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Targeted G-protein inhibition as a novel approach to decrease vagal atrial fibrillation by selective parasympathetic attenuation

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Aims The parasympathetic nervous system is thought to play a key role in atrial fibrillation (AF). Since parasympathetic signalling is primarily mediated by the heterotrimeric G-protein, $G\alpha_i\beta\gamma$, we hypothesized that targeted inhibition of $G\alpha_i$ interactions in the posterior left atrium (PLA) would modify the substrate for vagal AF.

Methods and results Cell-penetrating(cp)-$G\alpha_i1/2$ and cp-$G\alpha_i3$ C-terminal peptides were assessed for their ability to attenuate cholinergic-parasympathetic signalling in isolated feline atrial myocytes and in canine left atrium (LA). Confocal fluorescence microscopy indicated that cp-$G\alpha_i1/2$ and/or cp-$G\alpha_i3$ peptides moderated carbachol attenuation of cellular Ca^{2+} transients in isolated atrial myocytes. High-density epicardial mapping of dog PLA, left atrial pulmonary veins (PVs), and left atrial appendage (LAA) indicated that the delivery of cp-$G\alpha_i1/2$ peptide or cp-$G\alpha_i3$ peptide into the PLA prolonged effective refractory periods at baseline and during vagal stimulation in the PLA and to varying extents also in the LAA and PV regions. After delivery of cp-$G\alpha_i$ peptides into the PLA, AF inducibility during vagal stimulation was significantly diminished.

Conclusion These results demonstrate the feasibility of using specific G_i-protein inhibition to achieve selective parasympathetic denervation in the PLA, with a resulting change in vagal responsiveness across the entire LA.

1. Introduction

In recent years, the pulmonary veins (PVs) and posterior left atrium (PLA) have been shown to play a significant role in the genesis of atrial fibrillation (AF). AF treatment consists of either antiarrhythmic drug therapy, or radiofrequency ablation, or both. Antiarrhythmic drug therapy is the most common treatment, but agents suffer from suboptimal efficacy, tolerance, and safety.[1] PV isolation ablation has recently been judged superior to antiarrhythmic drug therapy.[2] Yet, AF frequently recurs after PV-isolation, and so repeated procedures are required that progressively damage the health of the myocardium.[3] Both shortcomings are because of our poor understanding of the underlying mechanisms of AF and other arrhythmias.

Aberrant autonomic signalling, particularly cardiac parasympathetic signalling, has been implicated as a key player in the genesis of AF as well as other arrhythmias.[4] We have recently shown that the PLA is more densely innervated than the rest of the LA in normal hearts, and has a unique parasympathetic profile that contributes to AF substrate.[5–7] Parasympathetic signalling in the heart is initiated upon presynaptic release of acetylcholine (ACh) from vagal nerve terminals. ACh in atrial myocytes primarily activates muscarinic type-2 receptors (M_2Rs), which preferentially transduce the vagal signal via intracellular coupling to heterotrimeric G_i-proteins ($G\alpha_i\beta\gamma$). Activated $G_i\alpha$ inhibits adenylate cyclase (AC)|cyclic-AMP(cAMP)|protein kinase-A(PKA) action, and $G\beta\gamma$ directly activates an inward rectifying K^+ current, $I_{K\text{-}ACh}$.[8] This slows sinus rate and atrioventricular conduction and shortens atrial refractoriness, the latter of which can be inherently susceptible to re-entry and AF.[9,10]

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Currently available MR antagonists acting at the MR orthosteric ligand-binding site have little if any subtype selectivity,[11] as this site has high sequence homology across the five known MR subtypes[12] (all varyingly present in heart[13]). Conversely, the G-protein interface of G-protein-coupled receptors (GPCRs) does not express such redundancy. Sequences in the extreme C-terminus, the extreme N-terminus, an αN-β1 loop, an α4–β6, and an α5 helix of the Gα subunit all variously contribute to the selectivity of particular GPCR|Gα interactions—e.g. $M_{2,4}$Rs and $M_{1,3,5}$Rs primarily coupled to $G\alpha_{i/o}$ and $G\alpha_{q/11}$, respectively.[14–16] Antagonists carefully designed to specifically act at one or more of these regions in $G\alpha_i$ should engender selective disruption of $M_{2,4}R|G\alpha_i$ coupling and even M_2R vs. M_4R selectivity. Further selectivity could be engendered via localized delivery of the peptides to their target organ–suborgan. Native-sequence and analogue peptides that mimic C-terminal region of various Gα subunits have shown promise in these regards.[17–21]

We therefore hypothesized that localized delivery of $G\alpha_i$ C-terminal peptides into the PLA would achieve the necessary suborgan-targeted disruption of $M_2R|G_i$ coupling to selectively antagonize vagal influences involved in AF genesis—i.e. vagal-selective 'pharmacological denervation' in the LA would reduce vagal-induced AF. The present study describes our efforts to test our hypothesis using two $G\alpha_i$ isoform-specific C-terminal peptides.

2. Methods

All procedures involving animals were performed using protocols approved by the Institutional Animal Care and Use Committee at Northwestern University, which conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1 $G\alpha_i$ protein expression in PLA, PV, and left atrial appendage

Relative $G\alpha_i2$ and $G\alpha_i3$ subunit isoform protein expression in the PLA, PV, and left atrial appendage (LAA) regions was assessed via western blot assay (see Supplementary material online for details).

2.2 Assay of cp-$G\alpha_i$ peptide effects on viability and CCh-attenuated Ca^{2+} transients in isolated atrial myocytes

Isolated feline or canine atrial myocytes were obtained by enzymatic digestion of pertinent left atrial regions. The use of feline rather than canine myocytes in many of the experiments of the present study (as specified) was simply a matter of cost and appropriate animal use considerations. Additional experiments using isolated canine atrial myocytes conducted in ongoing studies have indicated no significant differences in the physiological effects between feline and canine myocytes (using the same concentrations of peptides).

The toxicity of the cell-penetrating(cp)-$G\alpha_i1/2$ peptide and cp-$G\alpha_s$ peptide in isolated canine atrial myocytes was assessed using tetrazolium 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. Fluo-4 Ca^{2+}-fluorescence confocal microscopy image acquisition was used to visualize and record CaTs in isolated atrial myocytes as elicited by electrical field stimulation, of which the effects of cp-Gα peptides on parasympathetic-cholinergic signalling was assessed by determining alterations to carbachol (CCh)-attenuated CaTs in the absence or presence (±) of cp-Gα peptides. Comparisons of alterations to CCh-modulated CaTs by the cp-$G\alpha_i$ peptides were made with that of tertiapin-Q (a specific $I_{K\text{-}ACh}$ blocker) and that of atropine (a non-selective MR antagonist). All isolated myocyte experiments were conducted at room temperature (20–25°C). See Supplementary material online for details.

2.3 Assay of cp-$G\alpha_i$ peptide effects on LA refractoriness and AF inducibility/dominant frequency/organization

Canine preparation, open-heart surgery, and high-density epicardial mapping in the PLA, PV, and LAA to obtain regional-effective refractory periods (ERPs) and induce AF under baseline conditions or during left cervical vagal stimulation were performed as described in our previous studies.[5–7] FLAG-tagged cp-Gα peptides (200 nM–3 μM of cp-$G\alpha_i1/2$, cp-$G\alpha_i3$, or random-sequence cp-$G\alpha_{SCR}$ peptide) were injected into the PLA epicardium of the dogs followed by either sonoporation or electroporation. These latter dogs will be referred to as cpGPp dogs. See Supplementary material online for details of these procedures and for the corresponding ERP and AF inducibility and dominant frequency (DF) analyses.

