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Targeted nonviral gene-based inhibition of $G\alpha_{i/o}$-mediated vagal signaling in the posterior left atrium decreases vagal-induced atrial fibrillation

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Abstract

BACKGROUND—Pharmacologic and ablative therapies for atrial fibrillation (AF) have suboptimal efficacy. Newer gene-based approaches that target specific mechanisms underlying AF are likely to be more efficacious in treating AF. Parasympathetic signaling appears to be an important contributor to AF substrate.

OBJECTIVE—The purpose of this study was to develop a nonviral gene-based strategy to selectively inhibit vagal signaling in the left atrium and thereby suppress vagal-induced AF.

METHODS—In eight dogs, plasmid DNA vectors (minigenes) expressing $G\alpha_i$ C-terminal peptide ($G\alpha_i$ctp) was injected in the posterior left atrium either alone or in combination with minigene expressing $G\alpha_o$ctp, followed by electroporation. In five control dogs, minigene expressing scrambled peptide ($G\alpha_R$ctp) was injected. Vagal- and carbachol-induced left atrial effective refractory periods (ERPs), AF inducibility, and $G\alpha_{i/o}$ctp expression were assessed 3 days following minigene delivery.

RESULTS—Vagal stimulation- and carbachol-induced effective refractory period shortening and AF inducibility were significantly attenuated in atria receiving a $G\alpha_{i2}$ctp-expressing minigene and were nearly eliminated in atria receiving both $G\alpha_{i2}$ctp- and $G\alpha_{o1}$ctp-expressing minigenes.

CONCLUSION—Inhibition of both G_i and G_o proteins is necessary to abrogate vagal-induced AF in the left atrium and can be achieved via constitutive expression of $G\alpha_{i/o}$ctps expressed by nonviral plasmid vectors delivered to the posterior left atrium.

Keywords

Atrial fibrillation; Atrial fibrillation inducibility; Autonomic nervous system; Effective refractory period; Muscarinic cholinergic receptor; Pertussis toxin-sensitive G proteins; Vagal signaling

Introduction

Atrial fibrillation (AF) is the most common sustained rhythm disorder of the heart.[1,2] In view of the limitations of current treatment options, several investigators have indicated a

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need for novel therapies that target specific mechanisms underlying AF.[2] The autonomic nervous system—specifically the parasympathetic nervous system—is known to be involved in the genesis of AF[3,4] and may be a viable therapeutic target in patients with AF.[5,6]

In the atria, vagal-released acetylcholine (ACh) stimulates primarily type 2 muscarinic cholinergic receptors (M_2Rs), which activate heterotrimeric $G\alpha_{i/o}\beta\gamma$ proteins, with resulting dissociation of the $G\alpha_{i/o}$ subunit from $G\beta\gamma$. $G\beta\gamma$ activation of $I_{K\text{-}ACh}$ leads to significant abbreviation of action potential duration, thereby creating a substrate for reentry.[7] In proof-of-concept studies,[8] we previously demonstrated that atrial-selective attenuation of vagal signaling can be achieved by a $G\alpha_{i2}$ C-terminal peptide ($G\alpha_{i2}$ctp) delivered to the posterior left atrium (PLA).[9,10] This $G\alpha_{i2}$ctp putatively acts by selectively disrupting M_2R-$G\alpha_{i2}$ coupling, thus impeding $G\alpha_{i2}\beta\gamma$ signal transduction. Although encouraging, the utility of such a peptide-based pharmacotherapy requires sustained and controlled intracellular expression of peptide in the atrial myocardium. Moreover, it was clear in our previous study that inhibition of $G\alpha_{i2}$ did not completely abrogate M_2R/vagal signaling in the PLA. Indeed, multiple studies have suggested that other pertussis toxin–sensitive $G\alpha$ subunit isomers, particularly $G\alpha_o$ isomers, contribute to vagal signaling in the atria.[11,12] Hence, the present study describes our efforts toward achieving more constitutive administration of not only $G\alpha_{i2}$ctp but also $G\alpha_{o1}$ctp by incorporating their cDNA into plasmid expression vectors (minigenes), delivering them into canine PLA, and assessing their effects on cholinergic responsiveness.

Methods

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Northwestern University. The research conforms with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (Publication No. 85-23, revised 1996).

Minigene preparation

Cloning of $G\alpha_{i2}$ctp, $G\alpha_{o1}$ctp, and $G\alpha_R$ctp inserts into plasmid backbone—The corresponding cDNA sequences of the last C-terminal 11 amino acids of the $G\alpha_{i2}$ subunit, and that for the $G\alpha_{o1}$ subunit and that of random-ordered $G\alpha_{i2}$ctp ($G\alpha_R$p), were each separately cloned into a pFLAG CMV6a plasmid expression vector (Sigma-Aldrich, St. Louis, MO) to generate $G\alpha_{i2}$ctp-$G\alpha_{o1}$ctp- and $G\alpha_R$p-expressing plasmid constructs (minigenes) as described elsewhere[13] (see brief protocol in Online Data Supplement).

Transformation and plasmid purification—Plasmids were propagated in *Escherichia coli* and purified using Qiagen Mega-prep kits (Qiagen, Valencia, CA, USA), as described by the manufacturer. Details of transformation and plasmid purification are given in the Online Data Supplement.

