L-Type Ca\(^{2+}\) Current Downregulation in Chronic Human Atrial Fibrillation Is Associated With Increased Activity of Protein Phosphatases

T. Christ, MD; P. Boknik, PhD; S. Wöhrle, MSc; E. Wettwer, PhD; E.M. Graf, PhD; R.F. Bosch, MD; M. Knaut, MD; W. Schmitz, MD; U. Ravens, MD; D. Dobrev, MD

Background—Although downregulation of L-type Ca\(^{2+}\) current (\(I_{\text{Ca,L}}\)) in chronic atrial fibrillation (AF) is an important determinant of electrical remodeling, the molecular mechanisms are not fully understood. Here, we tested whether reduced \(I_{\text{Ca,L}}\) in AF is associated with alterations in phosphorylation-dependent channel regulation.

Methods and Results—We used whole-cell voltage-clamp technique and biochemical assays to study regulation and expression of \(I_{\text{Ca,L}}\) in myocytes and atrial tissue from 148 patients with sinus rhythm (SR) and chronic AF. Basal \(I_{\text{Ca,L}}\) at +10 mV was smaller in AF than in SR (−3.8±0.3 pA/pF, n=138/37 [myocytes/patients] and −7.6±0.4 pA/pF, n=276/86, respectively; \(P<0.001\)), though protein levels of the pore-forming \(\alpha_{1c}\) and regulatory \(\beta_{2a}\) channel subunits were not different. In both groups, norepinephrine (0.01 to 10 μmol/L) increased \(I_{\text{Ca,L}}\) with a similar maximum effect and comparable potency. Selective blockers of kinases revealed that basal \(I_{\text{Ca,L}}\) was enhanced by Ca\(^{2+}\)/calmodulin-dependent protein kinase II in SR but not in AF. Norepinephrine-activated \(I_{\text{Ca,L}}\) was larger with protein kinase C block in SR only, suggesting decreased channel phosphorylation in AF. The type 1 and type 2A phosphatase inhibitor okadaic acid increased basal \(I_{\text{Ca,L}}\) more effectively in AF than in SR, which was compatible with increased type 2A phosphatase but not type 1 phosphatase protein expression and higher phosphatase activity in AF.

Conclusions—In AF, increased protein phosphatase activity contributes to impaired basal \(I_{\text{Ca,L}}\). We propose that protein phosphatases may be potential therapeutic targets for AF treatment. (Circulation. 2004;110:2651-2657.)

Key Words: myocytes • fibrillation, atrial • calcium • phosphatases

Atrial fibrillation (AF) induces alterations in atrial electrophysiology that promote its own perpetuation, which has led to the concept of electrical remodeling.\(^1\) AF-induced remodeling is associated with a decrease in the atrial effective refractory period and a loss of physiological rate adaptation.

In humans, electrical remodeling is associated with changes in activity of several ion currents.\(^2\) During experimental and clinical AF, the amplitude of L-type Ca\(^{2+}\) current (\(I_{\text{Ca,L}}\)) decreases\(^3-8\) with corresponding reductions in mRNA and protein levels of the pore-forming \(\alpha_{1c}\) subunit.\(^3-8\) However, the hypothesis of transcriptionally mediated down-regulation of \(I_{\text{Ca,L}}\) was challenged in humans by recent studies that failed to detect changes in mRNA and protein levels of \(\alpha_{1c}\) and the regulatory \(\beta_{2a}\) subunits.\(^13,14\) Although reduced amplitude of \(I_{\text{Ca,L}}\) is a consistent finding in AF, the molecular mechanisms are not fully understood.

Functional regulation of Ca\(^{2+}\) channels relies on phosphorylation processes. Protein kinase A and C and the Ca\(^{2+}\)/calmodulin-dependent protein kinase II (PKA, PKC, and CAMKII, respectively) affect \(I_{\text{Ca,L}}\).\(^15,16\) In the heart, phosphorylation of \(I_{\text{Ca,L}}\) is counteracted by type 1 and type 2A phosphatases (PP1 and PP2A).\(^17\) Thus, the actual amplitude of basal \(I_{\text{Ca,L}}\) is determined by the balanced activity of kinases and phosphatases.