2.4 Assessments of cp-$G\alpha_i$ peptide incorporation canine LA

Anti-FLAG antibodies were used to assess for the presence of FLAG-tagged cp-$G\alpha_i$ via peptides in cpGPp dog PLA tissue homogenates via western blot, and thin sections via immunohistochemistry. Like assays were performed on cpGPp dog PV and LAA tissue for internal control comparisons (and to assess for diffusion of the PLA-delivered peptides), and on control dog PLA tissue for external control comparisons. See Supplementary material online for details.

2.5 cAMP in cpGPp vs. control dog LA tissue

PLA and LAA tissue sample explants from cpGPp and control dogs were homogenized as described above for western blotting (see Supplementary material online for details). The amounts of cAMP in multiple samples of PLA and LAA homogenates were then immediately assessed via competitive enzyme immunoassay using a kit from Cayman Chemicals. The assay was performed according to the manufacturer's instructions, similar to that as described previously.[22]

2.6 Statistical analysis

All data are presented as mean ± SEM, and statistical significance was at $P < 0.05$. See Supplementary material online for details on statistical analyses.

3. Results

There are three major isoforms of $G\alpha_i$ expressed in mammalian tissues—$G\alpha_i1,2$, and 3. In the heart, $G\alpha_i2$ is most highly expressed, followed by $G\alpha_i3$, with little detectable

$G\alpha_i1$.[23,24] Our western blot analyses confirmed higher relative $G\alpha_i2$ subunit protein expression when compared with $G\alpha_i3$ protein subunit expression in normal canine LA ($G\alpha_i1$ subunit protein was not detected), and indicated a trend for higher $G\alpha_i$ protein expression in the PLA and/or PVs than in the LAA (see Supplementary material online, *Figure S1*). Thus, only the actions of cp-$G\alpha_i1/2$ and cp-$G\alpha_i3$ peptides on vagal-cholinergic signalling were assessed in the present study.

3.1 Effects of cp-$G\alpha_i$ peptides on cholinergic modulation of CaTs in isolated atrial myocytes

As $G\alpha_i1/2$ peptide has already been shown to reduce $I_{K\text{-}ACh}$,[25] in the present study we have expanded upon this by investigating the effects $G\alpha_i$ peptides exert on atrial myocyte CaTs, particularly in light of the recent indications that Ca^{2+} cycling plays a major role in autonomic-induced arrhythmogenesis.[26–29] Acute CCh attenuation of CaTs is the result of M_2R-stimulated dissociation of $G\alpha_i\beta\gamma$ and subsequent membrane-delimited activation of $I_{K\text{-}ACh}$ by $G\beta\gamma$, which dramatically accentuates repolarization such that the atrial action potential is abbreviated. In preliminary experiments (Supplementary material online, *Figure S2*), we demonstrated that CCh (10 μM) does dramatically attenuate CaTs in isolated feline atrial myocytes. This effect was significantly reduced or eliminated by pre- and co-application of either tertiapin-Q (a selective $I_{K\text{-}ACh}$ antagonist) or atropine (a non-selective MR antagonist), thus indicating that its primary acute mediation via MR|$G\beta\gamma$-activated $I_{K\text{-}ACh}$. While $I_{Ca\text{-}L}$ is also decreased upon M_2R stimulation via suppression of AD|cAMP|PKA activation, which further accentuates repolarization and additionally diminishes the E-C coupling trigger, this action is much less immediate than the membrane-delimited $I_{K\text{-}ACh}$ activation. Indeed, the tertiapin-Q result indicates that the CCh-M_2R mediated decrease of $I_{Ca\text{-}L}$ evidently has little role in acutely diminishing CaTs in atrial myocytes.

After determining via MTT assay that concentrations of cp-$G\alpha_i$ peptide <5 μM induced little cytotoxicity in isolated atrial myocytes (Supplementary material online, *Figure S3*), we next examined the action of cp-$G\alpha_i$ peptides on CCh attenuation of CaTs in isolated feline atrial myocytes. In *Figure 1A*, example confocal X-t line-scan images and corresponding mean *F* vs. time profiles (below images) illustrate that the CCh attenuation of atrial CaTs (*Figure 1A, i*) was moderated after the myocyte was pre-exposed for 15–20 min via focal superfusion to 3 μM cp-$G\alpha_i1/2$ peptide (*Figure 1A, ii*), the effect of which was found to be statistically significant (*Figure 1A, iii*; $n=9$ cells). *Figure 1A, i–iii* illustrates our finding that cp-$G\alpha_i3$ peptide also effectively moderated CCh attenuation of atrial CaTs ($n = 9$ cells) (see Supplementary material online, *Figure S4*, for summaries of effects these peptides had on other characteristics of CCh-attenuated CaTs). However, *Figure 1C, i–iv* illustrates that co-administration of equal doses of cp-$G\alpha_i1/2$ and cp-$G\alpha_i3$ tended towards greater, though not significantly greater, moderation of CCh-attenuated CaTs than either peptide alone (*Figure 1C, iii*; $n = 4$ cells). Significant moderation of CCh attenuation of atrial CaTs in the presence of cp-$G\alpha_i1/2$ and/or cp-$G\alpha_i3$ was observed in 21 out of 28 isolated atrial myocytes tested. Longer cp-$G\alpha_i1/2$ or cp-$G\alpha_i3$ incubation times of 45–60 min did neither change the extent of CCh attenuation of CaTs, nor did incubation/application of peptides alone result in significant change in CaTs ($n = 6–9$ cells; data not shown). An example result from preliminary experiments illustrating similar cp-$G\alpha_i1/2$-moderation of CCh attenuation of CaTs in isolated canine atrial myocytes is presented in the Supplementary material online, *Figure S5*. Overall, these results demonstrate that cp-$G\alpha_i$ peptides can successfully attenuate M_2R-stimulated G_i-mediated effects on atrial myocyte E-C coupling as hypothesized, and moreover provided proof-of-principle with regards to proceeding with their *in vivo* testing.

Moderation of CCh-attenuated CaTs by the cp-$G\alpha_i$ peptides in these experiments was less than that by tertiapin-Q or atropine (recall Supplementary material online, *Figure S2*). But this was likely attributable to 10 μM CCh being used in the cp-$G\alpha_i$ peptide experiments (vs. 1 μM CCh used in the tertiapin-Q/atropine experiments) or to the requirement of cp-$G\alpha_i$ peptides achieving intracellular translocation (and avoid proteolytic degradation) to beget their action. Comparative cp-$G\alpha_i$ peptide vs. tertiapin-Q or atropine dose–response (the response being moderation of CCh attenuation of CaTs, which itself entails conducting a CCh dose–response) experiments were not deemed essential for this proof-of-principle study. Such experiments would not only entail very extensive effort, but problems of accurately determining intracellular dosages of cp-$G\alpha_i$ peptide would limit their interpretation. To address this, additional studies are planned wherein various non-cell-penetrating $G\alpha$ C-terminal peptides (i.e. $G\alpha_i1,2,3$, $G\alpha_o$, $G\alpha_q$, $G\alpha_s$) will be introduced into myocytes via patch-pipette, and comparative CaTs measurement will be made accordingly. Example results from preliminary experiments illustrating cp-$G\alpha_s$-moderated ISO-potentiation of CaTs in isolated canine atrial myocytes is presented in the Supplementary material online, *Figure S6*.

