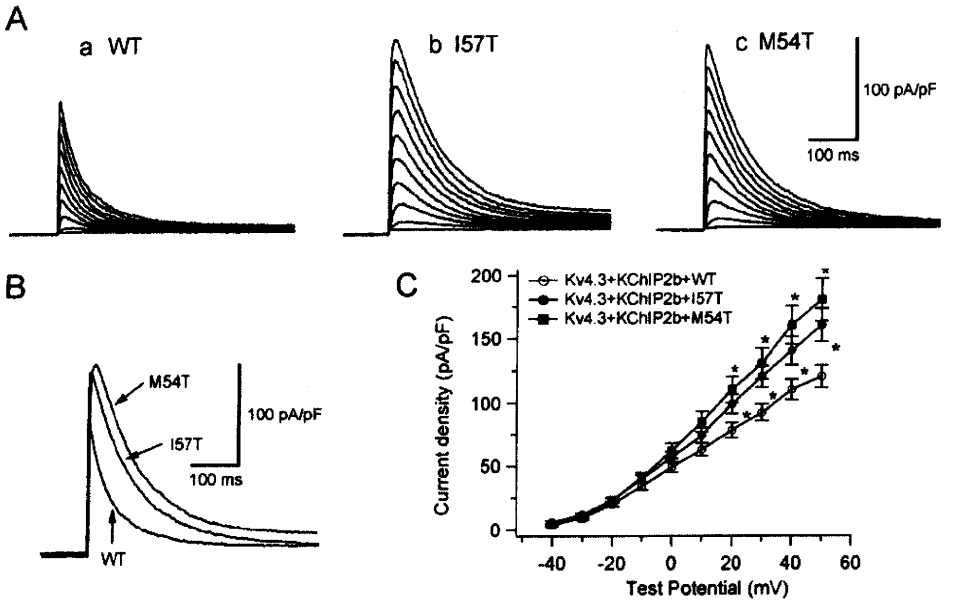

Figure 4 Two *KCNE2* transmembrane variants, I57T and M54T, increase the reconstituted Kv4.3 + KChIP2b channel current and slow its inactivation. **A:** Three sets of current traces elicited by depolarizing pulses for 500 ms from a holding potential of −80 mV to potentials ranging between −40 and +50 mV in 10-mV increments (same protocol as in experiments of Figure 1A). **B:** Superimposition of three original current traces recorded upon depolarization showing variant-related increase in peak outward current density. **C:** Current–voltage relationship curve showing average peak outward current densities (**P* <.05 vs Kv4.3 + KChIP2b + WT). WT = wild type.

KCNE2 co-expression also caused a positive shift (approximately +5 mV) of voltage dependence of steady-state inactivation. Steady-state inactivation was assessed using a double-step pulse method (Figure 2A, inset). Peak outward currents recorded at various levels of prepulse (Figure 2A) were normalized by that measured after a 500-ms prepulse at −90 mV and are plotted as a function of prepulse test potentials (Figure 2B). Half-inactivation potentials of steady-state inactivation, determined by fitting data to the Boltzmann equation (Eq. 2), were −46.0 ± 1.3 mV for Kv4.3 (open circles) and −40.8 ± 1.7 mV for Kv4.3 + *KCNE2* (filled circles, $P < .01$), consistent with the observation of Tseng's group.[13]

A double-pulse protocol (Figure 3A, inset) was used to test the effect of *KCNE2* co-expression on the time course for recovery from inactivation. Figure 3A shows the time course of recovery of Kv4.3 alone (open circles) and Kv4.3 + *KCNE2* (filled circles). Mean time constants for recovery from inactivation were not significantly different, indicating that co-transfection of *KCNE2* did not affect the time course of recovery from inactivation.

Effects of *KCNE2* on Kv4.3 + KChIP2b current and its gating kinetics

For human native cardiac I_{to}, KChIP2 has been shown to serve as a principal β subunit.[22–25] Accordingly, in another series of experiments, we examined the effect of WT and mutant *KCNE2* on Kv4.3 + KChIP2b current. Consistent with previous reports, in the presence of KChIP2, Kv4.3 currents showed a significantly faster recovery from inactivation (Figure 3B and Table 1).[26,27] Co-expression of WT *KCNE2* produced similar changes on Kv4.3 + KChIP2b current as on Kv4.3 current (Table 1). Kv4.3 + KChIP2b current recovery from inactivation was further accelerated: average time constant was 89.2 ± 6.5 ms for Kv4.3 + KChIP2b alone (open circles) and 60.2 ± 8.4 ms for Kv4.3 + KChIP2b + *KCNE2* (filled circles, $P < .05$). In 16 of 21 cells transfected with *KCNE2*, we observed an "overshoot" phenomenon, which is commonly seen during recording of native I_{to} in human ventricular myocytes.[28]

KCNE2 variants increase Kv4.3 + KChIP2b current and alter its gating kinetics

The I57T variant was first identified in an asymptomatic middle-aged woman with very mild QT prolongation.[6] In addition to this variant, the authors reported another *KCNE2* variant of the transmembrane segment (M54T) that was associated with ventricular fibrillation during exercise in a middle-aged woman. This patient appeared to show a wide range of QTc interval (390–500 ms). Therefore, we tested the functional effects of these two transmembrane *KCNE2* variants on Kv4.3 + KChIP2b currents.

The three panels of Figure 4A show three sets of current traces elicited by depolarizing pulses from a holding potential of −80 mV in cells co-transfected with WT (a), I57T (b), or M54T (c) *KCNE2*. Neither variant caused a significant shift of half-maximal activation voltage: −7.4 ± 1.4 mV (n = 8) for co-expression of WT *KCNE2*, −6.1 ± 1.5 mV (n = 8) for I57T, and −6.6 ± 1.6 mV (n = 8) for M54T. Both variants significantly increased I_{to} density: 125.0 ± 10.6 pA/pF in WT *KCNE2* (n = 21), 178.1 ± 12.1 pA/pF with I57T (n = 9), and 184.3 ± 27.9 pA/pF with M54T (n = 9, Figure 4C).

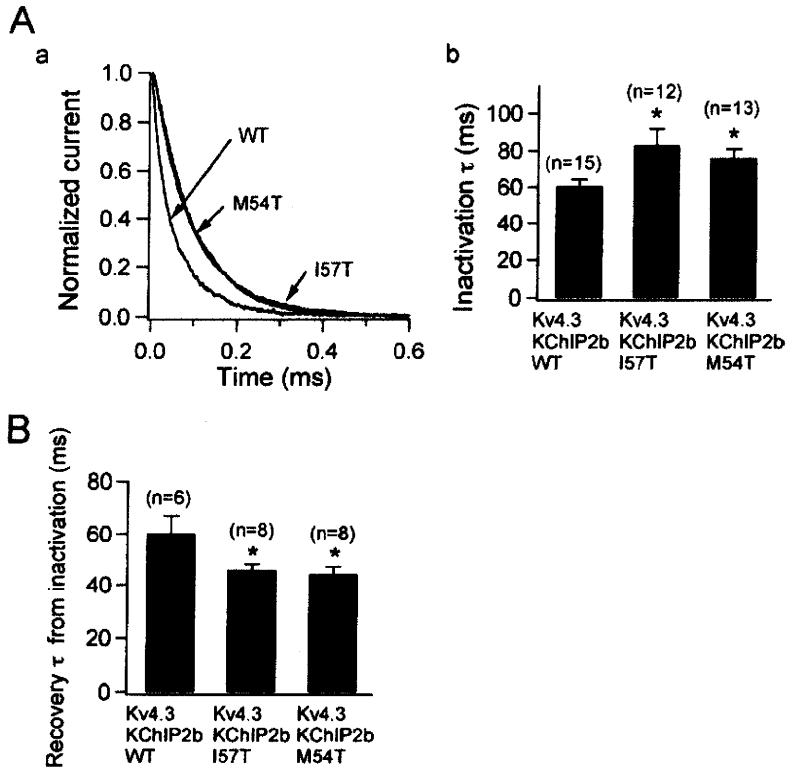

Figure 5 Two *KCNE2* variants slow inactivation kinetics and accelerate recovery from inactivation. **A, a:** Three current traces obtained from Chinese hamster ovary (CHO) cells transfected with wild-type (WT), I57T, and M54T *KCNE2* variant co-expressed with Kv4.3 and KChIP2b. Traces, which are normalized and superimposed, show that the variants slow inactivation. **A, b:** Time constants of decay at +20 mV for WT and variant *KCNE2* (*P <.05 vs Kv4.3 + KChIP2b + WT). Numbers in parentheses indicate numbers of observations. **B:** Time constants of recovery from inactivation recorded using a double-pulse protocol (*P <.05 vs Kv4.3 + KChIP2b + WT). Numbers in parentheses indicate numbers of observations.

Figure 5A shows the three traces depicted in Figure 4B normalized to their peak current level. This representation shows that the time course of inactivation of the two variant currents is slowed. The current decay was fitted by Equation 3 and the time constants (at +20 mV) summarized in Figure 5A, panel b. Finally, Figure 5B shows that the time constants of recovery of the two mutant channels from inactivation were significantly reduced. Thus, compared to WT *KCNE2*, recovery of reconstituted Kv4.3 + KChIP2b channels from inactivation was significantly accelerated with both I57T and M54T mutants.

Discussion

Kv4.3/KChIP2/MiRP1 complex can recapitulate the native I_{to}

In the present study, co-expression of WT *KCNE2* produced changes in kinetic properties (Figures 1–3 and Table 1) that led to close recapitulation of native cardiac I_{to}.[28,29] Notably, in addition to causing a positive shift of steady-state inactivation (Figure 2), *KCNE2* co-expression hastened the recovery of Kv4.3 + KChIP2b channels from inactivation (Figure 3). These modifications rendered Kv4.3 + KChIP2b channels more similar to native cardiac I_{to}, suggesting that *KCNE2* may be an important component of the native I_{to} channel complex. In contrast to a previous observation in HEK293 cells,[21] *KCNE2* co-expression decreased the current density of Kv4.3 and Kv4.3 + KChIP2b channel current in the present study, which seems to be a more reasonable result as the native I_{to} density reportedly was smaller in isolated human heart.[28] *KCNE2* co-expression has also been shown to reduce the density of Kv7.1[8,9] and HERG[6,7] channels.

Similar to the result of Deschenes and Tomaselli,[21] we failed to observe an overshoot during recovery from inactivation when *KCNE2* was co-expressed with Kv4.3 (Figure 3A), which is in contrast to the report of another group.[13] However, co-expression of *KCNE2* with Kv4.3 + KChIP2 channels produced an overshoot (Figure 3B), consistent with the report of Wettwer's group.[25] Wettwer et al also found that other *KCNE* subunits either were ineffective or induced only a small overshoot in CHO cells. Therefore, both MiRP1 and KChIP2 subunits are sufficient and necessary to recapitulate native I_{to} in the heart. Considering that the overshoot phenomenon has been described only in human ventricular I_{to} channels of the epicardial but not endocardial region,[28] these results may further implicate participation of MiRP1 and KChIP2 in the I_{to} channel complex in epicardium.

