Differential Protein Kinase C Isoform Regulation and Increased Constitutive Activity of Acetylcholine-Regulated Potassium Channels in Atrial Remodeling

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Rationale: Atrial fibrillation (AF) causes atrial-tachycardia remodeling (ATR), with enhanced constitutive acetylcholine-regulated K\(^+\) current (I\(\text{K}_\text{AChc}\)) contributing to action potential duration shortening and AF promotion. The underlying mechanisms are unknown.

Objective: To evaluate the role of protein-kinase C (PKC) isoforms in ATR-induced I\(\text{K}_\text{AChc}\) activation.

Methods and Results: Cells from ATR-dogs (400-bpm atrial pacing for 1 week) were compared to control dog cells. In vitro tachypaced (TP; 3 Hz) canine atrial cardiomyocytes were compared to parallel 1-Hz paced cells. I\(\text{K}_\text{AChc}\) single-channel activity was assessed in cell-attached and cell-free (inside-out) patches. Protein expression was assessed by immunoblot. In vitro TP activated I\(\text{K}_\text{AChc}\), mimicking effects of in vivo ATR, Discrepant effects of PKC activation and inhibition between control and ATR cells suggested isoform-selective effects and altered PKC isoform distribution. Conventional PKC isoforms (cPKC; including PKC\(\alpha\)) inhibited, whereas novel isoforms (including PKC\(\varepsilon\)) enhanced, acetylcholine-regulated K\(^+\) current (I\(\text{K}_\text{AChc}\)) in inside-out patches. TP and ATR downregulated PKC\(\alpha\) (by 33% and 37%, respectively) and caused membrane translocation of PKC\(\varepsilon\), switching PKC predominance to the stimulatory novel isoform. TP increased [Ca\(^{2+}\)]\(_i\) at 2 hours by 30%, with return to baseline at 24 hours. Buffering [Ca\(^{2+}\)]\(_i\) during TP with the cell-permeable Ca\(^{2+}\) chelator BAPTA-AM (1 \(\mu\)mol/L) or inhibiting the Ca\(^{2+}\)-dependent protease calpain with PD150606 (20 \(\mu\)mol/L) prevented PKC\(\varepsilon\) downregulation and TP enhancement of I\(\text{K}_\text{AChc}\). PKC\(\varepsilon\) inhibition with a cell-permeable peptide inhibitor-suppressed TP/ATR-induced I\(\text{K}_\text{AChc}\) activation, whereas cPKC inhibition enhanced I\(\text{K}_\text{AChc}\) activity in 1-Hz cells.

Conclusions: PKC isoforms differentially modulate I\(\text{K}_\text{AChc}\) with conventional Ca\(^{2+}\)-dependent isoforms inhibiting and novel isoforms enhancing activity. ATR causes a rate-dependent PKC isoform switch, with Ca\(^{2+}\)/calpain-dependent downregulation of inhibitory PKC\(\alpha\) and membrane translocation of stimulatory PKC\(\varepsilon\), enhancing I\(\text{K}_\text{AChc}\). These findings provide novel insights into mechanisms underlying I\(\text{K}_\text{AChc}\) dysregulation in AF. (Circ Res. 2011;21:00-00.)

Key Words: atrial tachycardia • protein kinase C isoforms • remodeling
protein kinases (PKs). Indirect evidence suggests that in AF patients, PKCε may contribute to I_{K_{AChC}} activation. The present study aimed to investigate the molecular basis of ATR-induced I_{K_{AChC}} channel activation in a dog model.

### Subjects and Methods

#### Animal Model

A total of 100 mongrel dogs (20–36 kg) were divided into control and ATR groups (including both preliminary studies and work presented in the article). Animal care procedures followed National Institutes of Health guidelines (publication 85–23, revised 1996) and were approved by the Animal Research Ethics Committee of the Montreal Heart Institute. The animal model was prepared as previously described in detail. Briefly, the atrioventricular node was ablated by radiofrequency-application. The right ventricle was paced at 80 bpm and the right atrium was paced at 400 bpm for 7 days. Open chest study was performed as previously described. On study days, dogs were anesthetized (morphine 2 mg · kg⁻¹ · h⁻¹ intravenous load, 29.25 mg · kg⁻¹ · h⁻¹ infusion) and mechanically ventilated. Right atrial effective refractory period was measured at basic cycle lengths of 150, 200, 250, 300, and 360 ms with 10 basic (S1) stimuli, followed by an S2 with 5-ms decrements (all pulses twice-threshold, 2 ms). AF (irregular atrial rhythm >400 bpm) was induced with 1- to 10-second atrial burst pacing (10–20 Hz, 4×threshold, 2-ms pulses). Mean AF duration was determined in each dog based on 10 AF inductions for AF <5 minutes and five inductions for 5 to 30 minutes of AF. AF >30 minutes was considered sustained; cardioversion was not performed and electrophysiological assessment was terminated (On-line Table I shows in vivo hemodynamic and electrophysiological data, available at http://circres.ahajournals.org).

#### Atrial Cardiomyocyte Isolation

Hearts and adjacent lung tissues were excised via a left thoracotomy and immersed in oxygenated Tyrode solution (mmol/L): NaCl, 136; KCl, 5.4; CaCl₂, 2; MgCl₂, 1; NaH₂PO₄, 0.33; glucose, 10; and HEPES 5 (pH 7.4, NaOH). Left atrial (LA) cardiomyocytes were isolated as previously described. Briefly, the proximal left circumflex coronary artery was cannulated and the LA was perfused with Ca²⁺-free Tyrode solution containing collagenase (100 U · mL⁻¹, Worthington type II). The cells were then either kept for immediate electrophysiology studies in storage solution (KCl 20, KH₂PO₄ 10, glucose 25, potassium glutamate 70, β-hydroxybutyric acid 10, taurine 20, EGTA 10, albumin 0.1% mannitol 40; pH 7.4, KOH) at 4°C or kept at 37°C in culture medium (M199 medium supplemented with 1% insulin-transferrin-selenium and 1% penicillin/streptomycin) for in vitro tachypacing. Cell aliquots were also snap-frozen for subsequent biochemical studies.

