Arrhythmia/Electrophysiology

Defective Cardiac Ryanodine Receptor Regulation During Atrial Fibrillation

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Background—Ca2+ leak from the sarcoplasmic reticulum (SR) may play an important role in triggering and/or maintaining atrial arrhythmias, including atrial fibrillation (AF). Protein kinase A (PKA) hyperphosphorylation of the cardiac ryanodine receptor (RyR2) resulting in dissociation of the channel-stabilizing subunit calstabin2 (FK506-binding protein or FKBP12.6) causes SR Ca2+ leak in failing hearts and can trigger fatal ventricular arrhythmias. Little is known about the role of RyR2 dysfunction in AF, however.

Methods and Results—Left and right atrial tissue was obtained from dogs with AF induced by rapid right atrial pacing (n=6 for left atrial, n=4 for right atrial) and sham instrumented controls (n=6 for left atrial, n=4 for right atrial). Right atrial tissue was also collected from humans with AF (n=10) and sinus rhythm (n=10) and normal cardiac function. PKA phosphorylation of immunoprecipitated RyR2 was determined by back-phosphorylation and by immunoblotting with a phosphospecific antibody. The amount of calstabin2 bound to RyR2 was determined by coimmunoprecipitation. RyR2 channel currents were measured in planar lipid bilayers. Atrial tissue from both the AF dogs and humans with chronic AF showed a significant increase in PKA phosphorylation of RyR2, with a corresponding decrease in calstabin2 binding to the channel. Channels isolated from dogs with AF exhibited increased open probability under conditions simulating diastole compared with channels from control hearts, suggesting that these AF channels could predispose to a diastolic SR Ca2+ leak.

Conclusions—SR Ca2+ leak due to RyR2 PKA hyperphosphorylation may play a role in initiation and/or maintenance of AF. (Circulation. 2005;111:2025-2032.)

Key Words: arrhythmia ■ atrium ■ fibrillation ■ calcium ■ ion channels

Atrial fibrillation (AF), the most common cardiac arrhythmia in humans, represents a major cause of morbidity and mortality.1 Despite the clinical importance of AF, the molecular mechanisms underlying this arrhythmia are poorly understood, and treatment options are limited.

It is well established that structural and electric remodeling, including shortening of atrial refractoriness, loss of rate-related adaptation of refractoriness,2–5 and shortening of the wavelength of reentrant wavelets, accompanies sustained tachycardia.6 This remodeling is likely important in the development, maintenance, and progression of AF. Previous studies suggest that Ca2+ handling may play a role in electric remodeling in AF.7–12

The cardiac Ca2+ release channel/ryanodine receptor (RyR2) is the major Ca2+ release channel required for excitation-contraction coupling in the heart. Protein kinase A (PKA) associates with RyR2 through its targeting protein mAKAP as part of a macromolecular signaling complex.13 PKA phosphorylation of RyR2 results in dissociation of the channel-stabilizing subunit calstabin2 (FK506-binding protein or FKBP12.6),13 which results in increased RyR2 open probability (Po) secondary to increased sensitivity to Ca2+ dependent activation.13 In normal hearts, PKA phosphorylation of RyR2 occurs as part of the “fight-or-flight” response, providing a mechanism for increasing excitation-contraction coupling gain in response to stress.13,14 In heart failure, chronic activation of the sympathetic nervous system leads to maladaptive PKA hyperphosphorylation of RyR2. As a result, channels become “leaky,” leading to depletion of sarcoplasmic reticulum (SR) Ca2+ stores required for excitation-contraction coupling and aberrant release of SR Ca2+ during diastole.13 The latter has been implicated as a trigger for fatal...
ventricular arrhythmias in humans with exercise-induced sudden cardiac death.\textsuperscript{15} 
Little is known about the relationship between structural and functional changes in the RyR2 macromolecular complex and atrial arrhythmias. We used a canine model of rapid atrial pacing–induced AF, as well as atrial tissues from patients with AF, to investigate the potential role of dysfunctional regulation of RyR2 in the pathogenesis of AF. We also investigated a potential mechanism of action of JTV519, a 1,4-benzothiazepine derivative recently shown to inhibit the ability to induce AF in a canine model,\textsuperscript{16} involving restoration of physiological RyR2 function.

