relation between late potential and quinidine administration was clear, the contribution of spontaneous ECG variation in the disappearance of late potential could not be excluded completely because the signal-averaged ECG was recorded only 1 time during the administration of quinidine.

In conclusion, quinidine eliminated the late potentials in a patient with Brugada syndrome. Signal-averaged ECG may be useful to detect subtle electrophysiologic effects of pharmacologic treatment, particularly with quinidine, as well as risk stratification in Brugada syndrome.

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Letter to the Editor

A novel mutation in FKBP12.6 binding region of the human cardiac ryanodine receptor gene (R2401H) in a Japanese patient with catecholaminergic polymorphic ventricular tachycardia

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Abstract

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an autosomal dominant inherited disorder characterized by adrenergic induced polymorphic ventricular tachycardias and associated with sudden cardiac death. The human cardiac ryanodine receptor gene (RyR2) was linked to CPVT. A 20-year-old male was referred to our hospital because of recurrent syncope after physical and emotional stress. Routine cardiac examinations including catheterization revealed no structural abnormality. Exercise on treadmill induced premature ventricular contraction in bigeminy and bidirectional ventricular tachycardia was induced during isoproterenol infusion. β-Blocking drug was effective in suppressing the arrhythmias. We performed genetic screening by PCR–SSCP method followed by DNA sequencing, and a novel missense mutation R2401H in RyR2 located in FKBP12.6 binding region was identified. This mutation was not detected in 190 healthy controls. Since FKBP12.6 plays a critical role in Ca channel gating, the R2401H mutation can be expected to alter Ca-induced Ca release and E–C coupling resulting in CPVT. This is the first report of RyR2 mutation in CPVT patient from Asia including Japan.
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Keywords: Arrhythmia; RyR2; Ca-induced Ca release; E–C coupling; Sudden death

1. Introduction

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a rare inherited arrhythmogenic disorder characterized by stress- and exercise-induced bidirectional or polymorphic ventricular tachycardias. It occurs in the structurally normal hearts and causes sudden death in childhood and adolescence. Recently, cardiac ryanodine receptor gene (RyR2) was linked to CPVT and also to arrhythmogenic right ventricular dysplasia type 2 (ARVD2) [1]. To date, 15 RyR2 missense mutations have been reported in Italian and Finnish CPVT families (six in ARVD2) [2–4].

2. Clinical case

The patient is a 20-year-old male and referred to the hospital because of recurrent syncope occurring during physical or emotional stress. The syncope first developed at his age of 10 years old. Before admission to our hospital, he had been followed by another hospital for 10 years and polymorphic VT followed by ventricular fibrillation was documented at electrophysiologic study. There is no family history of syncope and sudden death. Clinical evaluations

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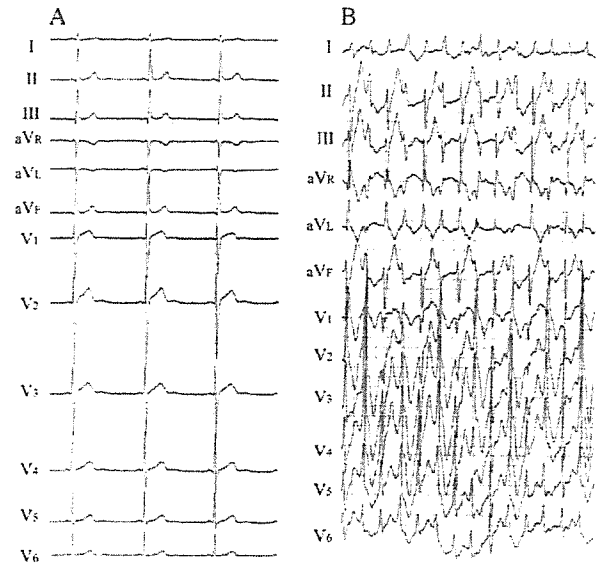

Fig. 1. ECG of the patient. (A) ECG of the patient at rest. (B) ECG of the patient after isoproterenol infusion. Bidirectional ventricular tachycardia was induced.

including cardiac catheterization revealed no structural abnormality. ECG at rest was normal including QT interval (Fig. 1A). Exercise stress test on treadmill induced PVC in bigeminy and bidirectional ventricular tachycardia was induced by isoproterenol infusion (Fig. 1B). The arrhythmia was suppressed by β-blocking drug, propranolol.

3. Genetic analysis

After a written informed consent was obtained for genetic analysis, genomic DNA was extracted from peripheral blood lymphocytes and amplified by standard PCR method. The primers for amplifying exons of the RyR2 genes are designed by Dr. N. Tiso, available at the Web site: http://telethon.bio.unipd.it/ARVDnet/molgen_arvd2.html. PCR products were analyzed by single-strand conformation polymorphism. Abnormal conformers were sequenced with ABI377XL genetic analyzer.

PCR–SSCP analysis of the proband revealed an abnormal band in exon 47 of RyR2 (Fig. 2) and such abnormal band was not observed in 190 genomic DNAs (380 chromosomes) from unrelated healthy individuals. DNA sequencing confirmed a G to A transition at the position of 7202 leading to amino acid change of histidine for arginine 2401, R2401H (Fig. 2). No other mutation was found in KCNQ1, KCNH2, SCN5A, KCNE1 and KCNE2 gene of this patient. Genetic screening for his family members was not available.

4. Discussion

RyR2 is ~ 565 kDa protein composing tetramer and requires FKBP12.6 protein for channel gating [5]. All of the previously reported mutations are missense mutations locating in three hot lesions; in FKBP12.6 binding region (2361–2496 amino acid region), Ca binding site and in transmembrane segments. FKBP12.6 binding region is

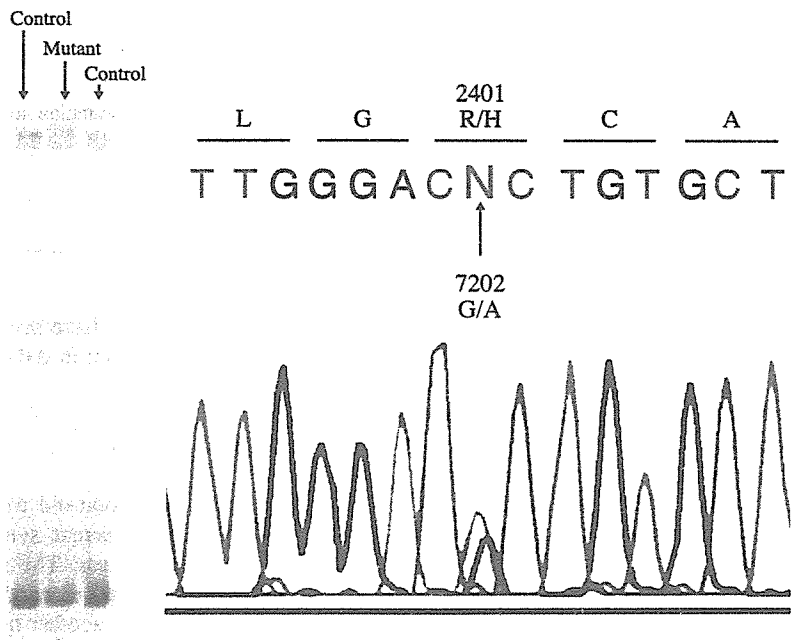

Fig. 2. SSCP and electropherogram of R2401H mutation. SSCP revealed an abnormal band (left) and electropherogram comfirmed G to A transition at the position of 7202 (right).

thought to stabilize the channel during diastole and mutations in this region lead to increased channel open probability and Ca overload [6]. It is also suggested that adrenergic stimulation leads to phosphorylation of RyR2 by protein kinase A (PKA), which dissociates FKBP from RyR and results in a Ca overload in the cells [5].

In summary, we present a novel RyR2 mutation in a Japanese CPVT patient. This R2401H mutation is located at the FKBP12.6 binding region of RyR2. The ventricular arrhythmia must be related to Ca overload and precipitated by adrenergic stimuli or inhibited by β-blocking drug. Further studies focused on Ca signaling pathway and E–C coupling are required.

