Differential phosphorylation-dependent regulation of constitutively active and muscarinic receptor-activated $I_{K_{ACh}}$ channels in patients with chronic atrial fibrillation

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Abstract

Objective: In chronic atrial fibrillation (cAF) the potassium current $I_{K_{ACh}}$ develops agonist-independent constitutive activity. We hypothesized that abnormal phosphorylation-dependent regulation underlies the constitutive $I_{K_{ACh}}$ activity.

Methods: We used voltage-clamp technique and biochemical assays to study $I_{K_{ACh}}$ regulation in atrial appendages from 61 sinus rhythm (SR), 11 paroxysmal AF (pAF), and 33 cAF patients.

Results: Compared to SR basal current was higher in cAF only, whereas the muscarinic receptor (2 μmol/L carbachol)-activated $I_{K_{ACh}}$ was smaller in pAF and cAF. In pAF the selective $I_{K_{ACh}}$ blocker tertiapin abolished the muscarinic receptor-activated $I_{K_{ACh}}$ but excluded agonist-independent constitutive $I_{K_{ACh}}$ activity. Blockade of type-2A phosphatase and the subsequent shift to increased muscarinic receptor phosphorylation (and inactivation) reduced muscarinic receptor-activated $I_{K_{ACh}}$ in SR but not in cAF, pointing to an impaired function of G-protein-coupled receptor kinase. Using subtype-selective kinase inhibitors we found that in SR the muscarinic receptor-activated $I_{K_{ACh}}$ requires phosphorylation by protein kinase G (PKG), protein kinase C (PKC), and calmodulin-dependent protein kinase II (CaMKII), but not by protein kinase A (PKA). In cAF, constitutive $I_{K_{ACh}}$ activity results from abnormal channel phosphorylation by PKC but not by PKG or CaMKII, whereas the additional muscarinic receptor-mediated $I_{K_{ACh}}$ activation occurs apparently without involvement of these kinases. In cAF, the higher protein level of PKCε but not PKCα, PKCβ1 or PKCδ is likely to contribute to the constitutive $I_{K_{ACh}}$ activity.

Conclusions: The occurrence of constitutive $I_{K_{ACh}}$ activity in cAF results from abnormal PKC function, whereas the muscarinic receptor-mediated $I_{K_{ACh}}$ activation does not require the contribution of PKG, PKC or CaMKII. Selective drug targeting of constitutively active $I_{K_{ACh}}$ channels may be suitable to reduce the ability of AF to become sustained.

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Keywords: Fibrillation; Atrium; Potassium channels; Kinases; Phosphatases

1. Introduction

Atrial fibrillation (AF) is the most frequent arrhythmia in the clinical setting. It is accepted that electrical and structural remodeling including abbreviated action potentials and refractoriness, hypertrophy and fibrosis promote the existence of reentry circuits and the progression of AF [1]. Studies in animal models and in patients with chronic AF (cAF) suggest that the G-protein-gated potassium current $I_{K_{ACh}}$ plays a crucial role in atrial arrhythmogenesis [2–6].

We have shown that the background inward rectifier potassium current $I_{K1}$ is higher in cAF and was associated
with smaller muscarinic receptor-mediated \( I_{K,\text{ACh}} \) activation [3–5]. Interestingly cAF patients exhibit agonist-independent constitutive \( I_{K,\text{ACh}} \) activity that contributes to the enhanced basal inward rectifier current [5]. In dogs a corresponding constitutively active \( I_{K,\text{ACh}} \)-like current exists which is up-regulated in response to atrial tachypacing [6,7]. Blockade of this current by the selective IK, ACh- blocker tertiapin resulted in action potential prolongation and suppression of inducible AF episodes [6]. Thus, identifying the culprit abnormalities in \( I_{K,\text{ACh}} \) regulation may lead to novel therapeutic targets for AF prevention and therapy.