Gene injection and *in vivo* electrophysiologic testing

Gene injection and electroporation—A total of 19 dogs (hounds) were used for this study (12 male, 7 female). Animals were premedicated with acepromazine (0.01–0.02 mg/kg) and induced with propofol (3–7 mg/kg). A median or lateral sternotomy was then performed under general anesthesia (inhaled) with isoflurane (1%–3%). Adequacy of anesthesia was assessed by toe pinch and palpebral reflex.

In a small number of pilot experiments (N = 6), 1 mg of $G\alpha_{i2}$ctp or $G\alpha_R$p minigene was injected subepicardially in the PLA (see Results). In the remaining experiments, 15–20 mg of ct-$G\alpha_{i2}$ctp (N = 5), 7–10 mg of $G\alpha_{i2}$ctp + 7–10mg of $G\alpha_{o1}$ctp (N = 3), or $G\alpha_R$p (N = 5)

minigenes was injected in the PLA. Minigenes were made up to a volume of 4 mL and injected at multiple sites (6–8 equally spaced sites, 0.5–1 cm apart; a volume of approximately 0.5 mL was injected at each site) in the PLA so as to cover the entire area between the pulmonary veins (PVs). The injected region is anatomically clearly demarcated (the four borders used are base of left atrial appendage, base of left inferior PV, interatrial septum, and atrioventricular groove) and is removed after gene injection. Immediately after gene injection, electroporation was performed at each site of injection as follows. Two gold-plated, needle-style electrodes (10-mm length each) were placed at each gene injection site on the PLA (interelectrode distance 5 mm). Electroporation with performed as previously described in the lung by Dean et al,[14] with eight pulses of 1 second at 120–150 V/cm^2 (ECM 830, Harvard Bioscience, Holliston, MA, USA).

After minigene delivery, the chest was closed and the animal allowed to recover. Vagal stimulation and electro-physiologic testing were not performed at baseline in order to minimize damage to the vagus nerve.

Terminal electrophysiologic study

Baseline study: Three days after the initial study, the chest was reopened. High-density plaques were applied to the left superior PV (8 × 5 electrodes, 2.5-mm spacing), PLA (7×3 electrodes, 5-mm spacing), and left atrial appendage (LAA; 7×3 electrodes, 5 mm spacing). The PV plaque was placed circumferentially around the vein while the other two plaques were laid flat on the PLA and LAA epicardium. Effective refractory periods (ERPs) were obtained from 5, 6, and 4 sites on the PV, PLA, and LAA plaque, respectively, at baseline.

Vagal stimulation: For vagal stimulation, the left cervical vagus nerve was isolated, a bipolar stainless steel electrode was attached to the nerve, and stimulation was performed at 20 Hz (15–20 V, 2–8 ms) (Grass S44G, Astromed Inc, West War-wick, RI, USA). A vagal response was defined as (1) sinus node slowing by at least 25% or (2) PR prolongation by more than 25% or 2:1 AV block.[13] ERP testing was performed in the presence and absence of vagal stimulation (VS).

Carbachol application: We also assessed for ERP shortening by direct application of carbachol (CCh), a nonselective MR agonist, to the PLA. CCh was injected under the subepicardium of the PLA in increasing doses of 3, 10, and 30 μM (also see *in vitro* CCh dose finding studies). After each dose, ERP testing was performed in the PLA. Long periods of AF were frequently encountered during atrial pacing at higher concentrations of CCh and thus precluded ERP testing at these higher doses.

AF inducibility: AF was defined as an atrial arrhythmia that was irregular in at least one of the recording electrodes. Regular atrial arrhythmias (eg, atrial flutter, atrial tachycardia) were excluded from AF analysis. AF inducibility was measured as the inducibility index and duration of AF episodes after a single extrastimulus.[8,15] As previously described, the inducibility index was defined as the number of AF episodes lasting more than 5 seconds induced by a single atrial extrastimulus divided by the total number of single atrial extrastimuli delivered to measure each ERP (at least three for each site).[8] The inducibility index was compared for each maneuver. Mean AF duration for each intervention was also assessed.

All data were acquired by a 128-channel mapping system (Prucka CardioLab, GE, Waukesha, WI, USA) at a sampling rate of 977 Hz. All AF episodes induced during extrastimulus testing were stored for offline analysis. After all ERPs had been obtained, minigene injection was performed as described later.

Offline electrogram analysis: Electrograms recorded during the maximum duration AF episodes obtained during extra-stimulus testing were analyzed with dominant frequency (DF) analysis. DF is an estimation of activation rate calculated as the frequency with the most power in the power spectrum. The power spectrum is obtained from the fast Fourier transform of an electrogram after rectification and low-pass filtering (20 Hz). These analyses were performed offline using Matlab (MathWorks, Natick, MA, USA).

Tissue explant assays

Upon finishing the *in vivo* portion of the study, euthanasia was achieved using a high dose of pentobarbital (>20 cc, fully saturated) to achieve a very deep plane of anesthesia, and the heart removed and perfused with cold cardioplegia solution. The left atrium and PVs were dissected, snap frozen, and subjected to further analysis as detailed in the following.