The present study tested the hypothesis that reduced \(I_{\text{Ca,L}}\) in patients with AF is associated with alterations in phosphorylation-dependent channel regulation.

Methods

Patients

The study was approved by the local ethics committee (No. EK114082202). Each patient gave written informed consent.

Right atrial appendages were obtained from 95 patients with SR and 53 patients with chronic AF (AF >6 months, Table). Significant differences between the groups were found for age, body mass index, incidence of coronary artery disease and/or valve disease, hyperlipidemia, and left atrial diameter. AF patients more often received digitalis, angiotensin receptor inhibitors, and diuretics, whereas \(\beta\)-blockers and lipid-lowering drugs were more frequently used in SR (Table).

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From the Department of Pharmacology (T.C., E.W., E.M.G., U.R., D.D.) and Cardiovascular Centre (M.K.), Dresden University of Technology, Dresden; Department of Pharmacology (P.B., W.S.), University of Münster, Münster; and Department of Cardiology, University of Tübingen, Tübingen (W.S., R.F.B.), Germany.

Correspondence to Dr Dobromir Dobrev, Fetscherstr. 74, 01307 Dresden, Germany. E-mail dobrev@rcs.urz.tu-dresden.de

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Patients’ Characteristics

<table>
<thead>
<tr>
<th></th>
<th>SR</th>
<th>Chronic AF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, n</td>
<td>95</td>
<td>53</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>69/26</td>
<td>30/23</td>
</tr>
<tr>
<td>Age, y</td>
<td>66.4 ± 0.8</td>
<td>70.6 ± 1.2*</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>27.4 ± 0.4</td>
<td>25.9 ± 0.6*</td>
</tr>
<tr>
<td>CAD</td>
<td>n 71</td>
<td>16*</td>
</tr>
<tr>
<td>MVD/AVD, n</td>
<td>11</td>
<td>14*</td>
</tr>
<tr>
<td>CAD+ MVD/AVD, n</td>
<td>12</td>
<td>23*</td>
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<tr>
<td>Hypertension, n</td>
<td>81</td>
<td>39</td>
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<tr>
<td>Diabetes, n</td>
<td>33</td>
<td>17</td>
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<tr>
<td>Hyperlipidemia, n</td>
<td>72</td>
<td>27*</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>59.3 ± 1.6</td>
<td>60.1 ± 1.6</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>15.5 ± 1.1</td>
<td>13.6 ± 0.8</td>
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<tr>
<td>LAD, mm</td>
<td>42.1 ± 0.6</td>
<td>50.6 ± 1.3*</td>
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<tr>
<td>LVEDD, mm</td>
<td>52.0 ± 0.8</td>
<td>51.8 ± 1.2</td>
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<td>IVS, mm</td>
<td>11.5 ± 0.3</td>
<td>11.1 ± 0.3</td>
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<tr>
<td>LVPW, mm</td>
<td>10.8 ± 0.2</td>
<td>11.1 ± 0.3</td>
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<tr>
<td>Digitalis, n</td>
<td>2</td>
<td>29*</td>
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<td>ACE inhibitors, n</td>
<td>62</td>
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<tr>
<td>AT1 blockers, n</td>
<td>5</td>
<td>9*</td>
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<td>β-Blockers, n</td>
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<td>35*</td>
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<td>Dihydropyridines, n</td>
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<td>6</td>
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<td>Diuretics, n</td>
<td>28</td>
<td>33*</td>
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<tr>
<td>Nitrates, n</td>
<td>34</td>
<td>16</td>
</tr>
<tr>
<td>Lipid-lowering drugs, n</td>
<td>61</td>
<td>24*</td>
</tr>
</tbody>
</table>

SR indicates sinus rhythm; AF, atrial fibrillation; CAD, coronary artery disease; MVD, mitral valve disease requiring valve replacement; AVD, aortic valve disease requiring valve replacement; LVEF, left ventricular ejection fraction; LVEPP, left ventricular end-diastolic pressure; LVEDD, left ventricular end-diastolic diameter; LVEDV, left ventricular end-diastolic volume; LVPW, left ventricular posterior wall thickness; ACE, angiotensin-converting enzyme; and AT, angiotensin receptor.