The molecular basis of constitutively active \( I_{K,\text{ACh}} \) in cAF is unknown. At the single channel level, agonist-independent \( I_{K,\text{ACh}} \) activity in cAF results from higher channel open probability without changes in amplitudes and open times. Earlier studies suggest that agonist-independent activation of \( I_{K,\text{ACh}} \) requires ATP [8–10]. Thus modified phosphorylation of \( I_{K,\text{ACh}} \) and regulatory proteins may contribute to constitutively active \( I_{K,\text{ACh}} \) in cAF. The \( I_{K,\text{ACh}} \) channel forms a macromolecular complex, allowing for local \( I_{K,\text{ACh}} \) regulation. The GIRK1 channel complex may comprise the catalytic subunits of protein kinases A (PKA) and C (PKC), the calmodulin-regulated protein kinase II (CaMKII), and the type-1 and type-2A protein phosphatases PP1 and PP2A [11]. The kinase/phosphatase signaling in the cellular microdomains of cAF patients is inhomogeneous resulting in increased, decreased or normal phosphorylation levels of proteins despite enhanced total PP1 and PP2A activity [12–14]. Thus, the quantitative and qualitative composition of the macromolecular channel complex may change during cAF resulting in abnormal phosphorylation-dependent \( I_{K,\text{ACh}} \) regulation.

The present study tested the hypothesis that alterations in phosphorylation-dependent channel regulation may contribute to constitutive \( I_{K,\text{ACh}} \) activity in cAF.

2. Materials and methods

2.1. Human samples

The study was approved by the local ethics committee of the university (No: EK790799) and each patient gave written informed consent. The investigation conforms with the principles outlined in the Declaration of Helsinki (Cardiovascular Research 1997;35:2–4).

Right atrial appendages were obtained from 61 patients with SR, 11 patients with paroxysmal AF (pAF) and 33 patients with cAF (cAF>6 months, Table 1 and supplementary data).

2.2. Electrophysiological recordings

Atrial myocytes were isolated using our previous protocol [15] and were suspended in storage solution (in mmol/L: KCl 20, KH₂PO₄ 10, glucose 10, K-glutamate 70, \( \beta \)-hydroxybutyrate 10, taurine 10, EGTA 10, albumin 1, pH=7.4). Membrane currents were measured with voltage-clamp technique. ISO-2 software (MFK) was used for data acquisition and analysis.

Borosilicate glass microelectrodes had tip resistances of 2–5 MΩ when filled with pipette solution (in mmol/L: K-aspartate 80, NaCl 8, KCl 40, Mg-ATP 5, EGTA 2, GTP-Tris 0.1, HEPES 10, pH=7.4). Myocytes were superfused with a solution containing (in mmol/L): NaCl 120, KCl 20, MgCl₂ 1, CaCl₂ 2, glucose 10, HEPES 10, pH=7.4 at 22–24 °C. Seal resistances were 4–8 GΩ. Series resistance and cell capacitance were compensated. Agonist-independent basal current was measured by applying a ramp pulse from −100 to +40 mV (Fig. 1A). Agonist-inducible \( I_{K,\text{ACh}} \) was stimulated with carbachol (CCh, 2 μmol/L) in the absence and presence of selective inhibitors of protein kinase G (PKG; KT5823, 1 μmol/L), PKC (bisindolylmaleimide-I [BIM-I] and its inactive form bisindolylmaleimide-V [BIM-V], 0.1 μmol/L each), CaMKII (KN-93 and its inactive form KN-92, 0.2 μmol/L each), protein kinase A (PKA; KT5720, 1 μmol/L), PP1/PP2A (okadaic acid, 1 μmol/L) and type-2B protein phosphatase calcineurin (PP2B; cyclosporin A, 10 μmol/L). All drugs were from Calbiochem.

### Table 1

Characteristics of patients used for the electrophysiological experiments

<table>
<thead>
<tr>
<th></th>
<th>SR</th>
<th>pAF</th>
<th>cAF</th>
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<tbody>
<tr>
<td>Patients, ( n )</td>
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<td>11</td>
<td>20</td>
</tr>
<tr>
<td>Gender, m/f</td>
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<td>9/2⁋</td>
<td>9/11⁋</td>
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<tr>
<td>Age, y</td>
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<td>71.8±2.2*</td>
<td>72.0±1.4*</td>
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<td>Body mass index, kg/m²</td>
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<td>26.6±1.2</td>
<td>27.8±0.9</td>
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<td>CAD, ( n )</td>
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<td>8*</td>
</tr>
<tr>
<td>MVD/AVD, ( n )</td>
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<td>1</td>
<td>8</td>
</tr>
<tr>
<td>CAD+MVD/AVD, ( n )</td>
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<td>6*⁡</td>
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<td>10</td>
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</tr>
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</tr>
<tr>
<td>Hyperlipidemia, ( n )</td>
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<td>11*</td>
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<tr>
<td>LVEF, %</td>
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<td>54.4±6.5</td>
<td>62.4±3.6</td>
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<tr>
<td>LAD, mm</td>
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<td>43.0±1.6⁡</td>
<td>51.3±1.9*</td>
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<td>11.7±0.5</td>
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<tr>
<td>Nitrates, ( n )</td>
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</tr>
<tr>
<td>Lipid-lowering drugs, ( n )</td>
<td>33</td>
<td>6</td>
<td>8*</td>
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</table>

