INTRODUCTION

This “Special Topic” series explores how regulators of G protein signaling (RGS) proteins share common, yet distinct roles at multiple levels, controlling autonomic regulation of heart function, and what critical questions remain for translational researchers examining the role of these pathways in conditions such as atrial fibrillation (AF). RGS proteins are currently being assessed as potential druggable targets. Although cholinergic AF has been investigated for decades, exact mechanisms are still controversial (Schotten et al., 2011). AF is highly complex involving multiple mechanisms for its initiation and sustaining the arrhythmia. The classical concept focused on the M2 receptor and its signaling cascade including RGS4, as these had been shown to have predominant effects on nodal function (heart rate and conduction block) as well as contractility. Recent evidence suggests that the M3 receptor may also play a role in initiation and perpetuation of AF. Mice lacking RGS2 (RGS2−/−) were found to have significantly altered electrophysiologically atrial responses and were more susceptible to electrically induced AF. Vagally induced or programmed stimulation-induced AF could be blocked by the selective M3R antagonist, darifenacin. These results suggest a potential surgical target (ICANS) and pharmacological targets (M3R, RGS2) for the management of AF.

Keywords: RGS proteins, autonomic nervous system, cholinergic, arrhythmia, atrial fibrillation, intrinsic cardiac autonomic nervous system, heart, M3 muscarinic receptor

HISTORICAL CONCEPTS OF AUTONOMICS IN THE HEART

The heart receives sympathetic and parasympathetic efferent innervation as well as the ability to process information internally via an intrinsic cardiac autonomic nervous system (ICANS). The ICANS is primarily located in five groups of atrial ganglionated plexi (GPs) which may represent areas of local control of cardiac muscle (Cardinal et al., 2009). Although close proximity of the neurons to the muscle does not mean exclusive control of the adjacent area, it is an important consideration, and is analogous to the enteric nervous system, which serves as the “local brain” in the intestinal tract (Furness et al., 2004). Subpopulations of intrinsic cardiac neurons express multiple neurotransmitters (Hassall and Burnstock, 1987); however, in Guinea pig posterior ganglia, choline acetyltransferase immunostaining of all neurons indicates major parasympathetic cholinergic input to the myocardium (Mawe et al., 1996).
ATRIAL FIBRILLATION

Atrial fibrillation, the most common clinical arrhythmia (Kannel et al., 1982; Benjamin et al., 1998), is characterized by having abnormal, irregularly irregular, rapid electrical activation. AF is a common health problem in the developed world (Savelieva and Camm, 2001). Its prevalence increases with age, from a prevalence of 0.02% of people of 18–39 years of age to 11.6% of people over 75 years of age (Nixon, 2011). As the population ages, AF will increase the burden on health care resources, both for its therapeutic management, and also due to its serious and costly complications: heart failure and stroke (Dries et al., 1998). Discussion of potential mechanisms for AF, as far back as 1870 (Efimov and Fedorov, 2005), has been separated into two basic themes; those stressing the primary role of the ICANS (referred to as neurogenic AF); Hoffa and Ludwig, 1850; Zipes et al., 1974; Coumel et al., 1978; Liu and Nattel, 1997; Chang et al., 2001; Nattel, 2004), or sometimes called “vagal AF” (Garrey, 1924) and those related exclusively to the role of the atrial muscle itself (referred to as myogenic AF). Both neurogenic (Hoffa and Ludwig, 1850; Zipes et al., 1974; Coumel et al., 1978; Liu and Nattel, 1997; Chang et al., 2001; Nattel, 2004) and myogenic (Vulpain, 1874; MacWilliam, 1887; Garrey, 1914; Moe and Abildskov, 1959; Moe, 1962; Moe et al., 1964; Allessie et al., 1985; Zipes, 1997; Jalife et al., 1998; Skanes et al., 1998; Mandapati et al., 2000) hypotheses have been proposed to account for arrhythmia mechanisms. The separation of AF mechanisms into strictly neurogenic and myogenic types is artificial as they certainly interact and share multiple mechanisms.