#### Culture and Tachystimulation

Freshly isolated LA cardiomyocytes from control animals were plated onto laminin-coated (20 μg/mL) 4-well culture dishes (Nunc) and maintained at 37°C, 95% O₂/5% CO₂. After 2-hour incubation, dead and unattached cardiomyocytes were removed and fresh medium was added. Cells were then paced at 1 or 3 Hz (studied in parallel for all experimental series) for 2 or 24 hours with square wave 5-ms pulses. In some experiments, 1 μmol/L BAPTA-AM or 20 μmol/L PD15060613 were added to the culture medium during 24-hour pacing or 10 μmol/L PKCε peptide inhibitor (ε-V1–2) or conventional PKC (cPKC) peptide inhibitor (β-C2–4) 14,15 for 1 hour before electrophysiological recording were used. After cell culture/pacing, cells were subjected to electrophysiological study or fast-frozen for subsequent biochemical study.

#### Single-Channel Recording

Single-channel currents were recorded in cell-attached and inside-out configurations (Axopatch 200A, Axon). Patch pipettes were prepared from 1.0-mm optical diameter borosilicate glass (Sutter Instruments), coated at the tip with Sylgard, and fire-polished with a microforge. When filled with pipette solution (mmol/L: KCl, 145; MgCl₂, 1; CaCl₂, 2; HEPES, 5; pH 7.4, KOH), tip resistances were approximately 8 to 10 mol/L·Ω and approximately 3 to 4 mol/L·Ω for cell-attached and inside-out configurations, respectively.

Bath solutions for cell-attached/patch studies contained (mmol/L: NaCl, 120; KCl, 20; MgCl₂, 1; CaCl₂, 2; glucose, 10; HEPES, 10, pH 7.4, NaOH). Bath (intracellular) solutions for inside-out studies contained (mmol/L) KCl 145, MgCl₂ 2 EGTA 2, and HEPES 5 (pH 7.4, Tris). Inside-out configuration was confirmed by: (1) persistence of gigaseal after membrane excision; (2) sporadic I_{K_{AChC}} openings; (3) inhibition of ATP-sensitive channels by ATP; and (4) reactivation of I_{K_{AChC}} channels by bath application of ATP and GTP or GTPγS. I_{K_{AChC}} rundown in inside-out patches was limited by adding (mmol/L) fluoride 5, vanadate 0.1, and pyrophosphate 10 to bath solutions. All recordings were performed at room temperature. Single-channel activity was recorded with previously detailed methods while holding at −100 mV. Electric noise was reduced by low-pass filtering at 1 kHz. ISO-2 software (MFk) was used for data acquisition and analysis.

#### Single-Channel Analysis

Data were digitized at 10 kHz. Amplitude and kinetic analyses were performed after leak current subtraction. Amplitude-histograms were fitted with Gaussian curves for open and closed channel states. Channel openings and closings were idealized by the 70% threshold method. The nPᵢ was calculated from idealized recordings of twenty...
Drugs
Chemicals were obtained from Sigma Chemicals unless otherwise indicated. Stock solutions of bisindolylmaleimide-I (0.1 mmol/L; Merck), diacylglycerol-kinase-inhibitor-I (R59022; 1 mmol/L), PD150606 (20 mmol/L), carbachol (CCh; 10 mmol/L), phenylephrine (1 mmol/L), BAPTA-AM (1 mmol/L), and phorbol-12-myristate-13-acetate (PMA; 0.1 mmol/L) were stored at -20°C until use.

Protein separations were performed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. Membranes were blocked in phosphate-buffered saline solution containing 0.05% (volume/volume) Tween-20 and 5% (weight/volume) nonfat dried milk. Membranes were incubated overnight at 4°C with primary antibodies diluted in phosphate-buffered saline containing 0.05% Tween-20 and 1% nonfat dried milk. After washing with phosphate-buffered saline Tween solution/1% nonfat dried milk, membranes were hybridized with horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were detected by ECL with BioMax MS films. Protein quantification was performed with Quantity-One software (Biorad) and expressed relative to GAPDH staining for the same samples on the same gels. For membrane localization experiments, the intensity of the cytosolic and particulate (membrane) immunoreactive bands were determined and normalized to the sum of their intensity (total content) for a specific sample. Data are expressed as percentages of total immunoreactivity.

Calcium Transients
Isolated cardiomyocytes were incubated with Indo-1 AM and pluronic F-127 (5 μmol/L and 100 μmol/L; Invitrogen) for 5 minutes and then superfused with Tyrode solution containing 1.8 mmol/L Ca2+ and then superfused with Tyrode solution containing 1.8 mmol/L Ca2+, 150 mmol/L NaCl, 25 mmol/L Tris, 5 mmol/L EGTA, 5 mmol/L MgCl2, 5 mmol/L KCl, 1 mmol/L Na2-2HPO4, 0.2 mmol/L Na3VO4, 20 mmol/L β-glycerophosphate, 0.5 mmol/L PMSF, 0.2 mmol/L aprotinin, 10 μg/mL pepstatin, 1 μg/mL leupeptin, 50 μg/mL microcin-LR, 1 mmol/L peptstatin, and Triton-X-100 1% (pH 7.34, HCl). After 30-minute incubation, homogenized samples were centrifuged (48 000 rpm, 10 minutes) to pellet debris and nuclei. Supernatant was collected. The pellet was resuspended in buffer-A supplemented with 1% Triton-X-100 (volume/volume) and incubated for 30 minutes on ice. Ultracentrifugation was again performed at 48 000 rpm. This second supernatant (particulate fraction) containing detergent-soluble proteins was collected.

