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Ca^{2+}/Calmodulin-Dependent Protein Kinase II (CaMKII) Increases Small-Conductance Ca^{2+}-Activated K^+ Current in Patients with Chronic Atrial Fibrillation

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Increased small-conductance Ca^{2+}-activated K^+ current (SK), abnormal intracellular Ca^{2+} handling, and enhanced expression and activity of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) have been found in clinical and/or experimental models of atrial fibrillation (AF), but the cumulative effect of these phenomena and their mechanisms in AF are still unclear. This study aimed to test the hypothesis that CaMKII increases SK current in human chronic AF.

Material/Methods: Right atrial appendage tissues from patients with either sinus rhythm (SR) or AF and neonatal rat atrial myocytes were used. Patch clamp, qRT-PCR, and Western blotting techniques were used to perform the study.

Results: Compared to SR, the apamin-sensitive SK current (I_{KAS}) was significantly increased, but the mRNA and protein levels of SK1, SK2, and SK3 were significantly decreased. In AF, the steady-state Ca^{2+} response curve of I_{KAS} was shifted leftward and the $[Ca^{2+}]_i$ level was significantly increased. CaMKII inhibitors (KN-93 or autocamtide-2-related inhibitory peptide (AIP)) reduced the I_{KAS} in both AF and SR. The inhibitory effect of KN-93 or AIP on I_{KAS} was greater in AF than in SR. The expression levels of calmodulin, CaMKII, and autophosphorylated CaMKII at Thr287 (but not at Thr286) were significantly increased in AF. Furthermore, KN-93 inhibited the expression of (Thr^{287})p-CaMKII and SK2 in neonatal rat atrial myocytes.

Conclusions: SK current is increased via the enhanced activation of CaMKII in patients with AF. This finding may explain the difference between SK current and channels expression in AF, and thus may provide a therapeutic target for AF.

MeSH Keywords: Atrial Fibrillation • Calcium-Calmodulin-Dependent Protein Kinase Type 2 • Phosphorylation • Small-Conductance Calcium-Activated Potassium Channels

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Background

Atrial fibrillation (AF) is the most common sustained tachyarrhythmia and is associated with increased risk of morbidity and mortality [1]. The structural and functional remodeling of ion channels in atrial myocytes promote the progression of atrial arrhythmias [2]. The pharmacological therapy of AF is limited by drugs with low efficacy and the risk of fatal ventricular arrhythmias. Development of the atrial-selective antiarrhythmic agents is important and plausible in the treatment and prevention of AF.

The functional role of small-conductance Ca^{2+}-activated K^+ (SK) channels has been documented in recent years [3]. The pivotal role of SK channels lies in the fact that SK channels are functionally more important in atria than in ventricles [4], and this finding may offer a pharmacological target for the treatment of atrial arrhythmias. However, the antiarrhythmic effect of SK channel inhibitors remains controversial. Global genetic knockout of the SK2 channel resulted in a significant prolongation of action potential duration (APD), predominantly in the late phase of the atrial repolarization and increased the susceptibility to AF [5]. In contrast, SK3 overexpression in mice induced a shortening of APD and predisposed the mice to inducible atrial arrhythmias [6]. SK2 current density was significantly increased in patients with chronic AF, which was reported for the first time by our group [7]. Qi et al. reported that SK channels played a crucial role in canine atrial repolarization [8]. In a pacing-induced rat AF model, intravenous application of NS8593, a highly selective SK channels blocker, reduced the duration of AF by 64.5% [9]. Pharmacological blockade of SK channels with the pore blocker ICA in acute myocardial infarction with development of ventricular fibrillation (VF) significantly decreased VT duration and returned monophasic action potential duration at 80% repolarization (MAPD80) to baseline [10]. Hsueh et al. demonstrated that SK channel blockade promoted arrhythmias in the canine left atrium, which could be attributed to the increase in APD heterogeneity [11]. Based on these studies, it appears that SK channels may play an important role in the genesis of atrial arrhythmias, but the details remain unclear.

Intracellular Ca^{2+} overload increases the incidence of Ca^{2+} sparks and enhances sarcoplasmic reticulum (SR) Ca^{2+} leakage, which is related to the occurrence and persistence of AF [12–14]. The Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional serine-threonine protein kinase and modulates myocytes membrane excitability by its action on ion channels. Gating of SK channels depends on the interaction between the pore-forming α subunits and calmodulin. Ca^{2+}-binding calmodulin leads to allosteric regulation of the sensitivity of channels to intracellular free Ca^{2+}. CaMKII activity is enhanced in clinical and experimental models of AF [15–17]. SK channels are upregulated via the enhanced activation of CaMKII in hypertrophic hearts of spontaneously hypertensive rats [18]. Therefore, we hypothesized that increased CaMKII activity enhances the intracellular calcium sensitivity of SK channels in human chronic AF, and the present study was designed to test this hypothesis.

Material and Methods

Sampling of human atrial appendage tissues

Right atrial appendage tissues were obtained from patients with either sinus rhythm (SR) (n=84) or AF (n=73) during open-heart surgery. AF was defined in patients with clinical diagnosis in which AF was sustained for ≥6 months. Atrial tissues used for electrophysiological experiments were from 64 patients with SR and 41 patients with AF, and the remaining tissues from 20 SR patients and 32 AF patients were used for qRT-PCR, Western blotting, and other laboratory studies. All experiment procedures were approved by the Ethics Committee of the Affiliated Hospital of Southwest Medical University. Each patient gave written informed consent.

Isolation of human atrial myocytes

Human atrial myocytes were isolated from the above-mentioned right atrial appendage tissues with a modified 2-step enzymatic digestion method [19]. Briefly, the obtained atrial samples were immediately brought to the laboratory in cold oxygenated cardioplegic solution (in mM: 10 taurine, 5 adenosine, 8 $MgSO_4$, 10 HEPES, 50 KH_2PO_4, 100 mannitol, 140 glucose, pH 7.4 with KOH). Tissue chunks were chopped into small pieces of about 1 mm^3 in size in cardioplegic solution, and then transferred into a Ca^{2+}-free solution (in mmol/l: 137 NaCl, 1 $MgSO_4$, 5 KH_2PO_4, 5 HEPES, 10 Taurine, 10 glucose, pH 7.4 with NaOH) containing proteinase (type XXIV, 5 units/ml) (Sigma) and collagenase V (150 units/ml) (Sigma), stirring for 35~45 min (37°C, 100% O_2). After this step was completed, the atrial pieces were transferred into the Ca^{2+}-free solution containing only collagenase V (150 units/ml) and stirred for 5~10 min (37°C, 100% O_2), and we repeated this step 2 times. Cardiomyocytes were harvested from the collagenase V solution by centrifugation at 400 rpm for 3 min. The atrial cardiomyocytes were kept in Kraft-Brühe (KB) storage solution (in mmol/l: 20 KCl, 10 KH_2PO_4, 25 glucose, 5 mannitol, 1 albumin, 70 L-glutamic acid, 10 β-hydroxybutyrate acid, 10 taurine, 0.5 EGTA, pH 7.2 with KOH) for at least 1 h before electrophysiology recording. Only rod-shaped elongated myocytes with clear cross-striations were selected for experiments.

Isolation of neonatal rat atrial myocytes

Atrial myocytes were isolated from the atria of 1~3-day-old SD rats. Briefly, hearts and then atria were excised from the rats in

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a sterilized fashion. The atrial tissues were washed with PBS (in mmol/l: 137 NaCl, 2.7 KCl, 4.3 Na_2HPO_4, 1.4 KH_2PO_4, pH 7.4 with NaOH) for 3 times. Then, the atrial tissues were kept in trypsin solution (0.25%, GIBCO, USA) overnight at 4°C. The next day, the atrial tissues were transferred to enzyme solution (collagenase type II, 0.5 mg/ml and bovine serum albumin, 5 mg/ml) at 37°C and shaken for 10 min and this was repeated 3 times. Dispersed cells were collected and then centrifuged for 3 min at 400 rpm. Cells were resuspended in DMEM (Gibco, USA) containing 20% fetal bovine serum and 1% penicillin-streptomycin. Cell suspensions were pre-plated for 2 h at 37°C with 5% CO_2 to remove fibroblasts. Atrial cardiomyocytes were plated on 3.5-cm plates and incubated in the above medium at 37°C with 5% CO_2 for 24 h, then treated with KN-92 or KN-93 for 48 h.