*P < 0.05 values from unpaired Student t test for continuous variables and from χ² test for categorical variables.

I_{Ca,l} Measurement

Atrial myocytes were isolated with a standard protocol. The solution for cell storage contained (in mmol/L) KCl 20, KH₂PO₄ 10, glucose 10, K-glutamate 70, ₇β-hydroxybutyrate 10, taurine 10, EGTA 10, and albumin 1, pH = 7.4. Whole-cell voltage-clamp I_{Ca,l} recordings were performed as previously described. ISO-2 software (MFK) was used for data acquisition and analysis. Borosilicate glass electrodes (Hilgenberg) had tip resistances of 2 to 5 MΩ. Series resistance and system capacitance were compensated. The bath solution contained (in mmol/L) TEA-Cl 120, CsCl 10, HEPES 10, Mg-ATP 4, Tris-GTP 0.4, EGTA 10, and CaCl₂ 3, pH = 7.2. Cell capacitances averaged 88.1 ± 1.9 and 106.1 ± 3.8 pF for SR (n = 276) and AF (n = 138) myocytes, respectively (P < 0.01). Seal resistances were 3 to 6 GΩ. Series resistance and system capacitance were compensated. The bath solution contained (in mmol/L) TEA-Cl 120, CsCl 10, HEPES 10, CaCl₂ 2, MgCl₂ 1, and glucose 10 (pH 7.4, with CsOH). Basal and norepinephrine (NE)- and isoproterenol (ISO)-activated I_{Ca,l} were measured at 37°C in the absence and presence of selective inhibitors of PKA (8-Br-Rp-cAMP, 100 μmol/L in the pipette), PKC (bisindolylmaleimide-I and its inactive form bisindolylmaleimide-V, 1 μmol/L each), CAMKII (KN-93 and its inactive form KN-92, 20 μmol/L each), and PP1/PP2A (okadaic acid, 1 μmol/L). All drugs were from Calbiochem and were applied via a rapid solution exchange system (ALA Scientific Instruments).

The data were not corrected for the calculated liquid junction potential (−15 mV; JPCalc, version 2.2).

Reverse-Transcription Polymerase Chain Reaction Analysis

Total RNA isolated from right atrial homogenates was reverse transcribed (Invitrogen) in the presence of random nucleotides. PCR experiments were performed in a thermocycler (Master cycler, Eppendorf) using standard PCR reaction mixes (Applied Biosystems) and 3-μL cDNA aliquots. Oligonucleotide sequences for α-actin, forward primer, GCCCCGAAACATGAGCAT; reverse primer, GAAATACCAACGCGTGAAGA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward primer, AACAGGCGACCCCACTCTC; reverse primer, GGAGGG-GGAGTACAGTGGTTG) were according to published sequences (GeneBank accession Nos. J00074, J2953, and J2642, respectively). The catalytic subunits of PKA (α, β, and γ) and PP2A (α and β) were detected with isoform-specific PCR primers as previously described.

Western Blot Analysis

The α₈, Ca²⁺ channel subunit and GAPDH were detected with primary antibodies (Biotrend, Köln, Germany) and anti-rabbit IgG (DAKO, Hamburg, Germany) and anti-mouse IgG (Sigma, Taufkirchen, Germany) as previously described. Antibodies against the β₁₃ subunit were raised in rabbit against an epitope mapping near the C-terminus (KKRNEAGENWRDNYIQ) and were affinity purified on the respective antigen column. The protein bands were visualized by using enhanced chemiluminescence (Pharmacia Bio-tech) and quantified using Quantity One Software (Bio-Rad).