KCNE2 variants may alter the arrhythmogenic substrate by modulating I_{to}

Heterologous expression in CHO cells was conducted to examine the functional effects of I57T and M54T variants on Kv4.3 + KChIP2 channels. Both I57T and M54T

KCNE2 variants significantly (1) increased peak transient outward current density (Figure 4), (2) slowed the decay of the reconstituted I_{to} (Figure 5A), and (3) accelerated its recovery from inactivation (Figure 5B). Both variants thus caused an important gain of function in human I_{to}. These sequence changes may play a role in modulating I_{to} and thereby predispose to some inherited fatal rhythm disorders.

Functional effects on I_{to} induced by I57T and M54T resemble each other, increasing I_{to} density and accelerating its recovery from inactivation. The gain of function in I_{to} opposes the fast inward Na^+ currents during phase 0 of the action potential, leading to all or none repolarization at the end of phase 1 and loss of the epicardial action potential dome, thus promoting phase 2 reentry and fatal ventricular arrhythmias.[30]

Another *KCNE2* variant (M54T) associated with fatal arrhythmias was first identified in a woman who had a history of ventricular fibrillation and varied QT intervals.[6] It is possible that her arrhythmia was also related to a gain of function in I_{to} secondary to this variation in *KCNE2*. Interestingly, the I57T variant has been reported to produce a loss of function of HERG or Kv7.1 channels, thereby predisposing to long QT syndrome,[6,8] indicating that the same *KCNE2* variant could cause two different cardiac rhythm disorders, similar to long QT syndrome and Brugada syndrome caused by *SCN5A* mutations.[31,32]

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P wave and the development of atrial fibrillation

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BACKGROUND Terminal P-wave inversion in lead V_1 representing left atrial overload has been considered a precursor of atrial fibrillation (AF).

OBJECTIVE The purpose of this study was to determine whether this P-wave morphologic characteristic can predict the development of AF.

METHODS Digital analysis of 12-lead ECGs was performed to enroll patients with P terminal force ≥0.06 s × 2 mm in lead V_1 from among a database of 308,391 ECG recordings. The prognostic value of ECG characteristics for developing AF was determined.

RESULTS A total of 78 patients (mean age 52 ± 19 years) with left atrial overload were chosen from among 102,065 patients in the database. During mean follow-up of 43 months, 15 (19%) patients developed AF (AF group) versus 63 (81%) patients who did not (non-AF group). No significant difference was noted between the AF and non-AF groups with regard to the area, duration, and amplitude of the P-wave terminal portion in lead V_1. In contrast, the area, duration, and amplitude of the P-wave initial portion in the same lead were significantly greater in the AF group than in the non-AF group (114.6 ± 73.0 μV × ms vs 73.1 ± 59.3 μV × ms, 42.2 ± 12.4 ms vs 35.7 ± 10.1 ms, and 94.0 ± 39.9 μV vs 68.8 ± 49.4 μV, respectively; $P < .05$ for each). Multivariate analysis confirmed that the area of the P-wave initial portion was independently associated with the development of AF (hazard ratio 4.02, 95% confidence interval 1.25–17.8; $P = .018$).

CONCLUSION P-wave initial portion in lead V_1 was an independent risk stratifier of AF development in patients with marked left atrial overload.

KEYWORDS Atrium; Electrocardiography; Fibrillation; Prognosis

ABBREVIATIONS **AF** = atrial fibrillation; **CI** = confidence interval; **ECG** = electrocardiogram; **LA** = left atrium; **RA** = right atrium

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Introduction

The P wave reflects electrical depolarization of both the right atrium (RA) and the left atrium (LA). When the P wave is biphasic in lead V_1, the positive initial portion and the negative terminal portion of the P wave represent depolarization of the RA and the LA, respectively.[1,2] Morris et al[3] reported that the magnitude of the negative terminal potion of the P wave, calculated as the algebraic product of the duration and amplitude (P terminal force) in precordial lead V_1, was significantly larger in patients with various valvular heart diseases than in normal subjects. In their study, the P terminal force was associated with mitral valve area and increased LA pressure. The magnitude of the P terminal force has been shown to be associated with LA enlargement as revealed by transthoracic echocardiography.[4,5] These findings suggest that the negative terminal potion of the P wave in lead V_1 is a sign of pressure and volume overload in the LA, which may lead to structural and functional remodeling in the LA. Because atrial fibrillation (AF) often occurs and/or recurs in the remodeled LA,[6] the increased P terminal force may underlie the generation of AF. The increased P terminal force is observed not only in valvular heart diseases but also in other heart diseases, including hypertension, myocardial infarction, and cardiomyopathy.[7,8] These disorders potentially underlie the generation of AF. However, little is known about whether P terminal force occurring in those disorders is associated with a prognostic risk for the development of AF. Prolonged P-wave duration is a useful predictor of AF development.[9,10] The signal-averaged P-wave electrocardiogram (ECG) has a significant role in identifying patients who are susceptible to paroxysmal AF and in predicting the progression from paroxysmal to permanent AF.[11] Measurement of signal-averaged P-wave duration requires a dedicated system, which is not widely available in general clinical practice. In contrast, standard 12-lead ECGs can be conveniently recorded, and automatic analysis of 12-lead ECG recordings yields information to clinicians. In our university hospital, more than 300,000 ECGs obtained from more than 100,000 patients are available for digital analysis. Using this large database, we performed a retrospective cohort study to investigate whether terminal P-wave inversion in lead V_1 predicts the development of AF.

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Methods

Database

We constructed a database for analyzing resting 12-lead ECGs recorded in our hospital, which is associated with the Shiga University of Medical Science. A total of 102,065 patients (49,286 females and 52,779 males) who had undergone ECG recordings between January 1983 and October 2008 were collected in our database, and a total of 308,391 ECG recordings were performed during this period. Twelve leads were simultaneously acquired. The 12-lead ECG was recorded for 10 seconds at a sweep speed of 25 mm/s and calibrated to 1 mV/cm in the standard leads. ECG signals were recorded at an interval of 2 ms (i.e., 500 Hz). Digital data were stored on a computer server with 12-bit resolution. From the database, patients who fulfilled ECG criteria of LA overload were chosen using the analysis software MUSE7.1 (GE Marquette Medical Systems, Inc., Milwaukee, WI, USA). Computer-processed ECGs defined LA overload criteria as follows. (1) ECGs displaying biphasic P wave in lead V_1 were chosen. (2) The P wave was divided into the positively deflected portion in the initial P wave and the negatively deflected portion in the terminal P wave. (3) The terminal P wave in lead V_1 with duration $\geq$0.06 second and amplitude ≤ -0.2 mV (i.e., P terminal force $\geq$0.12) was considered as meeting LA overload criteria in this study (Figure 1).

Study participants

From our database, 78 participants who had marked LA overload were selected and assessed for the development of AF. A control group of 234 participants who did not have LA overload also was selected (1:3 matching). Individual matching was performed accounting for confounders (age, gender, date when ECG was taken), and when control candidates numbered more than three, the three controls were chosen randomly from among the candidates. The research protocol was approved by the Ethical Committee of Shiga University of Medical Science (19–75).

Digital analysis of ECG

The MUSE7.1 software detected identical P waves using a template matching technique. A point that had an area $\geq$160 μV/ms from the baseline level was considered to be P-wave onset, and a point that had an area $\leq$160 μV/ms from the baseline level was considered to be P-wave offset. The duration, amplitude, and area of total P wave, initial P wave, and terminal P wave in lead V_1 were measured using matrix parameters available in MUSE7.1. P-wave area was constructed by integrating the duration and amplitude. Duration $\times$ amplitude of P-wave initial and terminal portions in lead V_1 were calculated as force values. These variables were composed using the average value of the P wave during 10 seconds of recording time. Because all measurements of 12-lead ECGs were performed digitally using MUSE7.1, neither intraobserver nor interobserver variability occurred in this study.

Statistical analysis

The occurrence of AF was set as an endpoint, and the prognostic factors for developing AF were explored in the analysis. Patients whose ECG exhibited AF during the follow-up period (AF group) were compared with patients who did not (non-AF group). The follow-up period was defined as the interval between the first day when an ECG with LA overload was recorded and the first day when an ECG displaying AF was recorded in the AF group, or the interval between the first day when an ECG with LA overload was recorded and the latest day when an ECG was recorded in the non-AF group. The occurrence of death from any cause during the follow-up period was assessed by mail questionnaire. Written informed consent was obtained from all patients. Data are given as mean $\pm$ SD or percentage, and group comparisons were made using t-test or Mann-Whitney test, as appropriate. Categorical variables were compared using the Fisher exact test. Comparison of AF occur-

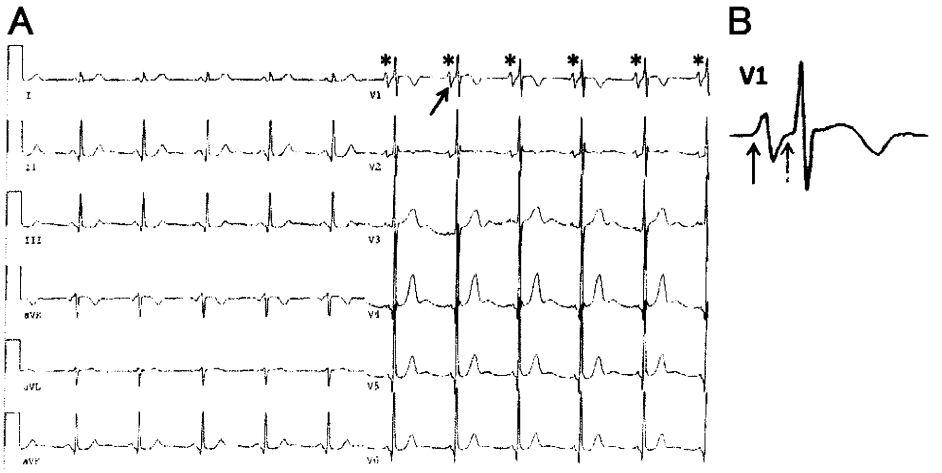

Figure 1 **A:** Twelve-lead ECG showing typical pattern of left atrial overload in lead V_1. *Red arrow* indicates P-wave negative terminal portion in lead V_1. *Asterisks* indicate P waves with identical morphology detected by template matching. **B:** Magnified ECG trace of lead V_1. *Blue arrow* indicates P-wave onset. *Green arrow* indicates P-wave offset.

rence between patients with LA overload and control patients was performed by logistic regression analysis and reported as odds ratio with 95% confidence interval (CI). Kaplan-Meier curves were used for determining the difference between two groups, and log rank test was used for examining the difference. Cox proportional hazard regression was used to estimate multivariate adjusted hazard ratios accounting for confounders (age, sex, cause of heart disease, ECG variables of P wave). All statistical tests were two-tailed, and $P < .05$ was considered significant.