*P<0.05 vs. SR, ⁡P<0.05 vs. cAF from ANOVA followed by Bonferroni multiple comparisons procedure for continuous variables and from \( \chi^2 \) test for categorical variables.

CAD, coronary artery disease; MVD/AVD, mitral/aortic valve disease; LVEF, left ventricular ejection fraction; LAD, left atrial diameter; LVEDD, left ventricular end-diastolic diameter; IVS, interventricular septum thickness; LVPW, left ventricular posterior wall thickness; ACE, angiotensin-converting enzyme; AT, angiotensin receptor.

Ba\(^{2+}\) (1 mmol/L) was applied in each myocyte and the currents were analyzed after subtraction of the resulting leak current. The myocytes were superfused with Tyrode’s solution, and the drugs were applied via an additional rapid solution exchange system (ALA Scientific Instruments, Long Island, NY, USA). To control for myocyte-size variability, the currents are expressed as densities (pA/pF).

2.3. Western blot analysis

Protein levels of calsequestrin (1:2500; Dianova), PKC\(\alpha\) (1:1000), PKC\(\beta_1\) (1:250), PKC\(\delta\) (1:250), and PKC\(\epsilon\) (1:500; all Santa Cruz) were quantified in atrial homogenates by Western blotting as described [16]. Immunological signals were visualized with anti-IgG-horseradish-peroxidase and enhanced chemiluminescence (Amersham) and quantified using Quantity One software (BioRad).

2.4. Molecular analysis of the \(G\beta_3\) gene

Direct activation of \(I_{K_ACh}\) is mediated by G-protein \(\beta\gamma\)-subunits. The G-protein \(\beta_3\) subunit gene (GNB3) contains a C825T-polymorphism, whereby homozygous 825T-allele carriers exhibit larger basal inward rectifier K\(^+\) current possibly due to enhanced signal transduction. To exclude confounding by homozygous 825T-allele carriers, all
patients were genotyped [15]. The patient sample includes homozygous and heterozygous C825-allele carriers only.

2.5. Statistical analysis

One-way ANOVAs were applied to determine the sources of K+-current and protein variation (SPSS version 12.0). Independent variables were rhythm status, selected clinical variables and medication (Table 1 and supplementary data). To test for interactions between rhythm status and clinical variables or medication, interaction terms were included in separate two-way ANOVA. Differences between group means for continuous data were compared by unpaired Student’s t-test or by one-way ANOVA and Bonferroni multiple comparisons procedure. Frequency data were analyzed with \( \chi^2 \) statistics. Data are means±SEM. \( P<0.05 \) was considered statistically significant.

3. Results

Significant differences between the groups were found for gender, age, coronary artery disease, valvular heart disease, hyperlipidemia and left atrial diameter. cAF patients more often received digitalis but less frequently lipid-lowering drugs. Diuretics were more often prescribed in pAF and cAF than SR (Table 1). With one-way
ANOVAs, AF was the only predictor of current densities and protein levels (not shown).

3.1. Basal current and CCh-activated $I_{K,ACh}$

Cell capacitances averaged $99.6\pm3.1$ pF ($n=108/48$; myocytes/patients) for SR, $80.9\pm6.5$ pF ($n=23/11$) for pAF and $109.3\pm5.3$ pF ($n=48/20$) for cAF myocytes ($P=N.S.$). Typical current traces and current amplitudes at $-100$ mV in the time course of an experiment are shown in Fig. 1A,B. Basal currents in SR and pAF were similar ($-12.8\pm0.7$ pA/pF, $n=108/48$ vs. $-14.1\pm1.5$ pA/pF, $n=23/11$), but smaller than in cAF ($-20.7\pm1.2$ pA/pF, $n=48/20$; $P<0.05$; Fig. 1C) confirming previous results [3–5,17,18]. The smaller basal currents in SR and pAF are in accordance with the resting membrane potential (RMP) being less negative in SR and pAF than in cAF ($-18.8\pm0.9$ mV, $n=86/48$ and $-15.6\pm1.6$ mV, $n=12/4$ vs. $-23.0\pm1.4$ mV, $n=48/20$; $P<0.05$; Fig. 1D) [3,4,17].