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Relationship Between Dominant Prolongation of the Filtered QRS Duration in the Right Precordial Leads and Clinical Characteristics in Brugada Syndrome

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SAECG and EPS in Brugada Syndrome. ***Background:*** **Electrical abnormalities in the RVOT may be involved in Brugada syndrome.**

Objectives: **We investigated the relationship between the signal-averaged ECG (SAECG) and electrophysiologic study (EPS), especially focusing on conduction delay in the outflow tract of the right ventricle (RVOT) and its contribution to clinical characteristics.**

Methods: **Twenty-four patients with Brugada syndrome (23 men and 1 woman; 61 ± 16 years old) were studied. We assessed the presence of late potential (LP) in SAECG and the filtered QRS duration in the right precordial leads (V_1 or V_2; RfQRS) and in the left precordial leads (V_5 or V_6; LfQRS) and the difference between them. In 18 patients, SAECG was evaluated for an LP on three separate occasions.**

Results: **SAECG was positive for LP in 15 patients at least once; and in 7 patients, SAECG was positive for an LP on multiple occasions, and 6 of 7 patients (86%) had a history of cardiac arrest. The difference between RfQRS and LfQRS was significantly greater in patients with cardiac arrest than in patients with syncope or in asymptomatic patients; 29 ± 10, 14 ± 11 ($P < 0.01$), and 7 ± 5 msec ($P < 0.001$), respectively. All patients were alive and one patient with cardiac arrest had an appropriate VF therapy delivered by the ICD.**

Conclusions: **The dominant prolongation of the filtered QRS duration in the right precordial leads may be related to the risk of arrhythmic event in Brugada syndrome.** *(J Cardiovasc Electrophysiol, Vol. 16, pp. 1311-1317, December 2005)*

Brugada syndrome, signal-averaged ECG, electrophysiologic study, RV outflow tract

Introduction

Brugada syndrome is characterized by ST-segment elevation in the precordial leads associated with ventricular fibrillation (VF). The changes in the electrocardiogram (ECG) are augmented by sodium channel blockers.[1-3] It has been suggested that loss of the action potential dome in the epicardium results in phase 2 reentry and polymorphic ventricular arrhythmias, and these have been demonstrated in an animal model.[4-7]

VF can often be induced by programmed electrical stimulation during an electrophysiologic study (EPS),[8,9] especially from the right ventricular outflow tract (RVOT).[10]

Kanda et al. demonstrated that the intraventricular conduction time from the RVOT to the lateral wall of the left ventricle was greater in Brugada syndrome patients with inducible VF than those without inducible VF; but the significance of this finding is unknown.[11] The body surface map or the signal-averaged electrocardiogram (SAECG) has shown delayed activation in the RVOT in Brugada syndrome,[12,13] which may play an important role in arrhythmogenesis. In the present study, we investigated the relationship between the results of SAECG with special attention to conduction delay in RVOT and the clinical characteristics of patients with Brugada syndrome.

Patients and Methods

Patients

From April 2000 to August 2003, we studied 24 consecutive patients with a Brugada-type abnormality in their ECG, including 15 symptomatic and 9 asymptomatic patients. All but 1 of the 24 patients was male (mean age: 61 ± 16 years; range: 25–81 years). The 15 symptomatic patients included 7 patients with a history of aborted cardiac sudden death and 8 patients with recurrent syncopal episodes. No patient had a family history of sudden death. There were no patients from the same family and in asymptomatic patients, type 1 or type 2 ECG were documented by their annual medical examination. All patients had a Brugada-type ECG in repeated recordings in the absence of antiarrhythmic drugs; type 1 ECG in 8 and type 2 ECG in 16. In patients who had type 2 ECG, pilsicainide (1 mg/kg in 5 minutes) provoked type 1 ECG.[14] Routine cardiac examinations, including echocardiography, left and right ventriculography, and coronary angiography were performed in all patients, but they showed no abnormalities.

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All patients did not have any electrolyte abnormality during admission. In 9 of 24 patients, we obtained informed consent and investigated the gene mutation in SCN5A, but the results turned out to be negative.

Signal-Averaged ECG (SAECG)

Late potential (LP) was studied in a standard manner using SAECG system (FDX-6521, Fukuda Denshi, Japan). Analysis of SAECG was based on quantitative time-domain measurements of the filtered vector magnitude of the orthogonal leads, X, Y, and Z. The QRS complexes were amplified, digitized, averaged (200–300 beats), and high-pass filtered (40 Hz). Three parameters were obtained using a computer algorithm: the filtered QRS duration (cutoff >114 msec), the root mean square voltage of the last 40 msec of the filtered QRS complex (RMS40, cutoff <20 μV), and the duration of the low-amplitude signal <40 μV at the terminal portion of the QRS complex (LAS40, cutoff >38 msec). The SAECG was considered positive for LP when two criteria (RMS40 <20 μV and LAS40 >38 msec) were fulfilled. Recordings were acceptable when electrical noise was <0.3 μV. In 18 patients, SAECG was measured on three different days to assess LP reproducibility and minimum RMS40 used as the lowest value of RMS40 in three measurement of SAECG (Table 1). The standard precordial leads, V_1–V_6 were amplified, digitized, averaged (200–300 beats), and high-pass filtered (40 Hz) and the filtered QRS duration was measured. The filtered QRS duration in V_1 or V_2 was considered to represent local conduction in the right ventricle (RfQRS) and compared with V_5 or V_6 that was considered to represent local conduction in the left ventricle (LfQRS). We used the longer filtered QRS duration in V_1 or V_2 and in V_5 or V_6 as RfQRS and LfQRS, respectively. The difference between RfQRS and LfQRS (RfQRS-LfQRS) was evaluated excluding 2 patients with complete right bundle branch block (cases 6 and 15). We also investigated the filtered QRS duration in precordial leads, RfQRS, LfQRS, and RfQRS-LfQRS, in the control group. The control group consisted of 7 healthy subjects and 3 patients with idiopathic ventricular tachycardia (VT) (verapamil-sensitive left VT in 1 and RVOT origin in 2): 9 males and 1 female (average age: 16–50, mean: 32 ± 10), who never had a Brugada-type ECG.

Electrophysiologic Study

EPS was performed in 22 of 24 patients after a written informed consent was obtained. No patient received any antiarrhythmic drugs. Three quadripolar electrode catheters with an interelectrode distance of 5 mm (6 F multipurpose catheters, USCI, Boston, MA, USA) were placed against the high right atrium, the apex of right ventricle (RVA) or the outflow tract of the right ventricle (RVOT), and the His bundle region through the right femoral vein. His-ventricular (HV) intervals were measured during constant right atrial pacing at a cycle length of 600 msec. Programmed ventricular stimulation was performed using two basic cycle lengths (400 and 600 msec) at the RVA and the RVOT with 2 msec of the width and at twice the late diastolic threshold. The number of extrastimuli was limited to 3 and the shortest coupling interval of any extrastimulus was limited to 180 msec. Rapid ventricular pacing up to a rate of 210 beats per minute was performed at the RVA and the RVOT. Stimulation was attempted first at the RVA and then at the RVOT if ventricular arrhythmias were not induced at the RVA. If ventricular arrhythmias were not induced by 1–2 extrastimuli, triple extrastimuli were introduced first

