Molecular Determinants of Altered Ca^{2+} Handling in Human Chronic Atrial Fibrillation

Ali El-Armouche, MD; Peter Boknik, PhD; Thomas Eschenhagen, MD; Lucie Carrier, PhD; Michael Knaut, MD; Ursula Ravens, MD; Dobromir Dobrev, MD

Background—Abnormal Ca^{2+} handling may contribute to impaired atrial contractility and arrhythmogenesis in human chronic atrial fibrillation (cAF). Here, we assessed the phosphorylation levels of key proteins involved in altered Ca^{2+} handling and contractility in cAF patients.

Methods and Results—Total and phosphorylation levels of Ca^{2+}-handling and myofilament proteins were analyzed by Western blotting in right atrial appendages of 49 patients in sinus rhythm and 52 cAF patients. We found a higher total activity of type 1 (PP1) and type 2A phosphatases in cAF, which was associated with inhomogeneous changes of protein phosphorylation in the cellular compartments, ie, lower protein kinase A (PKA) phosphorylation of myosin binding protein-C (Ser-282 site) at the thick myofilaments but preserved PKA phosphorylation of troponin I at the thin myofilaments and enhanced PKA (Ser-16 site) and Ca^{2+}-calmodulin protein kinase (Thr-17 site) phosphorylation of phospholamban. PP1 activity at sarcoplasmic reticulum is controlled by inhibitor-1 (I-1), which blocks PP1 in its PKA-phosphorylated form only. In cAF, the ratio of Thr-35–phosphorylated to total I-1 was 10-fold higher, which suggests that the enhanced phosphorylation of phospholamban may result from a stronger PP1 inhibition by PKA-hyperphosphorylated (activated) I-1.

Conclusions—Altered Ca^{2+} handling in cAF is associated with impaired phosphorylation of myosin binding protein-C, which may contribute to the contractile dysfunction after cardioversion. The hyperphosphorylation of phospholamban probably results from enhanced inhibition of sarcoplasmic PP1 by hyperphosphorylated I-1 and may reinforce the leakiness of ryanodine channels in cAF. Restoration of sarcoplasmic reticulum–associated PP1 function may represent a new therapeutic option for treatment of atrial fibrillation.

Key Words: atrial fibrillation □ calcium □ myosin binding protein C, cardiac, human □ phospholamban protein, human □ protein phosphatase-1 □ protein phosphatase-2A

Chronic atrial fibrillation (cAF) is accompanied by shortened atrial action potential (electrical remodeling) and impaired tissue structure (structural remodeling), which are important in maintenance and progression of atrial fibrillation (AF). Although it is generally accepted that abnormal Ca^{2+} handling contributes to arrhythmogenesis in AF, the underlying cellular mechanisms are poorly understood.

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Functional regulation of ion channels and modulator proteins of Ca^{2+} homeostasis and contractility relies on phosphorylation processes. Protein kinase A (PKA) and calmodulin-dependent protein kinase II (CAMKII) phosphorylation of L-type Ca^{2+} channel (I_{Ca,L}) subunits increases current amplitude. Phosphorylation of ryanodine receptor channels (RyR2) at the Ser-2809 (PKA site) and Ser-2815 (CAMKII site), respectively, enhances Ca^{2+} release. PKA and CAMKII phosphorylation of phospholamban (PLB) at Ser-16 and Thr-17, respectively, relieves sarcoplasmic reticulum Ca^{2+}–adenosine triphosphatase (SERCA2a) inhibition and enhances relaxation rate and contractility.