Results

Atrial fibrillation

A total of 78 patients (mean age 52 ± 19 years) who fulfilled ECG criteria of marked LA overload were selected from our database using the GE Marquette 12SL ECG analysis program and enrolled for ECG analysis in this study. Of these patients, 15 (19%) developed AF (AF group), whereas 63 did not present AF (non-AF group). The control group consisted of 234 patients who were well matched for age (52 ± 19 years) and gender (78 women and 156 men; Table 1). AF developed in 3 (1.3%)of 234 control patients. The incidence of AF in patients with marked LA overload was 15-fold higher than that in control patients ($P < .001$). The odds ratio for occurrence of AF in patients with LA overload compared with control patients was 18.3 (95% CI 5.15–65.3). The mean follow-up period of the control patients was significantly longer than that of the patients with LA overload (78 ± 73 months vs 43 ± 52 months; $P < .001$). Kaplan-Meier survival analysis is shown in Figure 2. The AF-free event rate was significantly higher ($P < .001$) in patients with LA overload than in control patients (hazard ratio 24.5, 95% CI 7.94–107.3).

Characteristics of the patients

The clinical characteristics of patients in the AF and non-AF groups are listed in Table 2. The mean follow-up period of the AF group and non-AF group averaged 45 ± 61 months and 43 ± 50 months, respectively ($P = .93$). No significant difference with regard to age and sex was disclosed between the AF and non-AF groups. The average age at ECG documentation of AF was 59 ± 13 years. In the AF group, 14 (93%) of 15 patients had structural heart diseases such as hypertension, myocardial infarction, valvular heart diseases, and nonischemic cardiomyopathy. In contrast, structural heart disease was present in 46 (73%) of 63 patients in the non-AF group ($P = .081$). The presence of hypertension was more frequent in the AF group than in the non-AF

Table 1 Comparison of characteristics of control patients and patients with left atrial overload

	Control	Left atrial overload
No. of patients	214	78
Age (years)	52.4 ± 19.3	52.4 ± 19.3
Male [n (%)]	156 (66.7)	52 (66.7)
Follow-up period (months)	78.0 ± 72.9*	43.3 ± 52.0

Values are given as mean ± SD unless otherwise indicated.
*$P < .001$ vs patients with left atrial overload.

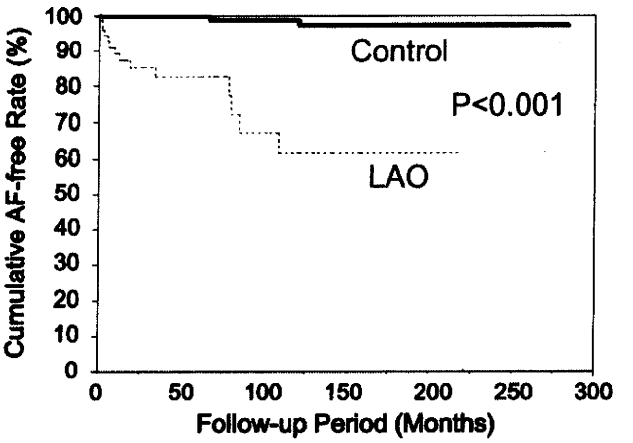

Figure 2 Kaplan-Meier estimates of atrial fibrillation (AF)-free event rate in patients with left atrial overload (LAO) and control patients. The difference between the two groups was significant ($P < .001$ by log rank test).

Table 2 Characteristics of the patients

Characteristic	AF group (n = 15)	Non-AF group (n = 63)	P value
Age (years)	55.8 ± 14.7	51.6 ± 20.3	.22
Gender (male/female)	10/5	42/21	1
Structural heart disease	14 (93)	46 (73)	.063
Hypertension	9 (60)	20 (31)	.045
Valvular heart disease	7 (47)	16 (25)	.12
Myocardial infarction	0 (0)	8 (13)	.06
Nonischemic cardiomyopathy	3 (20)	15 (24)	.66
Hypertrophic cardiomyopathy	3 (20)	7 (11)	.38
Dilated cardiomyopathy	0 (0)	8 (13)	.06
NYHA functional class I/II/III/IV	13/2/0/0	30/28/5/0	.80
Left ventricular ejection fraction (%)	63.2 ± 9.89	54.0 ± 18.5	.04
Antiarrhythmic drug			
Class IA	6	2	.01
Class IC	1	1	.32
Class III	1	0	.07
Diuretic	5	21	.70
Beta blocker	5	9	.32
Calcium antagonist	2	14	.44
Angiotensin II receptor blockade	1	2	.55
Angiotensin-converting enzyme inhibitor	3	8	.48
Nitrate	3	11	.81
Digitalis	5	15	.37
Oral anticoagulant	5	11	.14
Aspirin	3	5	.20

Values are given number, number (%), or mean ± SD.
AF = atrial fibrillation; NYHA = New York Heart Association.

group (odds ratio 3.2, 95% CI 1.01–10.3; P = .04), but other structural heart diseases showed no significant difference between the two cohort groups. Of note, the prevalence of both hypertension and valvular heart disease was significantly higher in the AF group (4/15 [26.7%]) than in the non-AF group (3/63 [4.8%]; odds ratio 7.3, 95% CI 1.4–37.1; P = .018).

Characteristics of ECG

ECG characteristics are listed in Table 3. No significant difference with regard to heart rate and frontal plane P-wave axis was seen between the AF and non-AF groups. The total duration of P wave in lead V_1 was significantly longer in the AF group than in the non-AF group. In contrast, the total amplitude (amplitude from top to bottom level) of the P wave in lead V_1 was not significant between the two groups.

For the two cohorts, we first evaluated the P-wave terminal portion in lead V_1, which was assigned as a marker for choosing patients from the database in the study. Table 4 (top) lists measurements of the P-wave terminal portion in lead V_1. The area of the P-wave terminal portion did not differ between the AF and non-AF groups. Neither the duration nor the amplitude of the P-wave terminal portion was different between the AF and non-AF groups. The same was true for the P-wave terminal force between the two groups. Because no significant difference in P-wave terminal portion in lead V_1 was observed between the AF and non-AF groups, we then estimated the initial portion of P wave in lead V_1. Table 4 (bottom) lists measurements of the P-wave initial portion in lead V_1. The area of the P-wave initial portion was significantly larger in the AF group than in the non-AF group. The duration of the P-wave initial portion was significantly longer in the AF group than in the non-AF group, and the amplitude of the P-wave initial portion was significantly higher in the AF group than in the non-AF group. Therefore, the P-wave initial force was significantly greater in the AF group than in the non-AF group.

AF development

Based on the significant association of the P-wave initial portion in lead V_1 with AF development, the AF-free event rate was estimated according to the area of P-wave initial portion. Using receiver operating characteristic analysis, the sensitivity and specificity of P-wave initial portion in response to developing AF were maximized by the area of P-wave initial portion of 65 (relative risk 4.0, 95% CI 1.2–13.1). Kaplan-Meier life-table analysis is shown in Figure 3. The area of the P-wave initial portion was associated with a significant difference of AF-free event rate between patients with area of P-wave initial portion ≥65 (n = 39) and those with area of P-wave initial portion <65 (n = 39; hazard ratio 4.02, 95% CI 1.25–17.8; P = .02). The rate of use of Class I antiarrhythmic drugs was identical between patients with area of P-wave initial portion ≥65 and those with area of P-wave initial portion <65 (10% vs 8%; P = .72). Because age is an important factor affecting the development of AF, the AF-free event rate was compared between patients <65 years old (n = 55) and those ≥65 years (n = 23). No significant difference was seen with regard to age (hazard ratio age ≥65 years to age <65 years = 2.39, 95% CI 0.72–7.19; P = .12). The AF-free event rate between patients with and those without hypertension was compared because hypertension was more prevalent in the AF group than in the non-AF group, but the presence of hypertension did not significantly affect the development of AF (hazard ratio of presence to absence of hypertension = 1.4, 95% CI 0.4–4.4; P = .54). In addition, no significant gender difference was found with regard to the AF-free

Table 3 Characteristics of ECG

Measurement	AF group	Non-AF group	P value
Heart rate (bpm)	69.0 ± 22.4	84.1 ± 19.3	.99
P-wave axis (°)	60.5 ± 20.5	61.9 ± 14.3	.62
P wave (ms) in lead V_1			
Total duration (ms)	126.7 ± 14.8	115.8 ± 16.7	.012
Total amplitude (μV)	310.7 ± 15.8	302.9 ± 64.9	.33

Table 4 Measurements of P wave in lead V_1

Measurement	AF group	Non-AF group	P value
Terminal Portion			
Duration (ms)	84.5 ± 15.0	80.1 ± 12.5	.123
Amplitude (μV)	−216.7 ± 20.1	−234.0 ± 40.0	.108
Area (μV × ms)	468.2 ± 155.0	477.7 ± 139.5	.41
Terminal force (s × μV)	18,491 ± 5,149	18,779 ± 4,584	.42
Initial Portion			
Duration (ms)	42.2 ± 12.4	35.7 ± 10.1	.018
Amplitude (μV)	94.0 ± 39.9	68.8 ± 49.4	.035
Area (μV × ms)	114.6 ± 73.0	73.1 ± 59.3	.011
Initial force (s × μV)	4,346.7 ± 2,712	2,650.3 ± 2,375	.0089

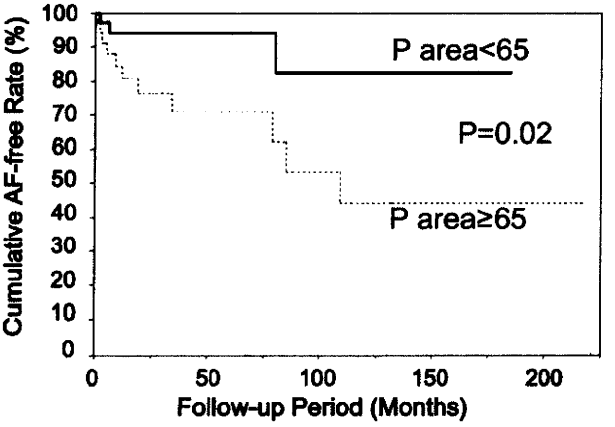

Figure 3 Kaplan-Meier estimates of atrial fibrillation (AF)-free event rate in patients with left atrial overload according to the area of P-wave initial portion in lead V_1. The AF-free event rate in patients with area of P-wave initial portion ≥65 μV × ms was significantly lower than in those with area of P-wave initial portion <65 μV × ms (P = .02).