TABLE 1

Clinical and Electrocardiographic Data

Patient Number	Sex/Age (Year)	Clinic	History	SCN5A	LP	LP Reproducibility	Minimum RMS40	V_1or$_2$ fQRS	V_5or$_6$ fQRS	V_1or$_2$–V_5or$_6$ fQRS Width	ST-Elevation Type	Therapy/ Follow-Up (Mo)
1	M/33	Cardiac arrest	0	NE	+	+	5	150	115	35	1	ICD/24
2	M/70	Cardiac arrest	0	NE	+	+	12	140	114	26	2	ICD/17
3	M/54	Cardiac arrest	0	–	–	0	21	135	120	15	2	ICD/23
4	M/58	Cardiac arrest	0	–	+	+	7	150	125	25	2	ICD/48
5	M/63	Cardiac arrest	0	–	+	+	14	149	117	32	2	ICD/46
6	M/74	Cardiac arrest	0	–	+	+	8	180	180	0	1	ICD/49
7	M/74	Cardiac arrest	0	–	+	+	3	160	115	45	1	ICD/52
8	M/71	Syncope	0	–	+	–	9	140	107	33	1	ICD/50
9	M/72	Syncope	0	NE	–	0	34	123	117	6	1	ICD/52
10	M/74	Syncope	0	NE	–	0	22	126	120	6	1	ICD/19
11	M/51	Syncope	0	NE	–	NE	29	139	135	4	2	ICD/13
12	M/25	Syncope	0	NE	–	0	24	142	135	7	2	ICD/50
13	M/81	Syncope	0	NE	+	NE	14	140	124	16	1	ICD/17
14	M/60	Syncope	0	NE	+	–	6	162	135	27	1	ICD/18
15	M/74	Syncope	0	–	+	–	14	170	162	8	2	ICD/52
16	M/64	Asymptomatic	0	NE	–	NE	24	135	120	15	2	ICD/15
17	M/47	Asymptomatic	0	NE	–	0	64	125	117	8	2	ICD/50
18	M/60	Asymptomatic	0	NE	+	–	9	125	120	5	2	Refused ICD/48
19	M/45	Asymptomatic	0	–	+	NE	12	149	135	14	2	ICD/47
20	M/53	Asymptomatic	0	–	–	NE	24	136	135	1	2	ICD/50
21	M/61	Asymptomatic	0	NE	+	NE	17	140	136	4	2	ICD/14
22	M/79	Asymptomatic	0	NE	–	0	41	120	110	10	2	No ICD/36
23	M/51	Asymptomatic	0	NE	+	+	10	140	130	10	2	Refused ICD/11
24	F/45	Asymptomatic	0	NE	+	–	5	126	125	1	2	ICD/20

LP = late potential; RMS = root mean square; fQRS = filtered QRS; + = positive; – = negative; NE = not examined; CRBBB = complete right bundle branch block; 0 = all negative.

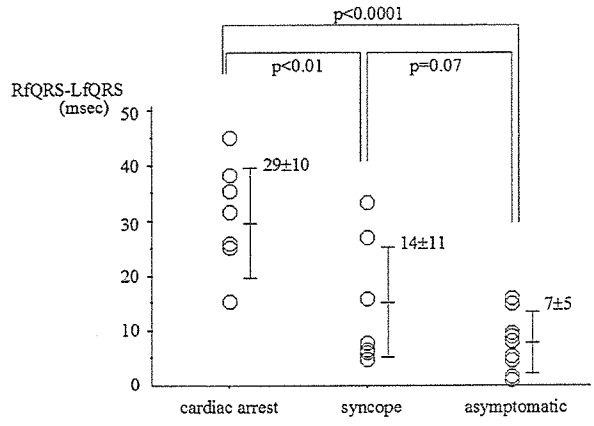

Figure 1. *Comparison of the difference between the filtered QRS duration in a right precordial lead (RfQRS) and a left precordial lead (LfQRS) in Brugada syndrome patients. The RfQRS-LfQRS is significantly longer in patients with aborted cardiac sudden death than in patients with syncope and asymptomatic patients.*

from the RVOT, and then from the RVA. The endpoints of programmed ventricular stimulation were either induction of sustained VT/VF or completion of the programmed stimulation protocol. The effective refractory period (ERP) was measured at a basic cycle length of 400 msec at both the RVOT and the RVA.

Conduction Time Between RVOT and RVA

We evaluated the conduction time from the RVOT to the RVA (CT-OA) after a single extrastimulus given at the RVOT, that is, we measured the conduction time between the stimulus artifact at the RVOT and the electrogram at the RVA at a basic cycle length of 600 msec.

Analysis

We divided the patients into three groups based on their clinical characteristics: patients with cardiac arrest (cardiac-arrest group, n = 7), patients with syncope (syncopal group, n = 8), and the asymptomatic patients (asymptomatic group, n = 9). We compared the following parameters among the three groups: LP reproducibility, RMS40, RfQRS, LfQRS, the difference between RfQRS and LfQRS, ERP, HV interval, and CT-OA. We also investigated the relationships among the HV interval, CT-OA, RfQRS, and RfQRS-LfQRS.

Statistical Analysis

Quantitative values are expressed as mean ± SD. Student's *t*-test for unpaired or paired values was used to compare parameters between two groups. Differences among three groups were evaluated using one-way analysis of variance (ANOVA) followed by the Bonferroni test. Linear regression analysis was used to determine the relationship between the CT-OA and the HV interval, and the relationship between

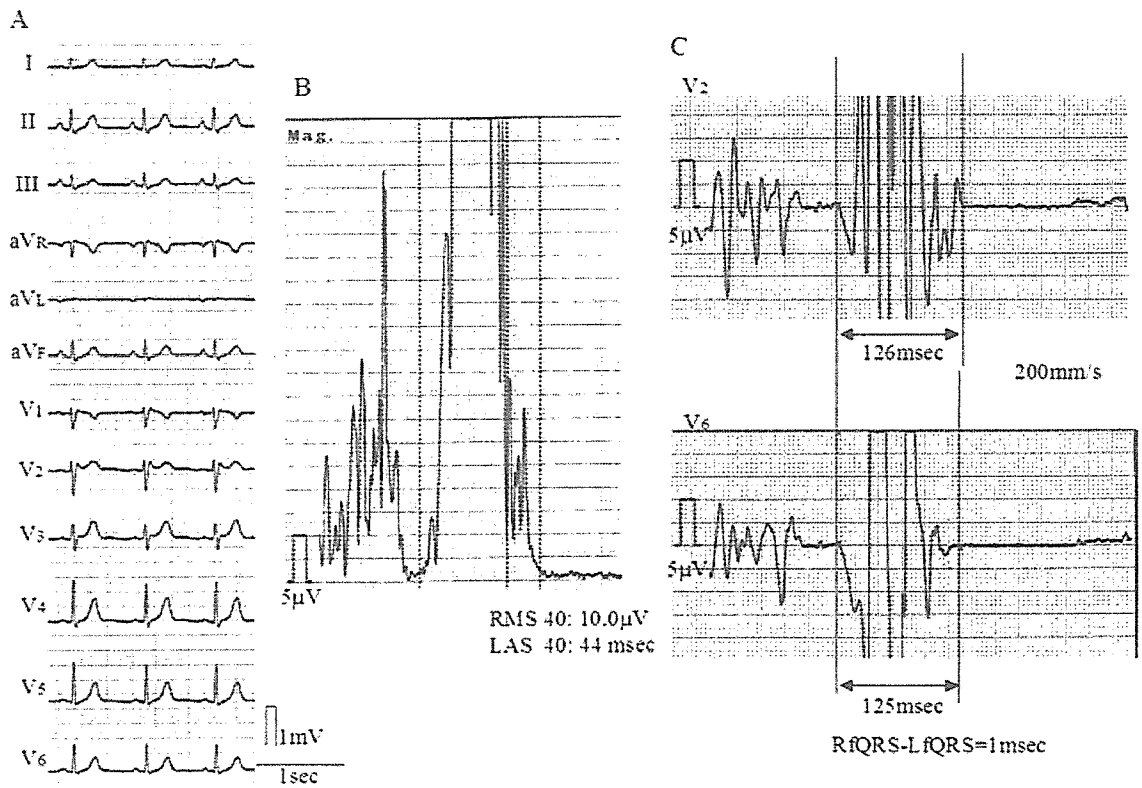

Figure 2. A: *A 12-lead ECG in an asymptomatic patient (patient 24) shows a type 2 Brugada ECG pattern.* B: *The SAECG is positive for LP with an RMS40 <20 μV, and an LAS40 >38 msec.* C: *The filtered QRS durations in the right precordial lead (V_2; RfQRS) and left precordial lead (V_6; LfQRS) are 126 and 125 msec, respectively, and the difference between them (RfQRS-LfQRS) is 1 msec.*