Application of CCh resulted in rapid initial increase (“Peak”) of $I_{K,ACh}$ followed by a decrease to a quasi steady-state level (“QSS”) despite continuous presence of CCh (“desensitisation”; Fig. 1B) [5]. There was no difference in $I_{K,ACh}$ desensitisation between the groups (QSS/Peak ratio: SR=0.58±0.01 [$n=108/48$], pAF=0.59±0.05 [$n=23/11$], and cAF=0.56±0.09 [$n=48/20$]; $P=N.S.$). However, the CCh-activated Peak- and QSS-$I_{K,ACh}$ were smaller in pAF and cAF than in SR (Peak-$I_{K,ACh}$: $-5.1\pm0.9$ pA/pF and $-6.3\pm1.0$ pA/pF vs. $-11.0\pm1.0$ pA/pF; QSS-$I_{K,ACh}$: $-2.5\pm0.4$ pA/pF and $-3.8\pm0.6$ pA/pF, $P<0.05$; Fig. 1C). The smaller CCh-activated $I_{K,ACh}$ resulted in less CCh-induced hyperpolarisation of RMP in cAF ($-7.3\pm1.1$ mV, $n=48/20$) and pAF ($-7.7\pm1.9$ mV, $n=12/4$) than in SR ($-12.6\pm0.9$ mV, $n=86/48$; Fig. 1D). With two-way ANOVA, test of interaction effects between coronary artery disease, valvular heart disease, hyperlipidemia, digitalis, diuretics, lipid-lowering drugs, gender, age or left atrial diameter and $I_{K,ACh}$ showed no significant interaction (not shown).

3.2. Effects of tertiapin on basal current and CCh-activated $I_{K,ACh}$ in pAF patients

In experiments with selective inhibitors, $I_{K,ACh}$ was stimulated twice (S1, S2) with 4 min CCh-free period in between. S1 served as internal control, S2 was measured in the presence of inhibitors [5]. Under control conditions $I_{K,ACh}$ was smaller during S2 than S1 suggesting incomplete recovery from desensitisation, but the degrees of $I_{K,ACh}$ desensitisation were similar between the groups (Fig. 2A).

In pAF, the selective $I_{K,ACh}$ channel blocker tertiapin (0.1–100 nM) during S2 reduced the S2/S1 ratio in a concentration-dependent manner without an effect on basal current (Fig. 2B) excluding constitutive $I_{K,ACh}$ activity.

3.3. Regulation of basal current and CCh-activated $I_{K,ACh}$ by protein phosphatases

Regulation of $I_{K,ACh}$ through phosphorylation is a dynamic process that reflects the actual balance between kinase and phosphatase activities at the various sites of phosphorylation in the atrium. Time course of a typical experiment and putative changes in the phosphorylation state of muscarinic receptors and $I_{K,ACh}$ channel is shown in Fig. 3. The balance between phosphorylated muscarinic receptor and $I_{K,ACh}$ channel is controlled by various kinases and phosphatases (bottom panel). Whereas channel phosphorylation increases $I_{K,ACh}$, concomitant phosphorylation of muscarinic receptors reduces $I_{K,ACh}$ (right panel, top). See text for further details.