Immunoblot: Protein Extraction
Fifteen isolated LA cardiomyocytes were pelleted, fast-frozen in liquid nitrogen, and homogenized in an extraction buffer containing Tris 25 mmol/L, EDTA 5 mmol/L, EGTA 5 mmol/L, NaCl 150 mmol/L, NaF 20 mmol/L, Na3VO4 0.2 mmol/L, β-glycerophosphate 20 mmol/L, PMSF 0.5 mmol/L, leupeptin 25 μg/mL, aprotinin 10 μg/mL, pepstatin 1 μg/mL, microcin-LR 1 mmol/L, and Triton-X-100 1% (pH 7.34, HCl). After 30-minute incubation, homogenized samples were centrifuged (3000 rpm, 10 minutes) to pellet debris and nuclei. Supernatant was collected and protein concentration was assayed with Bradford assay (Biorad). All protein samples on the same gels. For membrane localization experiments, the intensity of the cytosolic and particulate (membrane) immunoreactive bands were determined and normalized to the sum of their intensity (total content) for a specific sample. Data are expressed as percentages of total immunoreactivity.

Antibodies
Primary antibodies (1/2000) included polyclonal rabbit anti-PKCα (2683) from Cell Signaling, polyclonal rabbit anti-PKCβ (AB4132, ABCAM), polyclonal rabbit anti-PKCδ (sc-937; Santa Cruz), and monoclonal mouse anti-GAPDH (10R-G109a; Fitzgerald). Peroxidase-conjugated AffiniPure goat anti-rabbit IgG (11-035-144) and AffiniPure donkey anti-mouse IgG (70-035-151) from Jackson Immunoresearch were used as secondary antibodies.

Results
Constitutively Active I_{KCa} Channels
Online Figure IA (available at http://circres.ahajournals.org) shows typical cell-attached single-channel recordings. In the absence of cholinergic stimulation, constitutive I_{KCa} openings (I_{KCa}) occurred only sporadically in control cardiomyocytes. I_{KCa} openings were much more frequent in ATR cells. ATR did not affect single-channel conductance (Online Figure IB, available at http://circres.ahajournals.org), but increased open probability and frequency (Online Figure IC and D, available at http://circres.ahajournals.org). Mean open time was unaffected (Online Figure IE, available at http://circres.ahajournals.org). Cell tachypacing in vitro mimicked ATR effects (Online Figure II, available at http://circres.ahajournals.org).

Preparation of Cytosolic and Particulate Fractions
LA cardiomyocytes were pelleted and fast-frozen immediately after isolation. Cells were disrupted and homogenized using a Polytron device in 1 mL of ice-cold extraction buffer-A containing (mmol/L): HEPES, 20 (pH=7.5); EDTA, 5; EGTA, 5; NaF, 20; Na3VO4, 0.2; β-glycerophosphate, 20; benzamidine, 10; AEBSF, 0.5; leupeptin, 25 μg/mL; and DTT, 5. Homogenates were centrifuged (48 000 rpm, 30 minutes). The supernatant corresponding to the cytosolic fraction was collected. The pellet was resuspended in buffer-A supplemented with 1% Triton-X-100 (volume/volume) and incubated for 30 minutes on ice. Ultracentrifugation was again performed at 48 000 rpm. This second supernatant (particulate fraction) containing detergent-soluble proteins was collected.
Inside-Out Patches and I\textsubscript{KAChC}: Phosphorylation Dependence

After excision of the patch and formation of the inside-out configuration, constitutive I\textsubscript{KAChC} activity of ATR cardiomyocytes was strongly reduced (−93.8% ± 1.7%; n = 16/4; Figure 1A, middle panel) to a level not significantly different from control cardiomyocytes, pointing to contribution of a cytosolic factor to ATR-associated I\textsubscript{KAChC} increase. Under these conditions, the application of ATP and GTP (0.1 μmol/L and 3 mmol/L, respectively) was insufficient to restore open probabilities (Figure 1A, right panel) to levels seen in cell-attached configurations. Nevertheless, a statistically significant difference between control and ATR re-emerged, pointing to a role for channel phosphorylation in upregulation of constitutive function by ATR.

Previous studies of agonist-activated I\textsubscript{KACh} revealed that inhibition of phosphatases is necessary to prevent run-down in cell-free inside-out-patches.\textsuperscript{8,17,18} In addition, indirect evidence suggests that in AF patients PKC channel phosphorylation may contribute to constitutive I\textsubscript{KACh} increase. Under these conditions, the application of ATP and GTP (0.1 μmol/L and 3 mmol/L, respectively) was insufficient to restore open probabilities (Figure 1A, right panel) to levels seen in cell-attached configurations. Nevertheless, a statistically significant difference between control and ATR re-emerged, pointing to a role for channel phosphorylation in upregulation of constitutive function by ATR.