The phosphorylation level of Ca^{2+}-handling proteins is controlled by serine/threonine protein phosphatases (PPs) in a highly regulated manner. We have recently shown that reduced I_{Ca,L} amplitude in human cAF is associated with increased activity of type 1 (PP1) and type 2 (PP2A) phosphatases. Although increased PP activity is expected...
to dephosphorylate regulatory Ca\(^{2+}\)-handling proteins, phosphatase activity may be differently regulated in distinct cellular microdomains. For instance, PP1 is anchored to sarcoplasmic reticular RyR2 and PLB by the noncatalytic targeting subunits spinophilin and R\(_{\text{Glu}}\), respectively. Targeting to the close vicinity of these proteins enhances PP1 specificity and availability. Furthermore, PP1 is regulated by 2 cytosolic heat- and acid-stable proteins, inhibitor-1 (I-1) and inhibitor-2 (I-2). I-1 becomes active upon phosphorylation on Thr-35 by PKA. This results in inhibition of PP1 and enhanced PKA-mediated protein phosphorylation. However, PKC or mitogen-activated protein kinase–mediated phosphorylation of I-1 at Ser-67 converts I-1 in a less efficient substrate for PKA, which suggests an antithetic regulation of I-1 by PKA and other kinases, respectively. Thus, abnormalities in local signaling, ie, altered kinase/phosphatase balance in the cellular microdomains, may contribute to impaired atrial Ca\(^{2+}\) handling and contractility in AF.

The mRNA and protein levels of I\(_{\text{C,LL}}\), RyR2, calsequestrin (CSQ), Serca2a, PLB, and Tn-I were stable in human cAF. However, PKA phosphorylation of RyR2 was higher in cAF than in patients in sinus rhythm (SR), which is consistent with the larger spontaneous Ca\(^{2+}\) release from sarcoplasmic reticulum.

Reverse-Transcription Polymerase Chain Reaction Analysis

Total RNA was isolated from atrial tissue, and reverse-transcription polymerase chain reactions were performed as described previously. Primers were constructed according to published sequences (online-only Data Supplement, Table I).

Enrichment of I-1

I-1 was enriched from atrial tissue of SR (844±67 mg, n=6) and cAF patients (782±82 mg, n=7) by an optimized trichloroacetic acid (TCA) extraction procedure. This procedure yielded ~0.12 \(\mu\)g/mg tissue (n=13), with no significant difference between the

### Patient Characteristics

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<tr>
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<th>SR</th>
<th>cAF</th>
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<td>13*</td>
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CAD indicates coronary artery disease; MVD, mitral valve disease requiring valve replacement; A\(_{\text{V}}\), aortic valve disease requiring valve replacement; LVEF, left ventricular ejection fraction; LVEDP, left ventricular end-diastolic pressure; LAD, left atrial diameter; LVEDD, left ventricular end-diastolic diameter; IVS, interventricular septum thickness; LVPW, left ventricular posterior wall thickness; ACE, angiotensin-converting enzyme; and AT, angiotensin receptor.

*P<0.05, nonpaired Student \(t\) test for continuous variables and \(\chi^2\) test for categorical variables.

### Recombinant I-1 Protein

Recombinant I-1 protein was generated by cloning the complete cDNA of rat I-1 into the pGEX-AT expression vector (Stratagene, La Jolla, Calif). The plasmid was transformed into competent Escherichia coli BL21 (Novagen, Madison, Wis) to express I-1/glutathione-S-transferase protein. I-1/glutathione-S-transferase was purified from clarified bacterial lysate by affinity purification with glutathione-agarose beads. Loaded beads were washed 3 times, and recombinant I-1 was released by thrombin cleavage. Recombinant human I-2 was purchased from Calbiochem (EMD Biosciences, Inc, Darmstadt, Germany).

### Adenovirus Infection of Neonatal Rat Cardiomyocytes

For testing specificity of antibodies, neonatal rat cardiac myocytes were isolated and cultured, and for the I-1 studies, they were infected with an adenovirus encoding the full-length rat I-1 (Ad-I-1) as described previously.

### Western Blot Analysis

Total and phosphorylation levels of human I-1 at Thr-35 or Ser-67 (total protein 1:1000 and phospho-Ser-67 1:200, custom-made purified polyclonal antibodies [Eurogentec, Brussels, Belgium]; and
phospho-Thr-35, 1:2000 (Cell Signaling Technology Danvers, Mass.) and I-2 (1:2000; Calbiochem) were analyzed by immunoblotting as described previously. For testing specificity of total I-1, TCA extracts were preincubated with recombinant I-1 protein or preimmune serum. The specific signal at Thr-35 was verified by I-1, TCA extracts were preincubated with recombinant I-1 protein or preimmune serum. The specific signal at Thr-35 was verified by

The latter were also incubated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany) for dephosphorylation of the Ser-67 site.