Since the studies of Moe and colleagues in the 1950s, AF was shown to be composed of multiple re-entrant wavelets (Nattel, 2002). However, optical mapping studies of AF in isolated sheep atria have found that AF may involve both a high frequency “mother rotor,” and fibrillatory conduction of multiple wavelets spawned from the deterministic driver (Kleber, 2000; Jalife, 2003). Remodeling of the atria may also promote the propagation of multiple wavelets as “AF begets AF,” indicating that atrial remodeling promotes sustained disorganized activity (Morillo et al., 1995; Shen et al., 2011). AF relies on the interaction of triggers, perpetuators, and a vulnerable substrate for initiation and maintenance of the arrhythmia (Allessie et al., 2001). Triggers combined with dynamic substrates of structural heterogeneities, reduced refractoriness, enhanced spatial dispersion of refractoriness and abnormal impulse conduction initiate and perpetuate the arrhythmia (Nattel et al., 2001). Focal activity originating from the pulmonary veins has been suggested to account for more than 90% of AF triggers (Haissaguerre et al., 2000).

Abnormal impulse initiation and conduction contribute to the mechanism of AT (Figure 1), but are also involved in the initiation and maintenance of AF. The rapid rates during AF (Nattel, 2002) promote remodeling of the atria that potentiates AF duration (AF begets AF). There are, however, a number of factors that cause remodeling and “prime” the initial state, promoting rapid triggers (e.g., pulmonary vein ectopy) and susceptibility to fibrillatory conduction necessary for AF. These “clinical risk factors” (Rosiak et al., 2010) for the onset and development of AF are listed in Figure 2, for more detail see Dobrev and Nattel (2011). Two factors not mentioned in the Rosiak article are mechanical stretch and hyperthyroidism (Klein and Ojamaa, 2001; Souza et al., 2012). Although usually associated with other contributing factors such as hypertension or valve disease, stretch and stretch-induced channel conduction can lead to electrical instability. Indeed, interactions between mechanical stretch and cholinergic stimulation has been shown (Yamazaki et al., 2009). Although in embryonic and not the adult atria, there is evidence of interaction between M3 receptors and TREK-2, a stretch activated K+ channel (Kang et al., 2006). There are also interactions between mechanical stretch and gap junctions (Wang et al., 2000). These interactions can be very complex. For instance, Cx40−/− mice had a lower pacing induced breakdown frequency of 1–1 right atrial impulse conduction than wild type mice but did not present with AF (Bagwe et al., 2005). This indicates that the factors that promote fibrillatory conduction may not necessarily contribute to the mechanism driving AF and may even oppose it. On the other hand, slowed conduction in patients with somatic connexin 40 (Gollob et al., 2006) or connexin 43 mutations (Thibodeau et al., 2010) or mice with mutant connexin 43 (Tuomi et al., 2011) were associated with enhanced arrhythmia susceptibility.

Atrial fibrillation causes remodeling of the atria that promotes long lasting or even permanent AF (Thijssen et al., 2000). AF

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\text{FIGURE 1 | The leading circle, “wave length,” of re-entry in atrial fibrillation (AF). The size of a functional re-entry circuit (A), right, depends on the wavelength (WL) which is a product of conduction velocity (CV) and refractory period (RP). } \quad \text{WL} = CV \times RP.
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\[\text{short wavelengths allow multiple circuits (B, left), favoring AF. The wave rotating around a fixed point, rotor (C), lower, Jalife, 2003. Factors such which slow conduction enhance the potential for multiple smaller waves. Enhanced cholinergics decrease conduction velocity and/or reduce effective refractoriness. Modified connexins also slow conduction. Either, or a combination of both could promote the initiation and sustaining of AF.}\]
can be classified based on its duration and therapeutic approaches (Nixon, 2011; Yan and Kowey, 2011). Patterns of AF following the ACC/AHA/ESC guidelines include: (1) Paroxysmal (self-terminating), episodes that generally last less than or equal to 7 days (most less than 24 h); (2) Persistent (not self-terminating), usually more than 7 days, and (3) Permanent, cardioversion failed or not attempted. Either paroxysmal or persistent AF may be recurrent (Fuster et al., 2001).

Persistent AF is associated with diffuse macroscopic and histological atrial myocardial changes, i.e., remodeling. Atrial arrhythmia can also cause ventricular remodeling and, if protracted, cardiac failure. This in turn, can feed back on the atria and produce irreversible interstitial atrial fibrosis (Kalifa et al., 2003).