**Methods**

**Canine Model of AF**
All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Columbia University. Pacemakers were implanted in female adult mongrel dogs weighing 24 to 26 kg with the use of previously described techniques.\textsuperscript{17} Animals were anesthetized with thiopental sodium (17 mg/kg IV) and ventilated with isoflurane (1.5% to 2%) and O\textsubscript{2} (2 L/min). Active fixation leads were implanted in the right atrial (RA) appendage and right ventricular free wall and connected to an Itrel pulse generator and Thera 8962 pacemaker, respectively (Medtronic). Then 40% formaldehyde (0.1 to 0.3 mL) was injected into the pacemaker and left atrial appendage tissues were flash-frozen and stored at \(-80^\circ\text{C}.\)

**Human Atrial Samples**
All human studies were performed according to protocols approved by the Institutional Review Board of the New York Presbyterian Hospital and by the ethics committee of Dresden University of Technology. RA appendage tissue was obtained at the time of cardiac surgery as described previously\textsuperscript{18} from patients with chronic AF (>6 months; n=10), and patients in sinus rhythm (n=10).

**Immunoblotting**
Cardiac membranes were prepared, RyR2 was immunoprecipitated, and PKA phosphorylation was determined as described previously.\textsuperscript{13}

**Calstabin2 Rebinding Assay**
The 1,4-benzothiazepine derivative JTV519 was synthesized as described.\textsuperscript{19} RyR2 was immunoprecipitated from atrial SR (100 g) and washed with 1 \times kinase buffer. Immunoprecipitated RyR2 was phosphorylated with PKA (5 U) and 100 nmol/L Mg\textsuperscript{2+}-ATP at room temperature, and the reaction was terminated after 8 minutes by washing with ice-cold RIPA buffer. Recombinant calstabin2 (250 nmol/L) was incubated with PKA-phosphorylated RyR2 for 30 minutes at 30°C in the presence or absence of JTV519 (1 \mu mol/L). After they were washed with RIPA buffer, proteins were size fractionated by 15% SDS-PAGE and immunobotted for calstabin2.

**Immunoblotting**
Immunoblotting were performed as described\textsuperscript{13} with the use of anti-RyR2 (5029, 1:5,000), anti-calstabin2 (1:5,000), anti–PKA catalytic subunit (PKA, 1:500), anti–PKA regulatory subunit (RII, 1:500), anti–PP1 (1:500), anti–PP2A (1:500), anti–mAKAP (1:500), and a phosphospecific anti-RyR2 antibody that specifically recognizes RyR2 phosphorylated at Ser\textsuperscript{2809} (anti–RyR2-pSer2809, 1:5,000).

**RyR2 Single-Channel Recordings**
Single-channel recordings of RyR2 from canine LA tissue were performed under voltage-clamp conditions at 0 mV with the use of symmetrical ionic conditions as previously described.\textsuperscript{21} Single-channel recordings were made in the presence of 150 nmol/L [Ca\textsuperscript{2+}] and 50 nmol/L [Ca\textsuperscript{2+}] in the cis compartment.

**Statistical Analyses**
The Fisher exact test was used for dichotomous data, and the nonparametric Mann-Whitney test was used for continuous data. Statistical analyses were performed with the use of SPSS version 10, and a value of \(P<0.05\) was considered statistically significant.

**Results**
Patient demographics and clinical information are summarized in the Table. Although the mean ages of the 2 groups were similar, there were more men in the sinus rhythm group (although this difference was not statistically significant). A greater number of sinus rhythm patients had coronary artery disease alone, and the AF group represented a greater number of patients with valvular heart disease with or without concurrent coronary artery disease. Ejection fraction was similar and within the normal range for both groups. LA diameter and left ventricular end-diastolic dimension were significantly higher in the AF group than in the sinus rhythm group. Indices of cardiac hypertrophy were similar and within the normal range for both groups. Significantly more AF patients were taking digitalis and diuretics, and significantly more sinus rhythm patients were taking nitrates; otherwise, medical regimens were similar between the 2 groups.