Patch clamp experiment

Isolated human atrial myocytes were kept in a perfusion chamber for 10~15 min and then perfused with extracellular solution (in mmol/l: 140 *N*-methyl glucamine, 4 KCl, 1 $MgCl_2$, 5 glucose, 10 Hepes, pH 7.4 with HCl) at 1 ml/min. Under an inverted microscope (Olympus, Japan), cardiomyocytes with clear cross-striation were chosen for use in the electrophysiological experiment. Borosilicate glass electrodes (outer diameter 1 mm) were pulled using a micropipette puller (model P-97, Sutter Instrument, USA). The microelectrode had tip resistances of about 2~4 MΩ when filled with the pipette solution (in mmol/l: 144 potassium gluconate, 1.15 $MgCl_2$, 5 EGTA, 10 HEPES, pH 7.4 with KOH). SK current was recorded using a whole-cell configuration. In the presence of 5 mM EGTA, the MaxChelator program (*http://maxchelator.stanford.edu*) was used to calculate the amount of $CaCl_2$ that needed to be added to pipette solution to control the free intracellular Ca^{2+} level. SK current was measured using an EPC-10 amplifier (HEKA Instruments, Germany) and Patchmaster software (HEKA, Germany). When a giga-ohm seal was obtained, the cell membrane was ruptured using negative pressure to establish the whole-cell configuration. The series resistance was compensated up to 70~80%. The sampling frequency was 10 KHz. The SK channel current was identified as apamin-sensitive current (I_{KAS}). I_{KAS} was elicited by depolarizing step pulses from –130 mV to +60 mV for 300 ms with a holding potential of –60 mV. Data were acquired, stored, analyzed using Patchmaster and Pulse (HEKA Instruments), and plotted using Origin 8.0 software (OriginLab Corporation, USA).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from right atrial tissues (20 specimens from SR and 32 specimens from AF) using TRIzol reagent (Tiangen). The cDNA was generated from 1 μg of total RNA using the ReverTra-Plus kit (TOYOBO) and real-time PCR was carried out using the SYBR® green Real-time PCR Master Mix (TOYOBO) following the manufacturer's instructions. The relative gene expression levels of the KCNN1, KCNN2, and KCNN3 were normalized to the housekeeping gene (β-actin). The mRNA expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used were as follows:
KCNN1, forward 5'-TGGACACTCAGCTCACCAAG-3',
reverse 5'-TTAGCCTGGTCGTTCAGCTT-3';
KCNN2, forward 5'-GCATCGTGGTCATGGTCATC-3',
reverse 5'-TCTGCTCCATTGTCCACCAT-3';
KCNN3, forward 5'-CAGGTGGGAACCTGTGTCTT-3',
reverse 5'-CTTTGAGCACAGCAATGGAA-3';
β-actin, forward 5'-ACACTGTGCCCATCTACG-3',
reverse 5'-TGTCACGCACGATTTCC-3'.

Western blotting

Proteins were detected by Western blot analyses. Atria specimens were dissected into small pieces and then were frozen and ground in liquid nitrogen. The ground tissues were further homogenized using an electric homogenizer in ice-cold lysis buffer (in mmol/l: 20 3-Morpholinopropanesulfonic acid, 1 DTT, 250 sucrose) containing protease inhibitor (2 μg/ml) to prevent protein degradation (20 mg atrial tissues with 200 μl lysis buffer). Protein concentration was determined with BCA assay. Atrial tissue lysates (50 μg/lane) were resolved on a 10% SDS-polyacrylamide gel (SDS-PAGE) and then transferred to a PVDF membrane using a wet transfer system. The PVDF membrane was incubated with rabbit polyclonal anti-KCa 2.1 (SK1), anti-KCa 2.2 (SK2), anti-KCa 2.3 (SK3, Alomone Labs, Jerusalem, Israel) (dilution 1: 500), rabbit polyclonal anti-CaM (Santa Cruz Biotechnology, Santa Cruz, USA) (dilution 1: 1000), rabbit polyclonal anti-CaMKII (Abcam, Cambridge, UK) (dilution 1: 1000), rabbit polyclonal anti-pCaMKII (Thr^{286}, Cell signaling, USA) (dilution 1: 1000), anti-pCaMKII (Thr^{287}, Abcam, Cambridge, UK) (dilution 1: 1000), and rabbit polyclonal anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, USA) (dilution 1: 2000) overnight at 4°C. The PVDF membrane was then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, USA) (dilution 1: 50 000) for 30 min at room temperature. The immunoreactive bands were developed with an enhanced chemiluminescent detection kit (Gel imaging system, Bio-Rad). The image was acquired and analyzed by Quantity-one software.

Measurement of intracellular free Ca^{2+} concentration

Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) of atrial myocytes was measured after cells were loaded with 5 μmol/l of fluorescent Ca^{2+} indicator Fura-2-acetoxymethyl ester (Fura-2/AM, Invitrogen), and then the fluorescence was excited at 340 nm and 380 nm by the TILL photonics spectrofluorometer (TILLvisION 3.3, TILL Inc, Germany) and fluorescence microscope (DMI3000, Leica, Inc., Germany). The F340/380 ratio

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Table 1. Patient information.

Patient parameters	SR		CAF	
	n	%	n	%
Patients, n	84		73	
Gender, M/F	39/45	45.91/54.13	35/38	47.64/52.46
Age, y	49.34±2.46	–	56.51±4.13	–
CAD, n	7	8.33	9	12.32
MVD/AVD, n	63	75.00	65	89.04
CAD+MVD/AVD, n	3	3.57	5	6.84
NYHA functional class, n				
I	0	0	0	0
II	25	29.76	3	4.10**
III	54	64.48	50	68.49
IV	5	5.95	20	27.39**
Echocardiography				
LVEF, %	53.21±2.26	–	56.47±2.34	–
LAD, mm	35.42±2.67	–	48.54±3.57**	–
LVEDD, mm	50.53±1.68	–	52.56±2.67	–
IVS, mm	10.14±0.18	–	11.42±0.79	–
LVPW, mm	12.48±1.46	–	10.43±0.97	–
Medication, n				
Digitalis ACE inhibitors/ARBs	6	7.14	8	10.95
β-blockers	4	4.76	4	5.48
Diuretics	25	29.76	38	52.05*
Lipid-lowering drugs	10	11.9	12	16.44

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. * $P<0.05$, ** $P<0.05$ *vs*. SR – Fisher exact test for categorical variables.

was calculated, which represents the relative intracellular free Ca^{2+} fluorescence intensity. The fluorescence ratio values were used to calibrate $[Ca^{2+}]_i$ using the equation: $[Ca^{2+}]_i=K_d(F_d/F_s)\times(R-R_{min})/(R_{max}-R)$, where K_d was the dissociation constant of Fura-2/AM binding Ca^{2+} interaction, R_{max} was the F340/380 value under the condition Fura-2 fully binding Ca^{2+} (10 mmol/L), R_{min} was the F340/380 value under the Ca^{2+}-free condition (Ca^{2+} 0 mmol/L with 20 mmol/L EGTA), and F_D and F_S, respectively, represented the fluorescence intensity at 380 nm under Ca^{2+}-free and Ca^{2+}-Fura-2 saturate binding (10 mmol/L $CaCl_2$).