![Fig. 3. Working model of phosphorylation-dependent $I_{K,ACh}$ regulation in the atrium. Time course of a typical experiment and putative changes in the phosphorylation state of muscarinic receptors and $I_{K,ACh}$ channels. The balance between phosphorylated muscarinic receptor and $I_{K,ACh}$ channel is controlled by various kinases and phosphatases (bottom panel). Whereas channel phosphorylation increases $I_{K,ACh}$, concomitant phosphorylation of muscarinic receptors reduces $I_{K,ACh}$ (right panel, top). See text for further details.](image-url)
signal transduction (Fig. 3). Upon addition of CCh during S1, direct binding of liberated G\(\beta\)\(\gamma\)-subunits to GIRK1/GIRK4 causes rapid activation of \(I_{K, ACh}\) (1) [10] followed by desensitisation, the fast phase of which (green) is associated with channel dephosphorylation (2) [19,20], whereas the intermediate phase (blue) involves progressive receptor phosphorylation by a G-protein-coupled receptor kinase (GRK) which uncouples the receptor from the G-protein (3) [21]. During the CCh-free period (4) channel phosphorylation and receptor dephosphorylation recover only incompletely leading to a smaller CCh-activated \(I_{K, ACh}\) during S2 [5]. Thus, phosphorylation of muscarinic receptors or \(I_{K, ACh}\) channels modulates \(I_{K, ACh}\) in a reciprocal manner, with receptor phosphorylation decreasing and channel phosphorylation increasing current amplitude.

Since total PP1 and PP2A activity is higher in cAF than in SR [13,14] we investigated the contribution of phosphatases to the regulation of \(I_{K, ACh}\), constitutively active and CCh-activated \(I_{K, ACh}\) channels. Selective phosphatase inhibitors were applied 3 min before and during S2 (Fig. 4A). In SR, basal current was affected neither by the PP1/PP2A-inhibitor okadaic acid (1 \(\mu\)mol/L) nor by the calcineurin inhibitor cyclosporin A (10 \(\mu\)mol/L; Fig. 4A,B) and there was no change in RMP with either inhibitor (Fig. 4C). In contrast, inhibition of PP1 and PP2A led to a small but significant reduction of basal current in cAF (Fig. 4A,B), which was

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**Fig. 4.** Effects of phosphatase inhibitors on basal current and CCh-activated \(I_{K, ACh}\). A, Original time course of CCh-activated \(I_{K, ACh}\) and effect of okadaic acid (OA, 1 \(\mu\)mol/L) in both groups. B and C, Mean changes of basal current and RMP by OA and cyclosporin A (CsA, 10 \(\mu\)mol/L) in SR and cAF. D and E, Effects of OA and CsA on the S2/S1 ratio of Peak- and QSS-\(I_{K, ACh}\) and on CCh-induced changes in RMP in either group. Numbers indicate myocytes/patients. *\(P<0.05\) vs. corresponding values in SR or cAF.
associated with a significant change of RMP leading to less negative potentials (Fig. 4C).

Discriminating the contribution of phosphatases to $I_{K,ACH}$ is impeded by concomitant modulation of receptor phosphorylation (Fig. 3). PP2A (but not PP1 and calcineurin) is a component of the M$_2$-receptor complex [11] and PP2A inhibition is expected to shift the balance in favor of receptor phosphorylation by GRK resulting in reduced $I_{K,ACH}$ activation (Fig. 3) [21]. In the presence of okadaic acid, CCh-activated Peak- and QSS-$I_{K,ACH}$ were $\sim$35% lower in SR, whereas cyclosporin A was without effect (Fig. 4D). In cAF, however, okadaic acid did not affect CCh-activated $I_{K,ACH}$ suggesting potentially impaired function of GRK (Fig. 4D). The CCh-induced changes in RMP exhibited large inter- and intra-patient variability (Fig. 1D). Therefore, in analogy to the S2/S1 analysis of CCh-activated $I_{K,ACH}$, the corresponding effects of CCh on RMP were expressed as the ratio of the CCh-induced changes in RMP during S1 and S2, i.e. the CCh-sensitive change in RMP ($\Delta$RMP) during S2 is divided by the corresponding value during S1 ($\Delta$RMP(S2)/$\Delta$RMP(S1)). In SR, the okadaic acid-associated decreases of CCh-activated $I_{K,ACH}$ resulted in corresponding changes in RMP (Fig. 4E).

### 3.4. Regulation of basal current and CCh-activated $I_{K,ACH}$ by protein kinases

In SR, the PKG-inhibitor KT5823 (1 μmol/L) reduced basal current by 3.9±1.1 pA/pF ($n$=9/4), though without significant changes in RMP (Fig. 5A–C). Since basal current in SR consists of $I_{K,1}$ only [5], our results suggest that $I_{K,1}$ requires channel phosphorylation by PKG. The PKC-inhibitor BIM-I (0.1 μmol/L), the CaMKII-inhibitor KN-93 (0.2 μmol/L) and the PKA-inhibitor KT5720 (1 μmol/L) had no effect on basal current in SR (Fig. 5A,B). Accordingly, RMP remained unchanged (Fig. 5C).