PKC-Dependent Regulation of I\textsubscript{KAChC} in Control and ATR Cardiomyocytes

Physiologically, PKC is activated by stimulation of G\textsubscript{q} coupled receptors that activate phospholipase C. Phospholipase C hydrolyzes PIP\textsubscript{2} into inositol trisphosphate and DAG, which directly activates PKC.\textsuperscript{22–25} To control for possible time-dependent changes in I\textsubscript{KAChC}, all experiments involving pharmacological interventions included time-matched controls for comparison. To study the effect of PKC on I\textsubscript{KAChC}, we activated PKC by PMA (0.1 μmol/L), which mimics the action of DAG and nonselectively activates conventional as well as novel PKC isoforms. Consistent with previous studies showing that PKC-mediated I\textsubscript{KACh} phosphorylation decreases I\textsubscript{KACh} activity,\textsuperscript{26} PKC activation decreased I\textsubscript{KAChC} in control cells (Figure 2A, left). In contrast, for ATR cardiomyocytes, isoform-nonselective PKC activation significantly increased I\textsubscript{KAChC} open probability (Figure 2A, right panel). Similar enhancement of ATR cardiomyocyte I\textsubscript{KAChC} open probability was produced by increasing membrane DAG by inhibiting DAG kinase (Online Figure III, available at http://circres.ahajournals.org). Isoform-nonselective PKC inhibition with BIM-I (0.1 μmol/L; Figure 2B) also had distinct effects on ATR (significant reduction in open probability) versus controls (no change). These data suggest differential regulation of I\textsubscript{KAChC} by PKC in control versus ATR.
Isoform-Selective PKC Regulation of \( I_{K_{ACh}} \)

The PKC family contains many isoforms, including conventional isoforms (cPKCs), which are activated by Ca\(^{2+}\) and DAG, and novel isoforms (novel PKCs [nPKCs]), which require DAG, but not Ca\(^{2+}\), for activation. To dissect the effects of conventional versus novel isoforms, we applied recombinant isoforms of cPKCs and nPKCs to the intracellular membrane side of \( I_{K_{ACh}} \) channels in inside-out cell-free patches. GTP\(\gamma\)S (100 nmol/L) was added to augment \( I_{K_{ACh}} \) in control conditions to a level at which inhibitory effects could be appreciated (Figure 3). Application of conventional PKC isoforms (\( \alpha, \beta \), and \( \beta^\prime \)) to excise inside-out patches significantly reduced \( I_{K_{ACh}} \) in both control (Figure 3A, left panel) and ATR cardiomyocytes (Figure 3A, right panel). Novel PKC isoforms (\( \delta \) and \( \epsilon \)) activated \( I_{K_{ACh}} \) in both control (Figure 3B, left panel) and ATR (Figure 3B, right panel) cardiomyocytes in contrast to the well-known inhibitory effects of conventional isoforms on \( I_{K_{ACh}} \). Control experiments with scrambled PKC\(\alpha\) and PKC\(\epsilon\) showed no effect (Online Figure IV, available at http://circres.ahajournals.org).

Tachycardia-Induced PKC Isoform Protein Expression Changes

The results described indicate that cPKC isoforms inhibit, whereas nPKC isoforms increase, \( I_{K_{ACh}} \). In control cardiomyocytes, the cPKC (inhibitory) phenotype seems to predominate, whereas in ATR the balance is shifted, with stimulatory effects of nPKC isoforms enhancing constitutive \( I_{K_{ACh}} \) activity. We therefore compared PKC isoform expression in control versus ATR to determine whether the changes in function may have a basis in altered isoform expression. Figure 4A shows representative immunoblots and densitometric analyses of PKC isoform expression in isolated LA cardiomyocytes. The results show 37% lower expression levels for PKC\(\alpha\), but unchanged expression of other PKC isoforms, in ATR. These data suggest that reduced PKC\(\alpha\) expression may underlie the reduced cPKC role in ATR, but do not provide an explanation for the apparently increased effects of nPKCs. We wondered whether changes in subcellular PKC distribution could contribute to the changed PKC isoform response in ATR, because PKC translocation to the

Figure 2. Effect of protein kinase C (PKC) modulation on constitutive acetylcholine-regulated \( K^+ \) current (\( I_{K_{ACh}} \)). Representative recordings of \( I_{K_{ACh}} \) at baseline (0 minutes) and after application of 4\(\alpha\)-phorbol-12-myristate-13-acetate (PMA; 100 nmol/L; B), which mimics diacylglycerol (DAG) effects (A) or PKC inhibitor bisindolylmaleimide-I (BIM-I; 100 nmol/L; B), respectively. Lower parts show corresponding mean \pm standard error of the mean (SEM) open probability (\( P_o \)), together with time-matched controls (TMC). **\( P < 0.01 \), ***\( P < 0.001 \) vs corresponding baseline (0 minutes) values; **\( P < 0.01 \), ***\( P < 0.001 \). Numbers indicate myocytes/dogs.
membrane accompanies activation. We therefore performed immunoblot experiments on separated cytosolic and particulate (membrane) fractions from LA cardiomyocytes (Figure 4B). The relative expression of PKCα, PKCβII, and PKCδ in membrane and cytosolic fractions was not significantly altered by ATR. However, ATR increased the relative membrane expression of PKCε, pointing to translocation of PKCε from the cytosol. Because membrane translocation is needed for PKC phosphorylation of membrane proteins, this finding may explain the predominance of the nPKC (stimulatory) phenotype in ATR cardiomyocytes.