Protein expression of catalytic subunits of PP1α and PP2A was quantified as described. The structural subunit of PP2A (PP2A-A®) was assessed by a goat polyclonal antibody against the carboxy terminus of PP2A-A® of human origin (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) and alkaline phosphatase–conjugated rabbit-antigoat IgG (Sigma, St. Louis, Mo). To demonstrate the specificity of bands, antibodies for α₈, β₁₃, PP1, and PP2A-C were incubated with corresponding immunizing peptides before blotting. Immunologic signals were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech) and quantified in Storm 820 using ImageQuaNT Software (Molecular Dynamics).

Phosphatase Assay

Activity of phosphatases was measured in atrial homogenates as previously described.

Statistics

Univariate ANOVAs were applied to associate preoperative variables with expression and electrophysiological data (SPSS version 11.5). Concentration-response curves were fitted with software Prism (version 4.0). Differences between continuous data were compared by an unpaired Student t test. Frequency data were analyzed with χ² statistics. Data are given as mean±SEM. P < 0.05 was considered statistically significant.

Results

Expression and activity of proteins and amplitudes of I_{Ca,l} were related to selected clinical variables. With univariate ANOVAs, AF was the only predictor of protein expression, phosphatase activity, and electrophysiological parameters (data not shown).

Properties of I_{Ca,l} in SR and AF

Basal peak I_{Ca,l} at +10 mV was smaller in AF than in SR (−3.8 ± 0.3 pA/pF, n = 138/37 [myocytes/patients] and −7.6 ± 0.4 pA/pF, n = 276/86, respectively; P < 0.001). When expression and function of Ca²⁺ channels were compared in SR.
the same patients, reduced basal $I_{Ca,L}$ was not paralleled by decreased $\alpha_{1C}$ and $\beta_{2a}$ proteins (Figure 1, B through E). In addition, the maximum current was obtained at more positive potentials in AF than in SR (Figure 1F), suggesting phosphorylation-dependent changes in channel regulation rather than expression.23

Regulation of Basal $I_{Ca,L}$ by Kinases and Phosphatases

The phosphorylation state of Ca$^{2+}$ channels depends on the balance between kinases and phosphatases. Therefore, we measured $I_{Ca,L}$ in the presence of selective enzyme inhibitors. The PKA blocker 8-Br-Rp-cAMP (100 $\mu$mol/L), the PKC blocker bisindolylmaleimide-I, and its inactive form bisindolylmaleimide-V (1 $\mu$mol/L each) did not modulate basal $I_{Ca,L}$ in either group (data not shown). In contrast, the CAMKII inhibitor KN-93 (20 $\mu$mol/L), but not the inactive form KN-92, reduced basal $I_{Ca,L}$ by $\approx 40\%$ in SR but was ineffective in AF (Figure 2, A and B).

The lack of effect of KN-93 on basal $I_{Ca,L}$ in AF may be due to increased phosphatase activity. Indeed, the PP1/PP2A inhibitor OA (1 $\mu$mol/L) increased basal $I_{Ca,L}$ more effectively in AF than in SR (Figure 2, C and D). In the presence of the CAMKII blocker KN-93, OA failed to increase $I_{Ca,L}$ in AF ($-2.3 \pm 0.6$ pA/pF before and $-2.5 \pm 0.2$ pA/pF after OA, n=8/2, NS) and SR myocytes ($-8.2 \pm 0.9$ pA/pF before and $-8.5 \pm 0.5$ pA/pF after OA, n=13/3; NS), indicating that the OA-mediated $I_{Ca,L}$ increase involves activation by CAMKII.

Modulation of $I_{Ca,L}$ by NE and ISO

In both groups, exposure of myocytes to NE (0.01 to 10 $\mu$mol/L) increased $I_{Ca,L}$ with similar maximum effect (increase in peak $I_{Ca,L}$ at 10 $\mu$mol/L NE: $-6.8 \pm 1.3$ pA/pF,
n/H11005/13/5, AF versus /H11002/6.7/0.8 pA/pF, n/H11005/46/19, SR) and comparable potency (Figure 3). As expected from the higher phosphorylation state of the channels in the presence of NE,23 the maximum of the current–voltage relationship in SR and AF was shifted toward less positive potentials (Figure 3).