Lone or paroxysmal AF has been shown to be primarily initiated from the pulmonary veins and posterior left atrial regions (Morillo et al., 1995; Haisaguerre et al., 1998), where most of the GP are in contact with the left atrium. Both alterations in vagal responses and a significant increase in AF control (Pappone et al., 2000, 2004; Nademanee et al., 2004; Platt et al., 2004; Nakagawa et al., 2005a,b) were found in patients when the GPs were targeted (Sakamoto et al., 2010) or inadvertently modified during catheter ablation of AF sources in the pulmonary veins. Also, selective ablation of the GPs in pigs abolished the ability to acutely induce AF (Jones et al., 2008b) and reduced the acute inducibility in dogs (Nishida et al., 2011), which persisted in some animals up to 4 weeks following ablation in dogs (Nishida et al., 2011). Patterns of AF were not assessed (Machida et al., 2011). M3 muscarinic receptors were first identified in human atria in Hellgren et al. (2000). Early quantification of M1–M5 muscarinic subtype mRNAs in rat atria utilizing competitive RT-PCR indicated that M2 mRNA represented more than 90% of the total muscarinic mRNA in the atria, while M3 was less than 3% (Krejci and Tucek, 2002). More recently, quantitative PCR showed low level expression of M1, M4, and M5 receptors in the atria, but this may have been due to low level DNA contamination as RNA in most studies was not treated with DNase (Kitazawa et al., 2009). The functional consequence of co-expression of multiple muscarinic receptor types has been the focus of many studies discussed below, the most compelling of which use M2 and M3 receptor knockout mice.

AFTER 1 WEEK OF RAPID PACING, YEH et al. (2007) FOUND APPROXIMATELY A 50% DECREASE IN LEVELS OF mRNA ENCODING M2, M3, AND M4 RECEPTOR SUBTYPES IN BOTH THE LEFT ATRIAL APPENDIX AND LEFT PULMONARY VEIN REGION. IN CONTRAST, VOIGT ET AL. (2010) FOUND THERE WERE DIFFERENTIAL ALTERATIONS IN SAMPLES FROM ATRIAL APPENDICES OF PATIENTS WITH CHRONIC OR PAROXYSMAL AF. IN PAROXYSMAL AF, BASAL CURRENT WAS ~2-FOLD LARGER IN THE LEFT VERSUS RIGHT ATRIAL APPENDIX, INDICATING A LEFT-TO-RIGHT ATRIAL GRADIENT. IN BOTH ATRIA, Kir2.1 (I_{K1}) EXPRESSION WAS ~2-FOLD GREATER IN CHRONIC AF BUT COMPARABLE IN PAROXYSMAL AF VERSUS PATIENTS IN SINUS RHYTHM. Kir2.3 levels (I_{K,ACH}) WERE UNCHANGED IN CHRONIC AF AND THE RIGHT ATRIAL APPENDIX IN PAROXYSMAL AF BUT WERE 51% LOWER IN THE LEFT ATRIAL APPENDIX IN PAROXYSMAL AF. IN SINUS RHYTHM CARBAChOL-ACTIVATED I_{K,ACH} WAS 70% GREATER IN RA VERSUS LA. THIS RIGHT-TO-LEFT ATRIAL GRADIENT WAS DECREASED IN PAROXYSMAL AND CHRONIC AF CAUSED BY A SELECTIVE REDUCTION IN I_{K,ACH} IN RIGHT ATRIUM. SIMILARLY, IN SINUS RHYTHM, Kir3.1 AND Kir3.4 PROTEINS WERE GREATER IN RIGHT VERSUS LEFT ATRIUM AND DECREASED IN THE RIGHT ATRIUM OF PAROXYSMAL AND CHRONIC AF. Kir3.1 AND Kir3.4 EXPRESSION WAS UNCHANGED IN THE LEFT ATRIUM OF PAROXYSMAL AND CHRONIC AF (VOIGT ET AL., 2010).
In the heart, M2 receptors inhibit adenylyl cyclase (AC) activity via Goi and induce activation of the G protein coupled inward rectifier K⁺ channel (GIRK₁,₂,₃,₄ or I_K,ACb; Kovoor et al., 2001) by Gβγ released from Goi. Inhibition of adenylyl cyclase production of cAMP reduces the activity of I₁ (a CAMP dependent current) and the L-type Ca²⁺ current (ICa,L) through Goa. I_Ca,L is also regulated by Goi and nitric oxide (Burger et al., 2009a,b). Adenylyl cyclase 5 deficient mice have reduced I_Ca,L activity, while Goi2 knockout mice lack muscarinic regulation of I_Ca,L (Chen et al., 2001). M2 receptor inhibition of Goi decreases cAMP production, which reduces protein kinase A (PKA) activation (Kovoor et al., 2001). PKA, through phosphorylation, increases the activity of I_Ca,L and the rapid delayed rectifier potassium channel (IKr), which promotes repolarization from the plateau phase of cardiac action potential (Tamargo et al., 2004). The inhibition of IKr prolongs AP duration (Clark et al., 2002).