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Fig. 5. Effects of kinase inhibitors on basal current and CCh-activated $I_{K,ACH}$ in SR. A, Effects of PKG, PKC, CaMKII and PKA inhibitors (1 μmol/L KT5823, 0.1 μmol/L BIM-I, 0.2 μmol/L KN-93 and 1 μmol/L KT5720, respectively) on basal current. BIM-V (0.1 μmol/L) and KN-92 (0.2 μmol/L) served as negative controls. B and C, Effects of kinase inhibitors on basal current and RMP, respectively. Numbers indicate myocytes/patients. *$P<0.05$ vs. control.
Basal current in cAF involves $I_{K1}$ and constitutively active $I_{K,ACh}$ [5]. Thus any change in basal current could result from impaired regulation of either channel. KT5823 decreased basal current in cAF in the same order of magnitude as in SR confirming the positive regulation of $I_{K1}$ by PKG (Fig. 6A,B). KN-93 and its inactive analog had no effect on basal current or RMP. BIM-I, but not its negative analog, reduced basal current by $3.4 \pm 0.8 \text{ pA/pF} (n=7/3)$ and changed RMP to more positive potentials (Fig. 6A–C).

Lack of contribution of CaMKII, PKC, PKG and PKA to muscarinic receptor phosphorylation allows to investigate the contribution of these kinases to $I_{K,ACh}$ regulation without confounding effects on muscarinic receptor function (Fig. 3) [11,22]. In SR, KT5823, BIM-I and KN-93 reduced Peak- but not QSS-$I_{K,ACh}$. The inactive analogs were without effect (Fig. 7A). The kinase inhibitor-associated decreases of Peak-$I_{K,ACh}$ were paralleled by corresponding changes in RMP (Fig. 7B). Signal transduction via muscarinic receptors does not involve activation of PKA [23] and accordingly KT5720 did not affect CCh-activated $I_{K,ACh}$ (Fig. 7A). In cAF, however, the kinase inhibitors did not modulate CCh-activated $I_{K,ACh}$, and correspondingly, there was no change in RMP (Fig. 7A,B).

### 3.5. Protein levels of PKC isoforms

Levels of proteins were normalized to the tissue amount of calsequestrin, which was similar in SR and cAF (Fig. 8). The protein level of PKCε, but not PKCα, PKCβ1 or PKCδ, was $\sim 40\%$ higher in cAF than in SR (Fig. 8). Compared to SR the cAF patients had larger left atria and more frequently valvular heart disease but comparable other clinical parameters and medications (Supplementary data). There was no significant interaction effect between left atrial diameter or presence of valvular heart disease and cAF on PKCε (not shown). Thus, abnormal PKC activity and specifically PKCε is likely to contribute to constitutive $I_{K,ACh}$ activity.

### 4. Discussion

Evidence for abnormal phosphorylation-dependent regulation of constitutively active and CCh-activated $I_{K,ACh}$ is
provided by several observations: (i) basal current in SR, consisting of $I_{K1}$ only, was not affected by inhibition of protein phosphatases, PKC or CaMKII, whereas in cAF it decreased after inhibition of PKC (but not of CaMKII). (ii) The increased protein abundance of PKCε is likely to contribute to the promotion of constitutive $I_{K,ACh}$ activity by PKC; (iii) selective inhibition of PP1 and PP2A reduced CCh-activated Peak- and QSS-$I_{K,ACh}$ in SR but not in cAF suggesting impaired GRK function; (iv) the selective block of PKG, PKC or CaMKII decreased the CCh-activated Peak-$I_{K,ACh}$ without affecting QSS-$I_{K,ACh}$ only in SR but not in cAF. Our results indicate that the occurrence of constitutive $I_{K,ACh}$ activity in cAF may result from abnormal $I_{K,ACh}$ channel phosphorylation by PKC, whereas the additional muscarinic receptor-mediated $I_{K,ACh}$ activation apparently occurs without significant contribution of PKG, PKC or CaMKII.
4.1. Comparison with previous studies

We have recently shown that $I_{K,ACh}$ develops constitutive activity in cAF patients [5]. In dogs with pacing-induced tachycardia, atria and pulmonary veins develop a similar $I_{K,ACh}$-like component of basal current [6,7]. Blockade of these channels with the selective blocker tertiapin prevents induction of AF episodes [6] suggesting that $I_{K,ACh}$ is a major contributor to initiation and perpetuation of AF. However, pAF patients did not possess a tertiapin-sensitive component of basal current indicating that constitutive $I_{K,ACh}$ activity is a hallmark of cAF, but not of pAF, and thus most likely a consequence rather than a cause of the arrhythmia.