Table 5 Probability of AF development during follow-up based on clinical and ECG variables

	Hazard ratio	95% Confidence interval	*P* value
P area ≥65 μV × ms	4.07	1.16–19.4	.02
P area <65 μV × ms	1	—	—
Age ≥65 years	1.96	0.56–6.18	.28
Age <65 years	1	—	—
Hypertension	0.91	0.27–3.09	.87
No hypertension	1	—	—
Male	0.79	0.23–2.88	.71
Female	1	—	—

AF = atrial fibrillation; ECG = electrocardiographic.

event rate (hazard ratio of male to female 1.0, 95% CI 0.3–3.3; $P = .99$).

Multivariate analysis confirmed that the area of P-wave initial portion was independently associated with an increased propensity for development of AF (Table 5). After adjustment for age and gender, the hazard ratio for AF development was 4.07 (95% CI 1.16–19.4; $P = .02$). The level of the area of P-wave initial portion in lead V_1 was compared in patients with and those without hypertension. The area of P-wave initial portion in lead V_1 was not significantly different between patients with and those without hypertension (84 ± 59 vs 80 ± 67, respectively; $P = .80$) and was not significantly different between patients ≥65 years old and those <65 years (86 ± 67 vs 79 ± 62; $P = .69$). In addition, gender was not significantly related to the area of P-wave initial portion (male 83 ± 61, female 77 ± 70; $P = .68$), nor was left ventricular ejection fraction ($R^2 = 0.00048$, $P = .86$ by linear regression analysis).

Discussion

Since the early description of an asynchrony of atrial depolarization by Reynolds,[12] several studies reported P-wave abnormality suggesting LA enlargement.[13–15] In 1964, Morris et al[3] advanced this concept as representing LA overload. They proposed that P terminal force >0.04 second in duration and >0.1 mV in depth at lead V_1 was associated with hemodynamically strained LA in various valvular heart diseases. Since then, increased P terminal force in lead V_1 has been considered a probable precursor to development of AF, as patients with such disorders likely suffer from AF. In this study, we systematically tested in a large size of population the hypothesis that P wave with LA overload is linked to the development of AF. Consistent with previous epidemiologic studies,[16,17] AF occurred in a few percentage of control patients in this study but occurred at a substantially higher incidence in AF patients with LA overload. Our results confirmed that when LA overload was present, the magnitude of overload in the RA could be independently attributed to the development of AF, indicating that analysis of P wave in lead V_1 deserves consideration for predicting AF. This is an important for clinicians. The measurements of P wave in our study were performed using 12-lead ECG recordings, which are commonly available in clinical practice. Moreover, computer-based measurements were performed at high resolution for data analysis of P-wave variables, which provides precise reproducibility.

P wave and AF

A principal aim of this study was to establish the prognostic importance of the P wave in lead V_1. The terminal portion of the P wave in lead V_1 has been associated with electrical depolarization of the LA alone in humans[18] and in dogs.[19] Using angiocardiography, Miller and Spertus[20] showed a correlation of marked negative component in leads V_1 and V_2 with LA enlargement. Subsequently, Morris et al[3] showed a significant correlation of the magnitude of P terminal force with severity of hemodynamic abnormality. The P terminal portion in lead V_1 is composed of several factors: (1) anatomic shift of the LA to the posterior side by hemodynamic strain, (2) enlarged LA size, (3) LA hypertrophy, and (4) reduced conduction velocity in the LA.[8,21,22] These factors are also attributed to prolonged P-wave duration. We used a much larger P terminal force for patient selection in this study than did Morris et al. Therefore, it is reasonable to speculate that patients included in this study have a high probability of AF occurrence. Indeed, compatible with this assumption, patients with marked LA overload developed AF at a substantially higher rate than did control patients. This finding indicates that increased magnitude of P-wave terminal portion in lead V_1 is a useful marker for predicting the development of AF. Furthermore, in the current study, the increased P-wave terminal portion provided information on predictivity of AF when the P-wave initial portion in lead V_1 was additively estimated. Regardless of the magnitude of the P-wave terminal portion in lead V_1, however, the magnitude of the P-wave initial portion in lead V_1 was attributed to the development of AF. This finding indicates that overload in the RA may be critical to the development of AF, and atrial vulnerability to fibrillation is likely to increase when both atria are overloaded. In addition to LA overload, electrophysiologic abnormality in the RA may increase susceptibility to AF development. Although depolarization originating from the atrial septum and/or left atrium may participate in part of the P-wave initial portion, the P-wave initial portion in lead V_1 mainly represents depolarization of the RA. Thus, our data indicate the importance of evaluating whether or not the RA is overloaded when LA overload is present. Although Class I antiarrhythmic drugs were used more frequently in the AF group than in the non-AF group, the drugs were administered similarly between two groups dichotomized according to the area of P-wave initial portion, thereby indicating that overload in the RA is an independent prognostic marker of AF.

P-wave features observed in this study reflect electrophysiologic and structural remodeling of the atrium that predisposes to the development of AF. Increased P-wave duration results from either slow conduction or an enlarged atrium. The former shortens wavelength, and the latter provides a sufficient area for reentry to occur. These pathophysiologic changes are linked to the maintenance of AF.[6]

Increased intracardiac pressure of the left ventricle may cause LA remodeling, which is likely to occur in patients with structural heart disease. Disturbed transmitral blood flow due to elevated diastolic pressure in the left ventricle may induce heterogeneous distribution of the atrial refractory period. Structural remodeling, as occurs with interstitial fibrosis and connexin redistribution, causes anisotropic conduction or discontinuous propagation. In hypertrophied atrial myocytes, triggered activity, such as early and delayed afterdepolarizations, is prone to occur.[23,24] The present study showed that an increased magnitude of P-wave initial force in lead V_1 was associated with a higher rate of AF development. This finding suggests that when a substrate develops in the RA in addition to the LA, susceptibility to the development of AF may increase.

Study limitations

Because the retrospective cohort study was conducted using ECGs recorded in our hospital, several limitations are inherent. First, we determined AF development by reviewing past ECGs, but recordings of AF might have been missed if AF terminated spontaneously before the ECGs were recorded in the hospital. Because no AF can be documented during follow-up of a patient who suffered from transient AF, this patient was classified into the non-AF group, and the AF-free duration appears longer than the true AF-free duration. Second, in the present study, LA overload was defined based on the P-wave terminal portion in lead V_1. Although this ECG marker is representative of LA overload, surrounding tissue of the heart (e.g., fat and lung) may affect the amplitude and area of the P-wave terminal portion in lead V_1, indicating that how precisely the P-wave terminal portion reflected LA overload might differ depending on the individual. Third, because our study included patients who underwent ECG recording in our hospital, the risk of AF in the study population undoubtedly was greater than that in the general population. Therefore, this factor should be considered when our results are extrapolated to a broader population.

Clinical implications

AF is one of the most common cardiac rhythm disorders; however, useful ECG identification of patients at greatest risk for developing AF remains the preeminent challenge to physicians who care for AF-prone patients. Assessment of signal-averaged ECGs of P wave has served as the principal noninvasive means of determining AF risk. This method, which estimates vulnerability to AF, is fundamentally based on delayed conduction, which may provide the substrate for reentry. Consistent with signal-averaged ECG, our ECG parameters also reflect interatrial conduction disturbance. Our data indicate that P-wave analysis using standard 12-lead ECG recordings could successfully detect a risk stratifier of AF. In addition, our quantitative relationship between P wave and vulnerability to AF could be exploited to define the risk of AF development and determine which patients are most likely to benefit from preventive anticoagulant therapy. Our results suggest that coexistence of overload in the RA and the LA may be useful for evaluating some patients. For example, screening patients with palpitations might provide a means for identifying those at high risk for AF development. In order to make measurement of the P wave a widely available marker for patients, improvements of the automatic algorithm for analysis of 12-lead ECGs are needed to predict AF in a timely fashion.

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High prevalence of early repolarization in short QT syndrome

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BACKGROUND Short QT syndrome (SQTS) is characterized by an abnormally short QT interval and sudden death. Due to the limited number of cases, the characteristics of SQTS are not well understood. It has been reported recently that early repolarization is associated with idiopathic ventricular fibrillation and the QT interval is short in patients with early repolarization.

OBJECTIVE The purpose of this study was to study the association between early repolarization and arrhythmic events in SQTS.

METHODS The study consisted of three cohorts: SQTS cohort (N = 37), control cohort with short QT interval and no arrhythmic events (N = 44), and control cohort with normal QT interval (N = 185). ECG parameters were compared among the study cohorts.

RESULTS Heart rate, PR interval, and QRS duration were similar among the three study cohorts. Early repolarization was more common in the SQTS cohort (65%) than in the short QT control cohort (30%) and the normal QT control cohort (10%). Duration from T-wave peak to T-wave end was longer in the SQTS cohort than in the short QT control cohort, although QT and corrected QT intervals were similar. In the SQTS cohort, there were more males among patients with arrhythmic events than in those with a family history but without arrhythmic events. In multivariate models, early repolarization was associated with arrhythmic events in the SQTS cohort. ECG parameters including QT and QTc intervals were not associated with arrhythmic events in the SQTS cohort.

CONCLUSION There is a high prevalence of early repolarization in patients with SQTS. Early repolarization may be useful in identifying risk of cardiac events in SQTS.

KEYWORDS Arrhythmia; Electrocardiogram; QT interval; Repolarization; Sudden death

ABBREVIATIONS **QTc** = corrected QT interval; **SQTS** = short QT syndrome

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Introduction

The short QT syndrome (SQTS) is characterized by an abnormally short QT interval and increased risk of ventricular fibrillation and sudden death.[1,2] Similar to other arrhythmia syndromes, such as long QT syndrome and Brugada syndrome,[3] SQTS is a genetically heterogeneous disease, and, to date, five responsible genes encoding different ion channels have been identified.[3–7] Some inherited arrhythmia syndromes may share genetic backgrounds that result in overlapping arrhythmia phenotypes.[3]

Although early repolarization is generally considered benign,[8] it has been reported recently that early repolarization is associated with increased risk for sudden cardiac death in patients with idiopathic ventricular fibrillation.[9–12] Haissaguerre et al[9] reported that, among patients with idiopathic ventricular fibrillation, the QT interval was shorter in patients with early repolarization than in those without, suggesting an association between early repolarization and QT interval shortening. Evidence that mutations in calcium channel genes are associated with Brugada-type ST-segment elevation and abnormally short QT intervals further suggests a relationship between early phase repolarization abnormalities and short QT interval.[4] Here we report on our

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study of the prevalence of early repolarization and its association with arrhythmic events in SQTS.