In the atria, activation of I_K,ACb increases membrane K⁺ permeability, facilitating an outward current that hyperpolarizes the cell. There is a gradient of I_K,ACb current in the mouse atria that, when combined with the heterogeneous distribution of parasympathetic ganglia, may augment the dispersion of atrial refractoriness (Lomax et al., 2003). In mice (Nguyen et al., 2004), optically mapped action potential duration was shorter in the left than the right atrium (Nygren et al., 2004) and the cholinergic agonist, carbachol, reduced durations in all areas except the left atrial appendage. GIRK4 knock out (GIRK4−/−) mice which lack functional I_K,ACb channels have abnormal heart rate regulation and are resistant to carbachol-induced AF (Kovoor et al., 2001). In the SA node, I_K,ACb hyperpolarizes the membrane potential, and decreases the pacemaker pre-potential slope, which contributes ~50% to the in vivo bradicardic response. In summary, the functions of the M2 receptor include activation of I_K,ACb (hyperpolarization), inhibition of I_Kr (prolonging repolarization), and inhibition of I_Ca,L (negative chronotropy; Hashimoto et al., 2006).

On the other hand, M3 muscarinic receptors couple to Goq to activate PLC, PKC, and I_K,M3 (Shi et al., 2004). To date, I_K,M3 is the only identified K⁺ channel activated by Goq. I_K,M3 current is highly selective for K⁺ and has delayed rectifier properties with a relatively slow activation time constant (~150 ms at +50 mV), which is followed by a partial and slower decay (Wang et al., 2004). The tail current is characterized by an initial rapid rising phase followed by a slow decay with a mean time constant of ~170 ms (Shi et al., 2003). The waveform is similar to that of I_Kr; however, it is insensitive to the I_Kr blockers dofetilide and E-4031, and to the slow delayed rectifier K⁺ current (IK) inhibitor, chromanol 293B. I_K,M3 is blocked by the non-selective muscarinic antagonist, atropine (Wang et al., 2004), and the M3 selective antagonist, darifenacin (Wang et al., 2004, 2007a). M3 content is also altered in patients with chronic AF associated with mitral stenosis (Zhao et al., 2008) and in experimental chronic tachycardia in dogs (Yeh et al., 2007).

Of note, M3 receptor distribution in cardiac tissue was found to be mostly confined to the intercalated disk region (Wang et al., 2004), and thus was also suggested to regulate ventricular gap junction-mediated cell-to-cell conduction (Wang et al., 2007a). Physical and functional interactions between M3 and connexin 43 (Cx43) have been found in rat ventricular myocytes, which were impaired during myocardial ischemia (Yue et al., 2006). Gap junction remodeling is an essential component promoting atrial as well as lethal ventricular tachyarrhythmias (Dupont et al., 2001a,b; Gutstein et al., 2001; Danik et al., 2004; Shiroshita-Takeshita et al., 2007). The functions of the cardiac M3 receptors suggested so far include: (1) depressed inotropy; (2) protection against myocardial ischemia; (3) regulation of cell-to-cell communication, and (4) participation in the generation and maintenance of AF (Wang et al., 2004). Using the M3 receptor antagonist 4-DAMP Wang et al. (2004), concluded that the M3 receptor also mediates, in part, bradycardia elicited by vagal stimulation. However, the pKB of 4-DAMP for M3 is 8.9–9.3, only one order of magnitude greater the pKB for M2 receptors (7.8–8.4). Perhaps the weak inhibition of vagal bradycardia with high dose 4-DAMP used in Wang’s study was due to blockade of M2 rather than M3 receptors. On the other hand, vagally induced bradycardia was completely abolished in M2 muscarinic receptor (M2−/−) deficient mice but was not affected in M3 muscarinic receptor (M3−/−) deficient mice (Fishier et al., 2004), which does not support a role for M3 in bradycardia in the mouse. However, this does not exclude a role for M3-mediated modulation of atrial or AVN function, as analysis of other atrial or AVN functions was not done.

The functional consequence of co-expression of both M2 and M3 receptors in the atria was studied in M2−/−, M3−/−, and M2/M3 double knockout (M2−/−/M3−/−) mice (Kitazawa et al., 2009). In wild type mouse atria, carbachol-induced a negative inotropic response followed by a sustained positive inotropic response. M3−/− mice were devoid of the positive inotropic phase while M2−/− mice had only a positive inotropic response. M2−/−/M3−/− double knockout mice were devoid of any inotropic response (Kitazawa et al., 2009). The susceptibility to arrhythmia was not studied in these mice. However, these results strongly indicate the functional nature of M3 muscarinic receptors present in the atria.