The protein levels of CSQ (1:2500; Dianova, Hamburg, Germany), Tn-I (1:30000; Chemicon International, Temecula, Calif), phospho-Thr-I (1:30000; HyTest Ltd., Turku, Finland), Serca2a (1:2000; Santa Cruz Biotechnology, Santa Cruz, Calif), total PLB, Ser-16 and Thr-17 phosphorylated PLB (all 1:5000; Badrilla Ltd., Leeds, United Kingdom), PP1α (1:500; Biomol, Plymouth Meeting, Pa), total PP2A (1:100), affinity purified; Upstate Biotechnology, Lake Placid, NY), and methylated and demethylated PP2A (1:100 and 1:1000, respectively; Upstate), NCX1 (1:1000; Affinity BioReagents, Neshanic Station, NJ, and Golden, CO), total MyBP-C (1:5000; kind gift from Wolfgang Linke, University of Münster, Germany), and Ser-282 phosphorylated MyBP-C (1:100; Eurogentec) were quantified by Western blotting as described previously. For testing specificity of total PLB to Serca2a was higher in cAF than in SR patients (Figure 1A).

**Phosphorylation Levels of PLB in cAF Patients**

The cAF samples showed a higher Ser-16 (PKA site) and a nonsignificantly enhanced Thr-17 (CAMKII site) phosphorylation of PLB in cAF compared with SR (Figure 1B). The ratio of phosphorylated PLB to Serca2a was significantly higher for both phosphorylation sites in cAF.

**Phosphorylation Levels of Tn-I and MyBP-C in cAF Patients**

Total and PKA phosphorylation levels of Tn-I did not differ between the groups (Figure 2A). In contrast, total MyBP-C was higher, whereas phosphorylation level at Ser-282 was smaller in cAF than in SR, which resulted in an ~40% lower ratio of phosphorylated to total MyBP-C (Figure 2B).

**Protein Levels of NCX1 in cAF Patients**

Consistent with previous results, we found higher protein abundance of NCX1 in cAF than in SR patients (Figure 3).

**PP1 and PP2A Activity in SR and cAF Patients**

Because PLB is dephosphorylated mainly by PP1, the higher PLB phosphorylation suggests lower PP1 activity in cAF than in SR. We found, however, that the PP1- and PP2A-related activities were both significantly higher in cAF than in SR (Figure 4A). The stronger PP1 and PP2A activities in cAF were associated with increased protein levels of the corresponding catalytic subunits (Figure 4B).

**PP1- and PP2A-Anchoring Proteins in cAF Patients**

Reduced PP targeting to the RyR2 and PLB/Serca2a complexes may underlie altered PP function in cAF. However, the mRNA abundance of spinophilin and RGL, which target PP1 to RyR2 and PLB, respectively, was comparable in SR and cAF (Figure 5A). The same holds true for the mRNA level of the PP2A-anchoring protein PR130 (Figure 5B). Methylation of PP2A by methyltransferases alters its subcellular targeting and substrate specificity.

**PP1 Inhibitors in cAF Patients**

Because increased PLB phosphorylation in cAF may result from reduced microdomain PP1 activity, we analyzed the phosphatase inhibitors I-1 and I-2. Their transcript levels were similar in SR and cAF (Figure 6A). To quantify the protein level of I-1 in human atrium, a dilution of recombinant rat I-1 was probed with the I-1 antibody in parallel with a dilution of TCA extracts (Figure 6B). Under the assumption that the antibody detects human I-1 with similar affinity as rat I-1, we calculated a I-1 concentration of 92 fmol/mg protein (92 nmol/L). The latter is similar to the I-1 levels in human left ventricle (126 nmol/L) and is ~100-fold higher than the I-1 concentration required to inhibit PP1 by 50% (~1 nmol/L). Although the physical properties of I-1 and I-2 are similar, 1-2 proteins
were not detectable in either standard homogenates (not shown) or TCA extracts of atrial tissue (Figure 6B).

With the exception of larger left atria in cAF versus SR, the clinical characteristics of the patients used for determination of I-1 proteins were comparable in both groups (online Data Supplement, Table II). Total I-1 proteins tended to be lower in cAF than in SR (Figure 7A). Interestingly, the levels of Thr-35–phosphorylated to total I-1 were ≈10-fold higher in cAF than in SR (Figure 7A). The latter was associated with lower phosphorylation at Ser-67 (Figure 7A); the ratio of Ser-67–phosphorylated to total I-1, however, was unaltered in cAF. Figure 7B demonstrates specificity of the antibodies recognizing total, Thr-35–phosphorylated, or Ser-67–phosphorylated I-1.