Methods

This cooperative study consisted of three cohorts. (1) *SQTS cohort* included patients with SQTS referred to our institutions and patients with SQTS from previous reports. The diagnosis of SQTS was made if a patient with a short QT interval [corrected QT interval (QTc) by Bazett formula ≤330 ms] had an arrhythmic event including documented ventricular fibrillation, resuscitated sudden cardiac death, and syncope and/or had a family history of SQTS, or if a patient with a short QT interval (QTc ≤360 ms) had mutations in ion channel genes responsible for SQTS.[3,13] We searched in the electronic databases PubMed, EMBASE, and Cochrane for all published studies that examined patients with SQTS. The search was limited to the end of June 2009. Published studies were considered eligible if they included clinical characteristics of the patients and ECGs. All ECGs from patients reported in the literature were reanalyzed. Electrophysiologic study was performed in patients with SQTS based on the indication of each institution. (2) *Control cohort with short QT interval* (QTc ≤330 ms) *and no arrhythmic events* was selected from among 86,068 consecutive ECGs stored on the ECG database at Niigata University Medical and Dental Hospital from May 7, 2003 to July 2, 2009. Subjects who did not have arrhythmic events or cardiovascular disease and were not taking any medication were included in this cohort. (3) *Control cohort with normal QT interval* was also selected from the ECG database. This cohort consisted of subjects who were matched to the SQTS cohort for gender and age. Subjects who had normal QT interval (360–440 ms) and did not have cardiovascular disease or were not taking any medication were included in this cohort. Subjects with Brugada-type ST-segment elevation were excluded from all study cohorts.[3,9]

QT intervals were measured on lead V_2 with the tangent methods for determination of QT_{end} using a semi-automated digitizing program with electronic calipers by an experienced observer blinded to the clinical details of all subjects included in this study.[14,15] Early repolarization was defined as elevation of the J point noted as either as QRS slurring or notching ≥0.1 mV in more than two leads.[9]

Differences in parameters were analyzed using multivariable logistic regression models when SQTS cohort and control cohort with short QT interval were compared and analyzed using conditional logistic regression models when SQTS cohort and control cohort with normal QT interval were compared. All statistical analyses were performed with SPSS (version 12.0, SPSS, Inc., Chicago, IL, USA). Two-sided $P < .05$ was considered significant. Values are expressed as mean ± SD. The study protocol was approved by the Ethics Committee of Niigata University School of Medicine. To determine interobserver variability, a second observer made independent blinded QT interval determinations of all study subjects with short QT interval.

Results

Thirty-seven patients with SQTS were identified: 12 from our institutions and 25 reported in the literature,[2,5,6,14,16–25] Forty-four control subjects with short QT interval and 185 control subjects with normal QT interval also were identified (Table 1). The SQTS cohort consisted of 25 (68%) patients with symptoms, including 14 with cardiac arrest (3 sudden death, 11 resuscitated) and 11 with syncope. Genetic screening identified mutations in ion channels in 7 (41%) of 17 probands who were genetically screened (2 *KCNQ1*, 4 *KCNH2*, 1 *KCNJ2*). Among patients in our institutions and those reported in the literature, there was no difference with regard to gender, age, prevalence of family history, QT or QTc interval, or inducibility of ventricular tachyarrhythmia by electrical programmed stimulation.

Heart rate, PR interval, and QRS duration in the SQTS cohort were not different among patients in either the short QT control cohort or the normal QT control cohort (Table 1). QT and corrected QT intervals were shorter in the SQTS and short QT control cohorts than in the normal QT control cohort. Early repolarization occurred in 24 (65%) patients with SQTS (Figure 1). Interobserver variability between two investigators was 8.6 ms (95% confidence interval −0.5 to 17.7 ms) for QT interval and 9.0

Table 1 ECG parameters of study cohorts

	Patients with SQTS (N = 37)	Subjects with short QTc (N = 44)	Versus subjects with short QTc*		Subjects with normal QTc† (N = 185)	Versus subjects with normal QTc	
			OR (95% CI)	*P* value		OR (95% CI)	*P* value
Male gender [N (%)]	27 (73)	34 (77)	2.84 (0.72–11.2)	.14	135 (73)	—	—
Age (years)	30 ± 19	47 ± 23	1.05 (1.02–1.08)	.001	30 ± 19	—	—
Heart rate (bpm)	69 ± 393	65 ± 398	1.00 (1.00–1.01)	.3	70 ± 327	1.00 (1.00–1.00)	0.70
PR interval (ms)	138 ± 19	153 ± 38	1.01 (0.99–1.03)	.54	143 ± 24	0.99 (0.97–1.01)	0.18
QRS interval (ms)	86 ± 7	84 ± 8	0.97 (0.91–1.04)	.38	85 ± 7	1.01 (0.96–1.06)	0.74
QT interval (ms)	286 ± 36	286 ± 15	0.99 (0.97–1.01)	.28	367 ± 36	0.97 (0.96–0.98)	<0.001
QTc (ms)	308 ± 29	299 ± 21	0.98 (0.96–1.00)	.06	399 ± 24	0.97 (0.97–0.98)	<0.001

CI = confidence interval; OR = odds ratio; QTc = corrected QT interval; SQTS = short QT syndrome.

*Models were adjusted for gender and age.

†Gender and age were matched between patients with SQTS and subjects with normal QT interval.

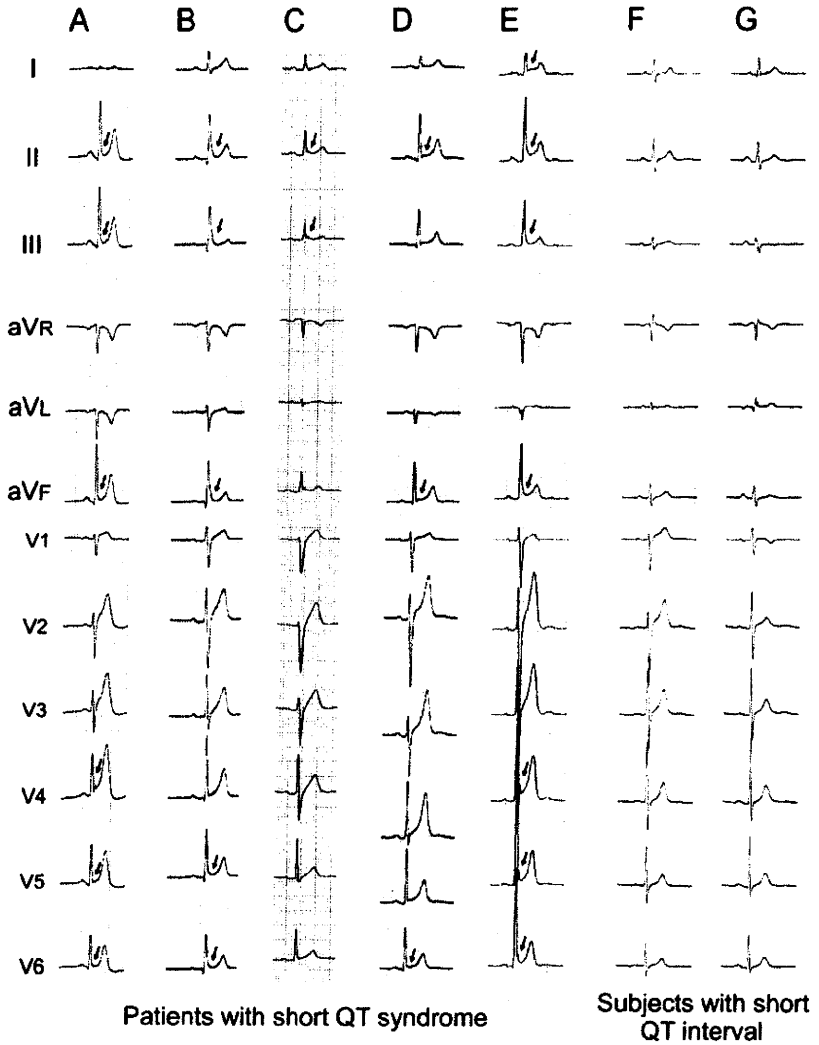

Figure 1 Early repolarization in short QT syndrome. ECGs were recorded from patients with short QT syndrome (**A:** 61-year-old woman; **B:** 30-year-old man; **C:** 38-year-old man; **D:** 31-year-old man; **E:** 22-year-old man) and control subjects with a short QT interval (**F:** 23-year-old man; **G:** 44-year-old woman). In each patient with short QT syndrome, early repolarization was evident in the inferolateral leads (*arrows*).

ms (95% confidence interval −0.6 to 18.7 ms) for QTc interval. The frequency of early repolarization was not different between patients in our institutions and those reported in the literature. Early repolarization was present in the inferior leads (II, III, aVF) in 9 patients, in the lateral leads (I, aVL, V_4–V_6) in 6 patients, and in both the inferior and lateral leads in 9 patients. Of 10 probands with early repolarization genetically screened, mutations were identified in 3 patients (1 *KCNQ1*, 2 *KCNH2*). Early repolarization was more common in the SQTS cohort than in the short QT control and normal QT control cohorts (Figure 2).

The association of early repolarization with arrhythmic events then was studied in patients with SQTS. In the SQTS cohort, there were more males among patients with arrhythmic events than among those with a family history but without arrhythmic events (Table 2). In multivariate models adjusted for gender and age, early repolarization was associated with arrhythmic events, although ECG parameters including QT and QTc intervals were not associated with arrhythmic events. Early repolarization remained associated with arrhythmic events after adjustment for age, gender, and QTc interval (P = .001). Electrophysiologic study performed in 18 patients with SQTS revealed no difference in inducibility of ventricular tachyarrhythmia between patients with arrhythmic events (73%) and those without arrhythmic events (71%).

QT interval parameters were compared between SQTS and short QT control cohorts because some of the parameters recently have been associated with SQTS.[26] Interval from T-wave peak to T-wave end (T_{peak} to T_{end}) was longer in the SQTS cohort than in the short QT control cohort even after heart rate correction using the Bazett formula, whereas QT interval, QTc interval, and interval from Q-wave to T-wave peak (QT_{peak}) were not different between the two cohorts (Table 3). Ratio of T_{peak} to T_{end} per QT was larger in the SQTS cohort than in the short QT control cohort.

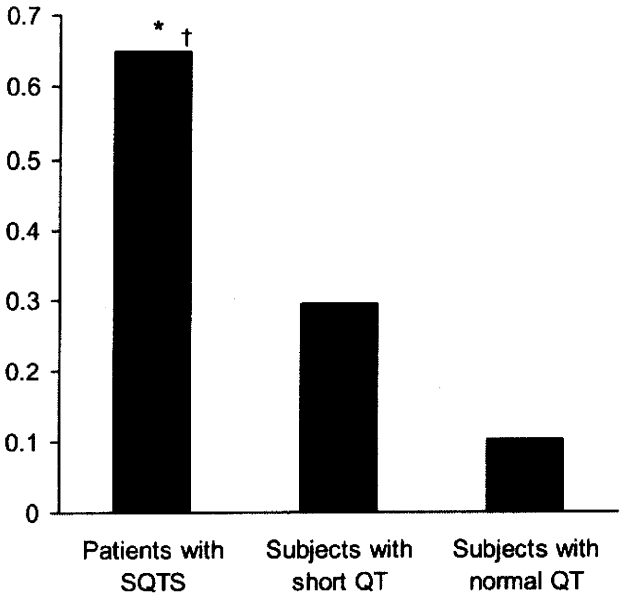

Figure 2 Frequency of early repolarization. Odds ratios (95% confidence intervals) for early repolarization in patients with short QT syndrome (SQTS) were 5.64 (1.97–16.15) and 16.58 (7.2–38.21) versus subjects with short QT interval and those with normal QT interval, respectively. *P = .001 vs subjects with short QT interval. †P <.001 vs subjects with normal QT interval.