Transgene expression

mRNA expression: The following primers to detect minigene-expressed mRNA were obtained from IDT (San Diego, CA, USA):

$G\alpha_{i2}$ctp: forward AGCTCAAGCTTATCAAGAACAACCT, reverse TACCGGATCCTCAGAAGAGGC

$G\alpha_{o1}$ctp: forward AGCTCAAGCTTATTGCCAACAACC, reverse GGTACCGGATCCTCAGTACAAGCC

$G\alpha_{R}$p: forward CAAGCTTAACGGCATCAAGTGC, reverse GGTACCGGATCCTCACAGCTT

Quantitative real-time polymerase chain reaction was performed to assess for expression of $G\alpha_{i2}$ctp, $G\alpha_{o1}$ctp, and $G\alpha_{R}$p expressing minigenes in the PLA following gene injection. GAPDH was used as a reference for sample normalization.

Western blotting: Anti-FLAG antibodies (Sigma) were used to assess for the presence of FLAG-tagged $G\alpha_{i2}$ctp in PLA tissue. Calsequestrin-2 was used as a loading control (see Online Data Supplement for details).

Immunohistochemistry: Thin sections (5 μm) of the PLA were obtained for hematoxylin and eosin staining and for immunohistochemistry (the latter to assess for FLAG-tagged $G\alpha_{i2}$ctp; see Online Data Supplement for details).

CCh concentration–Ca^{2+} transient response assay in isolated canine atrial myocytes

Myocyte isolation: Canine right atrial myocytes (from same hearts excised as described earlier) were isolated by colla-genase digestion via a coronary perfusion modified procedure previously described.[8,16] Dissociated myocytes were stored in normal Tyrode's solution at 4°C until use in confocal Ca^{2+} transient experiments as described.

Ca^{2+} transients acquisition and CCh administration: As previously described,[8] isolated atrial myocytes were incubated with 5–10 μM of the Ca^{2+} fluorescence dye fluo-4 (Invitrogen, Carlsbad, CA). Action potential (AP)-evoked Ca^{2+} transients were acquired as confocal X-t line-scan images of uncalibrated fluo-4 fluorescence at a scan rate of 1.92 ms/line-scan. Laser phototoxicity was minimized by scanning at <10% output transmission. Changes in Ca^{2+} transients in response to serial concentrations of acutely applied CCh (0.01–30 μM) were measured from multiple myocytes per isolation preparation (see online Data Supplement for details).

Statistical analysis

All data are reported as mean ± SE. Comparisons between $G\alpha_{i2}$, $G\alpha_{i2}$+$G\alpha_{o1}$, and $G\alpha_R$ dogs (for ERP, AF inducibility) were assessed for significant differences via analysis of variance. DF comparisons between $G\alpha_{i2}$ and $G\alpha_R$ dogs were made using unpaired *t*-tests. Before and after comparisons made in the same animals (eg, before and after VS or CCh) were assessed for significant differences via paired *t*-tests. $P \leq .05$ was considered significant.

Results

$G\alpha_x$ minigene injection dose vs $G\alpha_x$ minigene gene product expression

In pilot experiments (N = 6), 1–2 mg $G\alpha_{i2}$ctp minigene was injected into the PLA, followed immediately by electroporation. However, this pilot minigene injection dose resulted in low–modest $G\alpha_{i2}$ctp mRNA expression in the PLA (Online Supplemental Figure 1). An approximately 10× injection dose of minigene (15–20 mg) resulted in significantly greater $G\alpha_{i2}$ctp mRNA expression (see Online Supplemental Figure 1) and was used in all subsequent experiments where $G\alpha_{i2}$ctp minigene was tested alone. Assessment of mRNA expression in canine PLAs in which $G\alpha_{i2}$ctp mini-gene had been delivered ($G\alpha_{i2}$ctp minigene alone experiments) suggested a trend toward increased $G\alpha_{o1}$ mRNA compared to control PLAs (data not shown). Thus, additional experiments were conducted in which $G\alpha_{i2}$ctp mini-gene was delivered together with $G\alpha_{o1}$ctp minigene, and an injection dose of 7–10 mg of each minigene was used. As described later, clear electrophysiologic responses were obtained following these latter injection doses of minigenes.

Effects of $G\alpha_x$ minigenes on vagal-induced ERP shortening

The effects of PLA delivery of $G\alpha_{i2}$ctp minigene (N = 5) vs $G\alpha_{i2}$ctp + $G\alpha_{o1}$ctp minigene (N = 3) vs $G\alpha_R$p minigene (N = 5) on vagal-induced ERP shortening are shown in Figure 1A. Vagal-induce ERP shortening was significantly less in the PLA of $G\alpha_{i2}$ctp dogs vs $G\alpha_R$p dogs 19.5 ± 5.0 ms vs 43.6 ± 7.9 ms, $P < .05$). There was no significant difference in vagal-induced ERP shortening between $G\alpha_{i2}$ctp and $G\alpha_R$p dogs in the PV and the LAA. In comparison, in $G\alpha_{i2}$ctp+$G\alpha_{o1}$ctp dogs, vagal-induced ERP shortening was almost entirely eliminated in the PLA (with shortening significantly less than with $G\alpha_{i2}$ctp alone: 2.8 ± 1.5 ms vs 19.5 ± 5.0 ms, $P < .01$) and was also significantly attenuated in the PV and LAA (Figure 1A).