Discussion
In the present study, we demonstrate that increased total activity of PP1 and PP2A in cAF is associated with lower PKA phosphorylation of MyBP-C but with hyperphosphorylation of PLB. Thus, the changes in phosphorylation-dependent regulation in cAF are not homogeneous and point toward local regulation of kinase/phosphatase balance in cAF, ie, lower phosphorylation of MyBP-C at the thick myofilaments but preserved Tn-I phosphorylation at the thin myofilaments and enhanced PLB phosphorylation at the sarcoplasmic reticulum (Figure 8). These changes were accompanied by higher protein levels of NCX1. The different phosphorylation levels in the distinct compartments do not involve modified expression of PP1- and PP2A-anchoring proteins or impaired PP2A regulation through carboxymethylation. The decrease in MyBP-C phosphorylation is in agreement with the higher PP activity and may contribute to contractile dysfunction in cAF. The higher PLB phosphorylation appears to result from a stronger PP1 inhibition by PKA hyperphosphorylated (activated) I-1. This is predicted to render Serca2a more active and to increase Ca²⁺ loading of sarcoplasmic reticulum, leading to larger spontaneous Ca²⁺ release that may promote the occurrence of late after-depolarizations that trigger AF.
Comparison With Previous Studies

Although altered atrial Ca$^{2+}$ homeostasis contributes to electrical and contractile remodeling in AF, the underlying cellular mechanisms are poorly understood. Current concepts propose that disturbed function of Ca$^{2+}$ handling and myofilament proteins may result from changes in their phosphorylation levels. The higher PKA phosphorylation of RyR2 in cAF than in SR is associated with enhanced single RyR2 activity and larger spontaneous Ca$^{2+}$ release from sarcoplasmic reticulum. This together with the larger influx of Ca$^{2+}$ via $I_{Ca,L}$ (less influx of Ca$^{2+}$ per beat but more beats per time unit) is consistent with the Ca$^{2+}$-overload hypothesis in AF. Because cAF is associated with enhanced phosphorylation of PLB and reduced expression of sarcolin, another endogenous Serca2a inhibitor, Serca2a function is probably increased, compensating for the diastolic Ca$^{2+}$ “leak” through RyR2 channels.

The greater phosphorylation of PLB at Thr-17 in cAF compared with SR despite increased total PP activity is consistent with the stronger stimulation of CAMKII at increased heart rates and the higher CAMKII protein levels in cAF. The PKA hyperphosphorylation of PLB, however, was unexpected, because the increase in stimulation frequency does not affect the level of PKA phosphorylation at Ser-16. 

Figure 2. Protein and phosphorylation levels of Tn-I and MyBP-C in SR and cAF patients. A and B, Representative immunoblots of total Tn-I, phospho-Tn-I (cTnI-Phos), total MyBP-C (cMyBP-C-Total), phospho-MyBP-C (cMyBP-C-Ser282), and their densitometric analysis normalized to CSQ. Specificity of Ser282 signal is demonstrated by the increase of band density in response to isoproterenol (ISO). Same format as in Figure 1.

$^*$ $P<0.05$ vs SR.
Although modified phosphatase anchoring and reduced activity of cAMP-degrading phosphodiesterases may be involved,36,37 we suggest that the hyperphosphorylation of PLB and RyR2 may result from diminished local PP1 function due to enhanced I-1 activity, because the atrial concentration of I-1 and the 10-fold higher PKA phosphorylation levels reflecting its enzymatic activity are sufficient to shut down PP1 activity at the PLB/Serca2a complex. These results support the notion of compartmentation of protein kinase/phosphatase signaling in the atrium and that sarcoplasmic reticulum–associated PP1 activity is lower in cAF than in SR patients.