Discussion

SQTS is a recently discovered, very rare disease with an increased risk of sudden death.[2] Due to the limited number of cases, the characteristics of SQTS are not well understood. Therefore, we conducted a cooperative analysis of ECGs from patients with SQTS in our institutions and those reported in the literature and found that early repolarization is common in SQTS.

Early repolarization is a common ECG finding. It is present in 1% to 13% of the general population and usually is considered as a normal variant due to its benign long-term prognosis.[8,11,27–29] However, increasing evidence suggests that early repolarization is associated with arrhythmia.[9,27,30–34] Since 1985, we and other investigators have reported an association between early repolarization (or late depolarization) and sudden cardiac death.[30–32] A multicenter study including our institution recently showed that early repolarization is present in one third of patients with idiopathic ventricular fibrillation.[9] Early repolarization is associated with increased risk of sudden cardiac arrest in idiopathic ventricular fibrillation, and the amplitude of early repolarization increases before development of arrhythmic events.[9,10] In Brugada syndrome, which is characterized by J-wave and ST-segment elevation in the right precordial leads on ECG and sudden cardiac death,[3] early repolarization in the inferolateral leads is not uncommon and is associated with arrhythmic events,[34] although another report has shown negative results.[33] In our study, early repolarization in the inferolateral leads was frequently found in SQTS and, more importantly, was associated with arrhythmic events in SQTS. In addition to arrhythmia syndromes unassociated with structural heart disease, a high frequency of early repolarization in arrhythmogenic right ventricular dysplasia/cardiomyopathy has been reported.[27]

It has been suggested that SQTS and idiopathic ventricular fibrillation share clinical characteristics.[23] Short QT interval is frequently found in idiopathic ventricular fibrillation,[23] and QT interval is relatively short in patients with idiopathic ventricular fibrillation who have early repolarization.[9] Spontaneous and inducible ventricular fibrillation can be initiated by short-coupled premature ventricular beat in SQTS and idiopathic ventricular fibrillation.[21,35,36] The efficacy of isoproterenol and quinidine has been reported for both arrhythmia syndromes,[21,37] although the arrhythmogenic effects of isoproterenol in an experimental model of SQTS have been reported.[38] Our study showing an association of early repolarization with SQTS further supports the presence of common arrhythmogenic substrates in SQTS and idiopathic ventricular fibrillation.

A precise mechanism for ventricular fibrillation in SQTS is not known, but characteristic ECG abnormalities may reflect arrhythmogenicity. A prior study showed that the interval from T-wave peak to T-wave end is relatively long in SQTS, and our study replicated the results.[26] T-wave peak to T-wave end interval is considered to reflect transmural dispersion of repolarization, and relative prolongation of the interval in SQTS may indicate a high vulnerability to ventricular fibrillation.[39] An experimental model of SQTS

Table 2 Characteristics of SQTS patients with and those without arrhythmic events

	Patients with arrhythmic events (N = 25)	Patients without arrhythmic events (N = 12)	OR (95% CI)	P value
Male gender [N (%)]	21 (84)	6 (50)	10.44 (0.85–127.48)	.07
Age (years)	30 ± 19	23 ± 18	1.05 (0.99–1.12)	.13
Heart rate (bpm)	69 ± 393	76 ± 473	1.00 (1.00–1.01)	.38
PR interval (ms)	138 ± 19	134 ± 18	0.99 (0.95–1.04)	.84
QRS interval (ms)	86 ± 7	85 ± 10	0.93 (0.82–1.07)	.31
QT interval (ms)	286 ± 36	271 ± 40	1.00 (0.97–1.03)	.75
QTc (ms)	308 ± 29	306 ± 33	0.98 (0.94–1.02)	.33
Early repolarization [N (%)]	22 (88)	2 (17)	46.53 (4.52–478.79)	.001

CI = confidence interval; OR = odds ratio; QTc = corrected QT interval; SQTS = short QT syndrome.
Models were adjusted for gender and age.

Table 3 ECG parameters for study cohorts with short QT interval

	Patients with SQTS	Subjects with short QTc	OR (95% CI)	*P* value
QT_{peak} (ms)	211 ± 37	222 ± 19	0.99 (0.98–1.01)	.37
Corrected QT_{peak}	226 ± 32	234 ± 24	0.99 (0.98–1.01)	.56
T_{peak} to T_{end} (ms)	81 ± 21	67 ± 13	1.08 (1.03–1.13)	<.001
Corrected T_{peak} to T_{end}	89 ± 28	72 ± 17	1.05 (1.02–1.09)	.002
QT_{peak}/QT ratio (%)	27 ± 6	22 ± 4	0.83 (0.73–0.94)	.004

Models were adjusted for gender and age.
CI = confidence interval; OR = odds ratio; QTc = corrected QT interval; SQTS = short QT syndrome.

provides evidence that increased transmural dispersion of repolarization under short QT interval conditions results in ventricular tachyarrhythmia.[38] A tall peaked T wave is one of the characteristic ECG abnormalities in SQTS,[1] but the amplitude of the T wave is not different between patients with SQTS and subjects with short QT interval and no arrhythmic events, suggesting that a tall T wave is associated with a short QT interval but is not associated with arrhythmogenicity.[26] In SQTS, characteristic ECG abnormities are also found in the early repolarization phase. In patients with SQTS, the ECG shows a very short J-point to T-wave peak interval and no flat ST segment.[26] In our study, early repolarization was frequently found in SQTS and was associated with arrhythmic events. Whether the inferolateral J-point elevation reflects late depolarization or early repolarization is controversial, but this pattern has been considered repolarization because of slower inscription, spontaneous changes occurring concurrently with ST segment but not with QRS complexes, and absence of late potentials on signal-averaged ECG.[9,40] Taken together, the finding suggest that abnormalities in the early phase of repolarization create the arrhythmogenic substrate in SQTS.

Sex hormone and gender difference have an important role in the arrhythmia syndromes.[41–43] It is well known that the QT interval is affected by sex hormones, and the QT interval is longer in women than men.[44] Female gender is a risk factor for development of ventricular tachyarrhythmias in both congenital and acquired long QT syndrome.[41,42] On the other hand, Brugada syndrome is more prevalent in men than in women, and the male hormone testosterone is reported to contribute to male predominance in Brugada syndrome.[43] In this study, male gender was associated with arrhythmic events in SQTS and short QT interval was frequently found in men, suggesting a role of sex hormones in SQTS opposite to that in long QT syndrome. Recent evidence that the QT interval can be shortened by anabolic androgenic steroids and testosterone further supports this hypothesis.[45,46]

SQTS is a genetically heterogeneous disease with five responsible genes encoding ion channels: *KCNQ1*, *KCNH2*, *KCNJ2*, *CACNA2D1*, and *CACNB2b*.[3,4] An increase in outward current by gain-of-function mutations in potassium channels or a decrease in inward current by loss of function mutations in calcium channels may be responsible for SQTS.[3,4] Early repolarization was found in patients with mutations in *KCNQ1* and *KCNH2* and in those without mutations in the known genes, suggesting a heterogeneous genetic background for the association between short QT interval and early repolarization. To date, mutations in calcium channel genes (*CACNA2D1* and *CACNB2b*) have been identified in three probands with Brugada syndrome associated with a short QT interval, but early repolarization is not present in the inferolateral leads in any of them.[4] A recent study has identified a mutation in *KCNJ8*, an initial responsible gene for idiopathic ventricular fibrillation associated with early repolarization.[47] Although there are some similarities in phenotype between SQTS and idiopathic ventricular fibrillation with early repolarization, a common genetic background has not been identified.

Conclusion

Our study showed a high prevalence of early repolarization in patients with SQTS and an association of early repolarization with arrhythmic events. Early repolarization may be a useful marker for risk stratification of cardiac arrest in SQTS, although further investigation with longitudinal follow-up is required to evaluate our results.

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Characterization of the Rapidly Activating Delayed Rectifier Potassium Current, I_{Kr}, in HL-1 Mouse Atrial Myocytes

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Abstract HL-1 is the adult murine cardiac cell line that can be passaged repeatedly in vitro without losing differentiated phenotype. The present study was designed to characterize the rapidly activating delayed rectifier potassium current, I_{Kr}, endogenously expressed in HL-1 cells using the whole-cell patch-clamp technique. In the presence of nisoldipine, depolarizing voltage steps applied from a holding potential of −50 mV evoked the time-dependent outward current, followed by slowly decaying outward tail current upon return to the holding potential. The amplitude of the current increased with depolarizations up to 0 mV but then progressively decreased with further depolarizations. The time-dependent outward current as well as the tail current were highly sensitive to block by E-4031 and dofetilide (IC_{50} of 21.1 and 15.1 nM, respectively) and almost totally abolished by micromolar concentrations of each drug, suggesting that most of the outward current in HL-1 cells was attributable to I_{Kr}. The magnitude of I_{Kr} available from HL-1 cells (18.1 ± 1.5 pA pF^{-1}) was sufficient for reliable measurements of various gating parameters. RT-PCR and Western blot analysis revealed the expression of alternatively spliced forms of mouse *ether-a-go-go*-related genes (mERG1), the full-length mERG1a and the N-terminally truncated mERG1b isoforms. Knockdown of mERG1 transcripts with small interfering RNA (siRNA) dramatically reduced I_{Kr} amplitude, confirming the molecular link of mERG1 and I_{Kr} in HL-1 cells. These findings demonstrate that HL-1 cells possess I_{Kr} with properties comparable to those in native cardiac I_{Kr} and provide an experimental model suitable for studies of I_{Kr} channels.

Keywords Cardiac cell line · Potassium current · Potassium channel · Patch-clamp · HL-1 cell · siRNA

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Introduction

Cardiac delayed rectifier potassium current (I_K) is responsible for action potential repolarization and pacemaker activity and consists of multiple components with distinct time and voltage dependence and pharmacological properties. I_{Kr} is the rapidly activating, inwardly rectifying component of I_K, which can be isolated as a fraction specifically blocked by the class III antiarrhythmic methanesulfonanilide agents such as E-4031 and dofetilide (Sanguinetti and Jurkiewicz 1990). It is now well known that I_{Kr} is conducted by ERG1 (*ether-a-go-go*-related gene) potassium channels (Sanguinetti et al. 1995; Trudeau et al. 1995). Mutations in the human ERG1 (HERG) channel gene underlie the inherited long QT syndrome, a disorder of cardiac repolarization that predisposes affected individuals to life-threatening arrhythmias (Curran et al. 1995). In addition, I_{Kr} is sensitive to block by a diverse range of therapeutic agents (e.g., antihistamines, gastrointestinal prokinetic agents, psychoactive substances), and these adverse drug effects can induce acquired long QT syndrome (Roden et al. 1996).