Effects of $G\alpha_x$ minigenes on CCh-induced ERP shortening

To more specifically assess the action of $G\alpha_x$ minigene delivery on M_2R–$G_{i/o}$ protein coupling and resultant ERP shortening, exogenously applied CCh was used to induce ERP shortening in the PLA in $G\alpha_x$ctp dogs. To arrive at an appropriate CCh dose range for these *in vivo* experiments, we first determined the concentration–response relationship for CCh to attenuate Ca^{2+} transient peak amplitude in isolated canine atrial myocytes (see Online Supplemental Figure 2). The IC_{50} and maximum for this effect was 28 ± 8 nM and 3 μM CCh, respectively. At CCh >3 μM the effect faded, indicating agonist-induced acute M_2R desensitization.[17] Because our goal was to obtain maximum CCh effect in atrial tissue while minimizing agonist-induced M_2R desensitization, we arrived at a small *in vivo* test range of 3–30 μM CCh to assess ERP shortening following gene delivery *in vivo* (previous experience in our laboratory has indicated that *in vivo* dosage is ~3–10× the *in vitro* dosage). Moreover, as stated earlier, higher doses of CCh administered *in vivo* resulted in long periods of AF and therefore precluded ERP testing.

Accordingly, CCh (3–30 μM) was directly injected into the PLA. As expected, increasing doses of CCh caused progressively greater ERP shortening in the PLA, but as shown in Figure 1B, CCh-induced ERP shortening was significantly less in $G\alpha_{i2}$ctp compared to

$G\alpha_R$p dogs at lower CCh concentrations (3 μM, 10 μM). At 30 μM, there was no significant difference in ERP shortening between $G\alpha_{i2}$ctp and $G\alpha_R$p dogs. This indicates that 30 μM CCh stimulates M_2Rs sufficiently to overcome $G\alpha_{i2}$ctp inhibition of M_2R–G_{i2} signaling (because $G\alpha_{i2}$ctp acts as a competitive inhibitor to endogenous M_2R–$G\alpha_{i2}$ interaction). In $G\alpha_{i2}$ctp+$G\alpha_{o1}$ctp dogs, there was no significant CCh-induced ERP shortening at any dose of CCh (including the highest dose of 30 μM), with ERP shortening being significantly less than in the other two groups at each dose of CCh (Figure 1B).

Effects of $G\alpha_x$ minigenes on vagal- and CCh-induced AF

AF inducibility (in response to VS) was significantly less in $G\alpha_{i2}$ctp dogs vs $G\alpha_R$p dogs and was lowest (zero) in $G\alpha_{i2}$ctp+$G\alpha_{o1}$ctp dogs, with not a single episode of AF >5 seconds being induced in this group (Figure 2A). Mean AF duration (in response to VS) was also significantly less in $G\alpha_{i2}$ctp dogs vs $G\alpha_R$p dogs and was lowest in $G\alpha_{i2}$ctp+$G\alpha_{o1}$ctp dogs (Figure 2A). Similarly, AF inducibility and AF duration in response to CCh were significantly less in $G\alpha_{i2}$ctp dogs vs $G\alpha_R$p dogs but were lowest in $G\alpha_{i2}$ctp+$G\alpha_{o1}$ctp dogs (Figure 2B).

Effects of Gαi2 expressing minigene on AF characteristics

As previously shown by us and others, VS increases DF of AF.[8] When AF DF was assessed in $G\alpha_{i2}$ctp and $G\alpha_R$p dogs, the VS-induced increase in DF was significantly less in $G\alpha_{i2}$ctp dogs vs $G\alpha$Rp dogs (where significant DF was noted in the PLA and PV in response to VS; Figure 3A). Figure 3B shows examples of AF electrograms (with and without VS) from $G\alpha_{i2}$ctp vs $G\alpha_R$p dogs. AF DF could not be assessed in dogs receiving $G\alpha_{i2}$ctp+$G\alpha_{o1}$ctp dogs due to the very small number of AF episodes in these dogs and the very short duration of these episodes.

$G\alpha_x$ transgene expression in the left atrium and PVs

Figure 4A shows relative $G\alpha_x$ctp mRNA expression in the PLA, PV, and LAA after minigene delivery into the PLA. Robust expression for $G\alpha_{i2}$ctp, $G\alpha_{o1}$ctp, and $G\alpha_R$p minigenes was found in the PLA, with minimal expression in the PV (which was adjacent to the area of injection) and no expression in the LAA (remote from site of injection). Anti-FLAG Western blot analysis (Figure 4B) indicated $G\alpha_{i2}$ctp (FLAG-tagged) expression in the PLA but not in the LAA. Immunohistochemistry showed evidence of FLAG staining in both myocytes (Figure 4C, subpanel i) and nerve bundles (Figure 4C, subpanel ii) in the PLA. In contrast, no FLAG was detected in the LAA (Figure 4C, subpanel iii). Thus, $G\alpha_x$ctp minigene injection resulted in adequate $G\alpha_x$ctp translation at the site of minigene delivery.

Discussion

In this study, we demonstrate the feasibility and efficacy of a targeted nonviral gene therapy approach to AF. Using minigene constructs that were delivered to the PLA by direct injection + electroporation, we demonstrate that 3 days after gene injection, (1) vagal responsiveness in the normal PLA was attenuated by a $G\alpha_{i2}$ctp expressed *in situ* by a plasmid expression vector and (2) vagal responsiveness was almost entirely eliminated in the PLA and significantly attenuated elsewhere in the left atrium by a combination of minigenes expressing $G\alpha_{i2}$ctp and $G\alpha_{o1}$ctp, with a resulting dramatic decrease in vagal-induced AF.