Statistical analysis

Data are presented as mean ± standard error (SEM). Values "n" refer to the number of myocytes used for the patch clamp experiment or the number of patient atrial specimens for qRT-PCR and Western blotting, respectively. Analyses for the patch clamp study were carried out using Pulse (HEKA, Germany), Igor pro (USA), and Clampfit 10.0, Origin 8.0 (OriginLab, USA). Channel current was normalized by cell membrane capacitance to correct for differences in cell size. Differences between the SR group and AF group were compared using the grouped *t* test. For multiple group comparison, one-way ANOVA was used followed by the Bonferroni post-test. Categorical data were compared by Fisher's exact test. $P<0.05$ was deemed to be statistically significant. * and ** denote $P<0.05$ and $P<0.01$, respectively.

Results

Patient information

Patient information is presented in Table 1. Significant differences between the SR group and AF group were found in left

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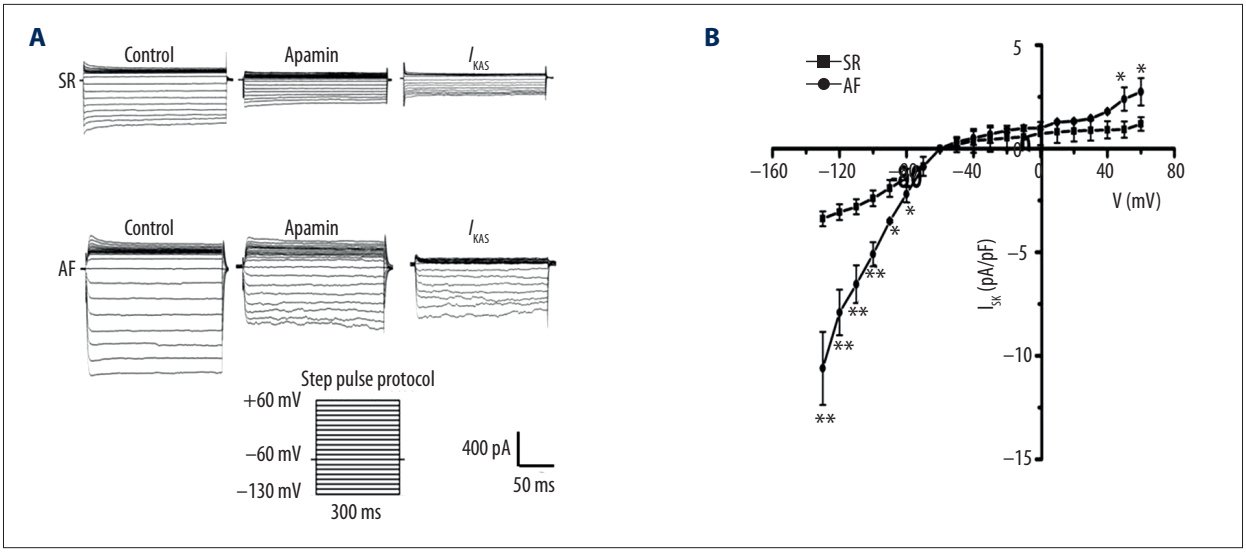

Figure 1. Patch clamp results showing the I_{KAS} in patients with SR and AF. (**A**) Representative currents recorded in atrial myocytes from SR and AF using voltage steps at a holding potential of –60 mV, step from –130 to +60 mV and in the absence or presence of 100 nmol/l apamin at 500 nmol/l $[Ca^{2+}]_i$. I_{KAS} was obtained using digital subtraction of the currents recorded in absence or presence of apamin. (**B**) Current-voltage relation curves of SK currents in atrial myocytes from SR (n=35 cells from 12 patients) and AF (n=24 cells from 9 patients). *P < 0.05, ** P < 0.01 *vs.* SR.

atrial diameter, cardiac function class (II and IV), and drug (diuretics) treatment. Patients did not show a significant difference with antiarrhythmic treatment.

I_{KAS} was upregulated in patients with AF

I_{KAS} was measured in atrial myocytes isolated from SR and AF patients with whole-cell configuration. Figure 1A shows the representative current traces recorded at 500 nmol/l free Ca^{2+} in the pipette solution. I_{KAS} was obtained at a holding potential of –60 mV, voltage steps were from –130 mV to +60 mV with 300 ms duration. In the chamber (extracellular) solution, *N*-methyl glucamine (NMG) was the dominant extracellular cation and Na^+ was omitted to inhibit I_{Na}. I_{KAS} was obtained using digital subtraction of the currents recorded in the absence and presence of 100 nmol/l apamin in the extracellular solution. Currents were normalized by cell membrane capacitance to correct for the cell size difference. Figure 1B was the current-voltage (I–V) relationship of I_{KAS} in atrial myocytes from SR and AF (n=35 cells from 12 SR patients and n=24 cells from 9 AF patients). It shows that atrial I_{KAS} was significantly increased in AF compared with SR (–6.54±0.90 *vs.* –2.80±0.36 pA/pF at –110 mV, P<0.01).

The mRNA and protein expressions of SK1, SK2, and SK3 were downregulated in the atrial tissues of AF patients

The pore-forming (α) subunit of SK channels are encoded by at least 3 genes – KCNN1 (SK1), KCNN2 (SK2), and KCNN3 (SK3) – in cardiomyocytes. To confirm whether the increase of atrial I_{KAS} in AF patients was consistent with the mRNA level change of the 3 SK channel subunits, we measured the mRNA levels of the 3 genes in SR and AF patients. The mRNA levels in the SR group were normalized to 1, then compared to the AF group. Figure 2A shows that KCNN1, KCNN2, and KCNN3 mRNA expression levels were all significantly decreased in AF (n=32) compared to that in SR (n=20) (P<0.05). The mRNA expression level of KCNN2 (Figure 2B, 2C) was higher than that of KCNN1 or KCNN3 in both the SR (n=20) and AF (n=32) groups (p<0.05). There was no significant difference between the mRNA levels of KCNN1 and KCNN3, regardless of being in the SR group or in the AF group (P>0.05).

To further address whether SK1, SK2, and SK3 protein expressions were also downregulated, as were their mRNA levels, we measured the atrial protein levels of SK1, SK2, and SK3 using Western blotting. The protein levels were normalized to that of GAPDH in each sample with Quantity-one software. Results (Figure 2D) showed that SK1, SK2, and SK3 protein expression levels were remarkably decreased in the AF group (n=32) compared with the SR group (n=20) (P<0.05). The SK2 protein expression level was higher than the SK1 and SK3 protein levels in both the SR and AF groups (P<0.05). No significant difference was observed between SK1 and SK3 protein levels in the SR group or in the AF group (P>0.05).

The Ca^{2+} sensitivity of atrial I_{KAS} was increased in AF patients

As shown above, we observed a contradictory phenomenon that atrial I_{KAS} was increased but SK1, SK2, and SK3 mRNA and protein levels were decreased in AF patients. In order to

