age 42 (Table 1). Although the proband initially responded to sotalol therapy, he gradually began to experience more frequent symptomatic recurrences of paroxysmal AF over a 7 to 8 year period. Flecainide was substituted for sotalol and has been effective at suppressing symptomatic AF. A detailed family history showed that most of the affected family members developed lone AF at a relatively young age and all presented with symptomatic paroxysmal AF (Table 1). To date, they have all been managed successfully with antiarrhythmic drugs. Evaluation of the ECG recordings showed that the corrected QT interval was normal during sinus rhythm for all family members studied (QT:  $412 \pm 25$  ms; QTc:  $431 \pm 6$  ms). Although two family members (III-5 and IV-1, Figure 2) have episodic palpitations that last up to 20 mins and occur every couple of months, we have not been able to document AF; therefore, we have classified their status as uncertain.

The *NPPA*-S64R proband in Vanderbilt AF 1111 (II:5) developed symptoms of palpitations, lightheadedness, and dizziness at age 36 and was diagnosed with paroxysmal lone AF at age 42 years when an event recorder documented an episode of AF associated with rapid ventricular response. As his AF episodes are short-lived and infrequent, occurring less than once per month, he has been successfully managed with only metoprolol. A family history (Figure 3)



revealed that the proband's older sister and mother also were diagnosed with paroxysmal AF at a relatively young age (44 and 50 years, respectively). The proband's mother (I:1) initially presented with rapid palpitations and was found to be in rapid AF but subsequently also developed hypertension and sick sinus syndrome necessitating a permanent pacemaker when she was 58 years old. Currently, the mother's AF is controlled with a combination of metoprolol and digoxin. The proband's sister (II-4) also has highly symptomatic paroxysmal lone AF that is responsive to flecainide. Two of the proband's children also describe infrequent palpitations. Although consistent with AF, to date we have not been able to document AF; therefore we have designated them as of uncertain phenotype. A number of individuals in generation III have declined genetic or clinical screening, so their AF status is unknown. ANP levels were compared between family members affected with AF who were mutation carriers and those unaffected and WT for the S64R variant and were found not to be significantly different ( $24 \pm 8.2$  pg/ml, N=3 [affected mutation carriers] vs.  $26 \pm 11$  pg/ml, N=6 [unaffected WT],  $P > 0.05$ ).

### The common *in vitro* phenotype - a large and rapidly-activating $I_{K_S}$

Expression of WT *KCNQ1* + *KCNE1* in CHO cells resulted in slowly-activating currents characteristic of cardiac  $I_{K_S}$ . Coexpression of *KCNQ1*-IAP54-56 with *KCNE1* generated currents that were much larger and activated much earlier than WT- $I_{K_S}$  (Figure 4); e.g., at +20 mV, peak current was  $75 \pm 8$  pA/pF (*KCNQ1*-IAP54-56) vs.  $25 \pm 5$  pA/pF (WT) after 5-sec pulses (n=7-8 each,  $P < 0.001$ ). Coexpression of WT and *KCNQ1*-IAP54-56 with *KCNE1* resulted in a phenotype intermediate between the two. Figure 4D highlights the accelerated activation seen with the variant.

Summary data for the *KCNQ1*-IAP54-56 mutant are presented in Figure 5 and Table 2. In addition to the increased amplitude (Figure 5A), there was a -21 mV shift in the voltage dependence of activation (Figure 5B), indicating that at plateau voltages the probability of channel opening is further enhanced in the mutant. The mutant accelerated both activation (Figure 5C) and deactivation (Figure 5D) over all voltages tested.

Application of WT *NPPA* peptide fragment (100 nM) to CHO-cells expressing WT *KCNQ1* + *KCNE1* yielded a moderate increase in cardiac  $I_{K_S}$  (Figure 6A and 6B; Table 2), in good agreement with previous studies [33]. However, a much more dramatic augmentation, remarkably similar to that observed with IAP54-56- $I_{K_S}$ , was observed with *NPPA* S64R peptide fragment (100 nM, Figure 6C). At +20 mV, peak current was  $78 \pm 10$  pA/pF (S64R) vs.  $23 \pm 8$  pA/pF (WT) after 5-sec pulses (n=7 each,  $P < 0.001$ ). Activating traces at +20 mV normalized to current amplitude at the end of the pulse from Figures 6A, 6B, and 6C are shown in Figure 6D to emphasize the changes in activation. The voltage dependence of activation and of deactivation are shown in Figures 6E and 6F respectively. As with *KCNQ1*-IAP54-56, the S64R fragment produced a ~20mV negative shift in the voltage-dependence of tail current (Figure 6F). The results for WT- $I_{K_S}$  and the effects of *KCNQ1*-IAP54-56 and *NPPA* S64R peptide fragment on  $I_{K_S}$  are summarized in Table 2.

ANP interacts with  $I_{K_S}$  via the ANP receptor initiation of a cGMP-dependent pathway present in mammalian cells (e.g., CHO cells) [33,34]. We hypothesized that the alterations in  $I_{K_S}$  magnitude and kinetics due to the *NPPA* peptide fragment are mediated through the ANP receptor. Guanylate cyclase, the putative ANP receptor [35], is known to be present on the membrane surface of CHO cells [34], and thus provided an appropriate model for testing this hypothesis. We pretreated  $I_{K_S}$ -expressing CHO cells with the competitive ANP receptor antagonist, anantin (500 nM), for 30 min prior to exposure to the *NPPA* S64R peptide fragment (100 nM) [36]. We found that pretreatment with anantin completely eliminated  $I_{K_S}$  augmentation and acceleration seen with the S64R fragment alone (Figure S-5 of the online data supplement) indicating that the effect of the S64R fragment on  $I_{K_S}$  is mediated by the endogenous ANP receptor. Further, to investigate the involvement of intracellular cGMP we

examined the effect of a cGMP analog, 8'-Br-cGMP, on  $I_{Ks}$ . Following acute exposure to 200  $\mu$ M 8'-Br-cGMP,  $I_{Ks}$  was increased in a similar manner to that observed with the S64R fragment (Figure S-6 of the online data supplement), supporting the notion that  $I_{Ks}$  modulation by the *NPPA* peptide fragment occurs via intracellular cGMP.