In vitro tachypacing activated I_{KACCH}, mimicking the effects of ATR (Online Figures I and II). We analyzed the PKC isoform expression changes associated with ATR in vivo and found that similar changes occur with in vitro LA cardiomyocyte tachypacing: total PKCα expression is downregulated with no change in PKCε expression (Figure 5B), whereas PKCs shows membrane redistribution and PKCα cytosolic/membrane portioning is unchanged (Figure 5C). Many manipulations required to further study the mechanisms of I_{KACCH} activation are impossible in vivo but feasible in the in vitro tachypaced system, which we therefore exploited.

Role of Tachycardia and Ca^{2+} Loading
Cell tachycardia induces atrial remodeling, with cellular Ca^{2+} loading implicated in several key signaling-pathways. We examined intracellular Ca^{2+} ([Ca^{2+}]_{i}) changes in our tachypaced cardiomyocytes and observed significant transient increases (Figure 6A, B), as previously reported. We therefore examined the role of increases in [Ca^{2+}]_{i} in mediating tachypacing effects on I_{KACCH}. Increasing cell Ca^{2+} loading by doubling the extracellular [Ca^{2+}]_{i} during the 24-hour pacing period activated I_{KACCH} in 1-Hz paced cells, mimicking the effects of ATR (Figure 6C). Buffering [Ca^{2+}]_{i} with the cell-permeable buffer BAPTA-AM during tachypacing suppressed I_{KACCH} activation (Figure 6D) and prevented PKCα downregulation (Figure 6E), while failed to prevent increased PKCε membrane localization (Figure 6F). Calpain is a Ca^{2+}-activated enzyme that cleaves cell proteins and can mediate effects of ATR. Inclusion of the cell-permeable highly selective calpain inhibitor PD 150606 attenuated I_{KACCH} activation (Figure 6G) and prevented PKCα down-regulation (Figure 6H) with cell tachypacing, pointing to calpain as a possible mediator of tachycardia-induced reductions in PKCα expression.

Effects of PKC Isoform-Specific Inhibition
The experiments described suggest that tachycardia activates I_{KACCH} by suppressing cPKC inhibiting effects and by enhancing nPKC activating effects on channel open probability. To assess the role of these isoforms more directly, we applied membrane-permeable isoform-selective peptide inhibitors, myristoylated forms of the cPKC inhibitor peptide -C2–4 and PKCε inhibitor peptide ε-V1-2, both during the last hour of pacing and in the bath. The cPKC inhibitor peptide significantly increased I_{KACCH} activity in 1-Hz paced cells, consistent with a tonic inhibitory influence of PKCα, but had little effect on 3-Hz paced cells, consistent with PKCα down-regulation (Figure 7A, left). However, the PKCε inhibi-
itor peptide did not significantly affect $I_{\text{KAC}}$ in 1-Hz paced cells, but suppressed $I_{\text{KAC}}$ in 3-Hz paced cells (Figure 7A, right). Corresponding effects were observed in cells isolated from control and ATR dogs (Figure 7B). These experiments confirm directly the predominant influence on $I_{\text{KAC}}$ of cPKC-mediated inhibition under control conditions and of PKCδ-mediated stimulation with ATR.

We previously reported that phenylephrine inhibits $I_{\text{KAC}}$ by a PKC-dependent mechanism. We exposed canine cardiomyocytes to maximally effective phenylephrine concentrations (1 mmol/L) and assessed the effect in the absence and presence of the cPKC inhibitory peptide. In the absence of cholinergic stimulation, $I_{\text{KAC}}$ open probability was very low and, even though mean $P_o$ was smaller after phenylephrine (Online Figure VA, available at http://circres.ahajournals.org), no statistically significant decrease was seen. After the addition of carbachol to activate $I_{\text{KAC}}$, clear statistically significant $P_o$ decreases were seen with phenylephrine (Online Figure VB, available at http://circres.ahajournals.org). In both situations, when phenylephrine was superfused in the presence of β-C2-4, no significant change was seen compared to results with β-C2-4 alone, indicating that the effects of phenylephrine are attributable to conventional PKC isoform activation.

**Discussion**

Studies in animal models and atrial cardiomyocytes from AF patients suggest that sustained rapid atrial activation increases the function of a constitutively active form of $I_{\text{KAC}}$. Because increased inward rectifier currents like $I_{\text{K1}}$ and $I_{\text{KAC}}$ abbreviate atrial action potential duration and remove Na⁺ current inactivation, they stabilize and accelerate high-frequency atrial reentrant sources. There is experimental evidence that enhanced constitutive $I_{\text{KAC}}$ activity contributes substantially to the maintenance of tachyarrhythmias in ATR-remodeled atria, and a highly selective $I_{\text{KAC}}$ inhibitor suppresses AF in ATR hearts.

In this study, we show for the first time to our knowledge that novel PKC isoforms activate $I_{\text{KAC}}$, which is in contrast to the well-known inhibitory PKC effect that we show to be attributable to conventional PKC isoforms. We also found...
that changes in the balance between the effects of PKC isoforms contribute to the increased constitutive I_{K_{AChC}} activity of ATR remodeled atrial cardiomyocytes: under control conditions, the cPKC (inhibitory) phenotype predominates, whereas in ATR the nPKC (stimulatory) phenotype prevails. At the molecular level, reduced expression of the cPKC isoform PKC\(_{\alpha}/H9251\) and increased membrane localization of the nPKC isoform PKC\(_{\varepsilon}/H9255\) appear to underlie the altered cPKC/nPKC balance in ATR. Isolated cell tachypacing results indicate that rapid cell firing per se is enough to alter PKC isoform balance and activate I_{K_{AChC}}, and that cellular Ca\(^{2+}\) loading is a key mediator, acting primarily by downregulating PKC\(_{\alpha}\) expression.