G PROTEIN COUPLED RECEPTORS IN THE HEART

G protein coupled receptors (GPCRs) are seven transmembrane domain cell surface receptors that bind extracellular ligands (hormones, neurotransmitters, and drugs) and transduce signals into the intracellular environment. Most sympathetic and parasympathetic receptors are members of the GPCR superfamily which couple to various downstream signaling cascades including those controlling adenylyl cyclase, Ca²⁺ homeostasis, and ion channels (Wieland et al., 2007). In the heart, GPCRs control the chronotropic (rate of contraction), inotropic (strength of contraction), lusitropic (rate of relaxation), and dromotropic (velocity of conduction) responses to acetylcholine and adrenaline (Wieland et al., 2007). Ligand binding stabilizes or induces a conformational change in its GPCR that activates a coupled intracellular heterotrimERIC G protein (Gαβγ). There are four families of mammalian heterotrimERIC G proteins including G₁α, G₁βα, G₁γα, and G₁2/13α with various subtypes, effectors, and expression profiles (Milligan and Kostenis, 2006).

RGS2 AND RGS4 IN THE HEART

Regulators of G Protein Signaling proteins were first identified as GTPase activating proteins (GAPs), accelerating the intrinsic
GTPase activity of Gα subunits, as the duration of G protein activation is primarily controlled by GTP hydrolysis. Also, RGS proteins can interfere with G protein binding to effector proteins, separate from their regulation of hydrolysis (Abramow-Newerly et al., 2006). Thus RGS proteins are important limiters of GPCR signaling. Currently, 20 different genes encoding RGS proteins have been identified and divided into four subfamilies, based on their sequence and structural similarities: R2A (RGS17, 19, and 20); R4/B (RGS1–5, 8, 13, 16, and 21); R7/C (RGS6, 7, 9, and 11), and R12/D (RGS10, 12, and 14; Bansal et al., 2007). The majority of RGS proteins are GAPs for Gαi/o and many also act on Gq/11\(^{\text{ii}}\); however, none are GAPs for Gs but some RGS-like proteins such as p115RhoGEF do have GAP activity on the Go11/12 subfamily. RGS2, 3, 4, 6, 10, RGSZ2, and GAIP are all found in atrial cardiomyocytes (Doupnik et al., 2001).

The ongoing control of cardiac function by GPCRs is itself modulated by RGS proteins. RGS-insensitive Gαq/G184S homozygous knock-in (Gα2GS/GS) embryonic stem cell-derived cardiomyocytes had enhanced responses to M2 muscarinic but not A1 adenosine receptor stimulation, while analogous Gαq/GS cardiac myocytes had enhanced A1 adenosine and M2 receptor mediated responses (Fu et al., 2006). Gαq stimulation of the intrinsic beating rate was almost completely abolished in Gα2GS/GS cells, likely due to a failure of AC activation resulting from an increase in countervailing inhibitory activity. Gα2GS/GS insensitive mutant mice also had pronounced (five-fold greater) muscarinic mediated bradycardia and third degree AV nodal block (Fu et al., 2007). These mutant mouse data indicate that in the SA and AV node, M2 receptors primarily couple through Gαi2 to inhibit cAMP production and activation of PKA (Fu et al., 2006, 2007). Adenosine 1 and 3 receptor overexpressing mice have also have prolonged AV nodal conduction (Kirchhof et al., 2003; Fabritz et al., 2004), which may involve receptor mediated inhibition of adenyl cyclase 5/6 (AC5/6), primarily coupling through Gαo (Fu et al., 2006, 2007). Susceptibility to atrial arrhythmia has not been examined in these mice. It is currently believed that specific RGS proteins may couple to specific signaling pathways within the cell.