Having established the role of the ANP receptor in modulating the interaction between the *NPPA* peptide fragment and  $I_{Ks}$ , we sought to determine the effects of different concentrations of the WT fragment and mutant S64R fragment on  $I_{Ks}$  channels. We conducted dose-response experiments with WT fragment and S64R fragment concentrations ranging from 0.1 to 100 nM. While WT fragment increased  $I_{Ks}$  in a dose-dependent manner with an  $IC_{50}$  value of  $8.6 \pm 1.0$  nM, the enhancement of the current by S64R mutant was much more potent with an  $IC_{50}$  value of  $1.1 \pm 0.2$  nM (Figure S-7 of the online data supplement). Also, the mutant markedly accelerated the  $I_{Ks}$  activation.

### Computational simulation of mutant $I_{Ks}$

A 17-state Markov model for  $I_{Ks}$  was implemented and fit to the WT and variant  $I_{Ks}$  current profiles described above. The most apparent differences between the two fits were in the transitions between open states:  $O1 \rightleftharpoons O2$  and  $O1 \rightleftharpoons O2$  transition rates  $[\psi, \omega]$  were  $0.52$  and  $0.59$   $ms^{-1}$  for variant  $I_{Ks}$  and  $2e-4$  and  $2e-7$   $ms^{-1}$  for WT- $I_{Ks}$ , respectively, at  $-10$  mV transmembrane potential (Table S-1 of the online data supplement).

### Computational simulation of human atrial AP

Atrial AP simulations with implementations of WT- $I_{Ks}$  or variant  $I_{Ks}$  were then compared (Figure 7A). At CL 1000 ms, open state  $I_{Ks}$  channel occupancy was larger for variant  $I_{Ks}$  which led to 4-fold increase in peak current versus WT- $I_{Ks}$  (0.5 pA/pF for variant  $I_{Ks}$  vs. 0.12 pA/pF for WT- $I_{Ks}$ ) (Figure 8A) despite a lower overall conductance for the variant  $I_{Ks}$  (variant  $G_{Ks} = 0.86 \times$  WT  $G_{Ks}$ ; Tables S-1.3 and S-1.4). While the mean closed state occupancies for variant  $I_{Ks}$  and WT- $I_{Ks}$  were similar, there was greater open state occupancy for variant  $I_{Ks}$  (6% for variant  $I_{Ks}$  vs. 1.4% for WT- $I_{Ks}$ ), due to a much more dynamic transition of channels from the closed state, through the activated state, into the open state (Figure 8B-D). By contrast, WT- $I_{Ks}$  channels showed relatively little state variation over the course of the AP. The 4-fold increase in peak variant  $I_{Ks}$  compared to WT- $I_{Ks}$  resulted, as expected, in a decrease in steady state AP duration (188 ms for variant  $I_{Ks}$  vs. 297 ms for WT- $I_{Ks}$  at CL 1000 ms) for all pacing CLs (Supplement Figure S-4).

During pacing at CL 1000 ms, the dome during phase II of the AP was less pronounced with variant  $I_{Ks}$  (Figure 7A). With simulations of premature atrial stimuli, the dynamic nature of these morphologic differences was accentuated. With double premature atrial stimuli at 400ms coupling interval, the dome was blunted for both WT- $I_{Ks}$  and variant  $I_{Ks}$  with the first premature stimulus (PS1). However, the dome was restored with the second premature stimulus (PS2) for the WT- $I_{Ks}$  but not the variant  $I_{Ks}$  simulation.

The dome during phase II of the AP reflects a period where the depolarizing effect of the L-type calcium current ( $I_{CaL}$ ) outweighs the repolarizing potassium currents, primarily  $I_{Ks}$ , the rapid delayed rectifier potassium current ( $I_{Kr}$ ), and the ultra-rapid delayed rectifier potassium current ( $I_{Kur}$ ) (Figures 7B-E). For both WT- $I_{Ks}$  and variant  $I_{Ks}$ , loss of the dome with PS1 is due to loss of this phase II calcium current. Failure of the PS2 dome to recover in the simulation with variant  $I_{Ks}$  reflects lack of calcium current recovery as a consequence of the altered AP trajectory imposed by variant  $I_{Ks}$ .

The repolarizing current  $I_{Kur}$  showed similar dynamics (though in the opposite direction) as  $I_{CaL}$  with smaller variation and hence smaller effect than those seen with  $I_{CaL}$ . In this simulation,  $I_{Kr}$  did not contribute significantly to repolarization dynamics.

## Discussion

In this study we identified mutations in *KCNQ1* (IAP54-56) and *NPPA* (S64R) in moderate-sized Caucasian kindreds with early onset familial AF and normal QT intervals. Although both these genes have previously been linked with familial AF, this is the first study to demonstrate that augmented potassium current is a shared phenotype across these diverse genetic defects associated with familial AF.  $I_{Ks}$  gain-of-function mutations have previously been reported for potassium channel defects, but the notion that mutations in disparate genes, *KCNQ1* and *NPPA*, lead to the same arrhythmia phenotype raises the possibility of subtype specific therapy for familial AF. The *KCNQ1*-IAP54-56 variant was also identified in a 2.1% of healthy African-American individuals, suggesting it may be a common risk allele in some populations.