**Composition of the Atrial RyR2 Macromolecular Complex**
We have previously shown that in ventricular myocardium, RyR2 forms a macromolecular complex, allowing for local regulation of RyR2 function.\textsuperscript{13} As in ventricular RyR2, the atrial RyR2 macromolecular complex is composed of calstabin2, the catalytic subunit of PKA, the PKA regulatory subunit (RII), mAKAP, and the protein phosphatases PP1 and PP2A (Figure 1A).

When immunoprecipitated RyR2 was incubated with \([^{32}\text{P}]\text{ATP}\) and PKA or cAMP, RyR2 was phosphorylated in a manner completely reversible by the specific PKA inhibitor PKI\textsubscript{1,24} (PKI), providing evidence that atrial RyR2 is substrate for PKA phosphorylation and that PKA is directly associated with the channel complex (Figure 1B).

Consistent with previous findings from ventricular myocardium demonstrating that PKA phosphorylation of RyR2 reduces the binding affinity of calstabin2,\textsuperscript{13,15,22} PKA phosphorylation of atrial RyR2 specifically reduced the amount of calstabin2 in the RyR2 macromolecular complex as determined by immunoprecipitation in a manner completely inhibited by PKI (Figure 1C).
PKA phosphorylation of RyR2 from humans with and without chronic AF was determined by immunoblotting with a phosphospecific anti-RyR2 antibody (anti–RyR2-pSer2809). PKA phosphorylation of immunoprecipitated RyR2 was increased by 220% in RA tissue from humans with AF compared with patients in sinus rhythm (Figure 2C; n=10 for AF, n=10 for control; P<0.001). Calstabin2 bound to RyR2 was decreased by 57% in AF relative to controls in these patients (Figure 2C; n=10 for AF, n=10 for control; P<0.001).

**RyR2 Channel Dysfunction in AF**

To determine the functional consequences of the RyR2 PKA hyperphosphorylation observed in AF, RyR2 single-channel measurements were performed in planar lipid bilayers with the use of channels from LA tissue of control dogs and dogs with chronic AF. Although none (0 of 11) of the atrial RyR2 channels from control dogs demonstrated increased P_{o}, (P_{o}>0.01), 15 of 17 channels (88%) from AF dogs showed increased P_{o} at 150 nmol/L cytosolic Ca^{2+}, conditions that simulate diastolic Ca^{2+} concentrations in the heart when RyR2 channels should be tightly closed (Figure 3). The average P_{o} was 0.36±0.06 in AF dogs versus 0.01±0.002 in control (P<0.001), the average gating frequency (F_{o}) was 22.5±4.1 s^{-1} in AF versus 1.7±0.6 s^{-1} in controls (P<0.0002), and the average open time (T_{o}) was 8.09±1.36 ms in AF versus 5.9±1.06 ms in controls (P=NS). At 50 nmol/L cis [Ca^{2+}], channels from AF dogs showed significantly increased P_{o}, relative to controls (0.169±0.06 for AF, n=5; 9 channels versus 0.004±0.002 for control, n=4; 4 channels; P<0.02), whereas F_{o} and average T_{o} were similar between groups. These data suggest that in AF, PKA hyper-
phosphorylation increases the Ca\(^{2+}\) sensitivity of RyR2 at low cytosolic Ca\(^{2+}\) concentrations. All data are reported as mean±SEM.

**JTV519 Increases Calstabin2 Binding to RyR2 in AF**

We sought to determine whether the novel therapeutic agent JTV519 could increase the binding of calstabin2 to PKA-hyperphosphorylated RyR2 from atrial tissue. Consistent with similar experiments performed with the use of ventricular RyR2, recombinant calstabin2 (250 nmol/L) was unable to bind to PKA-phosphorylated atrial RyR2 (Figure 4A).\(^{15}\) In the presence of JTV519 (1 \(\mu\)mol/L), however, calstabin2 (250 nmol/L) was able to bind to PKA-phosphorylated atrial RyR2 channels (Figure 4A).