Contribution of kinases to NE-activated \( I_{Ca,L} \) differed between SR and AF. 8-Br-Rp-cAMP did not change NE-activated \( I_{Ca,L} \) in SR but blocked this current by 60% in AF (\( P<0.05 \), Figure 4, A and B). In SR, bisindolylmaleimide-I increased NE-activated \( I_{Ca,L} \) by 100% compared with control but was ineffective in AF. KN-93 inhibited the NE-activated \( I_{Ca,L} \) by 19% in SR and by 38% in AF (\( P<0.05 \)), suggesting larger contribution of CAMKII to NE effects in AF (Figure 4, A and B). Bisindolylmaleimide-V and KN-92 had no significant effect. The ineffectiveness of 8-Br-Rp-cAMP on NE-activated \( I_{Ca,L} \) in SR may be due to opposite effects of \( \alpha \)- and \( \beta \)-adrenoceptor–mediated signal transduction. Therefore, we repeated the experiments with ISO, which activates \( \beta \)-adrenoceptors only. In the presence of 8-Br-Rp-cAMP, ISO-activated \( I_{Ca,L} \) was 31% smaller in SR and 51% smaller in AF than in its absence (Figure 4C).

**Expression and Activity of Phosphatases**

Because of the evidence for enhanced phosphatase activity in AF, we also studied the expression of the catalytic subunits of PP1 and PP2A in their various isoforms. Unexpectedly, the mRNA levels of all isoforms were lower in AF than in SR, though statistical significance was reached for PP1\( \alpha \) only (Figure 5). Because mRNA and protein levels do not always correlate, we also measured PP1 and PP2A proteins. In accordance with the higher impact of PP2A on channel regulation,17 protein expression of the catalytic subunit of this phosphatase was larger in AF than in SR, whereas protein levels were similar for PP1 and the structural PP2A subunit (Figure 6). Correspondingly, phosphatase activity was higher in AF than in SR (Figure 6B).

**Comparison With Previous Studies**

Several studies in human atria reported smaller \( I_{Ca,L} \) amplitude in AF than in SR. Although reduced \( \alpha_{1c} \) expression is an attractive molecular mechanism,10–11 others did not confirm this hypothesis.13,14 In the present study, reduced \( I_{Ca,L} \) was not paralleled by decreased channel expression (Figure 1). Moreover, the maximum of the current–voltage relationship of \( I_{Ca,L} \) in AF was shifted to the right, suggesting altered phosphorylation-dependent \( I_{Ca,L} \) regulation.23

The \( \alpha_{1c} \) and \( \beta_{2a} \) subunits possess several phosphorylation sites for PKA and PKC15 and possibly for CAMKII. Interestingly, the latter kinase is regulated by membrane voltage.16 Using selective kinase inhibitors, we found that in SR basal \( I_{Ca,L} \) is not modulated by PKA or PKC. Interestingly, the CAMKII blocker KN-93 reduced basal \( I_{Ca,L} \) in SR but not in AF, suggesting that CAMKII may modulate basal \( I_{Ca,L} \) in SR.
only. However, reduced CAMKII activity is not a likely explanation because protein levels of CAMKII were higher in AF than in SR.24 Because KN-93 prevents the calmodulin binding to CAMKII,25 calmodulin abundance or activity may be affected in AF. Modulation of $I_{\text{Ca,L}}$ by CAMKII requires the cytoskeleton.26 Thus, we cannot exclude the possibility that abnormalities of cytoskeletal proteins contribute to the lack of effect of CAMKII on $I_{\text{Ca,L}}$ in AF.

Further work is needed to verify these hypotheses.