RGS2, a member of the R4/B subfamily, the second most highly expressed RGS protein in the heart (Doupnik et al., 2001), is a selective GAP for Gαq (Zou et al., 2006). This selectivity does not reflect any exceptional affinity for the latter but rather a relatively weak RGS2 affinity for Gαi/o (Ingi et al., 1998; Cladman and Chidiac, 2002) which is due to the substitution of three conserved amino acid residues in its RGS domain that decrease binding affinity to Gαi/o but not Gαq (Heximer et al., 1999). RGS2 also produces a poorly understood inhibitory effect on Gq-activated AC signaling (Sinnarajah et al., 2001; Roy et al., 2006b). The mechanism by which such inhibition occurs does not appear to involve a GAP effect, as RGS2 fails to alter the GTPase activity of either free (Ingi et al., 1998) or receptor-activated Gq (Roy et al., 2003). Some evidence suggests that the inhibitory effect may involve a physical interaction of the N-terminus of RGS2 with the C1 catalytic loop of AC5 (Salim et al., 2003; Beazely and Watts, 2006); however other studies suggest that RGS2 may bind directly to Gq (Tseng and Zhang, 1998; Ko et al., 2001). In HEK293 cells, GFP-RGS2 tended to localize to the nucleus but was recruited to the plasma membrane by the co-expression of either Gq or most adenylyl cyclase isoforms, and BREI (bioluminescence resonance energy transfer) interactions between RGS2 and Gαq are not inhibited by adenylyl cyclase (Roy et al., 2006a). RGS2 appears to be important in the desensitization of Gq- and Gs-mediated GPCR receptor signaling as it is upregulated by both and moreover it can mediate cross-desensitization between Gq and Gs (Sinnarajah et al., 2001; Roy et al., 2006b).

Removal of RGS2’s inhibitory effect on Gq signaling increases Gq-dependent signaling activity in cardiomyocytes (Zhang et al., 2006). Interestingly, RGS2 has also been shown to be an important target in cardiovascular disease (cardiac hypertrophy and hypertension; Tsang et al., 2010). RGS2\(^{-/-}\) mice have a mild to moderate hypertensive phenotype (Heximer et al., 2003; Gross et al., 2005; Tank et al., 2007) that is suggested to be mediated by vascular changes, possibly through angiotensin II, endothelin and/or α-adrenergic receptors, rather than being due to cardiac alterations, although cardiac “contractility,” measured by left ventricular dP/dt, was not determined (Oliveira-dos-Santos et al., 2000; Heximer et al., 2003). Altered autonomic function and effects mediated via the kidneys may also contribute to elevated blood pressure in these animals (Gu et al., 2009). An increase in mean arterial pressure of ~10 mm Hg was found with Telemetry, while heart rate was unchanged, indicating a resetting of the baroreceptor reflex (Gross et al., 2005). This was confirmed by directly measuring baroreflex sensitivity calculated by cross-spectral analysis of heart rate variability. However, heart rate variability was not different in RGS2\(^{-/-}\) compared to RGS2\(^{+/+}\) mice (Gross et al., 2005), indicating that the cardiac phenotype was not explained by a baroreflex mediated enhancement of vagal nerve activity. Since RGS2 is a selective regulator of Gq signaling that can inhibit signaling via M3 receptors in other cell types (Tovey and Willars, 2004; Karakoula et al., 2008), it follows that RGS2 may be an important regulator of M3 muscarinic signaling in the atria. Notably, the scaffolding protein spinophilin, which is present in cardiomyocytes (Bers, 2004), binds to both RGS2 and M3 muscarinic receptors, and thus it may facilitate the inhibition of M3 muscarinic receptor-activated Gq signals, as has been observed with analogous α1-adrenergic signals (Wang et al., 2007b).

RGS4 regulates SA nodal function (Cifelli et al., 2008) via inhibition of parasympathetic signaling and \(I_{\text{K,ACb}}\) activity. In mice expressing LacZ under the control of the RGS4 promoter, high expression of LacZ-RGS4 was found in the SAN will little expression was observed in the surrounding right atrium. RGS4-null (RGS4\(^{-/-}\) mice had lower baseline heart rates and greater increases in heart rate following atropine blockade. High expression of RGS4 (demonstrated by LacZ staining), was also seen in the AV node and RGS4\(^{-/-}\) mice were highly susceptible to carbachol-induced AV nodal conduction block when treated with isoproterenol. However, there is limited electrophysiological data describing the location of conduction block, and results were obtained principally in Langendorff hearts (removed from in vivo autonomic influences).

As RGS4 was found only in the SA and AV nodal region, the RGS protein regulating parasympathetic signaling and \(I_{\text{K,ACb}}\) activity in the atrium remained, until recently, unknown. RGS6-deficient (RGS6\(^{-/-}\)) atrial myocytes exhibited a significant reduction in
the time course of $I_{K,ACH}$ activation and deactivation. RGS6$^{-/-}$ mice also displayed bradycardia and AV nodal conduction block indicating that there is at least partial redundancy in RGS protein function in vivo.