Gene therapy in AF: Prior experience in modification of autonomic signaling via G-protein–related pathways

In an innovative approach, Donahue et al[18,19] used an ad-enoviral vector overexpressing $G\alpha_i$ to suppress AV conduction and thereby slow heart rate during AF. Our approach differs from that of Donahue et al in that instead of increasing $G\alpha_i$ activity in the AV node to decrease ventricular rates during AF, we inhibited M_2R–$G\alpha_{i/o}$ interactions in the left atrium with nonviral minigene-expressing $G\alpha_{i/o}$ctps, with the intent of modifying the autonomic substrate in a region of the heart (PLA) that is considered critical to the genesis of AF. Indeed, disruption of M_2R–$G\alpha_i$ coupling caused decreased ERP responsiveness and AF inducibility, which became much more apparent upon the additional disruption of M_2R–$G\alpha_o$ coupling. Moreover, disruption of M_2R–$G\alpha_i$ coupling caused significant attenuation of vagal-induced increase in DF (of the AF that was induced).

Redundancy of G-protein– coupling to M_2Rs in the atrium

It is well established that M_2R signaling in the atria is transduced by pertussis toxin–sensitive $G_{i/o}$ proteins. However, because six GTP-binding $G\alpha$ subunit isoforms are known ($G\alpha_{i1,2\&3}$, $G\alpha_{o1,2\&3}$), the specific identity of the $G\alpha_{i/o}$ isoform(s) that couples to atrial M_2RS has not been unequivocally established.[11,12,20,21] Indeed, $G\alpha_o$ has been found to be co-localized with $G\alpha_i$ in the porcine atrium in a 1:1 ratio[22] and can activate $I_{K\text{-}ACh}$ as efficiently as $G\alpha_i$.[23] Such findings corroborate ours in the present study in that inhibition of both $G\alpha_i$ and $G\alpha_o$ seems to be required for complete disruption of M_2R responsiveness in the left atrium. To our knowledge, this is the first time disruption of M_2R–$G\alpha_o$ coupling has been attempted and shown to significantly contribute to a decrease in vagal-induced AF in the large animal heart.

Gene delivery in the atrium: Viral vs nonviral approaches

Both viral and nonviral delivery methods have relative advantages for use in myocardial gene delivery.[24,25] Importantly, a nonviral approach results in a reduced inflammatory and immune response *in vivo*[26] and therefore has a more favorable safety profile. Recent improvements in physical delivery methods (eg, sonoporation, electroporation) have allowed increasing levels of gene transfer and expression with naked DNA, nearing that of viral vectors.[26] In this study, we demonstrate that $G\alpha_{i/o}$ctps constitutively expressed via nonviral DNA vectors delivered into the PLA followed by electroporation results in attenuated vagal/M_2R-induced ERP shortening and AF.

Current viral approaches may have a potential for long-term gene expression and so may be suited for the treatment of a chronic condition such as AF. For a nonviral gene therapy approach to be a viable option for AF, (1) significantly longer-term gene expression in the atrium than that achieved in the current study would have to be obtained and (2) long-term safety study studies would need to be conducted. Ongoing experiments in our laboratory suggest that longer-acting promoters allow gene expression at least up to a few weeks.[27] Longer-term studies need to be performed in order to further investigate the feasibility of a nonviral approach for AF gene therapy.

Other issues to be considered prior to translation of above approach to clinical AF

Of note, $I_{K\text{-}ACh}$ is the major effector of M_2R stimulation in normal hearts, with a constitutive (agonist-independent) form of $I_{K\text{-}ACh}$ ($I_{K\text{-}ACh}$c)[28] becoming more pronounced in patients with chronic AF.[7] Nonetheless, $I_{K\text{-}ACh}$c does appear to be G-protein dependent.[28] Moreover, as recently shown by us,[29] left atrial parasympathetic responsiveness, although diminished in structural heart disease, still appears to play an important role in the maintenance of AF.

Also important is the presence of right atrial-left atrial gradients in $I_{K\text{-}ACh}$ activation.[30] Local gene therapy may change these gradients, with potentially maladaptive consequences during long-term treatment.

The effect of $G\alpha_{i/o}$ inhibition on Excitation-Contraction (E-C) coupling also needs to be assessed, especially in light of previous studies suggesting that sympathovagally induced changes in Ca^{2+} release contribute to arrhythmogenesis.[10,31]

Study limitations

With the CMV promoter, gene expression was limited in the current study and was assessed only up to 3–4 days. Longer-term studies, essential for such a gene-based approach to be translated to patients with AF, were not performed because cardiac expression in large animal hearts with the CMV promoter may be limited to 1 week.[26] However, in preliminary studies, we recently achieved longer-term gene expression with naked DNA using alternative, longer-acting promoters.[27]

The effect of $G\alpha_o$ inhibition alone on vagal responsiveness was not assessed in this study. However, based on the near complete inhibition of vagal responsiveness seen in response to combined $G\alpha_o$ and $G\alpha_i$ inhibition (as opposed to only partial inhibition noted with $G\alpha_i$ inhibition alone), it appears that $G\alpha_o$ is at least as important as, if not more important than, $G\alpha_i$ in mediating cholinergic responsiveness in the canine left atrium.