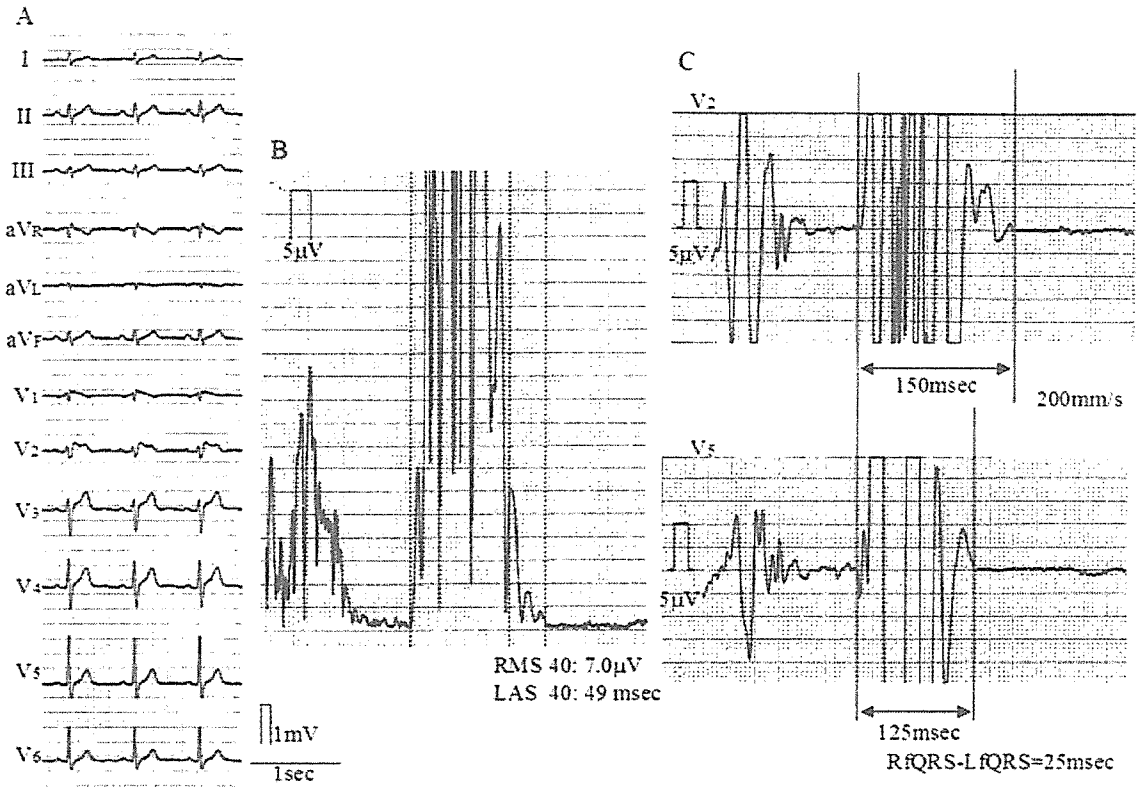

Figure 3. A: *A 12-lead ECG for a patient (patient 4) with aborted cardiac sudden death shows a type 2.* B: *The SAECG is positive for LP.* C: *The RfQRS (V_2) and the LfQRS (V_5) are 150 and 125 msec, respectively, and the RfQRS-LfQRS is 25 msec.*

CT-OA and RfQRS, and the difference between RfQRS and LfQRS. A P value <0.05 was considered statistically significant.

Results

SAECG (Table 1)

Including the repeated test, the SAECG was positive for LP at least once in 6 of 7 (86%), 4 of 8 (50%), and 5 of 9 (55%) patients in the cardiac-arrest, syncopal, and asymptomatic groups, respectively (P = 0.33). In 18 patients LP was measured three times and 13 patients had reproducible results (LP was positive in 7, and negative in 6). In the other 5 patients, results of LP were variable according to each examination: 4 of 5 patients were LP positive in 2 of 3 SAECG and the remaining one patient was LP positive in 1 of 3 SAECG. In 86% (6/7 patients) with cardiac arrest, LP was present on all three occasions. The RMS40 was smaller in the cardiac-arrest group than the syncopal or asymptomatic groups (10 ± 6, 19 ± 10, and 26 ± 20 μV, respectively), but the differences were not significant among the three groups (P = 0.106). The RfQRS was 147 ± 8, 138 ± 13, and 132 ± 9 msec for the cardiac-arrest, syncopal, and asymptomatic groups, respectively, and it was significantly longer in the cardiac-arrest group than in the asymptomatic group (P = 0.011). In contrast, the LfQRS was not significantly different among the three groups (117 ± 4, 123 ± 10, and 123 ± 9 msec, respectively). Consequently, the difference between RfQRS and LfQRS was significantly greater in the cardiac-arrest group than the other two groups; 29 ± 10, 14 ± 11 (P < 0.01), and 7 ± 5 (P < 0.001) msec, respectively (Fig. 1). In the control group, RfQRS and RfQRS-LfQRS were 126 ± 6 msec and 2.2 ± 2.9 msec, respectively, and these values were smaller than those of patients in the asymptomatic group. Figures 2 and 3 show typical recordings from the cardiac-arrest and asymptomatic groups.

Electrophysiologic Findings (Table 2)

The averaged HV interval was 52 ± 8 msec in 22 patients; there were 11 patients with an HV interval above 55 msec. There were no significant differences in HV interval among the three groups (cardiac-arrest group, 53 ± 11 msec; syncopal group, 52 ± 7 msec; asymptomatic group, 51 ± 8 msec). There were no significant differences in the ERP at either the RVOT or the RVA among the three groups. The CT-OA was 69 ± 12 msec, and there were no significant differences among the three groups (cardiac group: 72 ± 19 msec; syncopal group: 72 ± 8 msec; asymptomatic group, 64 ± 7 msec).

In 20 of 22 patients (90%), VF was induced in 11 from the RVOT and in 2 from the RVA with double extrastimuli and in 4 from the RVOT and in 3 from the RVA with triple extrastimuli. The induction rate of VF was similar among the three groups.

The CT-OA measured during the EPS was well correlated with the HV interval: r = 0.755, P = 0.0003 (Fig. 4). However, there was no significant correlation between the CT-OA and RfQRS and RfQRS-LfQRS (Fig. 5).

TABLE 2
Electrophysiologic Study Data

Patient Number	HV (msec)	Inducible VF (Site, Number of Extrastimuli)	ERP (msec)		Conduction Time RVOT-RVA (msec)
			RVOT	RVA	
1	60	VF (RVOT, 2)	220	210	98
2	42	VF (RVA, 3)	210	220	65
3	55	—	210	200	60
4	67	VF (RVOT, 2)	200	220	91
5	35	VF (RVOT, 2)	190	200	48
6	50	VF (RVOT, 2)	—	—	—
7	62	VF (RVOT, 3)	210	210	74
8	45	VF (RVOT, 2)	200	200	68
9	57	VF (RVA, 2)	200	210	86
10	53	VF (RVOT, 2)	230	210	64
11	55	VF (RVOT, 2)	200	200	76
12	60	VF (RVA, 3)	210	210	70
13	NE	NE	NE	NE	NE
14	NE	NE	NE	NE	NE
15	43	VF (RVOT, 3)	—	—	—
16	58	VF (RVOT, 3)	—	—	—
17	52	VF (RVOT, 3)	210	190	69
18	49	VF (RVOT, 2)	210	190	66
19	58	VF (RVOT, 2)	230	220	74
20	57	VF (RVA, 3)	200	190	66
21	37	VF (RVOT, 2)	220	240	57
22	49	—	200	200	56
23	56	VF (RVA, 2)	200	200	66
24	40	VF (RVOT, 2)	—	—	—
Mean ± SD	53 ± 8		209 ± 12	197 ± 46	69 ± 12

HV = His-ventricle interval; ERP = effective refractory period; NE = not examined; VF = ventricular fibrillation; RVOT = right ventricular outflow tract; RVA = right ventricular apex; 1 = single stimulus; 2 = double stimulus.