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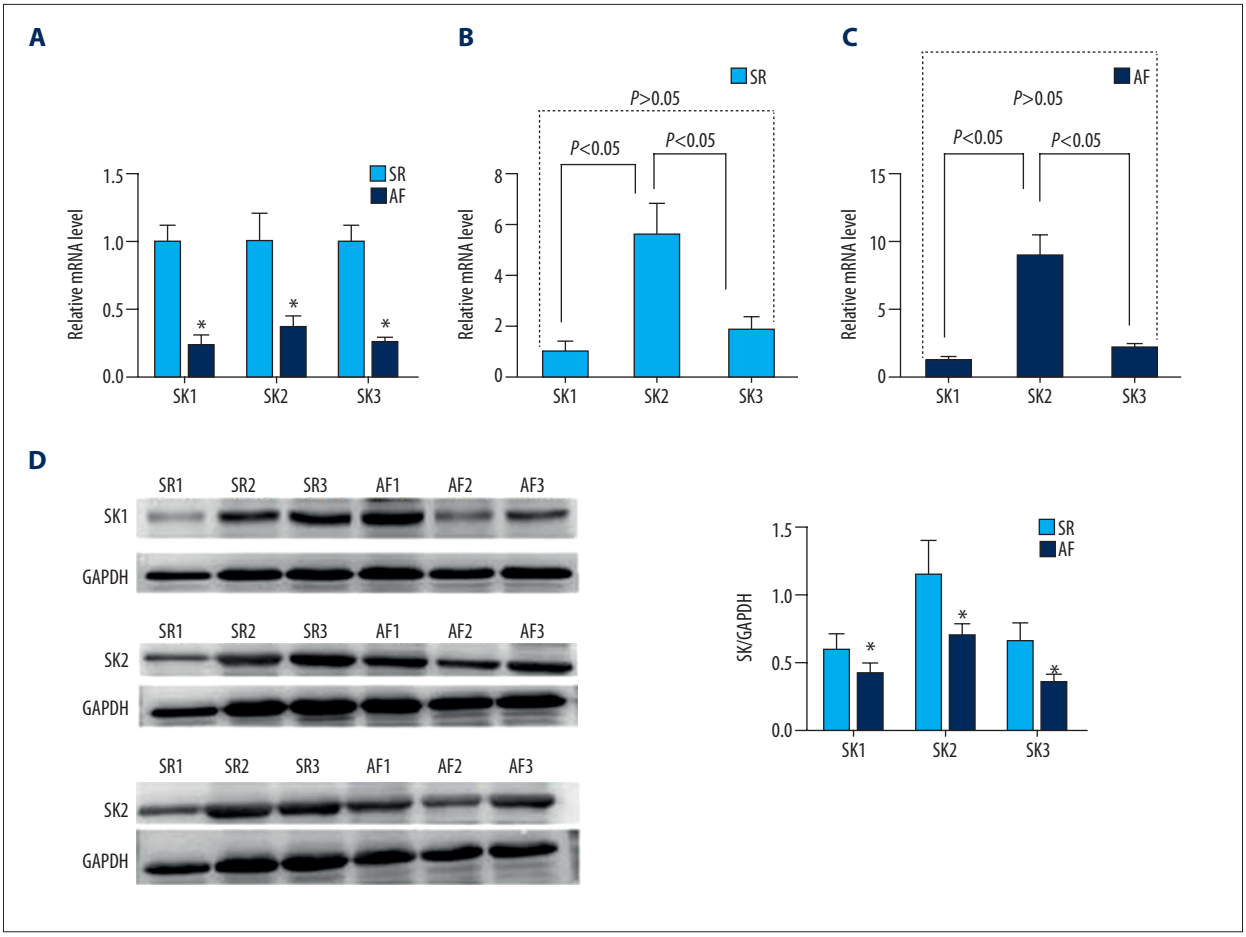

Figure 2. qRT-PCR and Western blotting results showing KCNN 1–3 mRNA levels and K_{Ca} 2.1/2.2/2.3 proteins levels in the atria of SR controls (n=20) and AF patients (n=32). (**A**) mRNA levels of KCNN1, KCNN2, and KCNN3 in SR and AF. (**B**) mRNA expression differences of KCNN1, KCNN2, and KCNN3 in SR group. (**C**) mRNA expression differences of KCNN1, KCNN2, and KCNN3 in AF group. (**D**) K_{Ca} 2.1–2.3 (SK1–3) proteins expression changes in SR (n=20) and AF (n=32). * P<0.05 *vs.* SR.

explore the underlying mechanisms, we measured the calcium sensitivity of I_{KAS} at the variant $[Ca^{2+}]_i$ levels (0, 0.2, 0.3, 0.5, and 1 μmol/l). The steady-state Ca^{2+} response curve of I_{KAS} (Figure 3) in variant $[Ca^{2+}]_i$ was fitted with the Hill equation: $y=1/(1+[K_d/\chi]^n)$. Here, K_d was the concentration of $[Ca^{2+}]_i$ at half-maximal activation and n is the Hill coefficient. K_d value was lower in AF (236±4 nmol/l) than that in SR (337±8 nmol/l) (P<0.01). Hill coefficients were not significantly different between SR (3.12±0.32) and AF (3.41±0.67). The steady-state Ca^{2+} response curve was left-shifted in AF compared to SR.

Intracellular Ca^{2+} overload in the atrial myocytes from patients with AF

Cytosolic free Ca^{2+} signals were measured by loading Fura-2/AM. Fluorescence was alternately excited at 340 nm and 380 nm. Figure 4A shows that the Ca^{2+} fluorescence intensity of atrial myocytes in AF group was stronger than that in the SR group. Fluorescence ratio values (F340/380) were used to calculate relative $[Ca^{2+}]_i$ by the equation: $[Ca^{2+}]_i=K_d\ (F_d/F_s)\times(R-R_{min})/(R_{max}-R)$. Figure 4B shows that $[Ca^{2+}]_i$ was significantly higher in the atrial myocytes of AF patients (247.3±16.3 nmol/l, n=13 cells) than that of SR patients (168.4±19 nmol/l, n=15 cells) (P<0.01).

Effects of CaMKII inhibitor KN-93 and CaMKII inhibitory peptide AIP on I_{KAS} in patients with SR and AF

To further elucidate why the Ca^{2+} sensitivity of SK channels was increased in AF, we detected the effects of CaMKII inhibitor on I_{KAS}. KN-93 (2-[N-(2-hydroxyethyl)]-N-(4methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine) has been identified as a selective inhibitor of CaMKII by competitive blockade of calmodulin binding to the kinase and has long been used to investigate the possible roles of CaMKII in cardiac myocytes under physiological and pathophysiological conditions. KN-92 (2-[*N*-(4-methoxybenzenesulfonyl)] amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine, phosphate)

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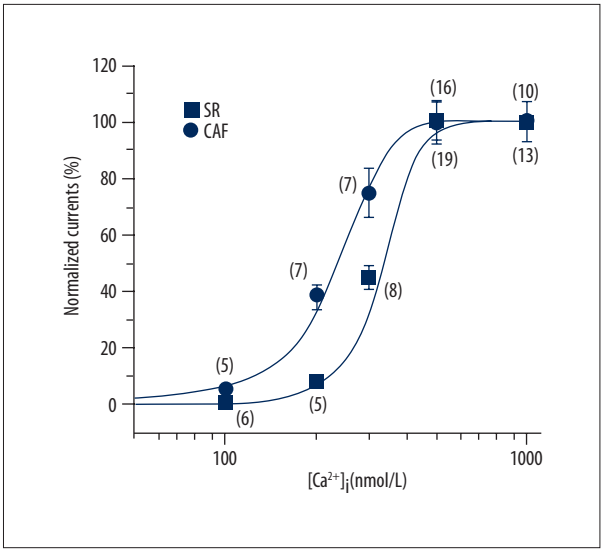

Figure 3. Steady-state Ca^{2+} sensitivity curve of SK currents in SR and AF. SK current was normalized to maximal currents with a free Ca^{2+} of 1 μmol/l. Data were fitted with Hill equation: $y=1/(1+[K_d/\chi]^n)$, where *y* represented the normalized I_{KAS} and *x* was the concentration of intracellular free Ca^{2+}; K_d value was the concentration of intracellular free Ca^{2+} at half-maximal activation; *n* was the Hill coefficient. Numbers in parentheses indicated the number of atrial myocytes with successful recording.

is an inactive analog of KN-93. Autocamtide-2 related inhibitory peptide (AIP) is a highly specific membrane-permeable peptide inhibitor of CaMKII. I_{KAS} was evoked by ramp-pulse from –130 mV to +60 mV at a holding potential of –60 mV with 500 ms duration. Atrial myocytes were pretreated with KN-93 (1 μmol/l) or KN-92 (1 μmol/l); then, apamin was added to the bath solution. The digital subtracted currents were the remaining I_{KAS}. As shown in Figure 5A, KN-92 did not exert a significant effect on I_{KAS} in the SR and AF groups, but KN-93 showed significantly inhibitory effects on I_{KAS} in the SR and AF groups. The inhibitory effect of KN-93 on I_{KAS} was significantly stronger in the AF group than in the SR group (Figure 5C). KN-93 inhibited I_{KAS} by 69.12±7.33% at –110 mV (n=6 cells from 4 patients) in the AF group, but only inhibited it by about 34±3.2% (n=5 from 4 patients, $P<0.01$) in the SR controls (Figure 5C). The time-course of inhibition of I_{KAS} by KN-93 or apamin showed that the inhibition of I_{KAS} by KN-93 or apamin was reversible by washout. (Figure 5B).