In contrast to the situation at sarcoplasmic reticulum, PKA phosphorylation of MyBP-C at thick myofilaments was lower in cAF than in SR, whereas phosphorylation levels of Tn-I were similar in both groups. The 2 PKA sites of Tn-I are not phosphorylated exclusively by PKA but also by protein kinases C and G.38 Thus, increased activity of these kinases in cAF may counteract the higher PP activity at the thin myofilaments. Phosphorylation of MyBP-C by PKA increases the number of myosin heads simultaneously attaching to actin, enhances the maximum force development, and decreases myofilibrillar Ca\(^{2+}\) sensitivity, accelerating relaxation.39 The lower PKA phosphorylation of MyBP-C in cAF may increase myofilibrillar Ca\(^{2+}\) sensitivity4 and thus muscle stiffness, resulting in reduced force of contraction17,22,23 and slowed relaxation kinetics. Hence, the impaired myofilament function may contribute to the contractile dysfunction in cAF and mask the effect of increased Serca2a and NCX1 activity on relaxation kinetics.17 Further studies are needed to verify this hypothesis.

The increased total PP1 and PP2A activity in cAF appears to be the consequence of higher protein levels of the catalytic subunits, but it is unlike our previous finding of unaltered PP1\(\alpha\) proteins in cAF.5 The reason for these inconsistent observations is currently unknown but may be due in part to an uncontrolled state of the cardiac diseases and/or patients’ medication.

### Study Limitations

The present study did not address the signaling pathways that lead to higher PKA phosphorylation of I-1. Phosphorylation of I-1 at Ser-67 was unaltered in cAF, which renders a reduced contribution of this counterbalancing mechanism unlikely.12 One possible explanation could be related to the renin-angiotensin system. During AF, the atrial levels of angiotensin II increase,40 which may result in stronger activation of presynaptic AT\(_1\)-receptors, with the concomitant release of norepinephrine from cardiac sympathetic nerve endings.41,42 Thus, the PKA hyperphosphorylation of I-1 may involve increased \(\beta\)-adrenoceptor signaling, with a concomitant increase in PKA activity.43

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**Figure 3.** Protein quantification of NCX1 in SR and cAF patients. Same format as in Figure 1. *\(P<0.05\) vs SR.

**Figure 4.** Serine/threonine PP1 and PP2A activity and their protein levels in SR and cAF patients. A, PP activity was assessed with phosphorylase A as substrate and quantified in nanomoles of \(^{32}\)Pi released per milligram of protein per minute. Okadaic acid (3 nmol/L) was used to differentiate between PP1 and PP2A activities. B, Representative immunoblots of PP1\(\alpha\) and PP2A and their densitometric analysis. Specificity of PP2A bands is demonstrated by incubation of the affinity-purified PP2A antibody with a corresponding immunizing peptide. Same format as in Figure 1. *\(P<0.05\) vs SR.
However, the cAF patients in the present study had no indices of increased sympathetic tone and were not in clinical heart failure, which is consistent with the normal blood levels of catecholamines in AF patients. Further studies are required to identify the molecular mechanisms of higher PKA phosphorylation of I-1 in cAF.

The determination of I-1 proteins was limited by the small sample size, which does not allow for a direct extrapolation of the results to the general AF population. Finally, we cannot exclude that human atria in vivo express I-2 proteins that contribute to modified phosphorylation-dependent regulation in cAF.

**Potential Clinical Implications**

PP1 inactivates transcription factors and is a repressor of protein translation. Thus, the increased total PP1 and PP2A activity may contribute to modified protein expression in cAF. Consistently, the overexpression of PP1 and PP2A in mice resulted in ventricular hypertrophy, dilatation, and increased interstitial fibrosis, which were associated with reduced contractility.

To the best of our knowledge, the present study is the first study demonstrating expression and function (phosphorylation) of PP inhibitors in the atrium. Overexpression of constitutively active I-1 in mice reduced ventricular hypertrophy and fibrosis in heart failure models, which suggests that increased I-1 function could be protective. However, mice overexpressing the PKA catalytic subunit exhibit hyperphosphorylation of PLB and RyR2 and develop AF, and high basal PKA-dependent phosphorylation of PLB and RyR2 in rabbits drives rhythmic sarcoplasmic reticulum Ca^2+ oscillations and spontaneous beating of cardiac pacemaker cells, which was attributable to activation of NCX1. Thus, restoration of sarcoplasmic reticulum–associated PP1 function may normalize leaky RyR2 channels.