The functional effects of JTV519-induced calstabin2 binding to RyR2 were investigated by incorporating single channels into planar lipid bilayers. PKA phosphorylation of atrial RyR2 significantly increased channel activity (P\(_o\) 0.323±0.007; n=5) examined under conditions that mimic diastolic Ca\(^{2+}\) concentrations of 150 nmol/L cytosolic Ca\(^{2+}\) (Figure 4B). These abnormalities in channel function were not seen in the presence of PKI (P\(_o\) 0.006±0.002; n=5; P<0.01), indicating that the observed increase in channel P\(_o\) was specific to RyR2 phosphorylation by PKA. When PKA-phosphorylated channels treated with JTV519 (1 \(\mu\)mol/L) and calstabin2 (250 nmol/L) were examined, single-channel function was similar to that of nonphosphorylated RyR2 channels (P\(_o\) 0.008±0.004; n=5; Figure 4B). Taken together, these data suggest that by inducing increased binding of calstabin2 to PKA-phosphorylated RyR2, JTV519 served to significantly reduce RyR2 P\(_o\).

**Discussion**

Numerous studies have suggested that abnormal Ca\(^{2+}\) homeostasis may play a role in the electric and contractile remodeling accompanying sustained atrial tachycardia and AF. Although sarcolemmal ion channels, including the L-type Ca\(^{2+}\) channel,\(^{23}\) appear to be involved in the remodeling process accompanying atrial tachycardia and AF, they are unlikely to fully explain this phenomenon. Previous studies have demonstrated, for example, that loss of rate adaptation in AF cannot be fully explained by alterations in I\(_{Ca,L}\) and I\(_{to}\)\(^{24,25}\) and that tachycardia-induced changes in intracellular Ca\(^{2+}\) handling also contribute significantly to this important component of atrial electric remodeling.\(^{7,25,26}\)
Furthermore, atria from a canine model of pacing-induced AF demonstrated a loss of action potential duration rate adaptation and altered action potential characteristics that could be reversed by the presence of ryanodine, suggesting a role for aberrant intracellular Ca\textsuperscript{2+}/H\textsubscript{1} release.\textsuperscript{26} The specific proteins and mechanisms responsible for mishandling of intracellular Ca\textsuperscript{2+} in AF are, however, not known, and, to our knowledge, atrial RyR2 function has not been well characterized in AF tissues.

Altered Ryanodine Receptors in AF

The present study explored, for the first time, the alterations in regulation and function of the RyR2 macromolecular complex that occur in AF. We found that AF is associated with PKA hyperphosphorylation of RyR2 and partial depletion of the channel-stabilizing subunit calstabin2.\textsuperscript{16} Similar PKA hyperphosphorylation was observed both in a canine model of AF, in which there is no ventricular dysfunction, and in patients with AF and normal ventricular function. Taken together, our similar findings from 2 separate models suggest that AF is associated with PKA hyperphosphorylation of RyR2 in a manner that is specific to the arrhythmia and is not an epiphenomenon of concurrent ventricular dysfunction or other confounding factors.
In preliminary data (not shown), left ventricular assist device treatment, which is known to restore the degree of RyR2 PKA phosphorylation to normal in the ventricles of patients with heart failure,13 did not correct PKA hyperphosphorylation of atrial RyR2 in patients with AF in the setting of heart failure despite normalization of ventricular RyR2 PKA phosphorylation levels in the same patients. These findings suggest that in AF, PKA hyperphosphorylation of RyR2 may not result from β-adrenergic–mediated upregulation of PKA, as is known to be the case in the chronic hyperadrenergic state of heart failure.13 These results would be consistent with the finding of Schotten et al27 that the β-adrenergic receptor density and G protein expression levels were not altered in AF. Although the specific mechanisms by which RyR2 is PKA hyperphosphorylated in AF are not known at this time, this could result from either upregulation of PKA through non–β-adrenergic signaling pathways or changes in the activity of the protein phosphatases PP1 and PP2A, which we have shown in the present study to be associated with the RyR2 macromolecular complex in the atrium.