Follow-Up

An implantable cardioverter defibrillator (ICD) was implanted in 21 of 24 patients. Two asymptomatic patients, who had inducible VF at EPS, refused the ICD therapy. The other one asymptomatic patient was followed-up without the ICD because they had no family history of cardiac sudden death or noninducible VF. During a follow-up period of 33 ± 16 months, one patient with previous cardiac arrest (patient 4) had an episode of VF, which was terminated by the ICD.

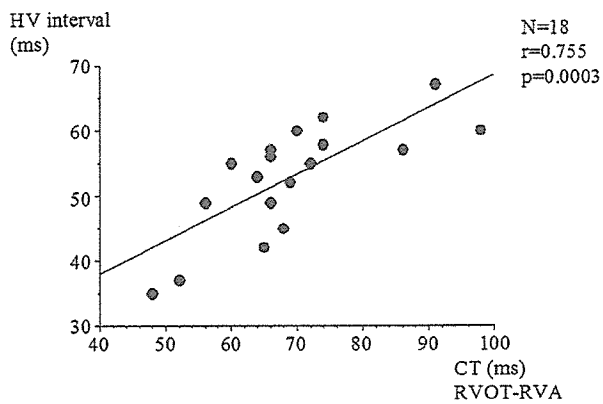

Figure 4. *Relationship between the conduction time (CT) between the RVOT and the RVA and HV interval. There was a significant correlation between them (r = 0.755, P = 0.0003).*

Discussion

In Brugada syndrome, local conduction delay at the RVOT has been detected by SAECG or at EPS[12,13,15] and the major finding of the present study was that the dominant prolongation of filtered QRS duration in the right precordial leads was related to the clinical characteristics: arrhythmic events. The presence of a consistent LP measured on different days was found in patients with a history of cardiac arrest. Although spontaneous episodes of VF have been shown to be triggered by premature ventricular contractions originating from the right ventricular free wall,[8,16] it is not known if local conduction delay in the RVOT plays a role in arrhythmogenesis in Brugada syndrome.

LP can be detected very frequently in Brugada syndrome and may serve as the basis of ventricular arrhythmias.[12,17] The presence of LP in the SAECG usually reflects delayed conduction in the ventricles; however, the mechanism of LP in Brugada syndrome is not fully understood. Nagase et al.[15] confirmed that the timing of a delayed potential in Brugada syndrome recorded from the epicardial surface of the anterior wall of the right ventricle was identical to that of LP recorded in the SAECG. Recent experimental studies have suggested that a prominent I_{to}-mediated phase 1 notch and a subsequent loss of action potential dome in epicardial cells, but not in the endocardial cells in the RVOT, give rise to voltage gradients across the ventricular wall, resulting in ST-segment elevation in the right precordial leads and causing VF due to the mechanism of phase 2 reentry.[4,6] Antzelevitch mentioned in editorial comment for Nagase's report[15] that the concealed occurrence of phase 2 reentry may contribute to the generation of delayed unipolar potential and LP in SAECG.[18] Both local conduction delay and concealed phase

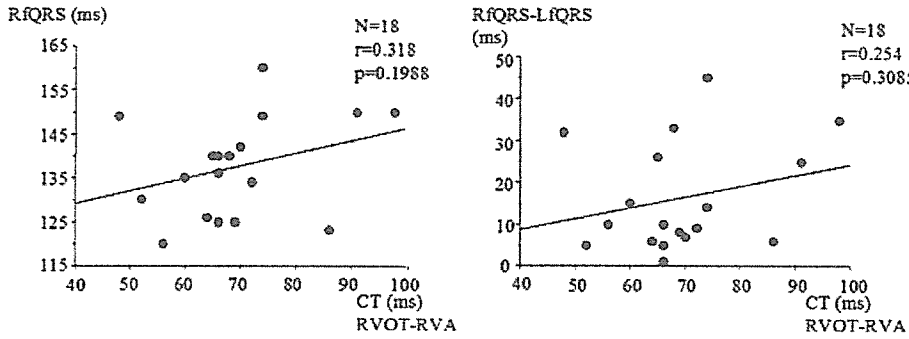

Figure 5. *Relationship between the conduction time (CT) between the RVOT and the RVA and the difference between RfQRS and LfQRS. There was no significant correlation between them. RfQRS = filtered QRS duration in the right precordial leads: LfQRS = filtered QRS duration in the left precordial leads.*

2 reentry might be arrhythmogenic, and further examination is needed to clarify which of them play a major role in cause of LP.

Brugada syndrome is often associated with a prolongation of the HV interval.[1,2] In the present study, there was a good correlation between conduction delay from the RVOT to the RVA and the HV interval but neither the conduction time nor the HV interval was correlated with the filtered QRS duration in the precordial leads or the presence of LP. We postulate that the prolongation of the filtered QRS duration in the right precordial leads represents a type of conduction abnormality that is not due to conduction delay like in the His-Purkinje system. In the present study as well as a previous study,[11] conduction delay including the HV interval was not related to clinical characteristics, and furthermore, LP by standard manner did not correlate to the clinical outcome in this study. This might suggest that the abnormality in RVOT (concealed phase 2 reentry or true conduction delay in RVOT) rather than a conduction abnormality in other parts of the heart might plays an important role in cardiac events.

In the present study, SAECG was positive for an LP in 63% of patients. In Brugada syndrome, the ECG patterns in V_1–V_3 show day-to-day variation, but it is unknown if LP show such day-to-day variation. Our data demonstrated that 7 of 18 patients were LP positive reproducibly, and 6 of 7 were patients with a history of cardiac arrest. Ikeda et al. reported that LP is a noninvasive risk stratification in patients with Brugada syndrome,[13] and whether or not the reproducibility of LP is useful in risk stratification of Brugada syndrome remains to be studied.

Limitations

In Brugada syndrome, symptomatic patients who have had syncopal episodes or aborted cardiac sudden death have a poor prognosis without ICD therapy. However, it is still controversial how to treat asymptomatic patients with a typical Brugada-type ECG. The results of EPS for patients with Brugada syndrome are conflicting.[19–22] LP may be useful to stratify the risk of Brugada syndrome; however, it is not fully studied in prospective and large number of patients. The main limitations in the present study are related to its retrospective nature and to the relatively small number of patients, and more number of patients with Brugada-type ECG, especially asymptomatic patients, are needed to clarify usefulness of filtered QRS duration in precordial leads for predictor. Although LP reproducibility tended to be higher in patients with a history of cardiac arrest in this study, statistical evidence could not be shown because of the small number of patients; so further study is required to clarify the role of LP reproducibility as a risk factor of Brugada syndrome.

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Low-dose isoproterenol for repetitive ventricular arrhythmia in patients with Brugada syndrome

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KEYWORDS
Isoproterenol;
Brugada syndrome;
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Aims Arrhythmic storm or repetitive ventricular arrhythmia (VA) has been occasionally observed in Brugada syndrome (BS). A beta-adrenergic stimulator [isoproterenol (ISP)] has been reported to suppress this arrhythmic storm in sporadic cases. Accordingly, we investigated the antiarrhythmic effects of ISP infusion in consecutive BS patients with arrhythmic storm or repetitive VA.
Methods and results Seven BS patients with arrhythmic storm were studied. Intravenous ISP was administered as a bolus injection (1–2 μg), followed by continuous infusion (0.15 μg/min). Arrhythmic storm or repetitive VA was suppressed immediately after the bolus administration of ISP, which was followed by continuous infusion of low-dose ISP for 1–3 days. In all patients, ST-elevation decreased in right precordial leads. In six of the seven patients, VA subsided after the discontinuance of ISP. RR interval was shortened and ST-elevation in right precordial leads was decreased after ISP bolus injection. ST-elevation in right precordial leads remained decreased during continuous ISP infusion, whereas the RR interval returned to the control level.
Conclusion Continuous administration of low-dose ISP may be effective for the suppression of repetitive VA occurrence in patients with BS.