**RGS2 and Atrial Arrhythmia in the Mouse**

In both RGS2$^{-/-}$ and C57Bl/6 wild type (WT, control) anesthetized mice (Tuomi et al., 2010) with body temperature maintained within the normal physiological range (36.5–38°C; Connolly and Lynch, 1981) and His bundle recording (Figure 3) used to validate correct positioning of the electrode (Figure 4), atrial effective refractory periods (AERPs) were heterogeneous, being longer in the high right compared to the mid right atrium. In addition, AERPs were significantly lower in RGS2$^{-/-}$ mice compared to WT mice. M2 receptor and M3 receptor mRNA was not different in RGS2$^{-/-}$ compared to WT mice. Carbachol reduced AERPs in both strains but strain differences persisted. In contrast, the AERPs were increased to similar levels in both strains with atropine. The selective M3 blocker, darifenacin, increased the AERP for the mid atrium of RGS2$^{-/-}$ and WT mice, and eliminated the strain-related differences (Tuomi et al., 2010), supporting a role of RGS2 in atrial function. Of interest, in paroxysmal AF there is a frequency gradient but in chronic AF, the frequency gradient in the left atrium has been shown to be absent (Lazar et al., 2006).

Since vagally induced bradycardia is mediated via M2 but not M3 receptors (Fisher et al., 2004), we compared the effects of cholinergic blockade using darifenacin and atropine on heart rate changes following vagus nerve stimulation. Stimulating the vagus decreased heart rate by 38 ± 6.8% in WT animals. This decrease was abolished by atropine but was unaltered in the presence of darifenacin (Tuomi et al., 2010). This provided evidence that this dose of darifenacin did not affect M2 signaling. Of note, this dose of darifenacin also terminated electrically induced fibrillation in connexin 43 mutant mice (Tuomi et al., 2011).

Both programmed electrical stimulation (Jones et al., 2001) and burst pacing (Jones et al., 2008a) were used to determine susceptibility to atrial arrhythmia induction. A single atrial stimulus induced AF (Figure 5). Overall, RGS2$^{-/-}$ mice were more susceptible to electrically induced arrhythmia (Table 1; Tuomi et al., 2010).

Atrial fibrillation is very rarely induced by single extrastimuli in large animal and human studies and was thus anticipated to be even less provokable in mice, due to their small size. Thus sensitivity to single extrastimuli in the mouse implies a highly vulnerable myocardial substrate. The duration of induced arrhythmia is also important with “sustained” AF being defined in large animal and human electrophysiological studies as lasting >30 s. Scaling of electrophysiological variables has been related to the $BM^{0.25}$ (Noujaim et al., 2004). Using this relationship, the duration equivalent is in the second range. However as there is no gold standard: indeed not even a convention, we concluded it best to use ranges. Hence, the data were analyzed based on susceptibility to pacing modality and grouped by the duration of the induced arrhythmia, lasting: <10 s; between 10 and 30 s, and >30 s. As expected, WT mice were virtually non-inducible with a single
FIGURE 5 | Programmed electrical stimulation-induced atrial fibrillation (AF) in a 1-month-old RGS2−/− mouse (A). Note the conduction block into the high right atrial (HRA) region denoted by the far-field (low amplitude) potential recordings during block. The duration of the arrhythmia was >200 s with an average atrial rate of ∼1600 bpm. A similar stimulation protocol failed to induce atrial fibrillation in wild type mice (B). LL1, limb lead 1. RV, right ventricular electrogram. HBE, electrogram from the His bundle region. HRA, recording from the high right atrium.

stimulus (4%, 1/25), compared to burst pacing (40%, 10/25). Carbachol increased the susceptibility to both single extrastimuli (33%, 4/12) and burst pacing (58%, 7/12). Carbachol also prolonged the fibrillation duration with only 2 of 12 untreated mice being sustained, while 5 of the 12 were sustained after carbachol. The atrial stimulation site also affected arrhythmia...
Table 1 | Incidence of AT/F induced by PES or burst pacing and maximum duration of induced AT/F with either PES or burst pacing induction.

| INDUCIBILITY          | Wild type | RGS2<sup>−/−</sup> |
|-----------------------|-----------|---------------------|
| PES                   | 4% (1/25) | 50% (11/22)         |
| Burst (50Hz, 400 ms pulse) | 40% (10/25) | 64% (14/22)         |

| DURATION               | Wild type | RGS2<sup>−/−</sup> |
|------------------------|-----------|---------------------|
| No response            | 60% (15/25) | 36% (8/22)          |
| <10 s                  | 20% (5/25)  | 23% (5/22)          |
| 10–30 s                | 16% (4/25)  | 18% (4/22)          |
| >30 s                  | 4% (1/25)   | 23% (5/22)          |

X<sup>2</sup> analysis of discrete values revealed significant differences in atrial tachycardia/fibrillation (AT/F) susceptibility with programmed electrical stimulation (PES; P < 0.05).

susceptibility. Burst pacing more readily induced arrhythmia from the mid right atrium of WT mice (40%, 10/25) compared to those from the high right atrial region (8%, 2/25, P < 0.05; Tuomi et al., 2010). This likely reflects the regional AERPs being significantly shorter in the mid-compared to the high right atrium. Electrical heterogeneity may be an important factor in providing a permissive substrate for wave break and re-entry initiation in the atrium.