**Functional Effects of PKA Hyperphosphorylation of RyR2 in AF**

The functional consequence of PKA hyperphosphorylation of RyR2 observed in the present study are increased P_o conditions that simulate diastole in the heart (low cytosolic Ca^{2+}). This abnormal channel function in AF is consistent with previous studies demonstrating that the loss of calstabin2 from RyR2 in the setting of PKA hyperphosphorylation results in leaky channels predisposed to diastolic Ca^{2+} leak secondary to an increased sensitivity to Ca^{2+}-induced Ca^{2+} release.21 Increased diastolic Ca^{2+} leak may lead to decreased SR Ca^{2+} content, which may contribute to a reduced amplitude of the Ca^{2+} transient.7 Moreover, calstabin2-depleted RyR2 channels are known to open and close (gate) stochastically, as opposed to in a coordinated fashion as single Ca^{2+} release units (coupled gating),28 which may also contribute to decreased atrial contractility in AF. Additional factors that contribute to depressed intracellular Ca^{2+} release in AF may include reduced expression and function of L-type Ca^{2+} channels on the sarcolemma and decreased SR Ca^{2+} reuptake into the SR.7,27 Although it has been reported that postrest potentiation may be preserved in AF, this would not exclude the possibility that there is a mild SR Ca^{2+} leak through calstabin2-depleted RyR2 because the reuptake system is known to play an important role in the postrest potentiation phenomenon.

Furthermore, both the increased RyR2 activity under diastolic conditions and reduced coupled gating would predispose to aberrant SR Ca^{2+} leak that could provide a trigger for AF. Evidence suggests that AF is typically initiated by premature atrial extrasystoles29,30 that are thought to result from afterdepolarizations.31 Extrasystoles are particularly likely to give rise to AF in the setting of a shortened atrial effective refractory period,32 which is a hallmark of atrial electric remodeling. Aberrant diastolic Ca^{2+} release from leaky PKA-hyperphosphorylated RyR2 has previously been shown to result in delayed afterdepolarizations (DADs) capable of triggering ventricular arrhythmias.15 Through a similar mechanism, leaky RyR2 could potentially contribute to the pathogenesis of AF by giving rise to DADs capable of triggering the arrhythmia.

The concept of aberrant Ca^{2+} release from RyR2 resulting in arrhythmogenic atrial activity is supported by a recent report that treatment of myocardial fibers from the pulmonary vein “myocardial sleeve” with ryanodine (0.5 to 2 μmol/L) resulted in pacing-induced spontaneous activity.33 Ryanodine at these concentrations would be expected to lock RyR2 in an open state, resulting in increased open probability, analogous to the increased open probability demonstrated in PKA-hyperphosphorylated RyR2 in the present study. Recently, other groups have reported that PKA phosphorylation of RyR2 does not dissociate calstabin2 from the channel14,35 or that calstabin2 is able to bind to a mutant recombinant RyR2-S2809D that mimics constitutively PKA-phosphorylated channels.56 The differences between our findings and these recent studies may be due to the presence of nonphysiological levels of excess calstabin2 in the experiments using recombinant RyR2 coexpressed with calstabin2 or when calstabin2 is added to PKA-phosphorylated channels. We have shown that PKA phosphorylation of RyR2 shifts the affinity of calstabin2 for the channel from ~200 to ~600 μmol/L. Given that the physiological levels of calstabin2 in the heart are ~200 μmol/L, this shift is physiologically significant and would effectively reduce the amount of calstabin2 bound to PKA-phosphorylated RyR2 in vivo in failing hearts, as we and others have shown.35,22,37,38 In experiments in which the much smaller calstabin2 is coexpressed with the 16-kb cDNA encoding RyR2, care must be taken to ensure that the ratios of the 2 cDNAs are such that vast overexpression of calstabin2 relative to RyR2 does not occur. In other experiments in which exogenous recombinant calstabin2 was added, it may be that the actual final concentration of calstabin2 was underestimated, especially when such measurements are determined by immunoblotting. Another possible difference between our studies and others’ conflicting studies is that in some experiments in which exogenous PKA was used to phosphorylate the channel, robust phosphorylation may not have been achieved, resulting in preserved binding of calstabin2 to the channel.