Cardiac $\beta$- and $\alpha$-adrenoceptors couple to different subtypes of G-proteins, thereby modulating PKA-, PKC-, and CAMKII-dependent processes that regulate $I_{\text{Ca,L}}$.15,16 Here, NE increased $I_{\text{Ca,L}}$ with similar potency and efficacy in SR and AF. The same holds true for maximum effect of ISO, confirming previous results.5 However, the effects of the kinase inhibitors differed between SR and AF. In both groups, block of CAMKII with KN-93 reduced NE-activated $I_{\text{Ca,L}}$, whereas inhibition of PKC with bisindolylmaleimide-I strongly increased the current in SR but not in AF. Our data are consistent with results in human atrial myocytes, where activation of PKC inhibits $I_{\text{Ca,L}}$.27 In SR, the PKA blocker 8-Br-Rp-cAMP had no effect on NE-activated $I_{\text{Ca,L}}$, which may be due to opposite effects of the $\alpha$- and $\beta$-adrenoceptor-mediated signal transduction on $I_{\text{Ca,L}}$, because 8-Br-Rp-cAMP reduced the ISO-activated $I_{\text{Ca,L}}$ both in SR and in AF. In AF, the NE-activated $I_{\text{Ca,L}}$ was inhibited by 8-Br-Rp-cAMP but not by bisindolylmaleimide-I, indicating impaired ability of PKC to modulate $I_{\text{Ca,L}}$ in AF.

The lack of effects of CAMKII and PKC on $I_{\text{Ca,L}}$ in AF suggests either limited kinase ability to phosphorylate the
channels or increased phosphatase activity. Blockade of PP1 and PP2A with OA increased basal $I_{Ca,L}$ to a greater extent in AF than in SR, and the difference in current density disappeared. The OA-induced $I_{Ca,L}$ increase was absent after blocking CAMKII with KN-93, suggesting involvement of this kinase. The stronger effect of OA in AF was compatible with increased PP2A-C protein expression and higher phosphatase activity. Increased PP2A-C expression was not associated with alterations of the structural PP2A-A subunit, suggesting that the former may directly target the channels.28 Thus, increased PP2A activity in AF appears to counteract stimulatory effects of CAMKII on $I_{Ca,L}$, resulting in reduced basal $I_{Ca,L}$.

**Study Limitations**

Here, we did not investigate channel phosphorylation, because measurement of the phosphorylation state of the channels was not feasible in cardiac myocytes.

**β-Blockers increase PP1 and PP2A expression in dog hearts.**29 However, their distributions were similar in the SR and AF subgroups. In rat hearts, expression and activity of PP1 and PP2A decline with age.22 Though patients were older in the AF group than in the SR group, age-related effects on phosphatase activity are unlikely because neither expression nor activity correlated with age.

Our results are not consistent with data at the single-channel level, where reduced $I_{Ca,L}$ expression was associated with increased mean open time of the single channels.12 The authors suggested reduced PP2A activity as the underlying mechanism, though the protein levels of PP1 and PP2A were unchanged.12 The reason for the discrepant observations is currently unknown. Differences between cell-attached and ruptured whole-cell voltage-clamp methods, uncontrolled state of the cardiac diseases, and/or patients’ medications could contribute to these discrepant findings. Therefore, we cannot exclude the possibility that in a subset of patients with AF, a reduction of $I_{Ca,L}$ expression and a compensatory increase in single-channel $I_{Ca,L}$ activity occur.

**Potential Significance**

Increased phosphatase activity may associate with enhanced dephosphorylation of other proteins, eg, those involved in excitation-contraction coupling.17 Because contractile dysfunction in AF may promote atrial thrombus formation,30 possible improvement of atrial contractility by blockade of phosphatases could reduce the risk of stroke in AF patients after cardioversion to SR. In addition, inhibition of phosphatases in AF may promote reversal of atrial remodeling.

Physiological function of cardiac potassium currents requires basal kinase activities.24,31 Thus, increased phosphatase activity may contribute to downregulation of potassium currents in human AF.
Conclusions
We conclude that in AF the ratio of protein kinase/phosphatase activity is altered in favor of increased phosphatase activity, resulting in lower basal $I_{Ca,L}$ activity. Thus, protein phosphatases may be potential drug targets for treatment of chronic AF.

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