One prevailing conceptual model proposed for AF pathogenesis describes reduced atrial refractory period as a substrate for re-entrant arrhythmias [37]. This model is supported by reports of gain-of-function mutations in genes encoding subunits of cardiac channels responsible for generating  $I_{Ks}$  (*KCNQ1/KCNE2*) and  $I_{K1}$  (*KCNJ2*) which are predicted to decrease AP duration [10,12,13]. Such understanding provides a therapeutic rationale for prolonging the atrial refractory period for the treatment of AF with antiarrhythmic drugs. In the present study, functional and simulation studies demonstrated that *KCNQ1* and *NPPA* variants are gain-of-function mutations that shorten the AP duration both at steady state and in the setting of PACs - the most common (> 90%) initiating event in the onset of paroxysmal AF [38] - and as such are a likely cause of AF in these families. Although a shortened AP duration would be expected to shorten the QT interval, this was not observed in affected family members carrying the *KCNQ1* variant. The mechanisms whereby some *KCNQ1* mutations produce AF and others generate ventricular phenotypes remains to be determined. One possible explanation for the phenotypic variability in *KCNQ1*-linked disease, including that seen in AF or long QT syndrome, may reflect chamber- or disease-specific interactions between the channel and its partners. The latter may be well-described proteins, such as  $\beta$ -subunits or entirely novel proteins.

Studies of kindreds with AF suggest a genetic basis for the condition, and mutations in several cardiac potassium channel genes have been linked to familial AF [12-14]. Although specific mutations in the *KCNQ1* gene have been observed in families with AF [10,39], the role of such mutations in AF remains unclear; often such isolated or “private” mutations are in residues of unknown function, effects on channel conductance are variable, and in most cases it may be difficult to discriminate rare polymorphisms of no functional significance from true mutations. However, the low prevalence of *KCNQ1* mutations in large AF cohorts, including ours, suggests that mutations in this gene are not a major cause of AF [40,41]. In both AF313 and AF1111, multiple family members presented with early onset lone AF, and our segregation analysis supports the novel *KCNQ1* and *NPPA* variants as being associated with AF. Although these variants were absent in the non-African-American control groups, and the *in vitro* data confirms that the variants are functional, we cannot completely exclude the possibility that the identified variants are not functional, risk-conferring polymorphisms in the Caucasian population. The *KCNQ1*-IAP54-56 variant however was identified in 2.1% of healthy African-American individuals, suggesting it may be a common risk allele in this population.

ANP, the primary biologically active peptide encoded by *NPPA*, is a circulating natriuretic hormone that plays an important role in regulation of intravascular blood volume and vascular tone through natriuresis, diuresis, and vasodilatation. It acts through stimulation of the



intracellular second messenger cyclic GMP. In congestive heart failure and hypertension, ANP is secreted and released into the circulation in response to atrial wall stretch. Importantly through cGMP signaling, ANP also modulates sodium, calcium, and potassium channel currents in cardiac myocytes [42-44].

Studies have shown that *NPPA* transcription and processing yields a separate biologically active protein fragment called vessel dilator that is distinct from ANP (Figure 1) [23,25]. Resequencing of *NPPA* identified a rare S64R variant that encodes for the vessel dilator in AF1111. The ANP assay does not directly measure the vessel dilator levels. However, one may reasonably infer that ANP concentration is an indirect measure of the fragment since proANP is the immediate progenitor of both. We found no significant differences in ANP levels between affected individuals with AF carrying the S64R variant and unaffected family members who were WT type for the variant, although elevated levels of ANP have been described in several different AF cohorts [45,46]. The mutant form of this *NPPA* protein fragment dramatically augments and accelerates cardiac  $I_{Ks}$ , an effect that shortens the atrial AP duration. Our study demonstrated that the mutant *NPPA* S64R peptide fragment-induced alterations in  $I_{Ks}$  magnitude and kinetics were initiated at the ANP receptor since the gain-of-function was prevented by application of the competitive ANP receptor antagonist anantin. The increased potency of the *NPPA* S64R peptide fragment compared to WT is evidence of increased affinity for the receptor and likely reflects the mechanism by which  $I_{Ks}$  augmentation is effected by the mutant fragment. With activity via the ANP receptor, the biologic activity of this *NPPA* protein fragment is likely similar to that of ANP, which has been shown in intact animal atria to cause a dose-dependent, autonomically-mediated shortening of atrial monophasic AP duration and the effective refractory period [47]. An augmented cardiac potassium current could thus provide a substrate for development of AF by shortening the atrial AP. It is likely, however, that not only mutations or rare variants in *NPPA* and cardiac ion channel genes but also variants in signaling pathways that link the two could increase the susceptibility to developing AF.

Studies have clearly shown that ANP not only modulates cardiac potassium currents but also sodium and calcium currents in cardiac myocytes [33,42,48,49]. As the *NPPA*-encoded vessel dilator has physiologic effects similar to mature ANP, it is quite likely that the mutant S64R peptide also modifies these channels as well. Although this study demonstrated that the mutant *NPPA* S64R peptide fragment modulates  $I_{Ks}$  and provides one possible mechanism for increased AF susceptibility, it does not rule out the possibility that other cardiac ion channels may also be contributing to the demonstrated effect on atrial AP duration. Ongoing studies are trying to determine the precise role of additional ion channels in modulating the effect on atrial AP duration.

Computational simulation of the effect of the  $I_{Ks}$  gain-of-function demonstrates the anticipated shortening of APs. In addition, simulations of premature stimulation reveal loss of the dome during phase II of the AP. This “triangularization” of the AP has been implicated as generating dispersion of repolarization time, and thus a fibrillation prone substrate, in Brugada Syndrome [50]. The atria are known to display marked heterogeneity of repolarization, in part as a function of region [51,52], so a similar mechanism seems likely here. While the mutant *NPPA* S64R peptide fragment yielded more dramatic  $I_{Ks}$  augmentation, the addition of WT fragment to WT- $I_{Ks}$  also resulted in modest  $I_{Ks}$  gain-of-function (Figure 6). These data suggest that *NPPA* activation, especially when exaggerated (e.g. due to atrial stretch), may augment  $I_{Ks}$  and thus predispose to AF; findings of increased ANP levels in patients with AF are also consistent with this hypothesis [53].