**Comparison With Previous Studies of Constitutive I_{K_{AChC}} and Its Regulation**

It is now well-established that ATR is associated with increased basal inward rectifier current in the absence of muscarinic receptor agonists.\(^{33-37}\) Although this change was initially attributed to upregulation of the background inward rectifier I_{K1}, more recent studies have shown a significant contribution of an inward rectifier current (I_{K_{AChC}}) with
properties of type 2 muscarinic-cholinoreceptor–dependent $I_{\text{KACb}}$ that is constitutively active in the absence of agonists.\textsuperscript{2–4} Consistent with previous work,\textsuperscript{5} we found here that tachycardia-induced enhancement of $I_{\text{KACb}}$ is related to increased open channel probability, without alterations in channel conductance or mean open time.

Little is known about the molecular control of constitutive $I_{\text{KACb}}$ activity in atrial cardiomyocytes. The similar changes in ionic current and single-channel properties of $I_{\text{KACb}}$ in human chronic AF and in the ATR dog model suggest a common molecular basis.\textsuperscript{2–5} The complex regulation of $I_{\text{KACb}}$ points to several possible mechanisms that could contribute to the development of constitutive activity. G\textsubscript{βγ} subunits activate $I_{\text{KACb}}$ channels by strengthening the interaction of PIP\textsubscript{2} with the channel subunits.\textsuperscript{18} In addition, development of constitutive $I_{\text{KACb}}$ activity in Kir3-overexpressing Xenopus oocytes is dependent on G\textsubscript{βγ} subunits.\textsuperscript{38–40} However, rate-related constitutive $I_{\text{KACb}}$ activity occurs independently of changes in muscarinic cholinoreceptor expression and the dissociation of G\textsubscript{α} and G\textsubscript{βγ} subunits.\textsuperscript{2–3} Accordingly, in the presence of phosphatase inhibitors, $I_{\text{KACb}}$ was only slightly reduced after inside-out formation and washout of ATP and GTP (Figure 1B), which are necessary for G-protein dissociation.

Because activation of $I_{\text{KACb}}$ requires ATP,\textsuperscript{5} modified phosphorylation-dependent channel regulation could contribute to constitutive $I_{\text{KACb}}$ activity. Supporting this idea, we found that in inside-out patches constitutive activity in the absence

Figure 6. Rate regulation of constitutive acetylcholine-regulated K\textsuperscript{+} current ($I_{\text{KACb}}$). A, Ca\textsuperscript{2+} transient recordings from cardiomyocytes subjected to 2-hour or 24-hour stimulation. B, Systolic and diastolic [Ca\textsuperscript{2+}]\textsubscript{i}. ***$P<0.001$. C, $I_{\text{KACb}}$ open probability in cells subjected to 24-hour stimulation at 1 Hz with 2 or 4 mmol/L [Ca\textsuperscript{2+}]\textsubscript{i}. ***$P<0.001$. Effect of 1-μmol/L BAPTA-AM during 24-hour 1-Hz and 3-Hz pacing on constitutive acetylcholine-regulated K\textsuperscript{+} current ($I_{\text{KACb}}$) open probability (D), protein kinase C (PKC)\textsubscript{α} expression (E) and PKC\textsubscript{α} distribution (F). Effect of 20-μmol/L PD 150606 during 1-Hz and 3-Hz pacing on $I_{\text{KACb}}$ open probability (G) and PKC\textsubscript{α} expression (H). ***$P<0.001$, ****$P<0.001$ control vs treatment. ##$P<0.001$, ###$P<0.001$ (1 Hz vs 3 Hz). All data are mean±standard error of the mean (SEM).
of exogenous ATP and GTP was only observed after inhibition of protein phosphatases (Figure 1). Previous publications from our group showed that β1-adrenoceptor–mediated activation of PKA increases constitutive IKACh.23 Voigt et al10 compared the effect on IKACh of inhibiting CaMKII, PKA, PKC, and PKG in cardiomyocytes from sinus rhythm versus AF patients. The only differential effect observed was that of PKC inhibition, which produced greater effects in AF patient than in sinus rhythm patient cells. Previous studies in expression systems,26,41 neonatal rat cardiomyocytes,27 and canine atrial cardiomyocytes23 showed inhibitory effects of PKC phosphorylation on IKACh channels, contrasting with the indirect evidence for increased IKACh associated with PKC activation in human atrial cardiomyocytes. Our findings indicate that this discrepancy is attributable to differential expression of cPKC and nPKC isoforms, which we found to exert divergent effects on IKACh activity.

Novel Findings and Potential Significance

The present study constitutes the first detailed assessment of the differential molecular regulation of constitutive IKACh channel function in remodeled atria. Figure 8 provides a schematic representation of our principal findings. We noted that the upregulation of constitutive activity is critically dependent on channel phosphorylation, as evidenced by a requirement for ATP/GTP or protein phosphatase inhibition to reproduce ATR activation in cell-free inside-out...
membrane-patches. We also report for the first time to our knowledge differential regulation of \( I_{\text{KACH}} \) by distinct classes of PKC isoforms. Furthermore, we found a role for this differential regulation in a disease paradigm increased constitutive \( I_{\text{KACH}} \) activity in AF-related atrial remodeling. Because PKC regulates a wide variety of cardiac functions, these findings have potential implications for the molecular regulation of cardiac physiology over a broader range of conditions. Conventional PKC upregulation is important in a variety of ventricular remodeling paradigms, and PKC\( \alpha \) appears to play an important role in the pathophysiology of heart failure.