To further clarify the inhibitory effect of CaMKII blocker on I_{KAS}, we applied a membrane-permeable peptide inhibitor of CaMKII (AIP). I_{KAS} showed digital subtraction in the presence of 100 nmol/l apamin. Pretreatment with AIP and then apamin showed that the digital subtracted currents were the same as I_{KAS}. AIP (0.5 μmol/l) also showed a more potent inhibitory effect on I_{KAS} in the AF group (n=6 from 4 patients) than in the SR group (n=5 from 3 patients) ($P<0.01$) (Figure 6A, 6C). The time-course of inhibition of I_{KAS} by AIP or apamin showed the inhibition of I_{KAS} by AIP or apamin was also reversible by washout (Figure 6B). This shows that CaMKII inhibitor reversed the enhancement of SK channels during AF.

Autophosphorylation of CaMKII was involved in I_{KAS} activation in human AF

Ca^{2+} regulates the activity of SK channels via calmodulin-dependent binding. CaMKII is modulated by the Ca^{2+} binding calmodulin (CaM). Thus, we measured CaM and CaMKII expression levels in SR and AF patients. Figure 7A shows that AF patients had significantly higher CaM levels than in the SR control (n=8, $P<0.01$). Figure 7B shows that the expression of CaMKII was also remarkably increased in AF patients (n=8) ($P<0.05$). Autophosphorylation of CaMKII by an order of magnitudes increased the activity of CaMKII. We found no significant difference in the (Thr^{286})p-CaMKII expression level between SR and AF patients (n=8, $P>0.05$) (Figure 7C), while the (Thr^{287})p-CaMKII expression level of AF patients was significantly higher than in the SR controls (n=8, $P<0.01$) (Figure 7D). The ratio of (Thr^{287})p-CaMKII/total CaMKII in AF patients was also

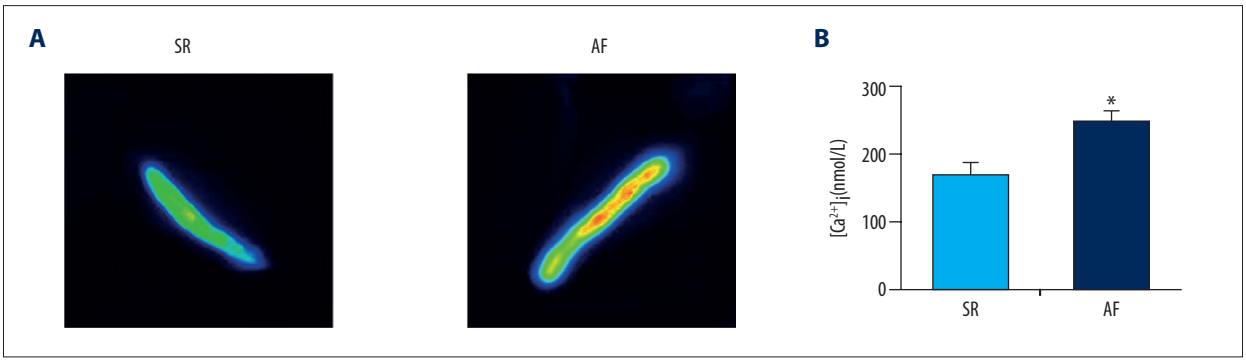

Figure 4. Diastolic free cytosolic Ca^{2+} levels in SR and AF. (**A**) Atrial myocytes loaded with 10 μmol/l Fura-2 AM measured by epifluorescence. (**B**) Diastolic free Ca^{2+} levels in SR (n=8 atrial myocytes from 4 patients) and in AF (n=10 atrial myocytes from 5 patients. * $P<0.05$ *vs.* SR.

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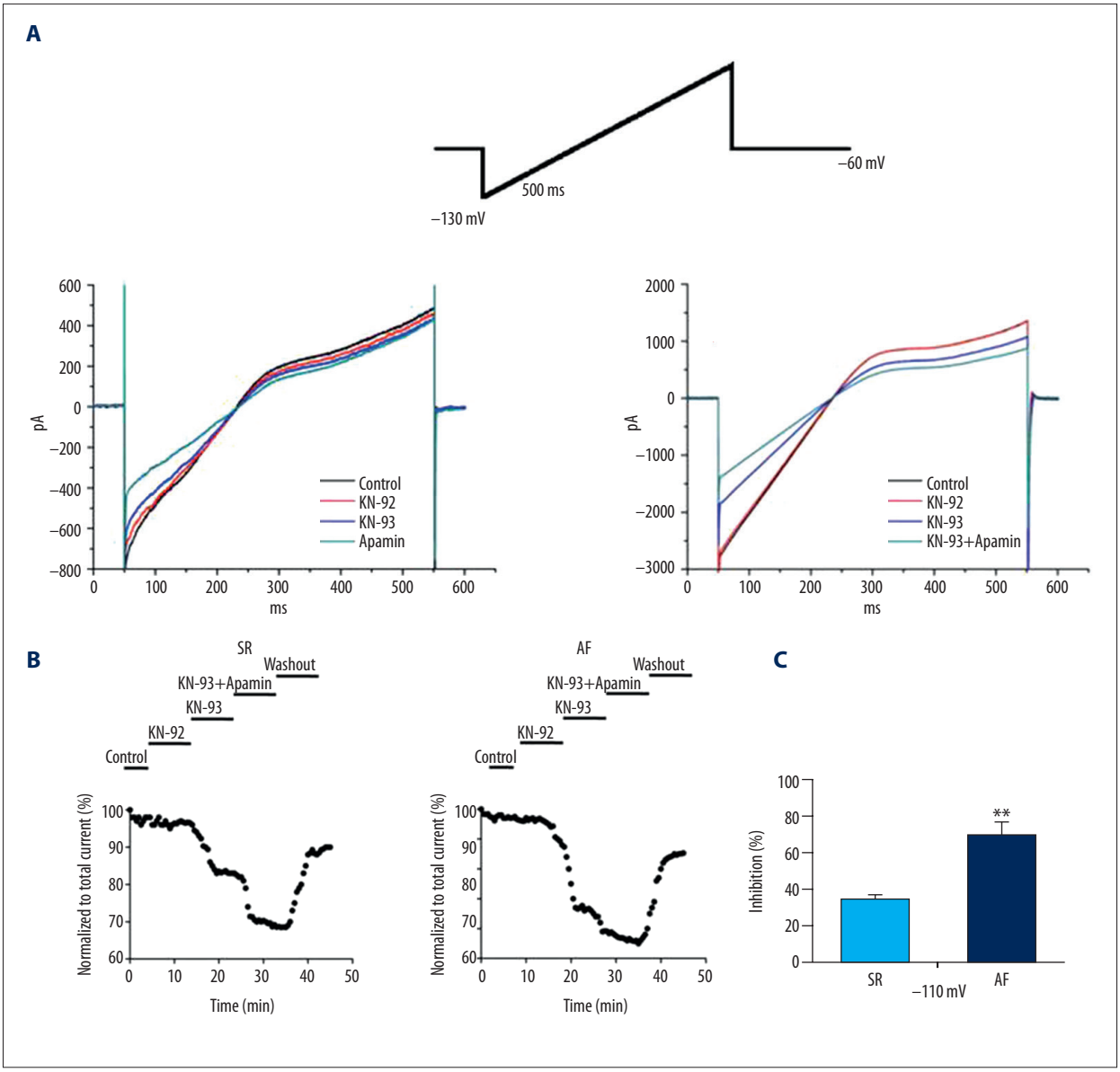

Figure 5. Effects of CaMKII inhibitor KN-93 on I_{KAS} in atrial myocytes from SR and AF groups. (**A**) Total K^+ currents recorded in the absence and presence of KN-92 (1 μmol/L), KN-93 (1 μmol/L), and KN-93 with apamin (0.1 μmol/L) in the extracellular solution with ramp-pulse protocol (test pulse from −130 mV to +60 mV, with holding membrane potential −60 mV). (**B**) Time-course of total K^+ current recorded with ramp protocol treated with absence and presence KN-92, KN-93, or KN-93 with apamin. (**C**) Inhibited percentage of KN-93 on I_{KAS} in SR and AF with holding potential at −110mV. ** P<0.01 *vs.* SR.

significantly higher than that in the SR controls (n=8, P<0.01). These results suggest that autophosphorylation of CaMKII at Thr287 induced CaMKII activation, which is associated with I_{KAS} enhancement and atrial arrhythmogenic remodeling.