3.2 Effects of cp-$G\alpha_i$ peptides on atrial refractoriness

In vivo actions of cp-$G\alpha_i$ peptides on parasympathetic signalling were assessed via open-heart electrophysiology measurements of vagal-attenuated atrial refractoriness. Changes in ERPs in the PLA, PVs, and LAA at baseline and during vagal stimulation (VS) were measured before and after the injection of cp-$G\alpha_i1/2$ (eight dogs), cp-$G\alpha_i3$ (four dogs) or cp-$G\alpha_{SCR}$ (three dogs) peptides followed by either sonoporation or electroporation (cp-$G\alpha_i1/2$ peptide+sonoporation in three of 15 dogs; cp-$G\alpha_i1/2$, cp-$G\alpha_i3$, or cp-$G\alpha_{SCR}$ peptide+electroporation in 12 of 15 dogs). The PLA was chosen as the site of peptide delivery because of our previous work demonstrating that this region has the highest parasympathetic innervation in the LA.[5–7] No atrial or ventricular arrhythmias were induced by the peptide injection ± sono/electroporation manoeuvres, and all animals remained haemodynamically stable throughout the experiments. ERP shortening in response to VS was comparable to that previously reported.[5,6] Hence forward, peptide 'delivery' encompasses injection+sono/electroporation.

Effects of FLAG-tagged cp-$G\alpha_i1/2$ peptide (0.2–3 μM) delivery into the PLA are shown in *Figure 2A*. Because peptide delivery was localized to sites in the PLA, effects of the peptide on atrial refractoriness were expected to be most prominent in the PLA. Indeed, the pronounced

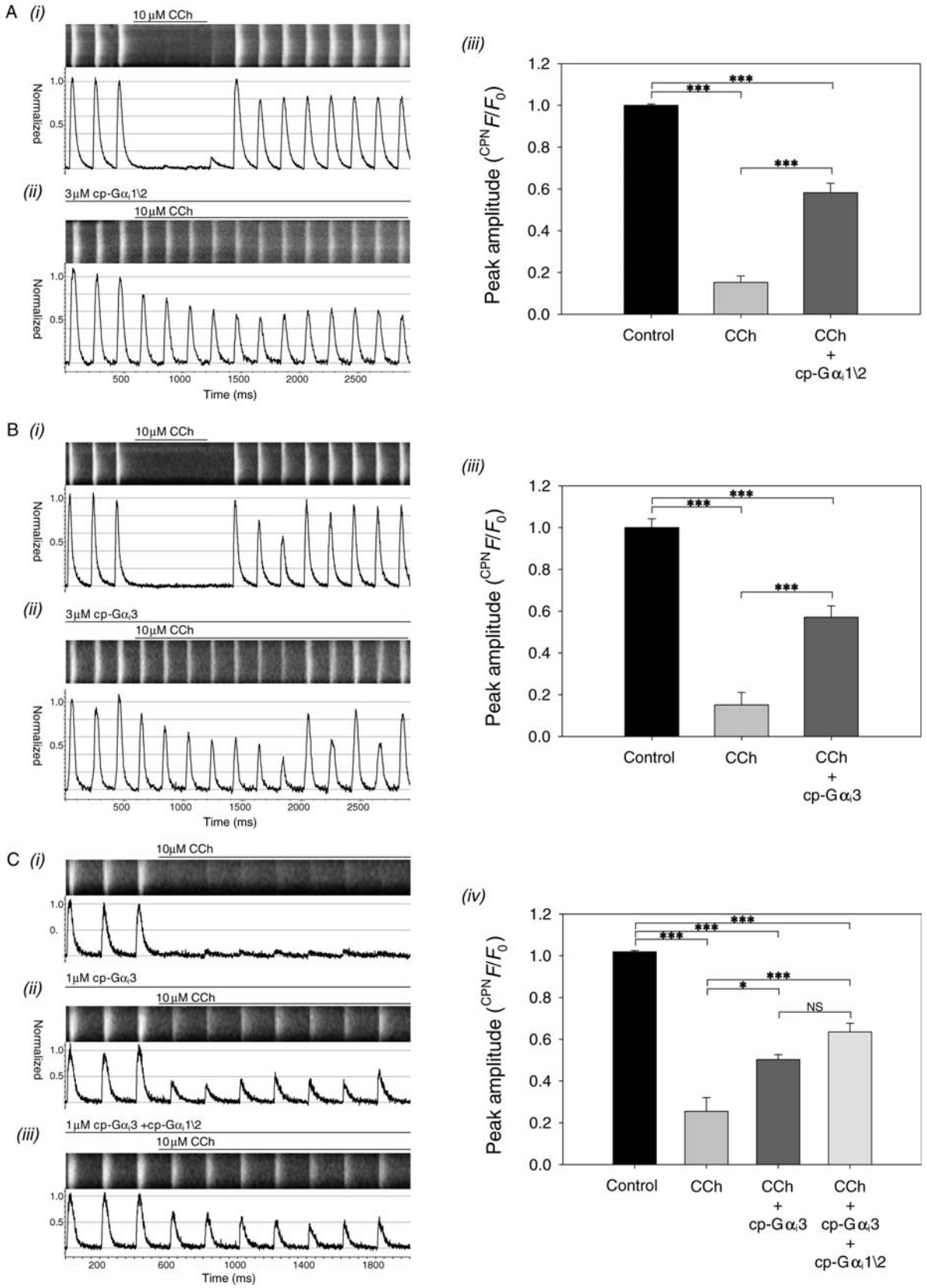

Figure 1 CCh attenuation of CaTs in atrial myocytes is blunted in the presence of cp-Gα_i peptides. (*A*) Serial confocal X-t linescan images (fluo-4 fluorescence) and the corresponding control (no CCh) peak-normalized fluorescence (^{CPN}F) vs. *t* profiles illustrating an example of an individual isolated feline myocyte paired responses to: (*i*) acute application of 10 μM CCh and subsequent washout-recovery in the absence of peptide; (*ii*) application of 10 μM CCh after pre-1 min and co-application of cp-Gα_i1/2 peptide showing blunting of the CCh action (CCh was applied for $\leq$3 s to ensure effect was sustained); and (*iii*) summary statistics bar graph of the paired responses from 10 cells in terms of peptide effect on CaT peak amplitude. (*B*) As in (*A*), but using cp-Gα_i3 peptide in a different myocyte (in *ii*). Summary statistics bar graph (in *iii*) compiled from nine cells. (**C**) As in (*A*), but in which a different myocyte was exposed first (in *ii*) to cp-Gα_i1/2 peptide, and then (in *iii*) to cp-Gα_i3 peptide. Summary statistics bar graph in (*iv*) compiled from four cells. NS, not significant. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