Because we performed vagal stimulation only at the terminal study and not at baseline, paired comparisons could not be made (for vagal stimulation) within each animal. However, paired comparisons were made for CCh.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

ACh	acetylcholine
AF	atrial fibrillation
CCh	carbachol
DF	dominant frequency
ERP	effective refractory period
$G\alpha_{i/o}$ctp	$G\alpha_{i/o}$ C-terminal peptide(s)
$G\alpha_{RP}$	$G\alpha$ random-sequence peptide
$I_{K\text{-}ACh}$	acetylcholine-activated inward rectifying potassium channel
LAA	left atrial appendage
M_2R	type 2 muscarinic cholinergic receptor

PLA	posterior left atrium
PV	pulmonary vein
VS	vagal stimulation

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Appendix

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.hrthm. 2011.06.018.

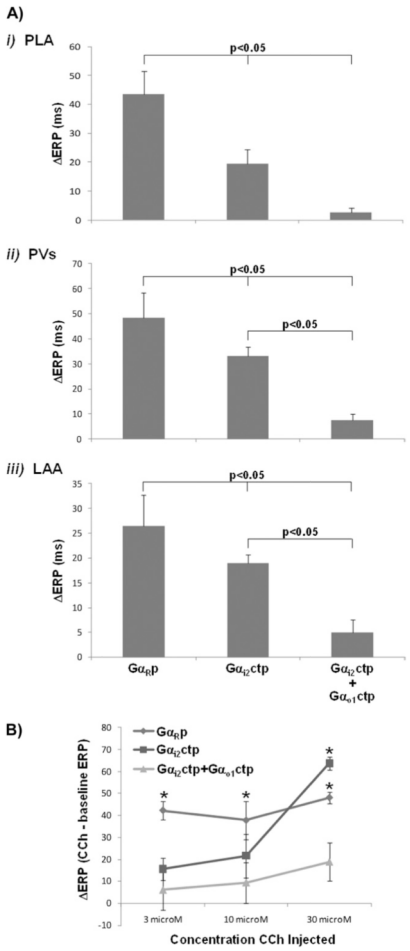

Figure 1.

Attenuation by minigene-expressing $G\alpha_{i/o}$ctp of vagal stimulation (VS)- and carbachol (CCh)-induced effective refractory period (ERP) shortening. **A:** *i:* Compared to $G\alpha_{R}$cpt, vagal-induced ERP shortening in the posterior left atrium (PLA) is attenuated in $G\alpha_{i2}$ctp dogs and is nearly eliminated in $G\alpha_{i2}$ctp+ $G\alpha_{o1}$ctp dogs. In $G\alpha_{i2}$ctp+ $G\alpha_{o1}$ctp dogs, vagal-induced ERP shortening was also significantly attenuated in the pulmonary vein (PV) *(ii)* and left atrial appendage (LAA) *(iii).* **B:** ERP shortening in the PLA in response to CCh. In $G\alpha_{R}$cpt dogs, significant ERP shortening was noted in response to each CCh concentration (3, 10, 30 μM). In contrast, in $G\alpha_{i2}$ctp dogs, ERP shortening was noted only at 30 μM CCh. In $G\alpha_{i2}$ctp+ $G\alpha_{o1}$ctp dogs, there was no significant ERP shortening at any dose of CCh. *P <.05 vs baseline ERP at terminal study.