Our findings illuminate previous observations regarding \( I_{\text{KACH}} \) regulation in clinical AF. Voigt et al.\(^{10}\) found significant reductions in \( I_{\text{KACH}} \) with PKC inhibition in AF samples without significant change in control samples, consistent with our observations in Figure 2B. This observation suggested that PKC promotes basal \( I_{\text{KACH}} \) opening in AF, opposite to the well-recognized inhibitory effect of PKC.\( ^{26,27} \) Our finding of ATR-induced altered PKC isoform balance, with reduced cPKC expression and increased nPKC, membrane localization in ATR demonstrated that these effects are attributable to rapid atrial activity per se, and we showed that PKC\( \alpha \) downregulation likely results from tachycardia-induced \( \text{Ca}^{2+} \) loading and calpain activation.

\( I_{\text{KACH}} \) enhancement in AF has emerged as a potentially interesting therapeutic target.\( ^{46} \) Whereas direct blockade of the \( I_{\text{KACH}} \) channel pore can suppress \( I_{\text{KACH}} \), off-target effects on \( I_{\text{KACH}} \) channels in the sinoatrial node and peripheral tissues (e.g., gastrointestinal tract, genitourinary system) may limit the value of this approach.\( ^{46} \) An improved understanding of the molecular basis for \( I_{\text{KACH}} \) upregulation in AF-related pathology might allow for more specific targeting of the upregulated current. Developing approaches to inhibit PKC and to exploit isoform-specific regulation in cardiac disease\( ^{25,44,47} \) may eventually permit the selective suppression of the ATR-induced \( I_{\text{KACH}} \) enhancement that helps to perpetuate AF.\( ^{3,32} \) ATR-induced atrial hypocontractility associated with AF is an important contributor to thromboembolic complications. Exploration of the consequences of ATR-induced PKC isoform changes for atrial contractile function therefore may be interesting.

**Potential Limitations**

The mechanisms underlying tachycardia-induced PKCe translocation to the membrane remain unclear and will require further investigation. Receptors for activated C-kinase constitute a family of membrane-associated PKC-anchoring proteins that are PKC isoform-specific.\( ^{48,49} \) They may play a role in altered PKC isoform expression and localization. Voigt et al.\(^{10}\) found increased atrial PKCe expression in AF patients, whereas we noted no change in total expression but increased relative membrane localization. This discrepancy could be attributable to technical differences (use of whole tissue in the Voigt study vs isolated cardiomyocytes in ours), species differences, and differences in the duration of atrial tachyarrhythmia, or in underlying heart disease. Increased membrane partitioning of PKC isoforms, commonly associated with their activation, is usually attributed to membrane translocation. Mechanistically, however, it could be attributable to translocation per se, enhanced degradation of cytosolic PKC, enhanced stability of membrane PKC, or even a localized posttranslational modification that alters antibody affinity.

In the present study, we investigated \( I_{\text{KACH}} \) behavior in vitro, with conditions (ion concentrations, temperature, and so others) that are different from those in vivo. In vitro assessment was essential to probe the behavior of single \( I_{\text{KACH}} \) channels and its response to pharmacological manipulation. Nevertheless, our data should be extrapolated with caution to in vivo conditions.

We examined LA cells because of the evidence for a prominent role of LA sources for AF perpetuation.\( ^{50} \) It remains to be determined whether similar mechanisms operate in the right atrium and various discrete atrial structures. Such an analysis, although potentially interesting, is out of the scope of the present study.

We have not identified enhanced phosphorylation of specific sites through which PKC isoforms modulate \( I_{\text{KACH}} \) in canine atrial cardiomyocytes. There are at least 12 PKC consensus sites on Kir3.1 and 3.4,\( ^{26} \) and PKCs could also be acting indirectly by phosphorylating Kir3.1/3.4-interacting proteins rather than channel proteins per se. Definitive identification of the PKC phosphorylation sites that modulate \( I_{\text{KACH}} \) particularly for the stimulatory effect of PKCe, would be of great interest for future work.

**Conclusions**

We have examined mechanisms underlying enhanced \( I_{\text{KACH}} \) function in ATR at the single-channel level in cell-attached and cell-free membrane patches. Our results identify altered PKC isoform-dependent phosphorylation in ATR as an important contributor to \( I_{\text{KACH}} \) activation in the absence of cholinergic agonists. Altered cPKC/nPKC regulation of \( I_{\text{KACH}} \) may constitute a novel atrial-selective and pathology-selective target for AF therapy.

**Acknowledgments**

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43. Sabri A, Steinberg SF. Protein kinase C isoform-selective signals that lead to cardiac hypertrophy and the progression of heart failure. Mol Cell Biochem. 2003;251:97–101.

Novelty and Significance

What is Known?

- Atrial fibrillation (AF) is the most common sustained cardiac rhythm disorder. It remodels atrial electric function in a way that promotes its own maintenance and causes increasing resistance to therapy.
- An important component of AF-related remodeling is increased ligand-independent (constitutive) activity of a ligand-gated potassium channel that normally operated by acetylcholine. Protein kinase C (PKC) modulates the activity of several ion channels.

What New Information Does This Article Contribute?

- Conventional (Ca2+ dependent) PKC isoforms inhibit, whereas the novel (Ca2+ independent) PKC isoforms enhance the activity of atrial acetylcholine-regulated potassium channels.
- Rapid activation of atrial cardiomyocytes to mimic AF reduces the expression of the conventional PKCα isoform and enhances membrane localization of the novel PKCε isoform, shifting the balance of PKC action toward channel activation.
- Tachycardia-induced changes in PKCα isoform expression require cell Ca2+ loading, likely via the Ca2+ dependent proteolytic enzyme calpain.