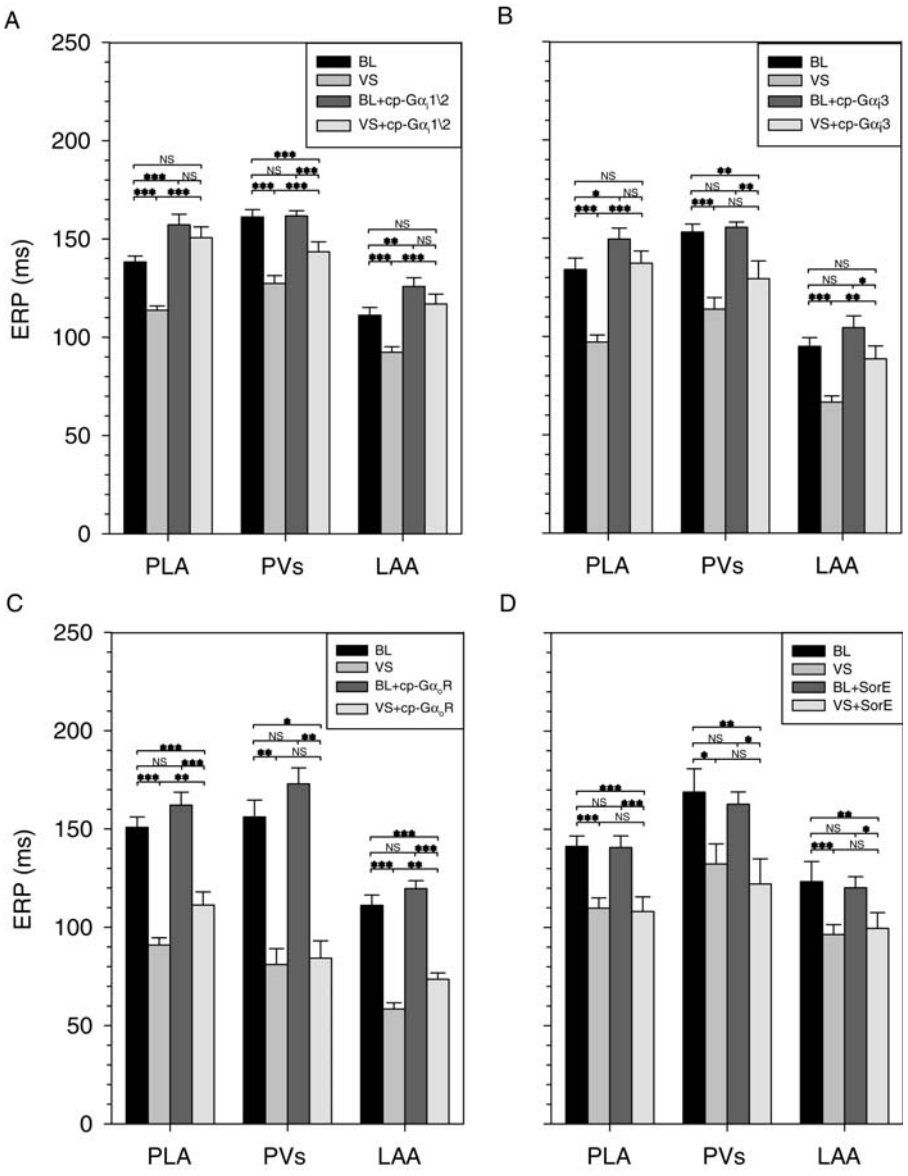

Figure 2 Effect of cp-$G\alpha_i$ peptides on left atrial ERPs. Summary statistics for ERPs at baseline (BL) and during VS in the PLA, PVs, and LAA are as follows: **(A)** $\pm$ cp-$G\alpha_i$1/2 peptide delivery (sono/electroporation-assisted) into the PLA **B)** $\pm$ cp-$G\alpha_i$3 peptide delivery (electroporation-assisted) into the PLA; **C)** $\pm$ cp-$G\alpha_o$R peptide delivery (electroporation-assisted) into the PLA; and **D)** $\pm$ sono- or electroporation of the PLA alone. NS, not significant. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

VS-induced ERP shortening was eliminated in the PLA after peptide delivery. In addition, ERPs at baseline in the PLA were prolonged—consistent with an attenuation of vagal tone—after peptide delivery. However, changes in atrial refractoriness after peptide delivery were not confined to the PLA. Elimination of VS-induced ERP shortening and prolongation of baseline ERPs also occurred in the LAA after peptide delivery. Moreover, ERPs during VS were prolonged in all left atrial regions after peptide delivery.

Effects of FLAG-tagged cp-$G\alpha_i$3 peptide (0.2–3 μM) delivery into the PLA were quite similar to that of cp-$G\alpha_i$1/2, as shown in *Figure 2B*. In the PLA, the pronounced VS-induced ERP shortening was eliminated and ERPs at baseline were prolonged after cp-$G\alpha_i$3 peptide delivery. Likewise, effects were not confined to the PLA, as VS-induced ERP shortening was reduced (though not eliminated) also in the LAA after peptide delivery. However, unlike the cp-$G\alpha_i$1/2 peptide results, prolongation of baseline ERPs after cp-$G\alpha_i$3 peptide delivery was evident only in the PLA.

The electrophysiological effects of these cp-$G\alpha$ peptides were found to be stable throughout the duration of our *in vivo* experiments in which the ERPs $\pm$ VS measured at 60–90 min after peptide delivery were within 10 ms of those measured 15–20 min after peptide delivery (no later measures were taken to avoid effect of hypothermia, fluid loss, etc.). Greater attenuation of VS-induced ERP shortening was not consistently evident upon increasing the injected concentration of either cp-$G\alpha_i$ peptide from 0.2–0.3 μM to 1–3 μM. But it should be noted that the inexactness of intracellular translocation inevitably associated with *in vivo* tissue delivery of peptides precludes accurate assessment of actual dosage achieved, and so precludes obtaining accurate dose–response relationships. Indeed, concomitant sono/electroporation was found necessary to