To further confirm the effect of CaMKII phosphorylation on SK2 channel activation in AF, we evaluated the effect of (Thr^{287})p-CaMKII on SK2 channel protein expression. Figure 8A shows that treatment with KN-92 (1 μmol/l, n=4) did not affect the expression of (Thr^{287})p-CaMKII expression, while KN-93 (1 μmol/l, n=4) significantly decreased the expression of (Thr^{287})p-CaMKII in the neonatal rat atrial myocytes (P<0.05). As expected, KN-93 significantly decreased the protein expression of SK2 channels in neonatal rat atrial myocytes (n=4, P<0.05) (Figure 8B).

Discussion

The major findings of this study were as follows: I_{KAS} was increased but the mRNA and protein expressions of SK channel

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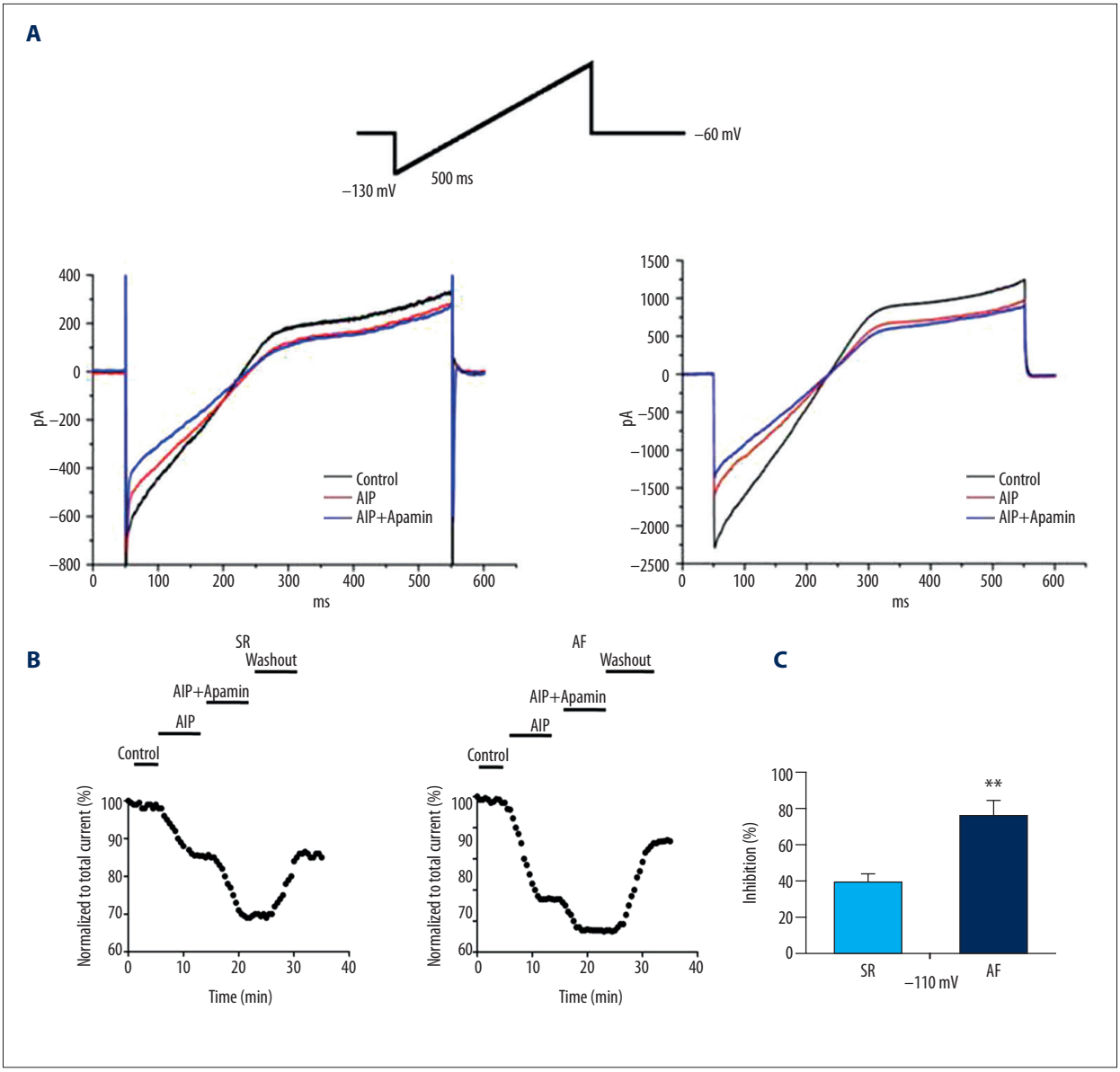

Figure 6. Effects of specific membrane-permeable CaMKII peptide inhibitor AIP on I_{KAS} in atrial myocytes from SR and AF groups. (**A**) Total K^+ currents recorded in the absence and presence of AIP (0.5 μmol/L), AIP with apamin (0.1 μmol/L) in the extracellular solution with ramp-pulse protocol (test pulse from −130 mV to +60 mV, with holding membrane potential −60 mV). (**B**) Time-course of total K^+ current recorded with ramp-pulse protocol treated with absence and presence AIP or AIP with apamin. (**C**) Inhibited percentage of AIP on I_{KAS} in SR and AF group with holding potential at −110 mV. ** $P<0.01$ *vs.* SR.

α subunits were decreased in patients with AF. This contradictory phenomenon was mainly due to CaMKII activation by autophosphorylation at Thr287, which increased calcium sensitivity of I_{KAS} and induced increased I_{KAS} in AF patients. Our study suggests an interesting role and mechanism of SK channels in AF pathogenesis.

Abnormal intracellular calcium handling is one of the major underlying mechanisms of arrhythmogenesis associated with AF [20]. Furthermore, sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) plays a crucial role in atrial arrhythmias by impaired $[Ca^{2+}]_i$ homeostasis, apparently by virtue of transcriptional downregulation, which becomes very important. Decreased Ica reduces action potential (AP) duration (APD) and APD rate adaptation, and accounts for much of the refractory period abbreviation and overexpression induced by suppressing ERP shortening and AF occurrence [21]. Abnormal SR function and diastolic Ca2+ leak triggered arrhythmias. SK channels play pivotal roles in regulating membrane excitability by manipulating intracellular Ca^{2+} levels and are more abundantly expressed