This study demonstrates the unique ability of computational simulation to explore the complex interplay between multiple ions, ion channels, ion exchangers and membranes underlying the

observed phenotype. AP simulation is also the most efficient means to address the AP consequences of individual mutations and has the great advantage that changes in the gating of other normal currents can be inferred. In this study, loss of the phase II dome for both WT- $I_{Ks}$  and variant  $I_{Ks}$  during premature atrial depolarization is shown to be primarily driven by a marked decrease in  $I_{CaL}$ . Recovery of the dome reflects rapid recovery of the calcium current - which is markedly attenuated in the simulation implementing variant  $I_{Ks}$ .

### Clinical Implications

Our study establishes that augmented potassium current with AP shortening and consequent altered calcium current is a common mechanism in diverse familial AF syndromes. As such, a mechanism-based approach targeting prolongation of the human AP can be proposed not only for AF probands but also family members diagnosed with the arrhythmia. Class III antiarrhythmic drugs such as sotalol would be one rational approach. The association of AP shortening with the establishment of a substrate for AF, as highlighted by our study of  $I_{Ks}$  augmentation, suggests a novel therapeutic role for agents that prolong the AP. For individuals or kindreds with AF mutations known to shorten AP, such therapeutic agents would theoretically be beneficial in preventing or terminating AF. As highlighted in our study, such agents may interact with the repolarizing channels directly (e.g. Class III Vaughan-Williams agents), but may also point to new agents that act indirectly on the channels.

### Limitations

Several limitations of the present study warrant consideration. First, the AF kindreds are small and therefore segregation analysis was limited. Second, the broad applicability of this study is limited because mutations in *KCNQ1* [40,41] and *NPPA* are rare in general and not a common cause of AF. However, identifying novel mutations in AF signaling pathways will provide insight into underlying pathophysiology of the arrhythmia and suggest other members of this biologic pathway as candidates for AF susceptibility. A third limitation is that comprehensive resequencing of the population-based controls was not performed. Although it is possible that additional novel *KCNQ1* and *NPPA* may have been identified, distinguishing pathogenic mutations from rare variants that have no relationship to the disease remains a challenge. Finally, computational simulation of human physiology is limited by the inherent difficulty in validating in human subjects the models upon which they are based. The models of WT- $I_{Ks}$  and variant  $I_{Ks}$ , while based on a good fit to the experimental data, are not necessarily unique; an alternate set of model parameters may yield similar fit to experimental data yet yield a different trajectory when applied to an AP simulation. The ultimate model validation would require *in vivo* measurement of individual APs and their comprising membrane currents in humans carrying the mutation being modeled - a technological feat that is not currently possible.

### Conclusion

In summary, we have for the first time to our knowledge uncovered a shared phenotype across two genetic defects that augment cardiac potassium current and are predicted to shorten cardiac atrial AP duration and predispose to the development of AF. The identification of a final common mechanism has implications for the potential therapeutic therapies for this common and morbid condition.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## ABBREVIATIONS

AF	Atrial fibrillation
AP	Action potential
NPPA	Natriuretic peptide precursor A
ANP	Atrial natriuretic peptide
WT	wild-type
$I_{Ks}$	slow delayed rectifier potassium current
$I_{CaL}$	L-type calcium current
$I_{Kr}$	rapid delayed rectifier potassium current
$I_{Kur}$	ultra-rapid delayed rectifier potassium current
I	Isoleucine
A	Alanine
P	Proline
C	Cysteine
PS1	First premature stimulus
PS2	Second premature stimulus
LQTS	long QT syndrome
CRN	Courtemanche-Ramirez-Nattel
CL	Cycle length