Introduction

Brugada syndrome (BS) is characterized by ST-segment elevation in right precordial leads and nocturnal ventricular fibrillation (VF).[1–3] These characteristics are modified by the autonomic nervous system.[4] For example, vagal stimulation augments ST-elevation and induces ventricular arrhythmia (VA).[5] In contrast, adrenergic beta stimulation attenuates ST-elevation and suppresses VA.[6–8] Malignant VA is often observed in BS, but repetitive VA including electrical storm is a rare phenomenon in this syndrome. It has been reported that electrical storm in some cases of BS was suppressed by treatment with low-dose isoproterenol (ISP). However, there have been few systematic studies on short-term effects of low-dose ISP infusion in patients with BS who had repetitive VA or electrical storm. This study was, therefore, carried out to determine the short-term effects of beta-adrenergic stimulation on VA in BS patients with repetitive VA.

Methods

Patients

The subjects were seven consecutive patients who had been referred to our hospital between March 2000 and May 2005 for evaluation and treatment after detection of ECG abnormalities compatible with BS. The mean age of the subjects was 47±9 years. All subjects had repetitive VA during or before admission. All patients had recurrent syncope episodes of unknown origin or had been resuscitated from cardiac arrest or VF. Only one case (no. 2) was a recurrent case, and all other cases were first attacks. Repetitive ventricular tachycardia (VT) occurred in one patient (no. 7). VF occurred during or before hospitalization in six patients (nos 1–6). All patients had several VF episodes or repetitive VT before ISP infusion (*Table 1*). Genetic analysis of the SCN5A gene was performed in all patients. Three patients had SCN5A mutation (nos 3, 5, and 7) (*Table 1*). There were no patients of the same family. Electrolyte levels, metabolic status, and results obtained by cardiac imaging techniques, including echocardiography and right ventriculography, were normal in all patients. There were no apparent triggering factors (i.e. fever) of VA in any patient.

Criteria for diagnosis of BS

Brugada-type ECG was defined by the report of the second consensus conference.[2] According to that report, there are three types (types 1–3) of repolarization pattern in Brugada-type ECGs. In this study, all patients showed Brugada-type 1 ECGs at least in one occurrence.

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Patients who showed type 1 ECG only after a class I antiarrhythmic challenge test were not included. One patient (no. 4) showed type 2 ECG in a general condition in right precordial leads, but showed type 1 ECG immediately before a VF attack (*Table 1*).

Intravenous injection of ISP

ISP was administered to all patients when frequent VA occurred during or before hospitalization. ISP was administered as a bolus injection intravenously at a dose of 1–2 μg, followed by continuous infusion at a dose of 0.15–0.30 μg/min until the next day. When VA recurred after stopping administration of ISP the next day, ISP was re-administered for 2 more days. Twelve-lead ECG was recorded before and during administration of ISP, and RR, PQ, QRS, and QT intervals and ST-levels were evaluated. ST-levels were measured at J points in leads V1 and V2. The difference between ST-levels before and after administration of ISP was calculated.

Statistical analysis

Values are expressed as mean ± 1 SD. Statistical analysis was performed using Wilcoxon's signed-rank test for paired values. A value of P less than 0.05 (two-sided) was considered statistically significant.

Results

VAs disappeared immediately after the bolus injection of ISP, followed by continuous injection in all patients (*Figure 1*). VA recurred in three patients when ISP administration was stopped the next day. ISP was re-administered for 2 more days in those patients. VA did not recur in two of those three patients when ISP administration was stopped on the fourth day. In the remaining one patient (no. 5), VF recurred when ISP administration was stopped on the fourth day. In this patient, 400 mg of oral quinidine sulphate was added to ISP injection and tapering of ISP was achieved. All patients felt transient palpitation after the initial injection of ISP, but no side effect was noted during the low-dose continuous administration of ISP.

RR interval was shortened and ST-elevation in leads V1 and V2 was decreased after ISP bolus injection. ST-elevation

Table 1 Patient characteristics

Patient no.	Sex/age (years)	Family history of SCD	Type of VA	Number of repetitive VA episodes	Point of time of repetitive VA episodes	ST-morphology	SCN5A mutation	Induced VA by PES	Duration of ISP therapy	ICD
1	55/M	(−)	VF	Two times/24 h	Night	Type 1	No	(+)	24 h	(+)
2	55/M	(+)	VF	Three times/24 h	Early evening and morning	Type 1	No	(+)	72 h	(+)
3	33/M	(+)	VF	Three times/48 h	Early evening and night	Type 1	Yes	(+)	72 h	(+)
4	41/M	(−)	VF	Two times/48 h	Day time	Type 1(2)	No	(+)	24 h	(+)
5	47/M	(−)	VF	Two times/24 h	Night	Type 1	Yes	(−)	120 h + quinidine	(+)
6	59/M	(−)	VF	Two times/24 h	Early evening	Type 1	No	(−)	24 h	(+)
7	42/M	(+)	RpVT	Two times/24 h	Early evening	Type 1	Yes	(+)	24 h	(+)

RpVT, repetitive ventricular tachycardia; SCD, sudden cardiac death; (+), VF was induced by programmed electrical stimulation (PES); (−), VF was not induced by PES.

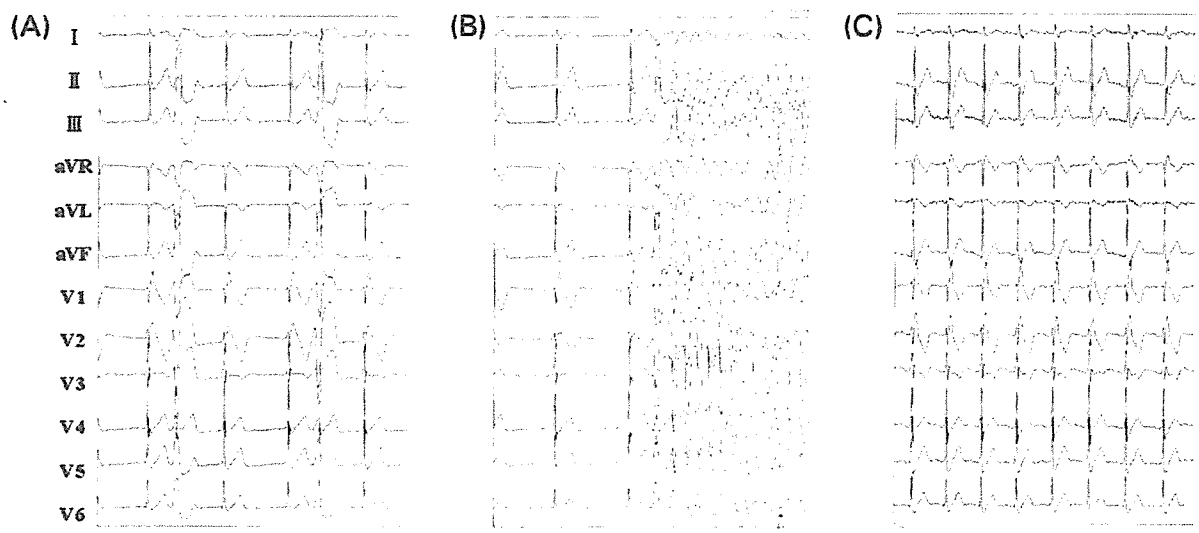

Figure 1 Occurrence of PVC and VF in case 5. (*A*) ECG before administration of ISP. PVC occurred, and QRS morphology of the PVC was left bundle branch block with inferior axis. (*B*) Onset of VF before ISP infusion. (*C*) Disappearance of PVC and VF after ISP infusion.