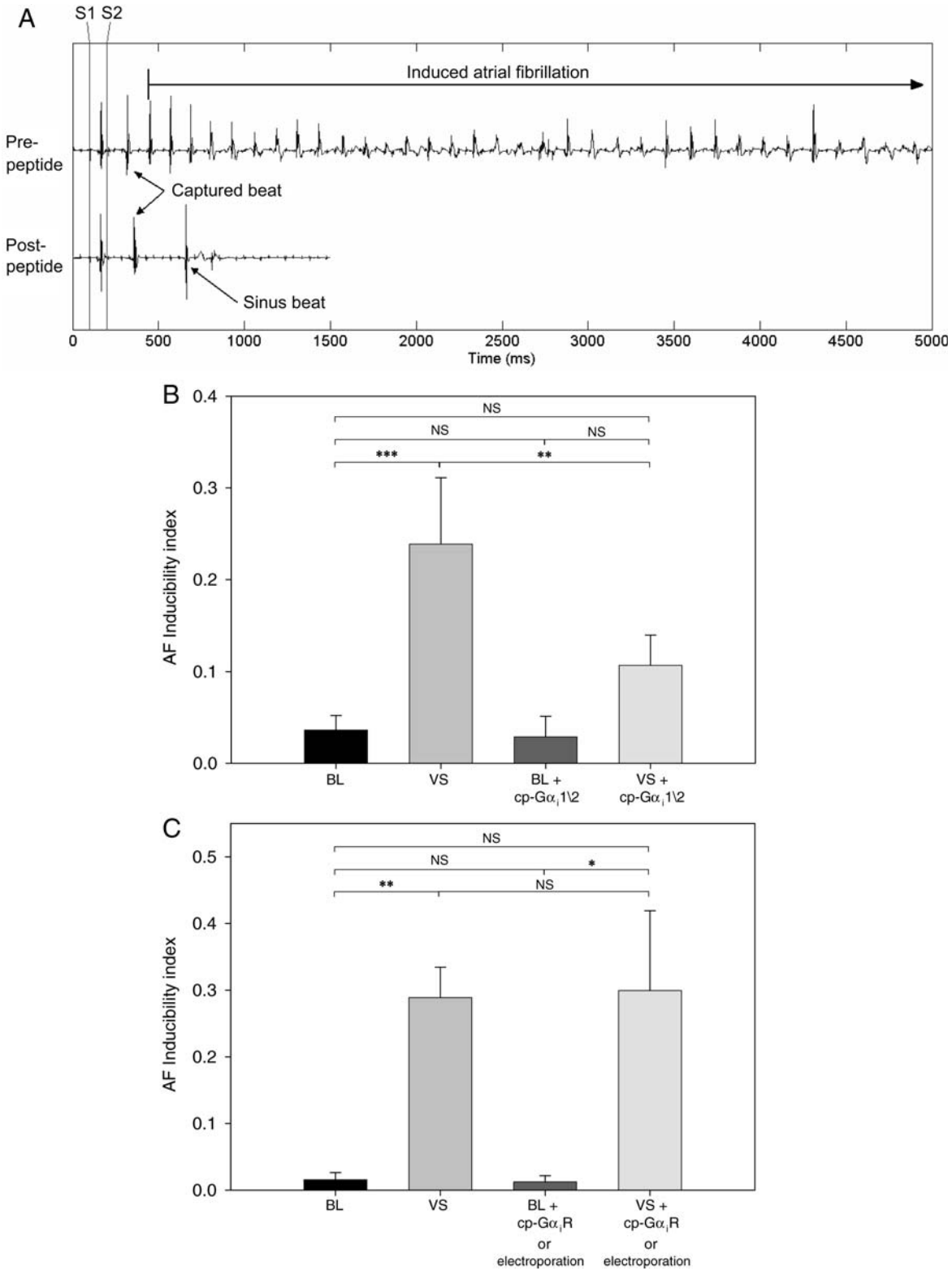

Figure 3 Effect of cp-Gα_i peptide on AF inducibility. (*A*) Example electrogram recordings from the same epicardial electrode in the PLA in which the upper tracing shows that after delivery of a single premature stimulus (S2) during VS, induced an episode of AF in the absence of cp-Gα_i1/2 peptide; whereas the lower tracing shows that in another episode of AF, this manoeuvre did not induce AF in the presence of cp-Gα_i1/2 peptide delivered into the PLA. (**B**) Statistical summary of AF inducibility ratio at baseline (BL) and during VS in the left atrium $\pm$ cp-Gα_i peptides delivery into the PLA (combinatorial average of cp-Gα_i1/2 and cp-Gα_i3 peptide results). (**C**) Statistical summary of AF inducibility ratio at baseline and during VS in left atrium $\pm$ cp-Gα_oR peptide delivery into the PLA or sono/electroporation of PLA (combinatorial average of results from these manoeuvres).

achieve intracellular transfer of the cp-Gα_i peptides, as only injection of cp-Gα_i1/2 into the PLA (three dogs) was found to be ineffectual (Supplementary material online, *Figure S7*). Sonoporation or electroporation alone of target PLA sites caused no overt permanent changes to electrogram characteristics, and histological analysis of PLA tissue

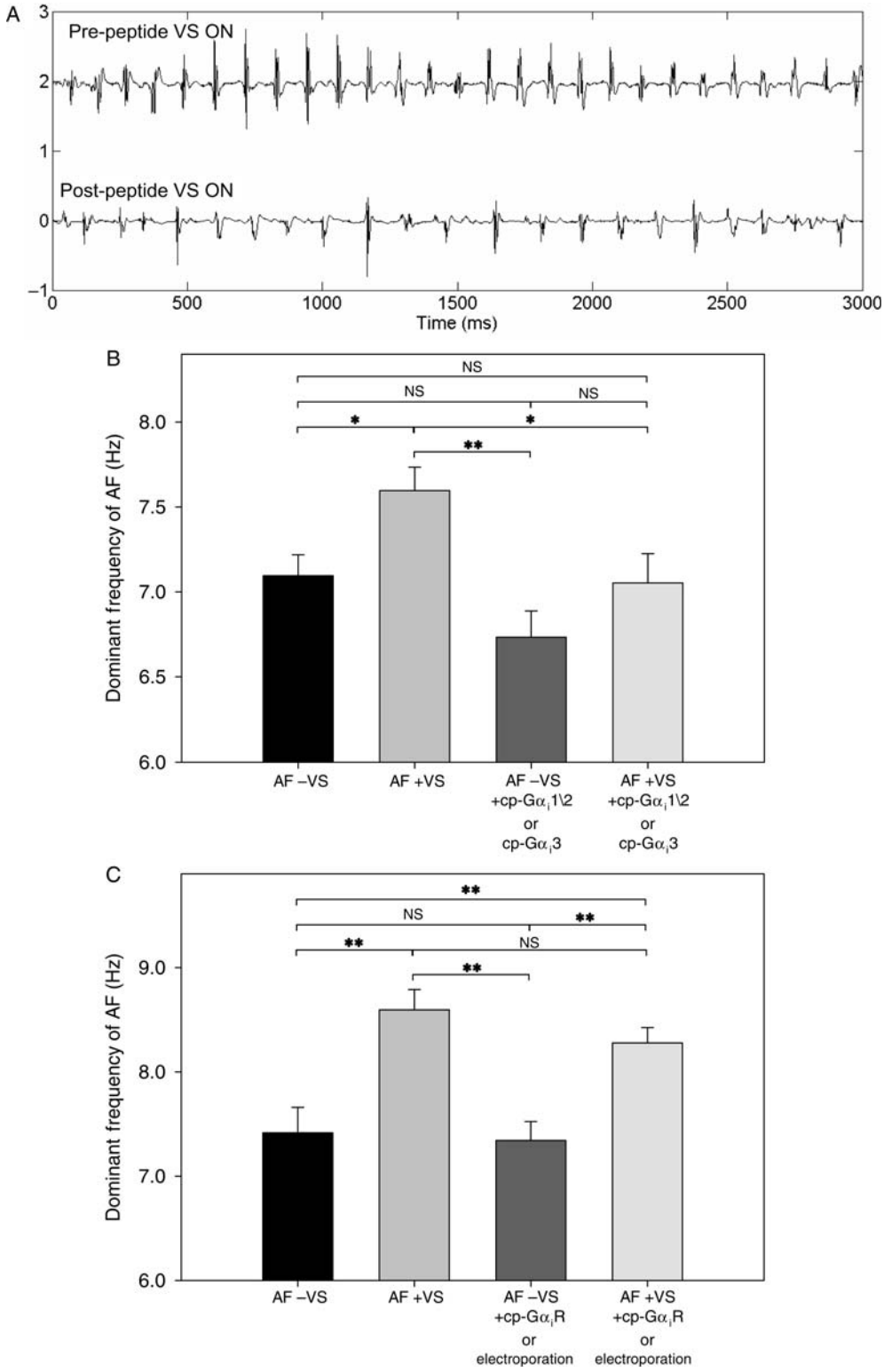

Figure 4 Effect of cp-$G\alpha_i$ peptide on the dominant frequency and organization of AF. (A) Examples of electrogram recordings from the same epicardial electrode in the PLA in which the upper tracing shows an episode of AF induced during VS in the absence of cp-$G\alpha_i$1/2 peptide; whereas the lower tracing shows another episode of AF induced during VS after cp-$G\alpha_i$1/2 peptide delivery into the PLA. Clearly, the most predominant frequency component, or dominant frequency (DF), of the AF episode in the presence of peptide is lower (has a slower periodicity) compared with that in the absence of peptide. (**B**) Statistical summary of the DF of AF in the absence of (AF−VS) and during VS (AF+VS) in the PLA, PV, and LAA regions ± cp-$G\alpha_i$1/2 peptide delivery into the PLA. (**C**) Statistical summary of DF of AF in the absence of and during VS in left atrium ± cp-$G\alpha_o$R peptide delivery into the PLA or sono/electroporation of PLA (combinatorial average of results from these manoeuvres).

sections taken after our *in vivo* electrophysiology experiments showed no evidence of myocyte necrosis (Supplementary material online, *Figure S8*). Accordingly, sono/electroporation of the PLA *per se* induced no significant effect on PLA, PV, and LAA ERPs at baseline or during VS (*Figure 2D*).