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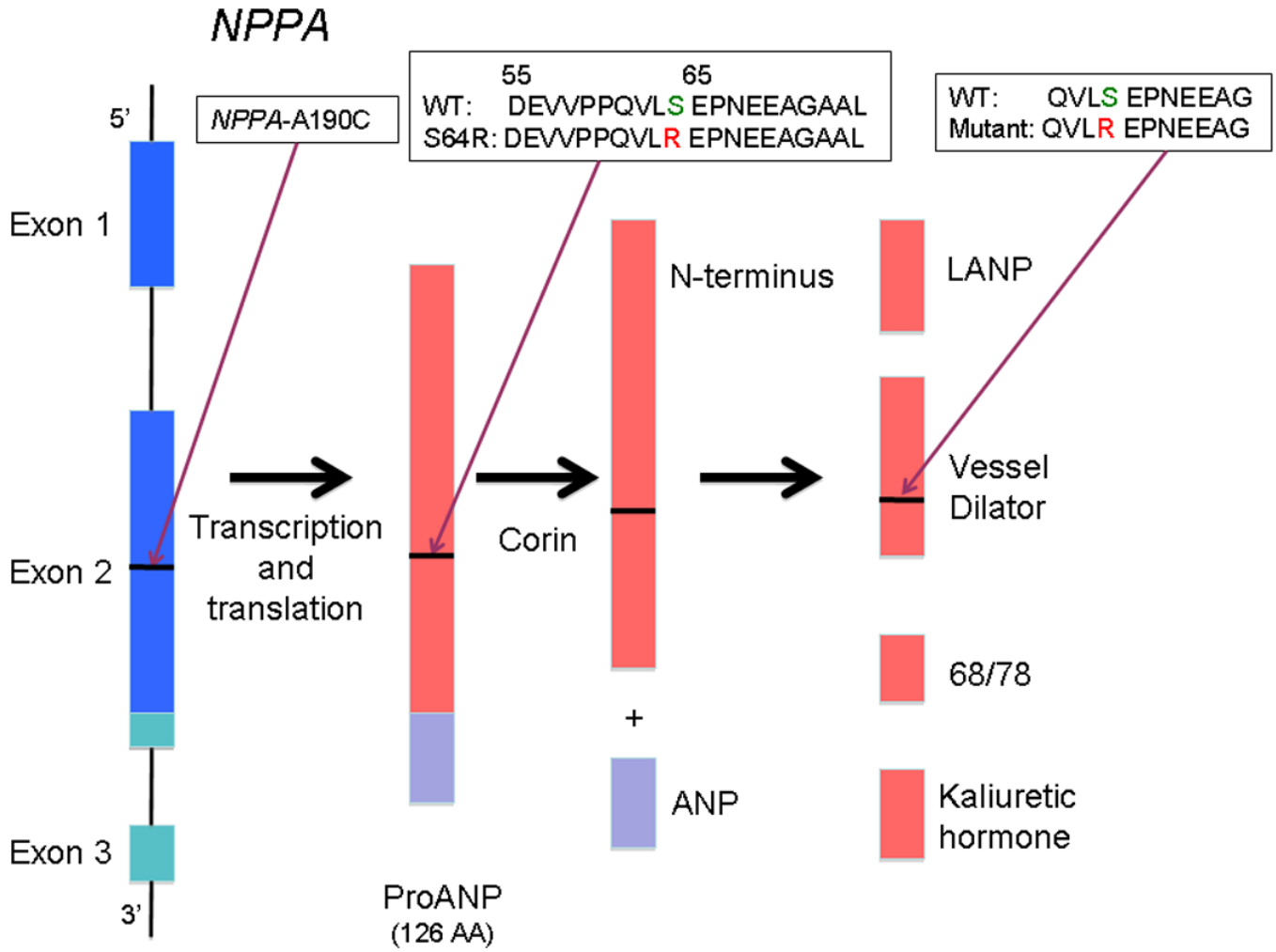
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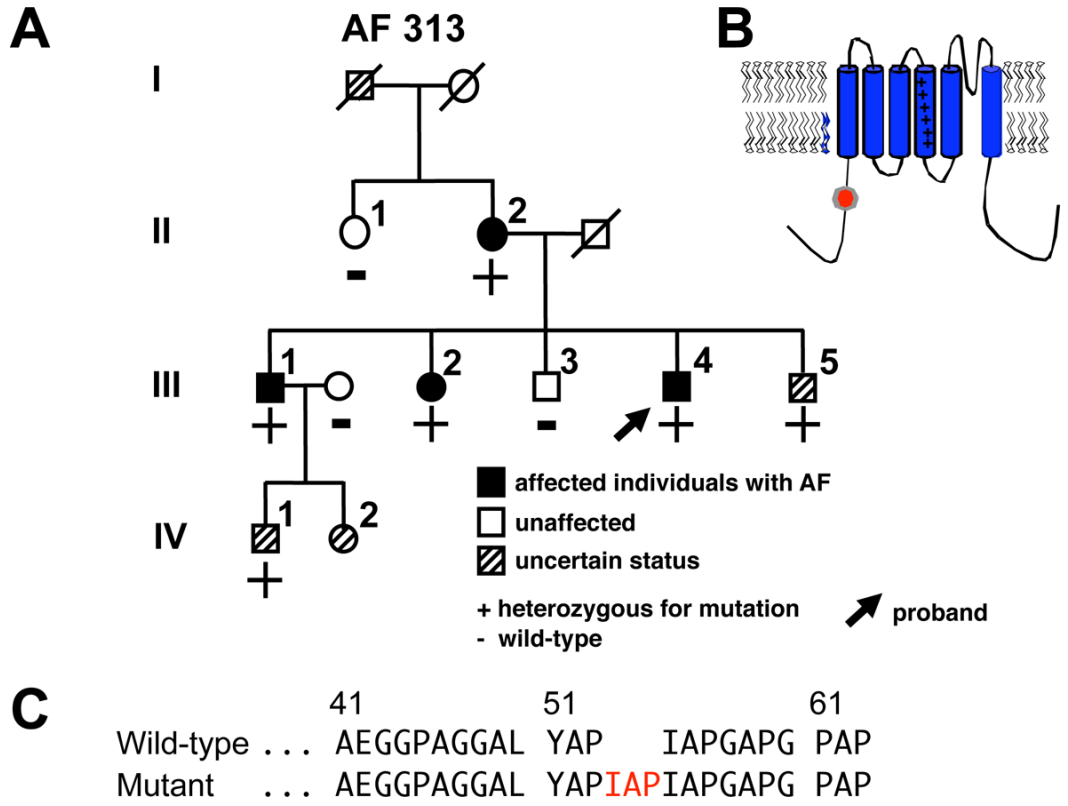
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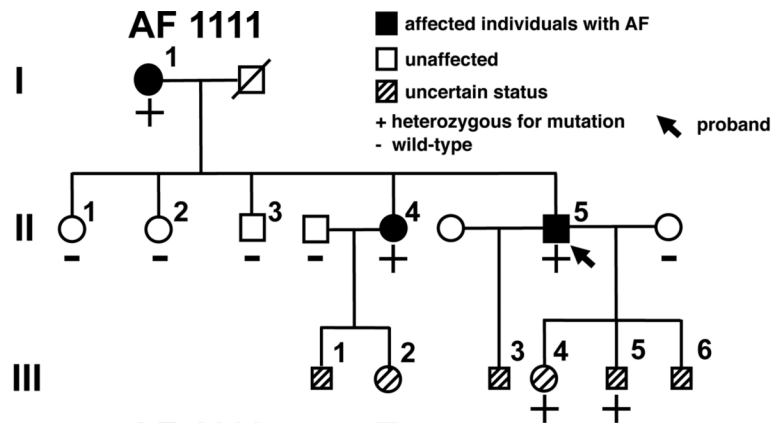
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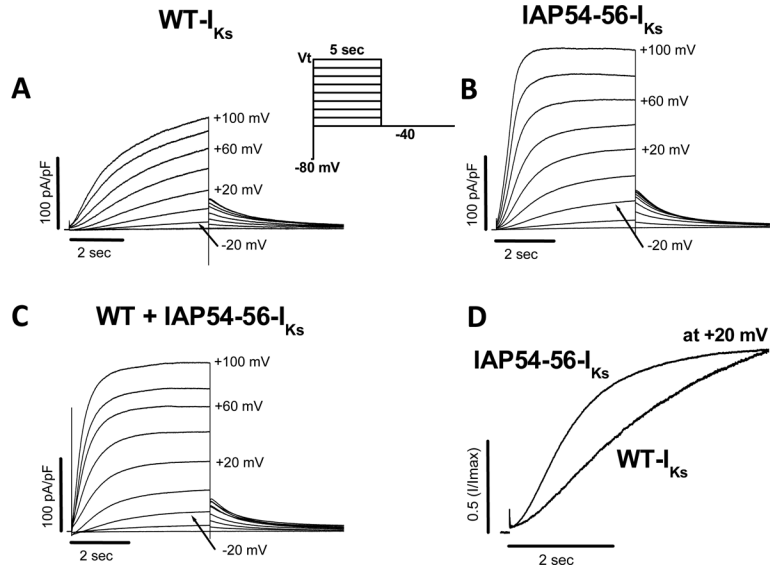
**Figure 1.** *NPPA* post-transcriptional processing. The initial 151-peptide product, PreproANP, is further processed into ANP and a collection of other protein fragments, including the N-terminal proANP 'vessel dilator' peptide fragment. The *NPPA*-A190C mutation leading to PreproANPS64R propagates into this vessel dilator peptide fragment. The amino acid sequences of the WT and mutant fragments are shown (inset). (Figure modified from Veseley, 2001 [19].)