Table 2 Electrocardiographic changes before and after ISP administration

	Mean ± SD			*P*-value for pairwise comparison		
	Control	Bolus	Continuous	Bolus vs. control	Continuous vs. control	Bolus vs. continuous
PQ (s)	0.18 ± 0.01	0.17 ± 0.02	0.17 ± 0.03	0.414	0.180	0.705
QRS (s)	0.10 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	0.396	0.317	0.739
QT (s)	0.37 ± 0.02	0.37 ± 0.01	0.38 ± 0.02	0.518	0.276	0.074
QTc (s)	0.40 ± 0.03	0.45 ± 0.03	0.42 ± 0.02	0.028	0.149	0.018
RR (s)	0.85 ± 0.10	0.68 ± 0.11	0.83 ± 0.09	0.018	0.307	0.018
ST(V1) (mV)	0.225 ± 0.094	0.079 ± 0.107	0.117 ± 0.103	0.027	0.034	0.276
ST(V2) (mV)	0.317 ± 0.093	0.064 ± 0.069	0.114 ± 0.024	0.017	0.018	0.131

Continuous parameters are expressed as mean ± SD.

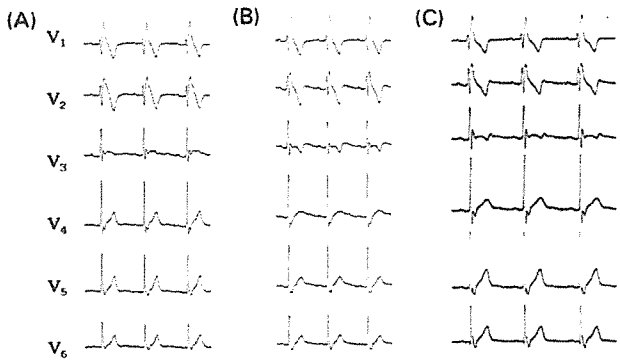

Figure 2 Electrocardiographic patterns before and during ISP infusion in case 5. (*A*) ECG before ISP infusion. Coved-type ST-elevation was observed in leads V1 and V2. RR interval was 0.84 s. (*B*) ECG after bolus ISP infusion. ST-elevation in leads V1 and V2 was decreased and RR interval was 0.68 s. (*C*) ECG during continuous ISP infusion. ST-elevation in leads V1 and V2 was decreased and RR interval was 0.90 s.

in leads V1 and V2 remained decreased during continuous ISP infusion, whereas the RR interval returned to the control level (*Table 2*; *Figure 2*). Decreased ST-elevation in right precordial leads continued until discharge without any drugs in patients without recurrence of VA (*Figure 3*). However, in early recurrence cases, ST-elevation re-appeared immediately after the discontinuance of ISP infusion (*Figure 4*).

Repetitive VA recurred in two patients 1 month (no. 2) and 2 years (no. 3) after VA attack. Treatment with disopyramide at 300 mg/day was started in one patient (no. 2), and this patient has not experienced VA attack for 4 years with disopyramide.

SCN5A gene mutation did not influence the response to ISP therapy in this study.

Discussion

It is well known that the autonomic nervous system activity influences the occurrence of VA and ST-elevation in patients with BS.[9,10] Increased vagal activity facilitates VA in patients with BS.[5,11] Attacks of VF usually occur during the night while sleeping, and the evaluation of autonomic nervous system activity using RR variability showed an increased high-frequency component immediately before VF.[12,13] Acetylcholine injection into the coronary artery increased ST-elevation in right precordial leads and induced VF in some patients.[14] In contrast, increased sympathetic nervous system activity prevents VA. Miyazaki *et al.*[4] reported that a beta-stimulant improved ST-elevation in some patients, and several case studies[6–8] have shown the effectiveness of ISP in patients with BS.

Clinical implication of low-dose continuous ISP

An implantable cardioverter defibrillator (ICD) is implanted in symptomatic patients with BS, especially those in whom VF has been detected. However, some patients have experienced an arrhythmic storm and frequent discharge from the ICD. It has been reported that some drugs (i.e. ISP and quinidine)[6–8,15–20] or catheter ablation for pre-mature ventricular contraction (PVC) is useful for the control of VF attack in such patients.[21] However, catheter ablation may be difficult when PVC does not appear during the catheter session. Considering that many patients will be free after short periods of arrhythmic storm, temporary intravenous administration of ISP while arrhythmias occur might be useful in BS patients with repetitive VA.

In patients with BS, antiarrhythmic drug (class Ia or Ic) challenge tests have frequently unmasked and augmented typical ST-elevation. Intravenous pharmacological tests are, therefore, increasingly being performed worldwide to diagnose BS. However, repetitive VA is often induced by sodium channel blocker administration in patients with BS.[22] We believe that ISP should be used as an emergency drug and administered immediately if VA occurs in a drug challenge test.

In the present study, we evaluated the effects of continuous administration of low-dose ISP for 1–3 days in seven consecutive patients with BS who had repetitive VA. We found that bolus injection of ISP suppressed VA in all patients and that the suppressive effects were maintained during continuous administration of ISP. The fact that six of the seven patients had no arrhythmia recurrence even after the termination of ISP administration for 3 days suggests that ISP might stabilize electrical activity within 3 days in a majority of patients with repetitive VA. When arrhythmic attack cannot be controlled by intravenous ISP therapy for 3 days, oral antiarrhythmic drugs should be considered. In our study, one patient in whom VF attack could

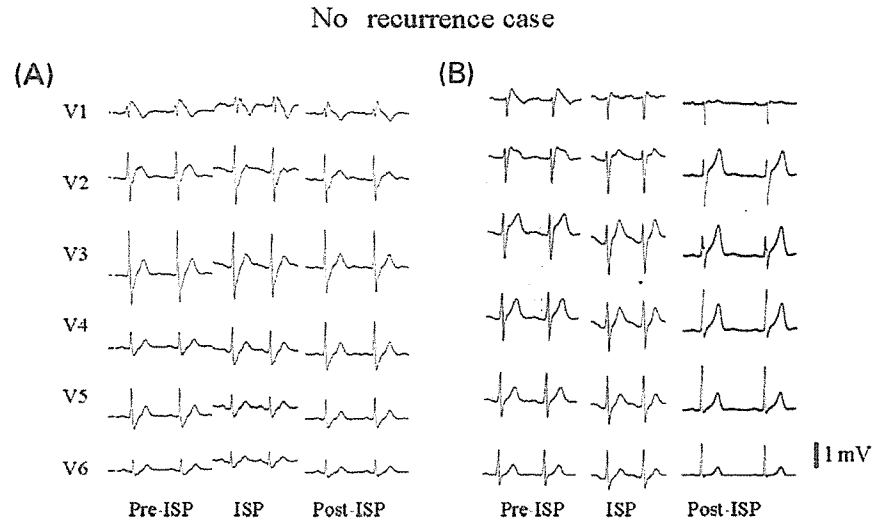

Figure 3 Electrocardiographic change after the discontinuance of ISP infusion in non-recurrence cases. Decreased ST-elevation in right precordial leads continued after the discontinuance of ISP infusion [(*A*) no. 1 and (*B*) no. 4].

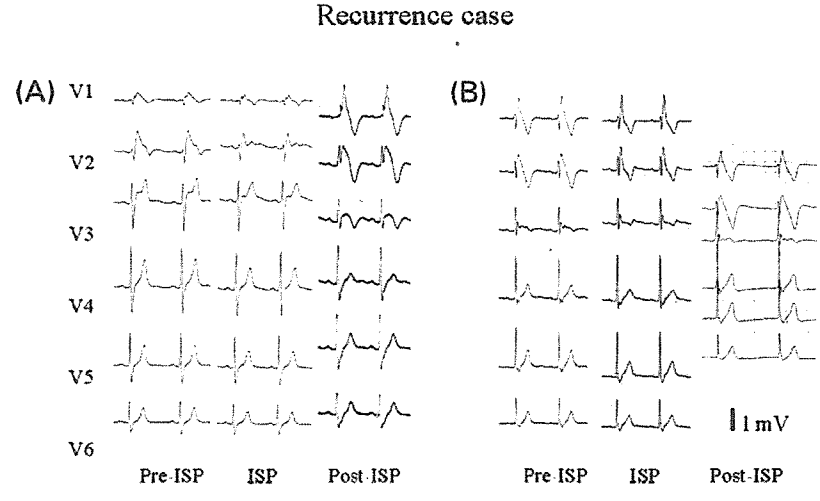

Figure 4 Electrocardiographic change after the discontinuance of ISP infusion in recurrence cases. ST-elevation in right precordial leads re-appeared immediately after the discontinuance of ISP infusion [(*A*) no. 2 and (*B*) no. 5].

not be controlled by ISP therapy for 3 days was administered oral quinidine sulphate for long-term control of arrhythmia.