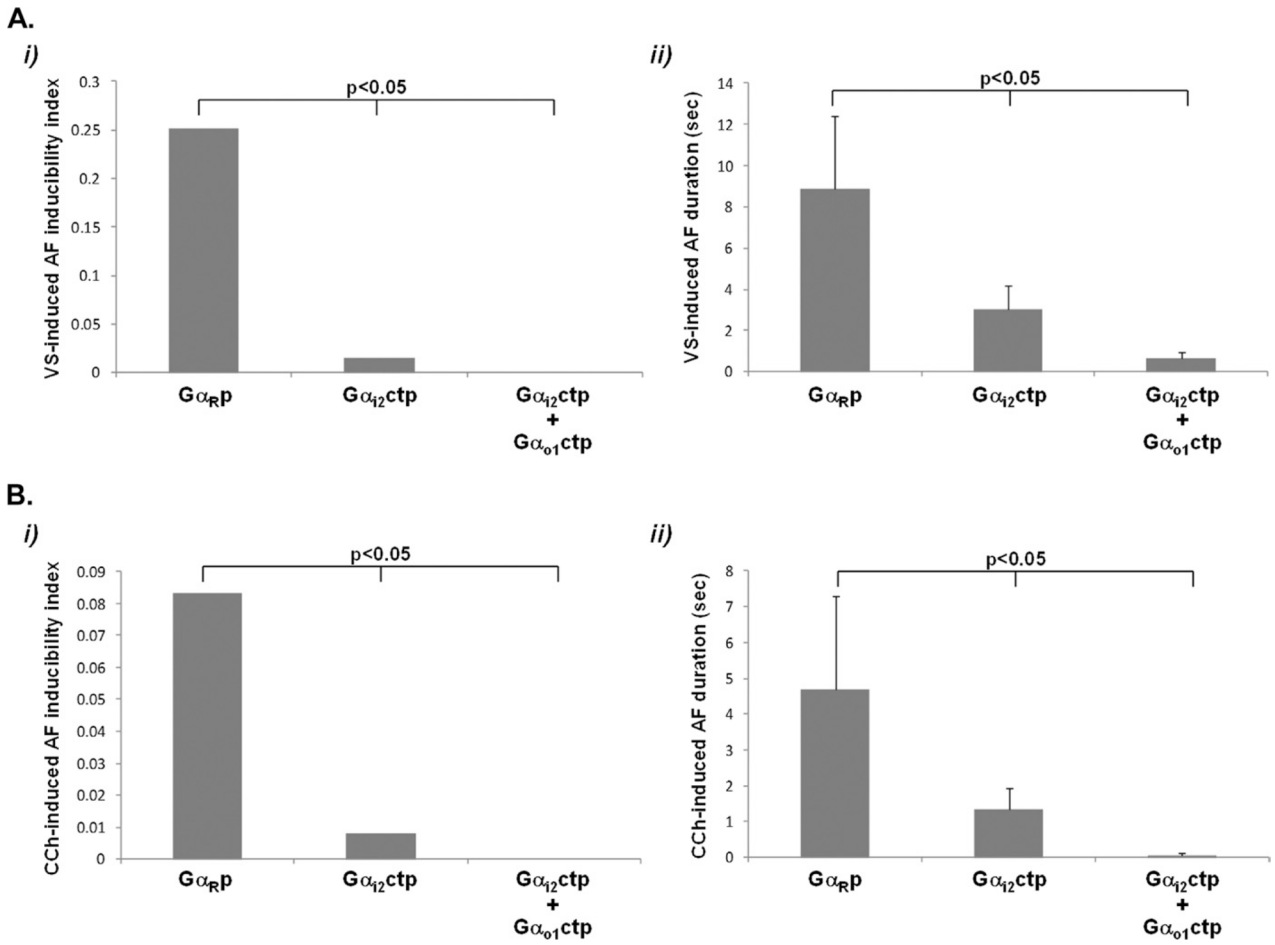

Figure 2.

Decrease in vagal stimulation (VS)- and carbachol CCh-induced atrial fibrillation (AF) by minigene-expressing $G\alpha_{i/o}$ peptide. **A:** Decrease in VS-induced AF in $G\alpha_{i2}$ctp and $G\alpha_{i2}$ctp + $G\alpha_{o1}$ctp dogs compared to $G\alpha_{R}$cpt. Both the AF inducibility index *(i)* and mean AF duration *(ii)* showed a progressive decrease in $G\alpha_{i2}$ctp and $G\alpha_{i2}$ctp+ $G\alpha_{o1}$ctp dogs, respectively. **B:** Decrease in CCh-induced AF in $G\alpha_{i2}$ctp dogs and in $G\alpha_{i2}$ctp+ $G\alpha_{o1}$ctp dogs compared to minigene-expressing $G\alpha_{R}$cpt. Both the AF inducibility index *(i)* and mean AF duration *(ii)* showed a progressive decrease in $G\alpha_{i2}$ctp and $G\alpha_{i2}$ctp+ $G\alpha_{o1}$ctp dogs, respectively.