**Figure 2.** *KCNQ1* mutation identified in a kindred with familial atrial fibrillation (AF). (A) Family pedigree of Vanderbilt AF 313. Solid symbols denote AF and striped symbols individuals of uncertain phenotype. Male subjects are shown as squares and female subjects as circles. The proband (arrow) and the presence (+) or absence (-) of the *KCNQ1*-IAP54-56 indel are indicated. (B) Location of the variant in the *KCNQ1* protein. (C) Portion of the *KCNQ1* N-terminus amino acid wild-type and mutant sequence.



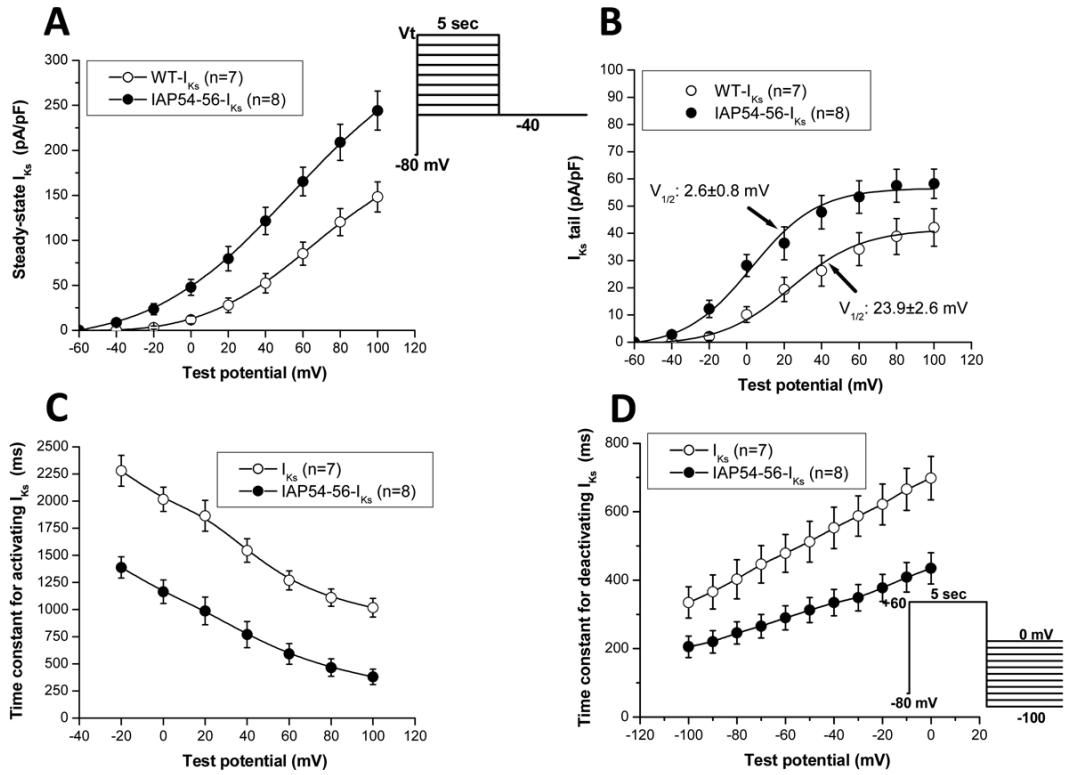
**Figure 3.** *NPPA* mutation identified in a kindred with familial AF. Shown is the family pedigree of Vanderbilt AF 1111. Symbols as in Figure 1A.