**Potential Therapeutic Implications**

AF has traditionally been treated with drugs that alter the excitability of the plasma membrane by blocking voltage-gated ion channels. The available therapeutic agents are, however, not specific for atrial electric activity and may significantly affect ventricular electrophysiology.39 The resulting proarrhythmic potential of some currently available antiarrhythmic drugs is a major factor complicating and limiting use of these agents.40 Nonpharmacological approaches to treating AF, including ablation therapy and implantable devices, have also yielded suboptimal results.39 The limitations in treating AF are in part due to an incomplete understanding of the molecular mechanisms underlying the arrhythmia. The present study demonstrates that the PKA signaling pathway, which normally regulates RyR2 function in response to stress, may be maladaptive in AF.
These findings suggest that components of the RyR2 macromolecular complex involved in regulation of channel function by PKA, including calstabin2, may provide novel and highly specific therapeutic targets for treating AF.

The 1,4-benzothiazepine derivative JTV519 has been shown to reverse the pathological loss of calstabin2 from the RyR2 channel complex and consequently to reduce the associated abnormal Ca^{2+} leak in heart failure.77,91 Similarly, in recent studies from our group, JTV519 prevented exercise-induced ventricular arrhythmias in calstabin2−/− mice by increasing the binding affinity of calstabin2 for PKA-phosphorylated RyR2, which stabilized the channel in the closed state and consequently prevented diastolic Ca^{2+} leak.19 The ability of JTV519 to repair a specific molecular-level defect in RyR2 Ca^{2+} handling makes it an intriguing candidate for a novel therapeutic agent. Calstabin2 binding to PKA-hyperphosphorylated RyR2 will reduce the open probability of the channel in diastole but will still allow the channel to open normally during systole, allowing for cardiomyocyte contraction. Thus, drugs like JTV519 that enhance the binding affinity of calstabin2 for RyR2 will improve the normal function of the channel (ie, stabilize the channel closed state and prevent a diastolic SR Ca^{2+} leak) without impairing the normal gating of the channel. It has been suggested that JTV519 acts as an allosteric modulator of RyR2, allowing for calstabin2 binding to the channel complex instead of acting as a pore blocker of RyR.19

Recently, JTV519 was shown to inhibit the ability to induce AF in a canine sterile pericarditis model of AF by Kumagai et al.10 The present study did not, however, define the molecular mechanism responsible for the antiarrhythmic effects of JTV519. On the basis of our present findings, combined with the work of Kohno et al,41 treatment with JTV519 could attenuate the generation of DADs by PKA-hyperphosphorylated RyR2 and restore physiological intracellular Ca^{2+} handling. By eliminating a potential trigger for AF and partially attenuating the ionic remodeling process, restoration of RyR2 channel function by JTV519 may explain the ability of this novel therapeutic agent to inhibit the inducibility and maintenance of AF.

**Potential Limitations**

The remodeling process that accompanies AF is complex and affects multiple ion channels. In addition to RyR2, other components of intracellular Ca^{2+} handling, including the 1,4,5-inositol trisphosphate receptor (IP3R), and proteins involved in SR Ca^{2+} reuptake (SERCA2a, phospholamban) may play a role in AF. Altered IP3R expression associated with AF has been reported,42 and a study in isolated rat atrial myocytes has suggested IP3R as a source of DADs,43 suggesting that this channel may also contribute to the pathogenesis of AF.

Furthermore, the present study does not exclude the possibility that the changes in RyR2 that we found in AF are unrelated to the mechanism of the arrhythmia. However, our findings demonstrating that, in atrial tissue, JTV519, a compound previously shown to inhibit the inducibility and maintenance of AF, specifically restored the aberrancies in RyR2 regulation and channel function that we have described in AF suggest that RyR2 plays an important role in the mechanism underlying the arrhythmia.

**Conclusions**

The present study provides a potential novel molecular mechanism, involving regulation of RyR2 by PKA, for the dysfunctional intracellular Ca^{2+} handling believed to contribute to AF and suggests RyR2 as a potential source of the afterdepolarizations necessary to trigger AF. Furthermore, these findings suggest that RyR2 and components of the macromolecular signaling complex, including calstabin2, may be novel therapeutic targets for the treatment of AF.

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