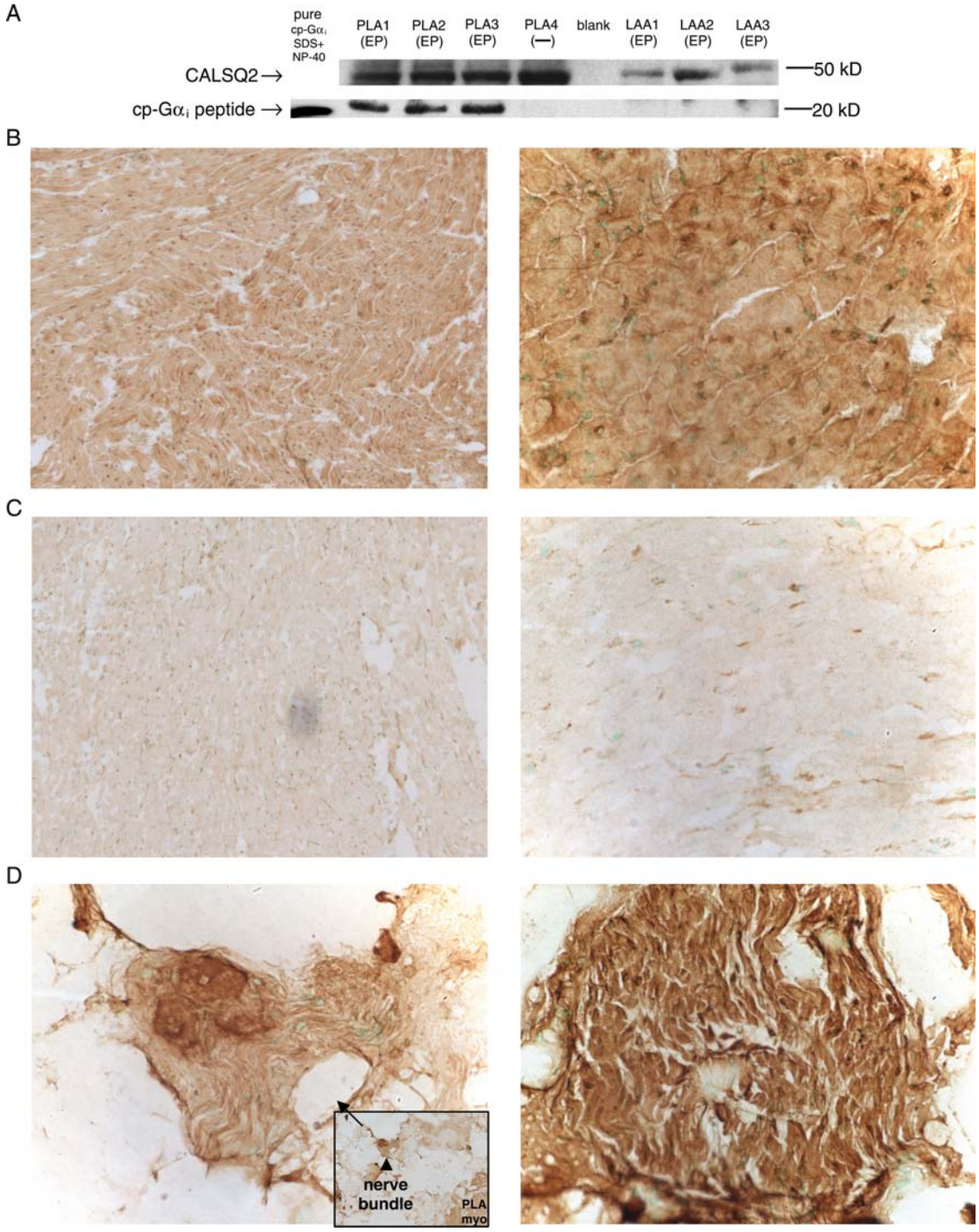

Figure 5 Retention of cp-Gα_i1/2 peptide in the PLA of cpGPp dogs. (*A*) Example of anti-FLAG western blots of samples as specified (above each lane): 'EP', atrial samples taken from dogs subjected to *in vivo* electrophysiology experiments in which FLAG-tagged cp-Gα_i1/2 peptide had been delivered into their PLAs; '–', atrial samples taken from dog in which no peptides had been delivered—i.e. negative controls; the amount of pure FLAG-tagged cp-Gα_i1/2 peptide used in lane 1 was 1 μg; 'numbers' to the right of PLA or LAA specifiers refer to the particular dogs. (*B*) (*Left*) 10X bright field image of a 5–10 μm thick cross-section (endocardium-to-epicardium) of the area of PLA that had been injected with 1 μM FLAG-tagged cp-Gα_i1/2 during an *in vivo* electrophysiology experiment (see text) showing marked brown anti-FLAG secondary horseradish-peroxidase (HRP) staining throughout; and (*right*) a region within the latter at 40X myocardium. (*C*) As in (*B*), but of a section of LAA—region remote from the PLA site of peptide injection—showing essentially only background HRP staining. (*D*) As in (*B*), but of a section of PLA near the area of peptide delivery that contained an autonomic nerve bundle within a fat pad (*left inset is 4X field at* $\frac{1}{3}$ *scale*); and the region within the latter at 40X showing ganglion cells within the nerve bundle. The marked brown anti-FLAG secondary HRP staining indicates that FLAG-tagged cp-Gα_i1/2 peptide had incorporated into this nerve bundle. Light green staining (methyl-green) in these sections denotes staining of cell nuclei. Photomicrographs shown were taken at identical camera settings/exposures.

Because Gα_i inhibitory peptides have an essential cystine in their sequence, it is possible that disulfide bond formations with other cystine-containing proteins in the PLA could cause effects on ERPs that are not specific to MR|G$_i$ action. To test for this, a cystine-containing Gα scrambled-sequence cp-peptide, cp-Gα_{SCR}, was delivered into the PLA

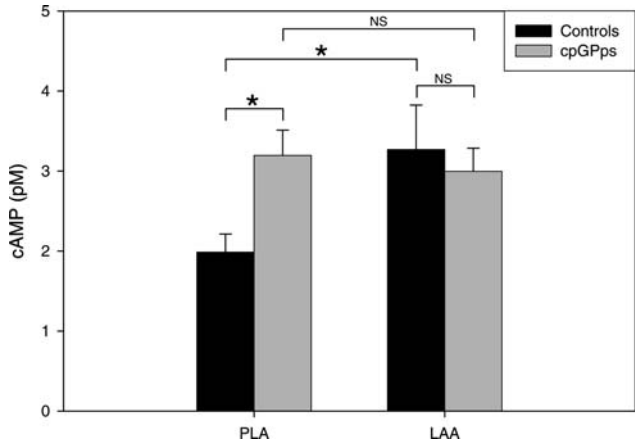

Figure 6 cAMP in cpGPp dogs vs. control dogs. Statistical summary of cAMP in PLA and LAA tissue explant homogenates from dogs whose PLAs had been injected (+electroporation) with cp-$G\alpha_i$1/2 peptide (cpGPp) compared with dogs whose PLAs were not injected with peptide (controls) during the *in vivo* electrophysiology experiments. LAAs were assayed as internal controls, particularly for cpGPp dogs.

of three dogs, and ERPs were subsequently measured in the PLA, PVs, and LAA regions as with the $G\alpha_i$ peptides. After cp-$G\alpha_{SCR}$ peptide delivery into the PLA, less shortening of ERPs during VS was evident in the PLA and LAA, but significant VS-induced ERP shortening was still evident (*Figure 2C*). This was in contrast to the insignificant VS-induced ERP shortening evident after delivery of either cp-$G\alpha_i$1/2 or cp-$G\alpha_i$3 peptides. This small effect observed after delivery of cp-$G\alpha_{SCR}$ into the PLA represent the minor non-specific action of the $G\alpha_i$ peptides and experimental manoeuvres on LA ERPs.