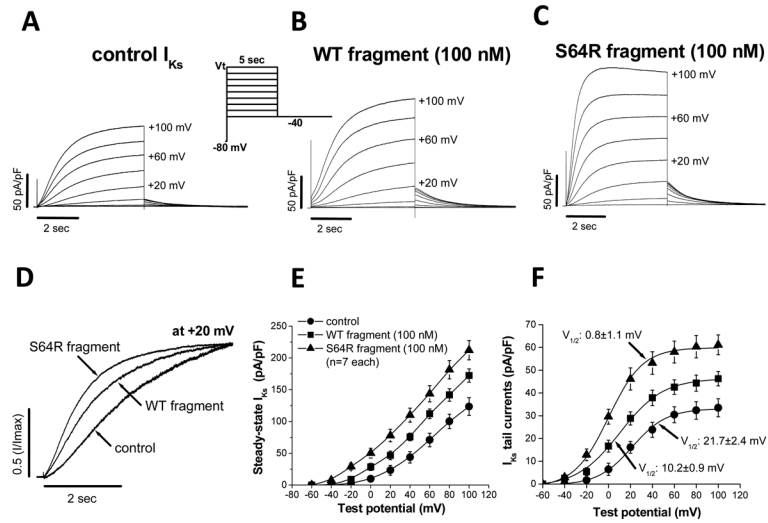
**Figure 4.**

Comparison of currents recorded from (A) wild-type (WT)-I<sub>Ks</sub> (*KCNQ1* + *KCNE1*), (B) IAP54-56-I<sub>Ks</sub> (*KCNQ1*-IAP54-56 + *KCNE1*) and (C) co-expression of the WT-I<sub>Ks</sub> + IAP54-56-I<sub>Ks</sub> channels in CHO cells during 5-sec depolarization steps from a holding potential of -80 mV to test potentials in the range -40 to +100 mV followed by repolarization to -40 mV (voltage protocol shown in inset). (D) Comparison of activation of WT and IAP54-56-I<sub>Ks</sub>. Traces obtained at +20 mV are redrawn from (A) and (B) at +20 mV.



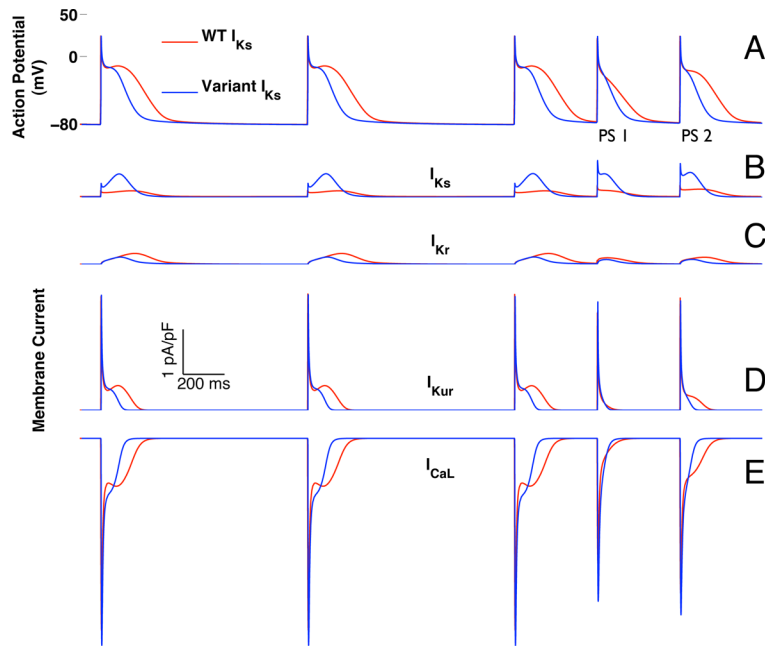


**Figure 5.** Current/voltage (I/V) relationships for WT- $I_{Ks}$  and mutant (IAP54-56- $I_{Ks}$ ) channels. **(A)** Activating  $I_{Ks}$  as a function of voltage, normalized to cell surface area and measured at the end of a 5-sec depolarizing pulse. **(B)** Normalized tail current amplitude at -40 mV as a function of depolarizing potential. Fits to Boltzmann distributions are shown. **(C)** Time constant of  $I_{Ks}$  activation as a function of voltage. Time constants of  $I_{Ks}$  deactivation as a function of voltage are shown in **(D)**.



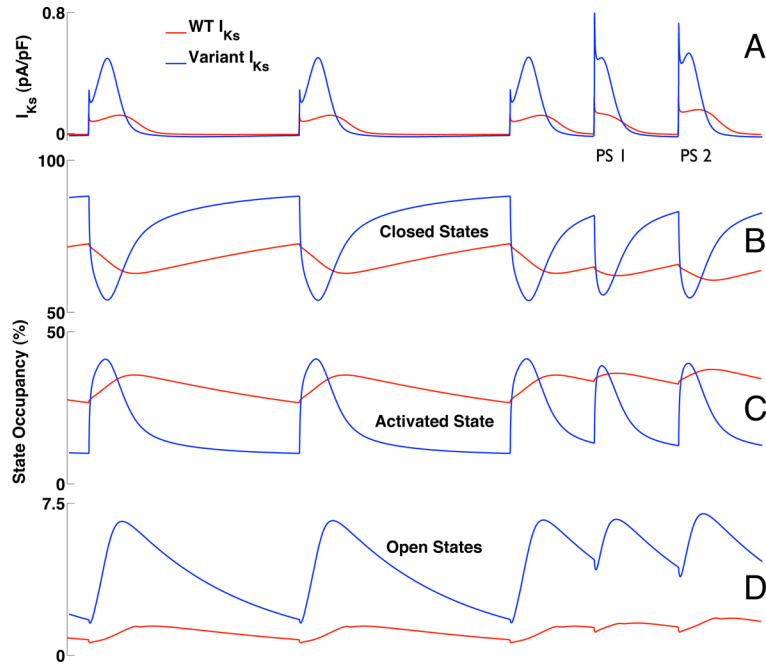
**Figure 6.**

Effects of WT *NPPA* peptide fragment and mutant *NPPA* peptide fragment-S64R on  $I_{Ks}$ . (A) control  $I_{Ks}$ . (B) Increased  $I_{Ks}$  on exposure to WT fragment. (C) Accelerated activation and further increase of  $I_{Ks}$  with S64R fragment. (D) Superimposition of activating  $I_{Ks}$  at +20 mV from panels (A), (B), and (C). (E) Steady state  $I_{Ks}$  normalized to cell surface area (measured at the end of a 5-sec depolarizing pulse). (F) Normalized tail current amplitude at -40 mV as a function of depolarizing potential. Fits to Boltzmann distributions are shown.