Taking advantage of molecular biological technology, functional analysis of reconstituted HERG channels in a heterologous expression system has provided information on the gating mechanisms, modulation and drug block of I_{Kr} channels. Nevertheless, current recordings from native channels are still important because several differences between native I_{Kr} and reconstituted HERG current have been revealed (Sanguinetti et al. 1995; Weerapura et al. 2002), possibly due to inadequate composition of channel proteins or lack of cardiac-specific environments in the heterologous expression system. AT-1 cells, a cardiac cell line derived from atrial tumor of adult transgenic mice expressing the simian virus 40 (SV40) large T-antigen targeted to atrial cardiomyocytes via the atrial natriuretic factor (ANF) promoter (Field 1988), have been often employed as a suitable source of native I_{Kr} channels (Liu et al. 1994; Yang and Roden 1996; Yang et al. 1994, 1995, 1997). Membrane current recorded from these cells displays phenotypical characteristics of cardiac I_{Kr} with minimal contamination of other time-dependent outward currents. Maintenance of AT-1 cells, however, is complicated and labored because it is impossible to passage these cells serially in vitro. They are maintained by serial propagation as a subcutaneous tumor in syngeneic mice and have to be used as primary cells (Delcarpio et al. 1991).

The HL-1 cell line was derived from subsequent development of AT-1 cells (Claycomb et al. 1998). Different from any other cardiac cell lines currently available, HL-1 cells can be repeatedly passaged in culture while maintaining a differentiated cardiac phenotype. They express many cardiac-specific proteins such as α-myosin heavy chain, ANF, α-cardiac actin and connexin 43 (Claycomb et al. 1998). Furthermore, several functional receptors, such as α_1-adrenergic and δ-opioid receptors, and intracellular signaling proteins required for phosphatidylinositol hydrolysis and the cyclic AMP synthesis pathway have been demonstrated in HL-1 cells (McWhinney et al. 2000; Neilan et al. 2000; Sartiani et al. 2002). Recent patch-clamp studies have revealed the existence of several cardiac membrane currents, including I_{Kr} as well as the hyperpolarization-activated nonselective cation current (I_f) and the L- and T-type Ca^{2+} currents ($I_{Ca,L}$ and $I_{Ca,T}$) (Claycomb et al. 1998; Sartiani et al. 2002; Xia et al. 2004; Zankov et al. 2009). Thus, HL-1 cells may be used as a model of cardiac cells for studying many features of ion channels in a cardiac-specific environment (White et al. 2004).

The present study characterizes I_{Kr} channels endogenously expressed in HL-1 cells. Whole-cell patch-clamp experiments demonstrate that I_{Kr}, defined as the E-4031-sensitive current, can be elicited in almost all cells with current magnitude of 0.1–1.5 nA suitable for high-quality recording, which allows us to analyze biophysical and pharmacological features extensively and reliably. In addition, alternatively spliced forms of mouse ERG1 (mERG1) are identified in HL-1 cells, and our RNA interference (RNAi) experiments suggest that these ERG1 isoforms indeed underlie I_{Kr}. Data obtained here will be helpful for future applications of HL-1 cells as a unique model to study cardiac I_{Kr} channels.

Methods

Culture of HL-1 Cells

The HL-1 cell culture (passage 36) was a kind gift from Dr. Claycomb (Louisiana State University Health Science Center, New Orleans, LA) who first established the cell line. Care of the HL-1 cells was described previously (Claycomb et al. 1998). Claycomb medium (JRH Bioscience, Lenexa, KS; catalog 51800), a commercially available medium specifically designed for the growth of HL-1 cells, was purchased. Before use, the Claycomb medium was supplemented with 10% fetal bovine serum (JRH Bioscience), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 0.1 mM norepinephrine (Sigma, St. Louis, MO) and penicillin–streptomycin (Nakalai Tesque, Kyoto, Japan). The supplemented Claycomb medium was prepared every 2 weeks and kept in the dark by covering the medium bottle with aluminum foil because it is highly light-sensitive. Cells were plated on T25 flasks (Techno Plastic Products, Trasadingen, Switzerland; 90025) precoated overnight with 0.00125% fibronectin (Sigma, F1141) in 0.02% gelatin (Difco, Detroit, MI; 0143-17-9) and maintained in supplemented Claycomb medium at 37°C in humidified 5% CO_2 and 95% air. The culture medium was changed daily. After full confluence, cells were dissociated by 0.05% trypsin/EDTA (Invitrogen). Isolated cells were then suspended in Claycomb medium supplemented with 5% fetal bovine serum and antibiotics, and the cell suspension was used for the patch-clamp experiments or split into new flasks for subsequent culturing.

Reverse Transcription-Polymerase Chain Reaction Amplification

HL-1 cells culture (passage 40) and atrial tissue dissected from adult mice were used for mRNA purification. Total RNA from each sample was extracted by the acid guanidium thiocyanate chlorofrom method (Chomczynski and Sacchi 1987). cDNA was synthesized from 5 μg of total RNA with 20 units of RAV-2 reverse transcriptase (Takara, Otsu, Japan) using random primers. PCR for mouse ERG1 isoforms (mERG1a, mERG1a′ and mERG1b) was

performed using the following primer sets reported previously (Clark et al. 2004) (from 5 to 3): ACA CCT TCC TCG ACA CCA TC (sense; position 621–641, accession AF012870) and GCA TCA GGG TTA AGG CTC TG (antisense; position 1405–1424, accession AF012871) for mERG1a, ACC ACT GGC ATA GGA CCA AG (sense; position 839–858, accession AF012870) and the same antisense as for mERG1a for mERG1a′, ATG GCG ATT CCA GCC GGG AA (sense; position 3952–3971, accession AF012871) and GAT GCC ATT GGT GTA GGA CC (antisense; position 8239–8258, accession AF012871) for mERG1b. The reaction included 0.4 µl of cDNA, 2.5 units of KOD dash polymerase (Toyobo, Osaka, Japan), 1 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 10 µg ml^{-1} BSA, 0.2 mM each of deoxynucleotide triphosphate and 4 pmol primers in 20 ml of 120 mM Tris–HCl buffer (pH 8.0). Amplification was conducted in a thermal cycler using 30 cycles consisting of denaturation at 98°C for 2 s, annealing at 55°C for 2 s and elongation at 72°C for 60 s. PCR products were identified in an ethidium bromide-stained 1.5% agarose gel by electrophoresis.

Western Blotting

HL-1 cells (passages 45–47) were washed with cold phosphate-buffered saline and resuspended in lysis buffer (50 mM Tris–HCl, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, pH 7.4) supplemented with a mix of protease inhibitors (Complete Mini; Roche, Mannheim, Germany). Cell lysate was centrifuged at 15,000 rpm for 5 min. Total protein was measured using the DC protein assay (Bio-Rad, Richmond, CA). For Western blot assay, 100 µg of total proteins were dissolved in 2 × SDS sample buffer (4% sodium dodecyl sulfate, 125 mM Tris–HCl, 12% 2-mercaptoethanol, 20% glycerol, 0.005% bromophenol blue, pH 6.8) and then sonicated and boiled for 5 min. Samples were resolved on 7.5% SuperSep gel (Wako, Osaka, Japan) and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was blocked in Tris-buffered saline (TBS; 10 mM Tris–HCl, 100 mM NaCl, pH 7.5) containing 0.1% Tween-20 and 10% nonfat dry milk for 1.5 h at room temperature and then incubated overnight at 4°C with a rabbit polyclonal anti-ERG1 antibody (Chemicon, Temecula, CA; AB5222) directed against the C terminus (amino acid residues 1121–1137, accession O08962) of rat ERG1, at a dilution 1:200. After washing with TBS-Tween 0.1%, the membrane was incubated with a horseradish peroxidase-conjugated secondary anitibody (Jackson ImmunoResearch, West Grove, PA; 1:5,000) for 1 h at room temperature. Signals were detected using an enhanced chemiluminescence system.

RNAi

Two Stealth small interfering RNA (siRNA) duplex oligonucleotides directed against all transcripts of the mERG1 gene and RNAi-negative control duplex oligonucleotide (ncRNA) were provided by Invitrogen. The siRNA sequences were as follows: siRNA-1, 5′-AGG CUG ACA UCU GCC UAC ACC UGA A-3′; siRNA-2, 5′-UGU CAU UCC GCA GGC GUA CAG ACA A-3′. HL-1 cell culture of nearly confluent (passages 42–45) was transfected with siRNA against mERG1 or nonspecific RNA (ncRNA, 50 pmol), together with a reporter plasmid DNA (pEGFP vector, 0.5 µg) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacture's instructions. Only GFP-positive cells 2 days after transfection were employed for electrophysiological experiments.

Patch-Clamp Recordings

Current recordings from HL-1 cells (passages 38–52) were performed using the whole-cell configuration of the patch-clamp technique (Hamill et al. 1981) with an EPC-8 patch-clamp amplifier (Heka, Lambrecht, Germany). Cells were dissociated from culture dishes by 0.05% trypsin/EDTA, suspended in Claycomb medium and stored at 4°C for a few hours before use. A small aliquot of cell suspension was transferred into a small (0.5 ml) recording chamber placed on the stage of an inverted microscope (TMD-300; Nikon, Tokyo, Japan). After settling to the glass bottom of the chamber (5–10 min), the cells were continuously superfused with normal Tyrode solution (containing appropriate drugs) kept at 25 ± 1°C or 35 ± 1°C, as indicated. Patch-clamp pipettes were prepared from glass capillary tube (Narishige, Tokyo, Japan) on a horizontal pipette puller (P-97; Sutter Instrument, Novato, CA), and the tips were then fire-polished by a microforge (MF-83, Narishige). Pipette resistance was 2–4 MΩ when filled with internal solution. Currents and voltages were digitized and voltage commands were generated through an ITC-16 AD/DA interface (InstruTECH, Long Island, NY) controlled by Pulse/Pulsefit software (version 8.54, Heka).

Data Analysis

Membrane capacitance (C_m) was calculated by fitting a single exponential function to the decay phase of the transient capacitive current in response to ±5-mV voltage steps (20 ms) from a holding potential of −50 mV. The current amplitude was divided by C_m to obtain the current density (pA pF^{-1}). Linear regression analysis was used for correlations. The voltage dependence of current activation and inactivation was determined by fitting the normalized

tail current (I_{tail}) vs. test potential (V) to a Boltzmann function expressed by $I_{tail} = 1/(1 + \exp[(V_{1/2} - V)/k])$ and $I_{tail} = 1/(1 + \exp[(V - V_{1/2})/k])$, respectively, where $V_{1/2}$ is the voltage at which the current is half-activated and k is the slope factor. The time constant for activation (τ_{act}) was determined from a single-exponential fit to the envelop of tail currents obtained after depolarizing pulses for varying durations, and time constants for deactivation (τ_{fast} and τ_{slow}) were obtained by fitting a two-exponential function to the time course of deactivating tail currents. Dose responses for drug block of currents were analyzed by fitting the relative amplitudes of tail currents (y/y_{max}) vs. the drug concentration ([D]) to a Hill function: $y/y_{max} = 1/\{1 + (IC_{50}/[D])^n\}$, where IC_{50} is the half-inhibitory concentration and n is the Hill coefficient. Data were expressed as mean ± SEM. Statistical analysis was performed by means of ANOVA and a post hoc Tukey test.