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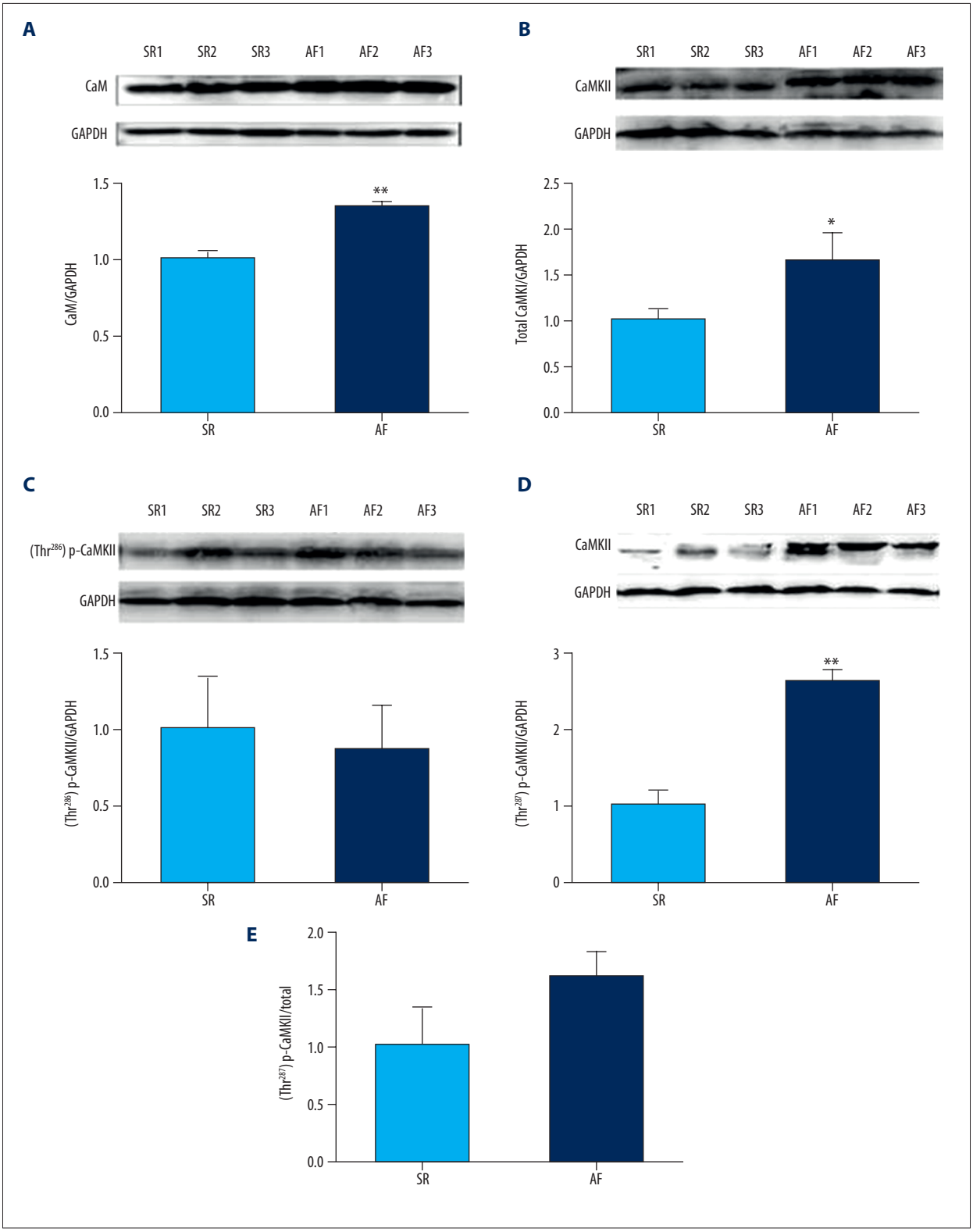

Figure 7. Protein levels of CaM, total CaMKII, and autophosphorylation of CaMKII at Thr286 and Thr287 phosphorylation sites (both n = 6 for SR and AF). (**A**) and (**B**) Note that CaM and CaMKII expression were increased in AF. (**C**) Phosphorylated CaMKII ((Thr^{286})p-CaMKII) expression in AF. (D) and (E) Phosphorylated CaMKII ((Thr^{287})p-CaMKII) expression and the proportion of autophosphorylation of CaMKII at Thr^{287} in total CaMKII in AF. * $P < 0.05$ represented the difference between SR and AF.

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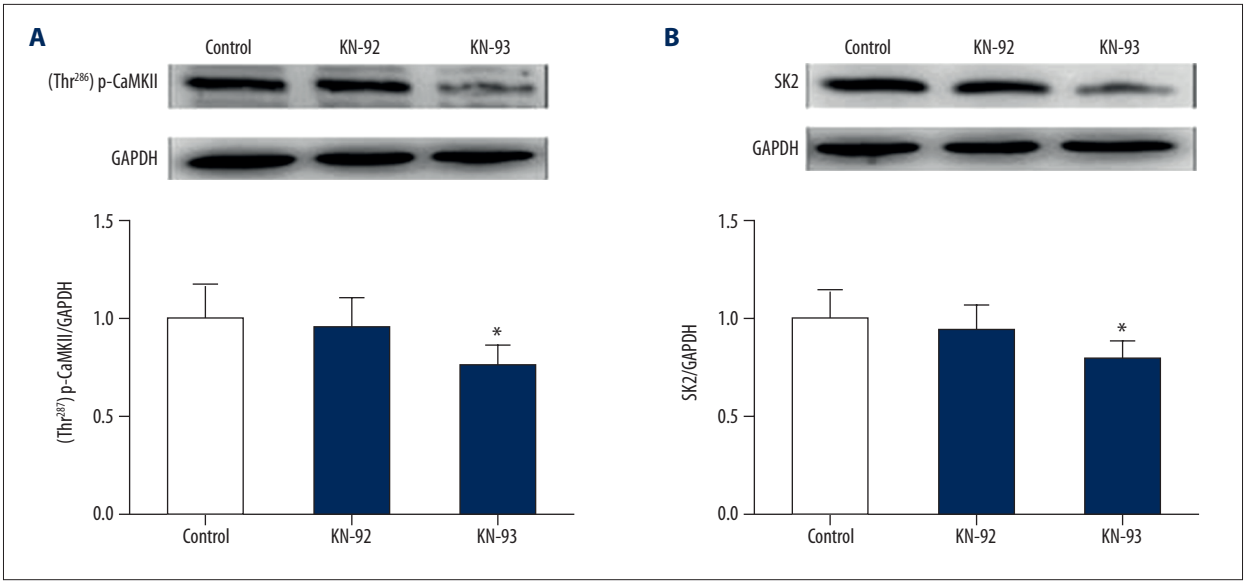

Figure 8. Protein expressions of (Thr^{287})p-CaMKII and SKca2.2 before and after treatment with KN-92 and KN-93 in neonatal rat atrial myocytes. (**A**) Effects of KN-92 and KN-93 on the expression of (Thr^{287})p-CaMKII in neonatal rat atrial myocytes (n=4). (**B**) Effects of KN-92 and KN-93 on the expression of SK2 protein. * P<0.05 *vs.* KN92.

in atrial myocytes than in ventricular myocytes. Nagy et al. demonstrated that SK channels are not active in ventricular myocytes of rat, dogs, and humans, and do not contribute to action potential repolarization [22]. SK channels are closely related with atrial arrhythmia and ventricular failing [23], so the functional role of SK channels in AF is questionable. Xu et al. first reported the functional role of SK channels in human atria and mouse hearts [3]. Ozgen et al. demonstrated that burst-pacing in rabbit pulmonary veins results in APD shortening, potentially by increasing I_{KAS} and upregulation of SK2 mRNA and protein expression [24]. Furthermore, rapid pacing induced SK2 channel protein trafficking from cytoplasm to cell membrane in cultured neonatal rat atrial myocytes. Overexpression of SK2 channels in mice results in shortening of the duration of spontaneous action potentials in atrioventricular nodes [25]. Ablation of the SK2 channels leads to APD prolongation and atrial arrhythmias [5]. These studies suggest that SK channels are important in atrial repolarization. Inhibition of SK channels prolongs atrial ERP and prevents and terminates AF *ex vivo* and *in vivo* [26]. Inhibition of SK channels also terminates pacing-induced AF of short duration and decreases AF duration and vulnerability, without affecting ventricular conduction and repolarization in horses [27]. Pharmacological inhibition of SK channels is terminated by vernakalant-resistant AF [28]. In the present study, we are the first to report the increased density of SK channel currents in human chronic AF, with the downregulation of expression of mRNA and protein levels of SK1, SK2, and SK3. Qi et al. demonstrated that SK current is enhanced by atrial tachypacing, suggesting that SK channel inhibition is a potential target for the treatment of AF [8]. In contrast to the above studies, Yu et al. found that SK currents are decreased concomitant with a significant decrease in protein and mRNA levels of SK1 and SK2. These variant findings may partially be due to species difference or patient heterogeneities [29]. The present study further shows that I_{KAS} was increased but the channel expression was decreased in patients with AF. This finding appears strange and need further investigation. Regardless if this finding, upregulation of I_{KAS} may contribute to atrial repolarization and AF susceptibility.