**Figure 7.**

Simulated double premature atrial depolarizations (400 ms coupling intervals) after a twelve-pulse pacing train (1000 ms cycle length). Data from simulations that include WT- $I_{Ks}$  are shown in red, and those incorporating variant  $I_{Ks}$ , based on IAP54-56, are in blue (A). The first atrial premature stimulus (PS1) simulated with variant  $I_{Ks}$  was shorter than that for WT- $I_{Ks}$  (126 vs. 193 ms). AP durations for the second premature stimulus (PS2) were 138 ms and 243 ms for variant  $I_{Ks}$  and WT- $I_{Ks}$ , respectively. In both simulations, PS1 exhibited loss of the dome associated with membrane repolarization. However, with WT- $I_{Ks}$  the dome was restored with PS2 while it remained absent with variant  $I_{Ks}$ . Changes in individual membrane currents are shown in (B)  $I_{Ks}$ ; (C)  $I_{Kr}$ ; (D)  $I_{Kur}$ ; and (E)  $I_{Ca-L}$ .



**Figure 8.**

$I_{Ks}$  and channel state occupancy during the simulation of Figure 7 (1000 ms pacing train followed by double premature atrial depolarizations with 400ms coupling interval). WT- $I_{Ks}$  is shown in red; variant  $I_{Ks}$  is in blue (A). Variant  $I_{Ks}$  exhibits much more dynamic variation in state occupancy during activation as channels transition from the closed states (B) through the activated state (C) into the open states (D). 'Closed States' refers to the sum of closed states R1 and R2; 'Open States' (D) refers to the sum of open states O1 and O2 (see Figure S-1A of the online data supplement).

**Table 1**  
Clinical phenotype of living genotype-positive individuals in Vanderbilt AF 313 and AF 1111.

AF kindred and pedigree number	Age at onset (yr)	Age at diagnosis (yr)	Rhythm	Symptoms	Echo	LA size (mm)	QT/QTc (ms)
<b>AF 313</b>							
II-2	48	52	PAF	Yes	LVH	46	368/430
III-1	46	54	PAF	Yes	Normal	44	390/440
III-2	48	56	PAF	Yes	Normal	42	428/430
III-4	38	42	PAF	Yes	Normal	36	440/436
III-5	44	-	NSR	Yes	Normal	40	430/420
IV-1	36	-	NSR	Yes	Normal	38	420/428
<b>AF 1111</b>							
I-1	42	44	PAF	Yes	LVH	44	390/405
II-4	46	50	PAF	Yes	Normal	45	410/420
II-5	36	42	PAF	Yes	Normal	42	380/410
III-4	28	-	NSR	Yes	Normal	41	390/410
III-5	30	-	NSR	Yes	Normal	42	400/415

PAF = paroxysmal atrial fibrillation; LA = left atrial; LVH = left ventricular hypertrophy; NSR = normal sinus rhythm.



Table 2

The effects of *KCNQ1* mutant IAP54-56, WT *NPPA* peptide fragment and mutant *NPPA* S64R peptide fragment on  $I_{Ks}$  properties.

	n	Activating $I_{Ks}$ (pA/pF, +20 mV)	$I_{Ks}$ tail (pA/pF)	$V_{1/2}$ (mV)	$\tau_{activation}$ (ms, +20 mV)	$\tau_{deactivation}$ (ms, -40 mV)
Control-1	7	25.2±5.1	18.9±4.5	23.9±2.6	1864.2±143.3	552.7±60.3
IAP54-56- $I_{Ks}$	8	75.1±8.2 <sup>†</sup>	36.4±6.1 <sup>†</sup>	2.6±0.8 <sup>†</sup>	987.4±127.5 <sup>†</sup>	334.5±38.6 <sup>†</sup>
Control-2	7	23.2±7.7	17.2±2.6	21.7±2.4	1859.8±125.7	564.5±53.2
WT <i>NPPA</i> fragment	7	53.1±9.2 <sup>‡</sup>	30.3±4.2 <sup>‡</sup>	10.6±1.3 <sup>‡</sup>	1448.8±106.4 <sup>*</sup>	414.9±37.5 <sup>*</sup>
Mutant <i>NPPA</i> S64R fragment	7	77.9±9.7 <sup>‡,  </sup>	46.2±5.9 <sup>‡,  </sup>	0.8±1.1 <sup>‡,  </sup>	850.3±83.1 <sup>‡,  </sup>	307.7±30.5 <sup>‡,§</sup>

Values are shown as mean±SEM; The activating  $I_{Ks}$  was elicited with 5-sec pulsing to +20 mV from a holding potential of -80 mV and the  $I_{Ks}$  tail was recorded upon pulsing back to -40 mV;

\*  $P < 0.05$  vs. control-2

<sup>†</sup>  $P < 0.001$  vs. control-1

<sup>‡</sup>  $P < 0.001$  vs. control-2

<sup>§</sup>  $P < 0.05$  vs. WT *NPPA* peptide fragment

<sup>||</sup>  $P < 0.001$  vs. WT *NPPA* peptide fragment; there was no statistical difference between two control groups.