The results of the present study point toward compartmentation of kinase/phosphatase signaling in the atrium (Figure 8). In cAF, increased total PP1 and PP2A activity is not translated into globally enhanced dephosphorylation of Ca^2+–handling and myofilament proteins. Although phosphorylation-dependent regulation of $I_{Ca,L}$ and...
MyBP-C was impaired, Tn-I phosphorylation at thin myofilaments is apparently preserved. In contrast, phosphorylation of sarcoplasmic RyR2 and PLB is higher in cAF than in SR. This is most likely due to enhanced PKA phosphorylation and therefore activity of inhibitor-1 of PP1, which prevents their dephosphorylation. Although the higher PLB phosphorylation may prevent sarcoplasmic reticulum Ca\(^{2+}\) depletion, it could reinforce the leakiness of RyR2, promoting AF. Thus, the higher phosphorylation of RyR2 and PLB together with the upregulation of NCX1 (present study\(^{17,21}\)) may represent the molecular correlate of triggered activity mediated by late afterdepolarizations.\(^{49}\)

We identified impaired MyBP-C phosphorylation as a potential novel molecular mechanism for contractile dysfunction, which is one major contributor to atrial thrombogenesis. Because positive inotropic drugs exhibit low efficacy and cause arrhythmias, restoration of MyBP-C...
phosphorylation may be a promising therapeutic target to improve atrial contractility in the postcardioversion period. The clinical consequences of hyperphosphorylated PLB are difficult to predict. Although disruption of the PLB gene (equivalent to hyperphosphorylation of PLB) results in improved cardiac function in mice, increased Serca2a function may be associated with higher vulnerability to arrhythmias. Thus, the hyperphosphorylation of PLB and the putative increase in Serca2a function probably compensate in part for the reduced contractility but at the expense of an increased propensity for atrial arrhythmias. Further study in clinically relevant animal models will be needed to address this issue.

Conclusions
We provide evidence that the fine-tuning of kinases and phosphatases is disturbed in cAF, resulting in impaired phosphorylation of MyBP-C but hyperphosphorylation of PLB. The former appears to involve increased PP1 and PP2A activity and may contribute to contractile dysfunction; the latter probably results from reduced sarcoplasmic reticulum–associated PP1 activity due to increased function of its endogenous inhibitor I-1. This may also contribute to the reported hyperphosphorylation of RyR2 that results in leaky RyR2 channels in cAF. The results of the present study provide new insights into the phosphorylation-dependent regulation of sarcoplasmic reticulum and myofilament function in cAF, which may help to design new therapeutic options for AF treatment.

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Disclosures
None.

References
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In chronic atrial fibrillation (cAF), abnormal Ca^2+ handling contributes to the pathophysiology underlying both arrhythmogenesis and contractile dysfunction. The molecular mechanisms, however, are poorly understood. We hypothesize that cAF may be associated with altered expression levels or phosphorylation status of key regulatory proteins. Despite increased total activity of phosphatases, protein phosphorylation status was inhomogeneous in various cellular compartments. For instance, in the sarcoplasmic reticulum, phosphorylation of phospholamban was increased at the protein kinase A and Ca^2+-calmodulin protein kinase sites. Phospholamban phosphorylation increases Ca^2+–adenosine triphosphatase activity and causes Ca^2+ overload of the sarcoplasmic reticulum, which results in spontaneous Ca^2+ release that induces late afterdepolarizations that may trigger cAF. Thus, a reduction of phospholamban phosphorylation and the subsequent normalization of Ca^2+–adenosine triphosphatase function may represent a new therapeutic option for cAF treatment. In the myofilament compartment, on the other hand, we found reduced protein kinase A phosphorylation of myosin binding protein C (MyBP-C). This regulatory protein is involved in the actin-myosin interaction, and reduced phosphorylation of MyBP-C is suggested to be a potential molecular mechanism for contractile dysfunction. Therefore, restoration of MyBP-C phosphorylation may be a promising therapeutic target for improvement of contractile function. Because reduced atrial contractility facilitates atrial thrombus formation, the associated risk of stroke in the postcardioversion period is expected to decline with this putative therapeutic concept.
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