3.3 Effects of cp-$G\alpha_i$ peptides on AF inducibility and frequency characteristics

Depicted in *Figure 3A* is an example of PLA epicardial electrogram recordings showing that under control conditions (no cp-$G\alpha_i$ peptide), a single extrastimulus delivered during VS induced an episode of AF (AF induced with VS nearly always terminated spontaneously after cessation of VS); whereas after cp-$G\alpha_i$1/2 peptide delivery into the PLA, a single extrastimulus interjected during VS failed to induce AF. The statistical summary of the evaluation of cp-$G\alpha_i$ peptides on AF inducibility is shown in *Figure 3B*, which indicates that interjection of single extrastimuli rarely induced AF at baseline (inducibility index = 0.03), but frequently did so during VS—40 AF episodes >5 s in duration (inducibility index = 0.17; i.e. more than five-fold that at baseline). However, after cp-$G\alpha_i$ peptide delivery into the PLA, AF inducibility in the presence of VS was significantly decreased—only 10 episodes >5 s in duration were induced (inducibility index = 0.05; i.e. a 69% reduction in AF inducibility during VS). Electroporation ± cp-$G\alpha_{SCR}$ peptide in the PLA did not decrease AF inducibility during VS (*Figure 3C*).

In addition to decreasing AF inducibility, delivery of cp-$G\alpha_i$ 1/2 or cp-$G\alpha_i$3 peptide into the PLA also altered the frequency characteristics of the AF that was induced. Depicted in *Figure 4A* are examples of PLA epicardial electrograms of AF episodes during VS in the absence and presence of cp-$G\alpha_i$1/2 peptide (delivered into the PLA) showing that the DF of AF is lower (slower periodicity) in the presence of peptide. As indicated by *Figure 4B*, statistical evaluation of the frequency characteristics of AF episodes indicated that after cp-$G\alpha_i$1/2 or cp-$G\alpha_i$3 peptide delivery into the PLA, there was a reduction in DF of AF during VS in the LA and the increase in DF during the elimination of VS (increase in DF of AF during VS was comparable to that previously reported).[30] Again, electroporation ± cp-$G\alpha_{SCR}$ peptide in the PLA did not cause such an effect (*Figure 4C*).

3.4 Confirmation of *in vivo* retention of cp-$G\alpha_i$ peptides

LA tissue samples were taken from three dogs after completion of the *in vivo* electrophysiology in which FLAG-tagged cp-$G\alpha_i$1/2, cp-$G\alpha_i$3 or cp-$G\alpha_{SCR}$ peptides had been delivered into their PLAs, and from a dog that had not been subjected to these peptide experiments. Tissue homogenates from the PLAs and LAAs from each of these dogs was then evaluated for the presence of FLAG-tagged cp-$G\alpha_i$1/2 peptide using anti-FLAG immunoassays. Anti-FLAG western blot assay (*Figure 5A*) indicated an intense band at approximately 20 kDa only for homogenates of PLA tissue samples taken at or near the site of peptide delivery in the *in vivo* electrophysiology experiments (note: the apparent molecular size of approximately 20 kDa for $G\alpha_i$ peptides in these western blots is consequent to its sodium dodecyl sulphate–polyacrylamide gel electrophoresis mobility being reduced in non-ionic detergent- and lipid-containing homogenates. This was conferred to the pure peptide-positive control by adding equivalent amounts of detergents contained in the homogenates to the sample. For details of Methods see Supplementary material online.

Further confirmation of retention of FLAG-tagged cp-$G\alpha_i$1/2 peptide in the PLA was indicated by above-background anti-FLAG immunohistochemical staining of endocardium-to-epicardium PLA cross-section tissue samples (*Figure 5B*). Conversely, only background levels of anti-FLAG immunohistochemical staining were seen in endocardium-to-epicardium LAA cross-section tissue samples (*Figure 5C*). Similar tissue retention was found for FLAG-tagged cp-$G\alpha_i$3 peptide (Supplementary material online, *Figure S9*). In addition, above-background anti-FLAG immunohistochemical staining was sometimes seen in autonomic nerve bundles in these PLA tissue sections (*Figure 5D*), indicating that cp-$G\alpha_i$ peptides can also associate with autonomic nerves in the PLA. This latter finding may underlie why ERPs in the LAA and PV regions (Section 3.4) often exhibited changes after localized delivery of cp-$G\alpha_i$ peptide into the PLA (a possible means by which this could occur is proposed in Section 4.2).

Western blot assays of injected peptides can only assure retention of the cp-$G\alpha_i$ peptides, not translocation; i.e. the peptides could simply be adsorbed to the extracellular side of cellular membranes and/or closely associated extracellular matrix. However, we have conducted preliminary trials of injecting FLAG-tagged $G\alpha_i$1/2 peptide-expressing minigene plasmid constructs into the PLA (+ electroporation), and assessed PLA tissue explants for peptide expression via western blot assay 24–72 h later (see Supplementary material online, *Figure S10*). Anti-FLAG western assay of such PLA tissue homogenates detected FLAG-tagged $G\alpha_i$1/2 peptide, which could only occur upon

intracellular translocation of the minigene. Such result provides strong support that our *in vivo* delivery method did impart intracellular translocation of cp-Gα_i peptides in the present study.

3.5 Level of cAMP changes in the PLA of cpGPp dogs

PLA and LAA tissue homogenates of tissue explants from cpGPp and control dogs were also assessed. As shown in *Figure 6*, cAMP levels in cpGPp dog PLAs were found to be significantly higher than that in control dogs, although cAMP levels in cpGPp dog LAAs were not different than that in control dogs. These results are consistent with cp_Gα_i1/2 peptide disruption of Gα_i-mediated inhibition of AC selective to the PLA, as would be expected by the target delivery of the peptide to the PLA in cpGPp dogs. Note that in control dogs, cAMP levels in LAA were higher than that in PLA, which seems in accordance with less Gα_i protein in LAA than in PLA.

4. Discussion

The major findings of this study are as follows: (1) both Gα_i2 and Gα_i3 protein isomers were found in canine LA, thus indicating the need to examine the effects of both cp-Gα_i2 and cp-Gα_i3 inhibitory peptides on LA cholinergic-parasympathetic signalling; (2) cp-Gα_i1/2 and/or cp-Gα_i3 peptides could moderate CCh-attenuated CaTs in isolated atrial myocytes; (3) injection + sono- or electroporation effectively delivered cp-Gα_i peptides into the PLA (albeit, also into constituent and/or neighbouring nerves); (4) delivery of cp-Gα_i1/2 or cp-Gα_i3 peptides into the PLA prolonged refractoriness in the PLA, and to differential degrees in the LAA and PV regions, both at baseline and during VS, but more so during VS; and (5) delivery of cp-Gα_i1/2 or cp-Gα_i3 peptides into the PLA significantly reduced vagal-induced AF. These findings demonstrate that isoform-specific Gα_i C-terminal peptides can be used to achieve selective disruption of parasympathetic-mediated M_2R/G_i-protein coupled signalling in the LA.