Solutions and Drugs

Normal Tyrode solution contained (mM) 140 NaCl, 0.33 NaH_2PO_4, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 5.5 glucose and 5 HEPES, pH adjusted to 7.4 with NaOH. The external solution for current recording was made by adding 0.4 μM nisoldipine (as 1 mM stock solution in ethanol) to normal Tyrode solution to eliminate $I_{Ca,L}$. In some experiments, the concentration of KCl was modified to 2 or 10 mM. The internal pipette solution contained (mM) 70 potassium aspartate, 50 KCl, 10 KH_2PO_4, 1 $MgCl_2$, 3 Na_2–ATP, 0.1 Li_2–GTP, 5 EGTA and 5 HEPES, pH adjusted to 7.2 with KOH. Liquid junction potential between the test solution and the pipette solution was measured at around −10 mV and corrected. In order to rule out possible contamination of $I_{Ca,L}$ in our data, all experiments were conducted in the presence of 0.4 μM nisoldipine (a generous gift from Bayer AG, Wuppertal-Elberfeld, Germany), which is specific blocker of $I_{Ca,L}$. E-4031 (Wako), dissolved in distilled water (1 mM) and dofetilide (a generous gift from Pfizer, Sandwich, UK), dissolved in acidified water (pH 4.0, 1 mM), were diluted down to the final concentration in the test solution.

Results

E-4031-Sensitive Current in HL-1 Cells

I_{Kr} was originally identified as a methanesulfonanilide-sensitive component of I_K in guinea pig cardiomyocytes (Sanguinetti and Jurkiewicz 1990). We recorded whole-cell membrane currents from single HL-1 cells before and after application of E-4031 and then analyzed a drug-sensitive current (Fig. 1). Possible participation of other voltage-dependent currents in our data was minimized; i.e., 0.4 μM nisoldipine was included in the bath solution to block $I_{Ca,L}$ (Xia et al. 2004) and membrane potential was held at −50 mV to inactivate $I_{Ca,T}$ (Xia et al. 2004) and avoid I_f activation (Sartiani et al. 2002). Figure 1a shows representative membrane currents in response to 1-s depolarizing (upper panel) and hyperpolarizing (lower panel) pulses to various test potentials, ranging between −80 and +40 mV in 10-mV steps from a holding potential of −50 mV. As shown in the upper panel of Fig. 1a, depolarizing steps activated time-dependent outward currents with amplitudes that increased with depolarization up to 0 mV and then progressively decreased as the potential became more positive (filled circles, Fig. 1d). After return of the membrane to the holding potential, slowly deactivating tail currents were elicited. In contrast, as shown in the lower panel, hyperpolarizing steps induced small-amplitude inward currents with a slight time dependence, which was possibly due to activation of I_f channels, and following depolarizing steps to the holding potential elicited transient inward currents, which may be attributed to activation of $I_{Ca,T}$. When E-4031 (5 μM) was applied to the bath solution, the time-dependent outward current during depolarizing steps as well as the tail current were almost completely abolished, whereas the inward current during the hyperpolarizing pulse was not significantly influenced (Fig. 1b). The currents after exposure to the drug were nearly time-independent and exhibited small conductance with slight outward rectification (open circles in Fig. 1d). E-4031-sensitive currents obtained by digital subtraction of current traces in the presence of drug from those before application of the drug are illustrated in Fig. 1c. The drug-free and the E-4031-sensitive currents showed very similar current–voltage relationships, and both currents have the characteristics of inward rectification at more positive potential than 0 mV, indicating that I_{Kr} is the dominant outward current in HL-1 cells.

The voltage dependence for I_{Kr} activation was determined by measuring the tail amplitude of E-4031-sensitive current. Figure 2a shows the initial part of tail currents elicited upon return of the membrane potential to −50 mV from the 1-s depolarizing steps to test potentials ranging from −40 to +40 mV. The tail current obviously activated at −30 mV and increased in amplitude for the steps up to +10 mV. In Fig. 2b, the amplitude of the tail currents was normalized to the maximum tail current amplitude and plotted as a function of the membrane potential. The $V_{1/2}$ and k, which were determined by curve fitting the data points to a Boltzmann equation, were −20.4 and 8.0 mV, respectively.

In Fig. 3, kinetic properties were determined by measuring time constants for apparent activation and deactivation of I_{Kr}. An envelope-of-tails test was used to assess

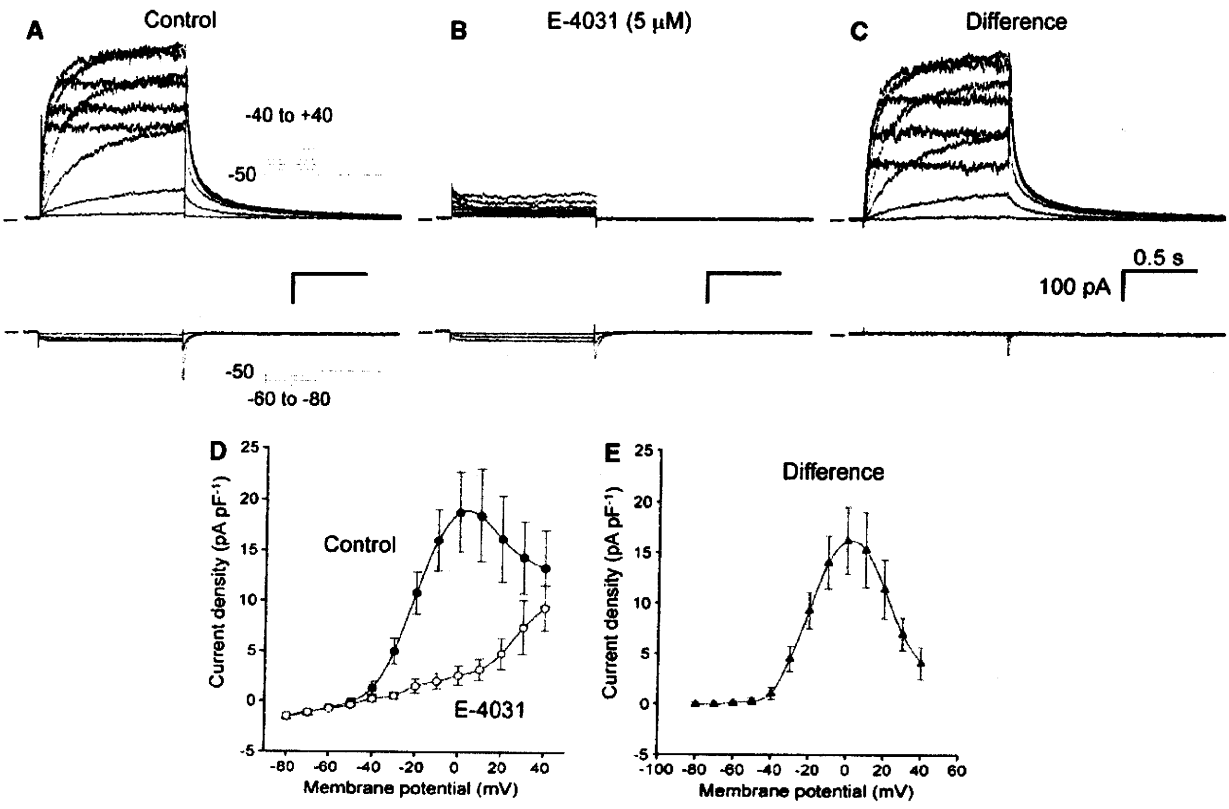

Fig. 1 E-4031-sensitive current recorded from isolated HL-1 cells. **a**, **b** Superimposed whole-cell membrane currents recorded from single HL-1 cell (passage 38) before (**a**) and after (**b**) exposure to 5 μM E-4031. The cell was held at −50 mV and given 1-s depolarizing (between −40 and +40 mV, *upper panel*) and hyperpolarizing (between −80 and −60 mV, *lower panel*) test pulses. The experiment was conducted at 35°C. **c** E-4031-sensitive current obtained from digital subtraction of two traces in **a** and **b**. **d** Average current–voltage relationships recorded before (*filled circles*) and after (*open circles*) exposure to E-4031. Current amplitudes measured just before the end of the 1-s pulses were plotted against the indicated membrane potentials. Values represent mean ± SEM of 10 HL-1 cells (passages 38–41). **e** Current–voltage relationship for E-4031-sensitive currents

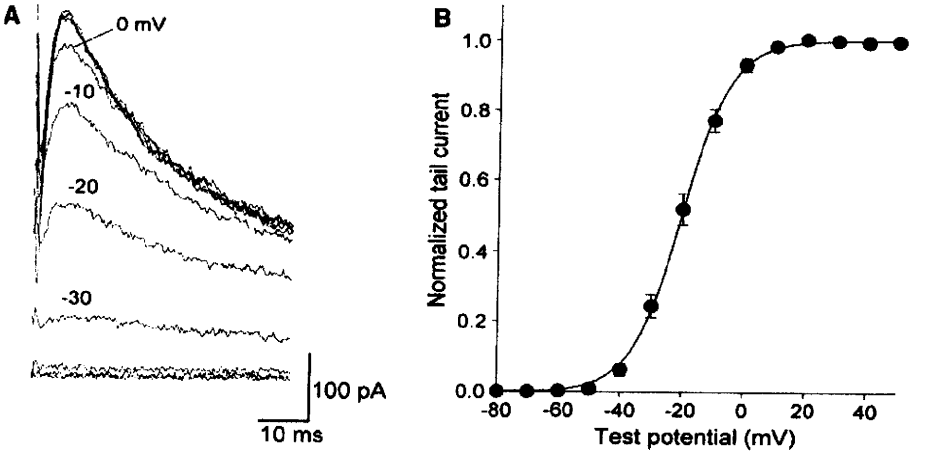

Fig. 2 Voltage-dependent activation of E-4031-sensitive current in HL-1 cells. **a** Representative tails of E-4031-sensitive current recorded from an HL-1 cell (passage 38). Tail currents were elicited on repolarization to −50 mV, following 1-s depolarization to +20 mV. **b** Voltage dependence of E-4031-sensitive current. Tail current amplitudes were normalized to the maximal value at +20 mV, and averaged data were plotted against the indicated test potentials. Values represent mean ± SEM of 10 HL-1 cells (passages 38–41). *Smooth curve* represents fitting of the data to the Boltzmann equation