Consistent with ~40% reduction in protein levels of the GIRK1 subunit [24] the muscarinic receptor-activated $I_{K,ACh}$ was ~40% smaller in pAF than in SR. The 10-fold lower potency of tertiapin in pAF (present study) than in cAF [5] suggests also impaired channel regulation. Thus modulation of atrial function by vagal excitation appears limited in pAF patients.

The molecular basis of constitutively active $I_{K,ACh}$ is incompletely understood. In cAF open probability of agonist-independent $I_{K,ACh}$ is higher than in SR without concomitant alterations of other current properties [5]. Lack of effect of the muscarinic receptor blocker atropine suggests also impaired channel regulation. Thus modulation of atrial function by vagal excitation appears limited in pAF patients.

The molecular basis of constitutively active $I_{K,ACh}$ is incompletely understood. In cAF open probability of agonist-independent $I_{K,ACh}$ is higher than in SR without concomitant alterations of other current properties [5]. Lack of effect of the muscarinic receptor blocker atropine demonstrates that constitutive $I_{K,ACh}$ activity is an agonist-independent process [5,7]. Increased receptor-independent dissociation of $\alpha$- and $\gamma$-subunits appears an unlike mechanism because neither pertussis toxin nor absence of GTP affected the $I_{K,ACh}$-like component of basal current in dogs [7]. These findings point to a modified regulation within the macromolecular $I_{K,ACh}$ complex which contains the catalytic subunits of PKA, PKC, and CaMKII, and PP1 and PP2A [10,11]. Thus, the quantitative and/or qualitative composition of this complex may change in cAF leading to abnormal phosphorylation-dependent $I_{K,ACh}$ regulation.

Activation of $I_{K,ACh}$ requires ATP which may modulate the channel through several mechanisms: (i) transphosphorylation between adenosine- and guanosine-nucleosides via nucleoside diphosphate kinase (NDPK) [8]; (ii) generation of phosphatidylinositol-4,5-bisphosphate (PIP2) via hydrolysis of ATP [25] and (iii) direct phosphorylation of the channels and/or their regulators by protein kinases [10,19]. Recent work, however, challenged the NDPK hypothesis as explanation for agonist-independent channel activation [26]. Although the levels of high-energy phosphates remain stable in cAF [27], high membrane levels of PIP2 underlying the constitutive $I_{K,ACh}$ activity cannot be excluded. The phosphorylation state of muscarinic receptors and $I_{K,ACh}$ channels is dynamically regulated by different kinase and phosphatase subtypes. Consistent with results in animals [19] inhibition of PP1/PP2A reduced CCh-activated $I_{K,ACh}$ in SR suggesting that the shift in the kinase/phosphatase balance to higher channel phosphorylation cannot compensate the stronger muscarinic receptor phosphorylation thereby limiting the activation of $I_{K,ACh}$ (Fig. 3). In contrast PP1/PP2A inhibition was without effect on CCh-activated $I_{K,ACh}$ in cAF indicating abnormal function of GRK.

The regulation of muscarinic receptor function does not involve phosphorylation by CaMKII, PKC and PKA (Fig. 3) [22]. This allows to investigate the contribution of these kinases to channel regulation without confounding effects on muscarinic receptor function. The GIRK1 and GIRK4 channel subunits possess phosphorylation sites for PKA, PKC, and CAMKII and possibly PKG [10,11] and regulation of CCh-activated $I_{K,ACh}$ was modulated by PKC, CaMKII and PKG but not by PKA. In SR, the kinase...
inhibitors reduced CCh-activated Peak-I_{K,ACH} without an effect on QSS-I_{K,ACH}. Since Peak-I_{K,ACH} reflects the direct binding of the Gi/γ-subunits to the channel and the strength of this binding depends on the degree of channel phosphorylation (Fig. 3) [10], PKG-, PKC- and CaMKII-mediated channel phosphorylation may stabilize the binding of Gi/γ-subunits to the channel increasing I_{K,ACH}. In contrast, the kinase inhibitors were without effect on CCh-activated I_{K,ACH} in cAF. This was unexpected, because the protein levels of CaMKIIßδ and PKCε are higher in cAF than in SR [28]. Also, we consistently found that the activity of PP1 and PP2A is higher in cAF than in SR and does not translate into homogeneous changes of protein phosphorylation [13,14]. Thus the lack of contribution of these kinases to CCh-activated I_{K,ACH} in cAF may result from a stronger channel dephosphorylation (Fig. 3). Alternatively, the channels may be hyperphosphorylated due to abnormal control of kinase/phosphatase signaling in the macromolecular complex [14] and inhibition of a single kinase may not reduce the channel’s phosphorylation state below the threshold required to impair CCh-activated I_{K,ACH}. Further work is needed to verify these hypotheses.