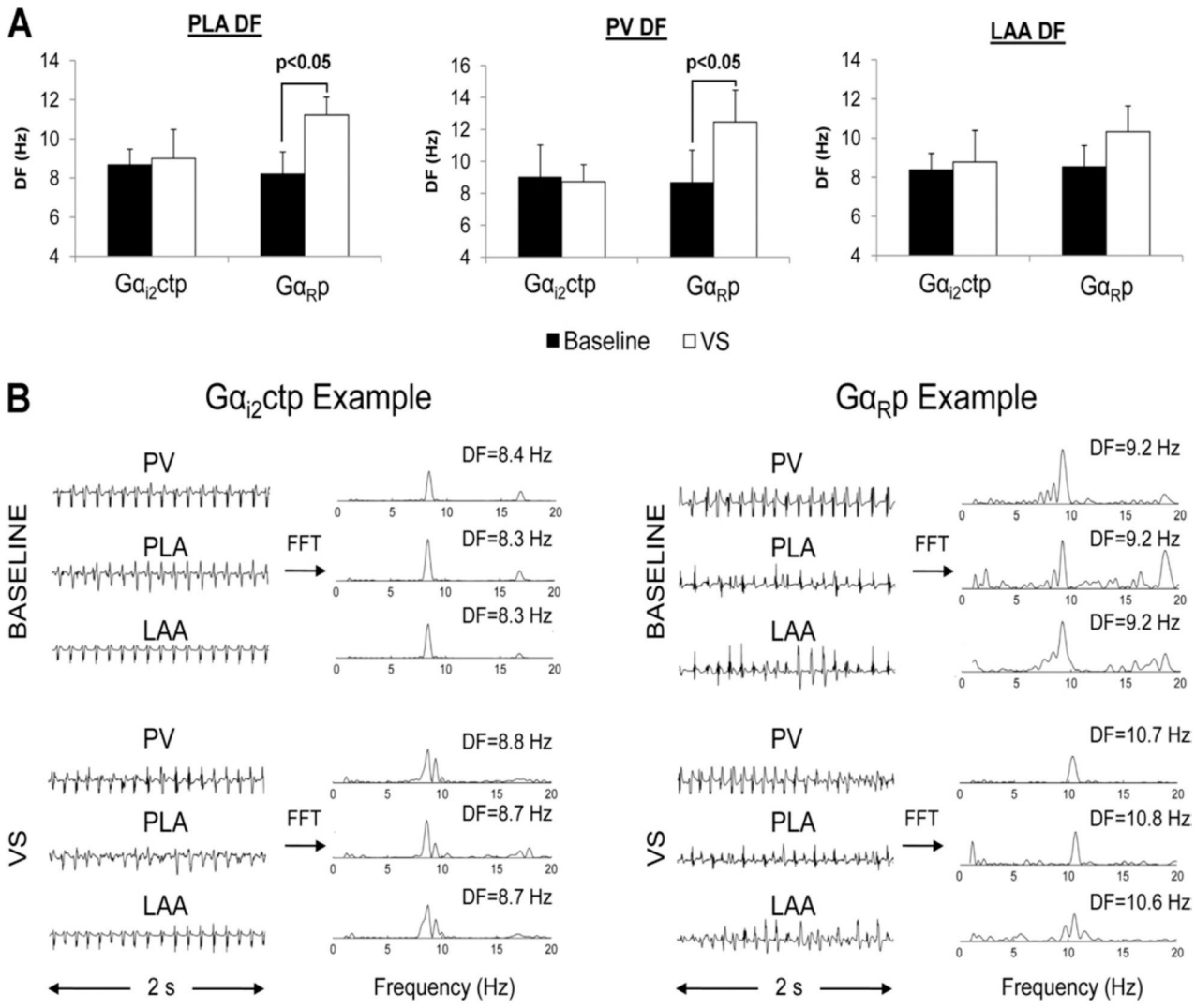

Figure 3.

Attenuation by minigene-expressing Gα_{i2}ctp of vagal stimulation (VS)-induced changes in atrial fibrillation (AF) dominant frequency (DF). **A:** VS led to a significant increase in AF DF in the posterior left atrium (PLA) and pulmonary vein (PV) of Gα_{R}cpt but not in Gα_{i2}ctp. No significant change in DF was noted in the left atrial appendage (LAA) in either Gα_{i2}ctp or Gα_{R}cpt dogs. **B:** Representative examples of AF electrograms recorded from the PV, PLA, and LAA and their corresponding power spectra. Electrograms recorded from the Gα_{i2}atp group show a modest increase in DF with VS compared to baseline. The Gα_{R}p group showed a much larger increase in DF.

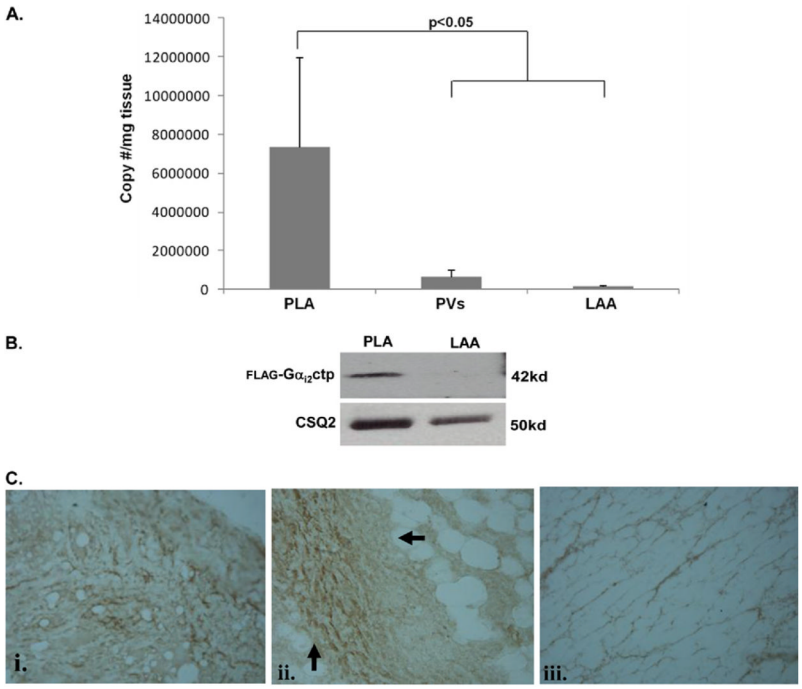

Figure 4.

Verification of Gα_x peptide transgene expression in the left atrium. **A:** Results of polymerase chain reaction on posterior left atrium (PLA), pulmonary vein (PV), and left atrial appendage (LAA) tissue. Transgene expression (for both Gα_{i2}ctp and G$\alpha_R$cpt minigenes) was noted in the PLA. There was minimal expression in the adjoining PV and no expression in the LAA. **B:** Results of Western blotting for FLAG-tagged Gα_{i2}ctp. FLAG-tagged peptide was detected in the PLA but not in the LAA. Calsequestrin Q is the loading control for each lane. **C:** Example of immunohistochemistry for FLAG-tagged Gα_{i2}ctp. FLAG-tagged peptide *(heavy brown stain)* was detected in PLA myocytes *(i)* and in nerve bundles in the PLA *(ii)* but not in the LAA *(iii).*