Rapid focal activity may initiate rotors due to the interaction of a high frequency propagating wave fronts with the refractory tail of the previous wave (Jafari and Pandit, 2005; Vaquero et al., 2008). Immediately after arrhythmia induction, local cycle lengths were often identical in the high right and His bundle regions. However, sometimes at onset, there was regional conduction block into the high right atrium (Figure 5), which could be accompanied by heterogeneity in local cycle lengths in RGS2<sup>−/−</sup> mice, with His bundle electrograms being shorter than those of the high right atrium. Over time, these intervals converged (Tuomi et al., 2010). This pattern is consistent with the concept of a drifting rotor that rapidly became anchored, although it may also indicate a tachycardia with a rapid rate at onset that slowed down as the driving mechanism stabilized. However, rapid onset may combine with the regional refractory heterogeneity to produce functional conduction block/slowing that eventually recovers at slower rates. Of note, the cycle length during arrhythmia was faster than the measured intrinsic AERP in the high right atrial region, suggesting the possibility of electronic interactions from a rotor core causing reduced refractoriness (Vaquero et al., 2008).

In summary, in addition to the previous focus on the role of M2 receptors in atrial arrhythmia, there is evidence that arrhythmia inducibility may be due to parasympathetic M3 receptors and their regulation by RGS2 knockout. RGS2 accelerates the intrinsic GTPase activity of Gaq, thus limiting the lifetime of the activated state, thereby regulating the Gaq coupled M3 receptor and its associated current, I<sub>K,M3</sub>. These data are the first we know that demonstrate a role for RGS proteins in atrial arrhythmia. The RGS2<sup>−/−</sup> mice were more susceptible to electrically induced AF, and there was a greater percentage of RGS2<sup>−/−</sup> mice with sustained AF. This strain dependent difference was maintained in the presence of carbachol, while atropine abolished the strain dependent differences. These findings suggest an alteration in muscarinic receptor-gated K<sup>+</sup> flux evidently due to an increase in M3 muscarinic receptor response per se.

CONCLUSION

With the aging of the population, there is an increasing drive to identify novel treatments for AF. This will require increased understanding of the signaling pathways and molecular regulators involved in arrhythmia induction, perpetuation, and atrial remodeling. Current medical management of cardiac arrhythmias depends primarily on ion channel blockade, most of which (~70%) are controlled by GPCRs; however, these agents may be proarrhythmic (The Cardiac Arrhythmia Suppression Trial (CAST) Investigators, 1989) and reduce the quality of life of the patients. Thus developing selective agents for cholinergic signaling may provide an effective treatment for AF initiation and perpetuation. Mutations in, or altered expression/function of a variety of GGS proteins could be involved in AF mechanisms in patients, and indeed, changes in RGS2 have been identified in several human cardiovascular phenotypes. Thus, targeting GGS proteins may be important for drug development (Roman et al., 2009). Selective M3R blockade, alone, or in combination with other anti-arrhythmic agents, may be useful for patients with AF. However, in mice, oral darifenacin exerted only transient binding to cardiac muscarinic receptors (Yamada et al., 2006), thus its use may be limited.

LIMITATIONS

While these results indicate a role for RGS2 and the M3 muscarinic receptor in promoting AF in the mouse, additional studies will need to directly determine the role of I<sub>K,M3</sub> activity. Also, it would be beneficial to examine the effects of novel I<sub>K,ACh</sub> antagonists NTC-801 (Machida et al., 2011) and fesoterodine, antagonist, acetacin (Li et al., 2008). Atrial vulnerability may also be due to a balance of autonomics (Scherlag et al., 2006; Jones et al., 2007). RGS2<sup>−/−</sup> mice have also been shown to have reduced renal sympathetic nerve activity compared to WT mice (Tank et al., 2007). Although, the lack of a heart rate difference between RGS2<sup>−/−</sup> and WT mice found in their earlier study (Gross et al., 2005) does not assist in establishing the role of the sympathetic nervous system in atrial susceptibility, the role of the balance between sympathetics and M3 responses remains to be determined. It is also recognized that it may not be possible to directly extrapolate from mouse experiments to the human.

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