Atrial remodeling caused by AF is an important contributor to the pathophysiology of arrhythmia; however, the underlying mechanisms are poorly understood. We examined enhanced activity of the acetylcholine-regulated potassium channel (I_{ACh}) in the absence of agonist (constitutive activity), which is an important component of this remodeling. We show, for the first time to our knowledge, that the conventional (Ca2+ dependent) and the novel (Ca2+ independent) isoforms have opposite effects on I_{ACh}, with conventional isoforms inhibiting and novel isoforms enhancing I_{ACh} channel activity in isolated membrane patches. We found that PKCα is downregulated by the rapid cell-firing rates associated with AF, and that this effect is Ca2+ dependent. It is likely mediated by the Ca2+ dependent proteolytic enzyme, calpain. In contrast, PKCε accumulates in the membranes of rapidly firing cells in a Ca2+ independent way. Thus, rapid cell-firing shifts the balance from the inhibitory conventional isoforms toward the stimulatory novel isoforms, thereby activating the channel. This knowledge helps in understanding the pathophysiology of AF and may allow development of novel therapeutic approaches.
Differential Protein Kinase C Isoform Regulation and Increased Constitutive Activity of Acetylcholine-Regulated Potassium Channels in Atrial Remodeling
Samy Makary, Niels Voigt, Ange Maguy, Reza Wakili, Kunihiro Nishida, Masahide Harada, Dobromir Dobrev and Stanley Nattel

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SUPPLEMENTAL MATERIAL
Online Figure I. Single-channel properties of constitutive $I_{K,ACH}$ in the atrial tachycardia remodeled (ATR) in-vivo dog model. Representative recordings (A), current-voltage relationship along with mean±SEM single-channel conductance (B), mean±SEM open-probability (Po; C), frequency of openings (fo; D) and open-time histogram (bin-width 0.4-ms) together with mean±SEM open-time constant (E). ***P<0.001 vs. corresponding controls. Numbers indicate myocytes/dogs.
Online Figure II. Single-channel properties of constitutive $I_{K,ACH}$ in isolated cardiomyocytes paced for 24 hours at 1 vs 3 Hz. Representative recordings (A), current-voltage relationship along with mean±SEM single-channel conductance (B), mean±SEM open-probability ($P_o$; C), frequency of openings ($f_o$; D) and open-time histogram (bin-width 0.4-ms) together with mean±SEM open-time constant (E). ***$P<0.001$ vs. corresponding controls. Numbers indicate myocytes/dogs.
Online Figure III. Effect of diacylglycerol (DAG) kinase inhibitor on $I_{K,ACHc}$.
Mean±SEM open probability ($P_o$) of constitutively active $I_{K,ACH}$ at baseline (0 min) and after 5-min application of R59022 (1-μmol/L) in control and ATR myocytes compared to time-matched controls. **$P<0.01$ vs. corresponding baseline (0 min) values, ##$P<0.01$.
Numbers indicate myocytes/dogs.
Online Figure IV. Effect of inactive (scrambled) purified PKC-isoforms on GTPγS-activated $I_{K,ACh}$. Single-channel open-probability in cell-attached (CA) configuration and after inside-out formation before (BL) and after application of scrambled (control) PKCα and PKCε (40-pg/μL each) peptides, respectively. ###$P<0.001$ vs. corresponding CA value. Numbers indicate myocytes/dogs.
Online Figure V. Effect of α-adrenoreceptor activation with phenylephrine on constitutive (A) and agonist-activated (10-μmol/L carbachol, B) $I_{K,ACh}$ in control-dog atrial myocytes. Mean±SEM open probability prior to (CTL) and after exposure to phenylephrine (PE; 1-mmol/L) and the selective cPKC-inhibitor (β-C2-4; 10-μmol/L) or a combination of both. ***$P<0.001$ vs. time-matched control (CTL). #P<0.05, ###P<0.001. Numbers indicate myocytes/dogs.
**Online Table I.** In-vivo hemodynamic and electrophysiological data

<table>
<thead>
<tr>
<th></th>
<th>Control ($n=31$)</th>
<th>ATR ($n=29$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP, mmHg</td>
<td>143±6</td>
<td>148±7</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>78±3</td>
<td>78±3</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>3.0±0.8</td>
<td>4.8±0.7</td>
</tr>
<tr>
<td>LAP, mmHg</td>
<td>5.0±0.8</td>
<td>6.0±0.7</td>
</tr>
<tr>
<td>RAP, mmHg</td>
<td>3.5±0.5</td>
<td>4.0±0.4</td>
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<tr>
<td>AERP (BCL 360 ms), ms</td>
<td>120±4</td>
<td>85±4***</td>
</tr>
<tr>
<td>AERP (BCL 300 ms), ms</td>
<td>121±4</td>
<td>86±4***</td>
</tr>
<tr>
<td>AERP (BCL 250 ms), ms</td>
<td>117±4</td>
<td>85±4***</td>
</tr>
<tr>
<td>AERP (BCL 200 ms), ms</td>
<td>109±4</td>
<td>83±4***</td>
</tr>
<tr>
<td>AERP (BCL 150 ms), ms</td>
<td>99±3</td>
<td>81±4***</td>
</tr>
<tr>
<td>DAF, sec</td>
<td>39±13</td>
<td>694±178**</td>
</tr>
</tbody>
</table>

BP indicates blood pressure; LVEDP, LV end-diastolic pressure; LAP, LA mean pressure; RAP, RA mean pressure; AERP, atrial effective refractory period; BCL, basic cycle length; DAF, duration of AF. **$P<0.01$** and ***$P<0.0001$** vs. control.