Inhibition of PP1/PP2A and calcineurin, and several protein kinases (PKA, PKC and CaMKII) did not affect basal current in SR. Phosphorylation of I_{K,1} channels by protein kinases results in current inhibition [29,30] and the PKG-inhibitor KT5823 reduced basal current in SR and cAF. This inhibition, however, was of similar magnitude in both groups suggesting potential block of I_{K,1} channels. In the kidney, PKG increases the amplitude of inward rectifier K\(^+\) current [31] pointing to the involvement of PKG in I_{K,1} regulation. Further studies are required to clarify the role of PKG in I_{K,1} regulation.

The CaMKII-inhibitor KN-93 had no effect on basal current in cAF rendering the contribution of CaMKII to either I_{K,1} or constitutively active I_{K,ACH} channels unlikely. Surprisingly, inhibition of PKC with BIM-I reduced basal current in cAF by ∼3.5 pA/pF. In human atria activation of Gqα-coupled receptors results in translocation of PKCδ and PKCe (but not PKCα or PKCβ) to the membrane [32], and the protein levels of PKCe are higher in cAF than in SR. Thus, the inhibition of basal current by BIM-I in cAF may result from abnormal contribution of PKCε. Since BIM-I was not effective on basal current in SR (only I_{K,1} present) and activation of PKC inhibits human I_{K,1} [33], the BIM-I-associated decrease in basal current in cAF must involve impaired regulation of constitutively active I_{K,ACH}. Thus while development of constitutively active I_{K,ACH} in cAF requires abnormal channel phosphorylation by PKC, the additional muscarinic receptor-mediated I_{K,ACH} activation apparently does not need the contribution of PKG, PKC or CaMKII.

4.2. Study limitations

Our results are not consistent with data from expression systems and neonatal myocytes where the increase in PKC activity inhibits I_{K,ACH} [11,34]. The reason for this inconsistent observation is unknown, though artificial systems lack important endogenous regulators, and the composition of the I_{K,ACH} channel differs between neonatal and adult myocytes [35]. The predominant subunit in neonatal rat myocytes is GIRK4 [35], whereas in diseased human atria GIRK1 predominates (unpublished data, 2002). Thus, species differences and the concomitant cardiac disease and/or patients’ medication could also contribute to the discrepant findings.

We studied the contribution of serine/threonine kinases only. However, mitogen-activated protein kinases (MAPKs) may also contribute to the regulation of I_{K,ACH}. PKCe is known to activate ERK1/2 [36,37] and may stimulate JNK and p38 [37] through an indirect increase of PKCδ [38]. Thus, we cannot exclude that MAPKs regulate I_{K,ACH} and that the effects of PKC on constitutively active I_{K,ACH} are mediated by stimulation of MAPKs. An inactive analog of KTS823 was not available to us. Thus, our results with this drug might be confounded by non-specific effects on I_{K,1}.

4.3. Clinical implications

In vivo, constitutively active I_{K,ACH} is expected to shorten action potential duration, to enhance atrial vulnerability to tachyarrhythmia and to sustain AF [1]. Here we demonstrate that the molecular basis of constitutively active I_{K,ACH} in cAF may involve abnormal phosphorylation-dependent regulation by PKC. To the best of our knowledge this is the first demonstration of increased PKCe expression in cAF. This may contribute to contractile dysfunction [39,40], hypertrophy and fibrosis, however, proof of these hypotheses warrants further investigations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cardiores.2007.02.009.

References