As we showed above, the increase of I_{KAS} was not paralleled with the upregulation of mRNA and protein expression of SK channels in AF, and even the changes of channel current and expression were contradictory and suggest unusual signaling that directs the differential channel expression and function, perhaps by changing channel Ca^{2+} sensitivity. It is known that abnormal intracellular calcium handling can change the expression and function of ion channels, which subsequently shortens the atrial ERP and leads to atrial electrical remodeling. Sun et al. demonstrated that Ca^{2+} overload in atrial tachyarrhythmia and inhibition of Ca^{2+} entry from L-type Ca^{2+} channels with verapamil attenuates short-term atrial tachycardia remodeling. Furthermore, Ca^{2+} is the main regulator of SK channels. In patients with AF, SK channel activation relies not only on the high $[Ca^{2+}]_i$, but also on the Ca^{2+} sensitivity of SK channels. We found higher $[Ca^{2+}]_i$ in the atrial myocytes of AF patients. Higher $[Ca^{2+}]_i$ activates the SK channels, especial in AF. Our results are consistent with the finding of Qi et al. [8], but conflict with the study of Yu et al. [28]. These contradictions of different studies may partially be due to differences in the basic cardiovascular diseases of the patient populations. Our

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finding supports that elevations of $[Ca^{2+}]_i$ and channel Ca^{2+} sensitivity are probably the key factors that modulate the activation of SK channels in AF. Moreover, the functional enhancement of SK channels is consistent with APD shortening.

Calmodulin (CaM) is a central mediator of Ca^{2+}-dependent signaling and targets various ion channels and signaling pathways in cardiomyocytes. SK channels are gated by elevation of $[Ca^{2+}]_i$. The structures of SK channels are heteromeric complexes that comprise 4 pore-forming α-subunits and the Ca^{2+}-binding protein calmodulin (CaM). Ca^{2+} binding to CaM induces conformational changes of α subunits, which mediate SK channel gating. CaM is confirmed as a Ca^{2+} sensor of SK channels [30]. Our results show that the $[Ca^{2+}]_i$ level and the expression levels of CaM were both upregulated in AF patients. Furthermore, CaM is a subunit of SK channels. Upregulation of CaM contributes to Ca^{2+} binding to the amino-terminal of CaM and subsequent conformational alterations in the α subunit of SK channels, and thus changes the activity of SK channels. Dysfunction of CaM leads to cell Ca^{2+} handling abnormalities, which consequently promotes ectopic activity and facilitates reentry and AF initiation and maintenance. In the atrial myocytes of AF patients, an increased affinity of Ca^{2+} to CaM can potentially contribute to allosteric regulation of SK channels, which induce I_{KAS} upregulation.

CaMKII is a multifunctional serine-threonine protein kinase that is abundantly expressed in hearts and is a predominant modulator of Ca^{2+}-linked signaling. CaMKII comprises 4 homologous isoforms (α, β, δ, and γ). CaMKIIδ is predominantly expressed in the heart. CaMKII activation depends on Ca^{2+}/CaM binding and then induction of substrate phosphorylation and autophosphorylation [1,31]. Dysfunction of CaMKII increases Ca^{2+} handling abnormalities. Wakili et al. demonstrated that the expression and activity of CaMKII are increased in dogs with pacing-induced atrial tachycardia [32]. Greaser et al. found that CaMKII is increased in goats with long-standing AF [33]. Our present study also confirmed that the expression levels of CaM and CaMKII were both increased in patients with AF. These results indicate that increased activation of CaMKII can be a consequence of AF; therefore, CaMKII activation is multifactorial and can result from AF itself or from AF-susceptible risk factors and diseases.

The activation of CaMKII by Ca^{2+}/CaM binding induced autophosphorylation of CaMKII [34] by more than 3 orders of magnitude, and the activation of CaMKII affects the CaMKII target substrate. Autophosphorylation extent significantly affects the activity of CaMKII. The isoforms of CaMKII all share autophosphorylation sites in the autoregulatory regions. Thr^{287}/Thr^{286} is the core regulatory domain in cardiomyocytes. Our present study showed that the levels of (Thr^{286})p-CaMKII did not yield a significant difference between AF and SR controls, while the total CaMKII, (Thr^{287})p-CaMKII, and the proportion of (Thr^{287})p-CaMKII in total CaMKII were all significantly increased in AF patients. It is likely that CaMKII autophosphorylation leads to remarkable CaMKII activation in AF.

SK channels are co-assembled complexes of α subunits (pore-forming) and the auxiliary subunit CaM. The activation of SK channels does not depend on the direct binding of Ca^{2+} to the channel, but rather depend on binding of Ca^{2+} to CaM. SK channels sense Ca^{2+} via CaM, which constitutively bind to the calmodulin-binding domain (CaMBD) of the channel C-terminal region. Our study showed that I_{KAS} was increased in AF. The inhibition of CaMKII by KN-93 or AIP reduced I_{KAS} both in the SR and AF groups; nevertheless, KN-92 had no effect on I_{KAS}. The inhibitory effect of KN-93 was stronger in AF patients than in SR controls. Our data presented here clearly show that CaMKII increased I_{KAS} in human AF. Increased CaMKII autophosphorylation enhanced the activation of CaMKII, especially in AF, as we showed in this study.

Factors involved in the elevation of $[Ca^{2+}]_i$ may be the modulators that alter the activation of SK channels. Of the molecular components, regulation of $[Ca^{2+}]_i$ occurs after Ca^{2+} binding to CaM to form the Ca^{2+}-CaM complex under the state of Ca^{2+} elevation. Mizukami et al. reported that SK channels are upregulated via the enhanced activation of CaMKII in the hypertrophic hearts of spontaneously hypertensive rats. SK2 has 4 potential sites for CaMKII phosphorylation [35]; KN-93 decreases the open probability, and the CaM inhibitor W-7 had a similar effect in gastrointestinal smooth muscles. Our results also demonstrated that inhibition of CaMKII decreased the expression of SK2 in neonatal rat atrial myocytes. Our study shows that increased SK channel currents in AF are not consistent with the expression level of the channels, but rather are consistent with the augmentation of SK channel sensitivity to $[Ca^{2+}]_i$ that resulted from increased autophosphorylation of CaMKII. AF is a constant process in which potentially lethal Ca^{2+} overload lead to excessive enhancement of I_{KAS} in AF, which results in changes of mRNA and protein expression levels. In the short term, I_{KAS} is consistent with the expression levels of SK subunits [8]. In the longer term, I_{KAS} is inconsistent with the expression levels of SK subunits, which may lead to compensatory reaction to counteract the increase in the currents. This compensatory reaction may be a crucial mechanism for atrial electrical remodeling.

Conclusions

Atrial SK channel current were significantly increased but the channel expression levels were decreased in AF patients. Atrial SK channels are more sensitive to $[Ca^{2+}]_i$ in AF patients, and this increased sensitivity is due mainly to CaMKII phosphorylation and $[Ca^{2+}]_i$ elevation. Our results suggest that CaMKII, especially its autophosphorylation, might become a pharmacological target for AF treatment.

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