4.1 Effects of cp-Gα_i peptides to attenuate CaTs in isolated atrial myocytes

In the present study, CCh attenuation CaT amplitude and duration in isolated atrial myocytes was significantly moderated in the presence of cp-Gα_i1/2 peptide and/or cp-Gα_i3 peptide. This is consistent with both Gα_i2 and Gα_i3 protein subunit isoforms being present in atrial myocardium;[23,24] albeit, moderation of CCh-attenuated CaTs in the presence cp-Gα_i3 peptide was equivalent to that in the presence of cp-Gα_i1/2 peptide, which was somewhat unexpected given that our western blot analyses indicated less Gα_i3 protein than Gα_i2 protein in LA. Moreover, the application of cp-Gα_i1/2 and cp-Gα_i3 peptides together did elicit a significant additive effect. While seemingly discrepant, similar findings have been reported in human and mouse atrium[24,31] or embryonic stem cell-derived cardiomyocytes,[32] which indicates that factors besides protein quantity govern particular $M_2R|G_i/G_o$-protein coupled signalling. But since the Gα_i1/2 and Gα_i3 C-terminal sequences differ only by two amino acids,[33] we cannot rule out the possibility that either peptide may interfere somewhat at either $M_2R|G\alpha_i$–protein interface. There is also the possibility of CCh-activated $MR|G_o,G_q$ protein-coupled signalling with respect to Ca_i^{2+} cycling and/or ion channel modulation[28,34,35] may become more apparent and/or pertinent when $MR|G_i$-protein coupled signalling is inhibited by cp-Gα_i peptides. These issues will be addressed in our ongoing studies examining the effects of not only Gα_i, but also Gα_o, Gα_q, and Gα_s C-terminal peptides via combined patch-clamp electrophysiology and confocal Ca_i^{2+}/CaT assays. Certainly, in light of recent findings indicating a major role of Ca_i^{2+} in both sympathetic β-adrenergic and parasympathetic/cholinergic-mediated promotion of re-entrant and trigger atrial arrhythmias,[36–39] the ability of these novel Gα inhibitory peptides to modulate atrial myocyte Ca_i^{2+} cycling underscores their potential role in the treatment of autonomically induced AF.

4.2 Effects of cp-Gα_i peptides to prolong refractoriness in the LA

In the present study, delivery of cp-Gα_i1/2 or cp-Gα_i3 peptide into the PLA resulted in significant prolongation of ERPs at baseline and during VS in the PLA. Interestingly, some of these effects were also noted in the LAA and to a lesser extent in the PV regions. Since left atrial ERPs were not significantly altered upon PLA sono/electroporation alone or upon injecting cp-Gα_{SCR} peptide into the PLA followed by electroporation, the changes in LAA and/or PV region ERPs that occurred after delivery of cp-Gα_i peptides into the PLA are perhaps most appropriately deemed 'distal' effects. Our anti-FLAG immunohistochemistry indicated no evidence that these distal effects were consequent to cp-Gα_i peptides inadvertently translocating directly into LAA and/or PV myocardium. But anti-FLAG immunohistochemistry did indicate that cp-Gα_i peptide could associate with nerve tracts in the PLA (*Figure 6D*). In previous studies, we have shown that autonomic nerve tracts to the LAA and PVs originate in, or otherwise pass through, the PLA,[5–7] and that the tropicamide (a muscarinic receptor antagonist) applied topically to the PLA resulted in the attenuation of vagal-induced shortening of ERPs not only in the PLA but also in the PVs and LAA.[6] Thus, since G_i-protein signal transduction in neurons is an incremental part of autoinhibitory negative feedback on neurotransmitter release,[40,41] G_i-protein inhibition resulting from cp-Gα_i peptide uptake by parasympathetic neurons and subsequent passive and/or active anterograde transport to presynaptic nerve endings (which would be diffusely hard to detect using anti-FLAG immunohistochemistry) would lead to ACh release overflow. Ongoing studies in our laboratory in which PLA-delivered Gα_i peptide-expression minigene plasma constructs are being assessed for resultant attenuation of vagal-induced shortening of and AF suppression entail scrutiny for any such 'distal' effects in the LAA and/or PVs.

4.3 Potential of Gα isoform-specific inhibitory peptides in AF therapy

The overall effect of cp-Gα_i peptides delivered into the PLA, whether proximal or both proximal and distal to the PLA, resulted in the prolongation of LA refractoriness and marked suppression of vagal-induced AF. These results clearly demonstrate that the targeted and selective inhibition of cholinergic-parasympathetic activity by these

peptides can produce the desired effect of attenuating vagal effects on AF genesis/maintenance. Further, these results may very well demonstrate that the utilization of cp-$G\alpha$ inhibitory peptides in therapy targeting autonomic-initiated/maintained AF represents progress towards achieving an alternative therapeutic strategy for controlling this type of AF—i.e. more precise targeting of the aberrant autonomic signalling involved in the genesis of AF without causing any significant non-selective side effects and damage to surrounding tissue (typically the case with ablation). Moreover, the capacity to make and utilize multiple types of $G\alpha$-protein isoform-specific $G\alpha$ inhibitory peptides (e.g. $G\alpha_o$, $G\alpha_q$, $G\alpha_s$, etc.) provides the possibility to precisely modulate various multiple upstream components of atrial autonomic signalling such that autonomic-mediated AF therapy could be tailored to particular AF genesis and/or maintenance mechanisms.

5. Study limitations

The *in vivo* studies were conducted using normal dogs, the results of which cannot be directly extrapolated to the human LA, or to pathological setting of AF. Further studies are therefore needed to assess the action of cp-$G\alpha_i$ inhibitory peptides in more 'pathological' settings.

Because of the significant expense associated with the use of a canine model, dogs were used only for the *in vivo* experiments described in the manuscript. Cat cells were felt to be suitable for confocal experiments with $G\alpha i$ peptide as previous studies have characterized $I_{K\text{-}ACh}$ and Ca_i^{2+} cycling in cat atrial myocytes,[39,42] as well as prior *in vivo* studies of vagal (or autonomic in general) response/control in cat atrium.[43–45]

In the current manuscript, in order to establish proof-of-concept, we used $G\alpha_i$ inhibitory peptides that were not specific to the $M_2R/G\alpha_i$ interface. Generalized $G\alpha_i$ inhibition, even in a localized area of the heart, is not likely to be appropriate for eventual clinical use. Future formulations of these peptides (being created in ongoing work in our laboratory) will only inhibit the interaction of $G\alpha$ with a specific receptor—e.g. $G\alpha_i$ interaction with the M_2-R. Interactions of $G\alpha_i$ with other receptors would therefore be unaffected. These selective peptides are expected to be more suitable for any future clinical use.

The contribution of the sympathetic innervation of the PLA and PVs to AF substrate was not examined in this study. The detailed interactions between the sympathetic and parasympathetic nervous systems within the LA—especially as they relate to the genesis of AF—need to be addressed in more detail in future studies.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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