Interestingly, in four of the six patients who were discharged without any medications, there was no recurrence of VT/VF for a period of 2 years after ISP therapy for arrhythmic storm. This indicates that the duration of arrhythmic storm in BS is transient in a majority of patients with repetitive VA.

Considering that ST-level re-elevated after the discontinuance of ISP infusion in recurrent cases, the response of ST-elevation to ISP withdrawal might be a predictor of recurrence.

Mechanism of the effectiveness of ISP

Yan and Antzelevitch[23] suggested that phase 2 re-entry is a mechanism of VA in patients with BS. Increased outward current or decreased inward current induces a change in the epicardial action potential, such as deepening of phase 1 notch and shortening of action potential duration, and excitation propagates as a difference in electrical voltage (phase 2 re-entry).[24,25] Beta-adrenergic stimulation induces increased inward calcium current and attenuates the excess of outward current, resulting in action potential change. Interestingly, in our study, the decreased ST-level in right precordial leads and the suppressive effect of VA were maintained, even though heart rate had returned to the control level during low-dose ISP therapy. This suggests that increased heart rate is not an important factor in the therapeutic effects of ISP and that the direct effect of ISP on the myocardium to increase inward current is important for therapeutic effects in patients with BS. Thus, reduction of ST-elevation in right precordial leads might be an indicator for prevention of repetitive VA in patients with BS.

Study limitations

It is well known that ECG parameters for patients with BS vary greatly depending on the time of day. Because we evaluated ECG only once a day, the possibility that the ECG changes in our study were due to this characteristic cannot be ruled out.

Another limitation is the fact that our study is observational and it is, therefore, difficult to know whether the arrhythmia actually responded to ISP or settled spontaneously.

As adjustment of multiple comparison was not considered in the analysis, a careful interpretation was required.

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Conflict of interest: none declared.

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Ginsenoside Re, a Main Phytosterol of *Panax ginseng*, Activates Cardiac Potassium Channels via a Nongenomic Pathway of Sex Hormones

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ABSTRACT

Ginseng root is one of the most popular herbs throughout the world and is believed to be a panacea and to promote longevity. It has been used as a medicine to protect against cardiac ischemia, a major cause of death in the West. We have previously demonstrated that ginsenoside Re, a main phytosterol of *Panax ginseng*, inhibits Ca^{2+} accumulation in mitochondria during cardiac ischemia/reperfusion, which is attributable to nitric oxide (NO)-induced Ca^{2+} channel inhibition and K^+ channel activation in cardiac myocytes. In this study, we provide compelling evidence that ginsenoside Re activates endothelial NO synthase (eNOS) to release NO, resulting in activation of the slowly activating delayed rectifier K^+ current. The eNOS activation occurs via a nongenomic pathway of each of androgen receptor, estrogen receptor-α, and progesterone receptor, in which c-Src, phosphoinositide 3-kinase, Akt, and eNOS are sequentially activated. However, ginsenoside Re does not stimulate proliferation of androgen-responsive LNCaP cells and estrogen-responsive MCF-7 cells, implying that ginsenoside Re does not activate a genomic pathway of sex hormone receptors. Fluorescence resonance energy transfer experiments with a probe, *SCCoR* (single cell coactivator recruitment), indicate that the lack of genomic action is attributable to failure of coactivator recruitment. Thus, ginsenoside Re acts as a specific agonist for the nongenomic pathway of sex steroid receptors, and NO released from activated eNOS underlies cardiac K^+ channel activation and protection against ischemia-reperfusion injury.

The earliest evidence of humans' use of herbs for healing dates back to the Neanderthal period (Winslow and Kroll, 1998; Goldman, 2001). In the late 20th century, concerns over the iatrogenic effects of conventional medicine and desire for more self-reliance led to increased interest in natural health, and use of herbal medicines again became popular (Winslow and Kroll, 1998; Goldman, 2001). Among the >20,000 herbal products that are currently on the market, ginseng root is one of the most popular herbs (Attele et al., 1999). Ginseng is known as a panacea (cure-all), and it exhibits a variety of actions, including modulation of immune responses and antineoplastic effects (Attele et al., 1999). Although estrogenic activities (Kim et al., 2004) and nitric oxide (NO) action (Gillis, 1997) have been suggested as a mechanism of ginseng's actions, the precise mechanism remains unknown, which is major hindrance for use of ginseng in modern medicine.

Ginseng root exhibits protection against cardiac ischemia-

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ABBREVIATIONS: NO, nitric oxide; NOS, NO synthase; eNOS, endothelial NOS; nNOS, neuronal NOS; E_2, 17β-estradiol; DHT, 5α-dihydrotestosterone; AR, androgen receptor; ERα, estrogen receptor-α; PR, progesterone receptor; FRET, fluorescence resonance energy transfer; *SCCoR*, single cell coactivator recruitment; LBD, ligand binding domain; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; P_4, progesterone; SMTC, *S*-methyl-L-thiocitrulline; L-NIO, L-N_5-(l-iminoethyl)ornithine; SH-6, D-2,3-dideoxy-myo-inositol 1-[(*R*)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate]; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; ICI182,780, fulvestrant; DMSO, dimethyl sulfoxide; PI3, phosphoinositide 3; pyrazole, 1,2,5-tris(4-hydroxyphenyl)-4-propylpyrazole; estren, 4-estren-3α,17β-diol; DMEM, Dulbecco's modified Eagle medium; $I_{Ca,L}$, L-type Ca^{2+} current; I_{Ks}, the slowly activating delayed rectifier K^+ current.

reperfusion injury (Gillis, 1997), a major cause of death in the West. We have demonstrated previously, in an in vivo rat model, that ginsenoside Re, one of the main constituents of *Panax ginseng*, prevents accumulation of mitochondrial Ca^{2+} in the heart during ischemia-reperfusion injury (Bai, 1993). We have also reported in isolated single cardiomyocytes that ginsenoside Re inhibits L-type Ca^{2+} current ($I_{Ca,L}$) and enhances the slowly activating delayed rectifier K^+ current (I_{Ks}), which we consider a possible mechanism underlying prevention of mitochondrial Ca^{2+} overload (Bai et al., 2003, 2004). Both inhibition of $I_{Ca,L}$ and activation of I_{Ks} by ginsenoside Re are attributable to NO actions, because NO trappers and NO synthase (NOS) inhibitors prevented ginsenoside Re-induced $I_{Ca,L}$ inhibition and I_{Ks} enhancement (Bai et al., 2004). However, the way in which ginsenoside releases NO is still an enigma. In the present study, therefore, we use NO-dependent I_{Ks} activation to unveil the mechanism by which ginsenoside releases NO. Results indicate that ginsenoside acts as a specific agonist for the nongenomic pathway of sex hormone receptors; it activates endothelial NOS (eNOS) and releases NO without activation of the genomic pathway.

Materials and Methods

The investigation was conducted in accordance with the rules and regulations of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Patch-Clamp Experiments. Single ventricular myocytes were harvested from adult female guinea pig hearts, and I_{Ks} was recorded with a perforated configuration of patch-clamp technique using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) as described previously (Bai et al., 2005b). I_{Ks} was elicited by a 3.5-s depolarizing pulse from a holding potential of −40 mV to various test potentials between −30 and +50 mV in 10-mV increments at 0.1 Hz. All experiments were performed at 36 ± 1°C.

External solution was K^+-free solution containing 135 mM NaCl, 0.33 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 0.53 mM $MgCl_2$, 5.5 mM glucose, and 5.0 mM 2-HEPES (pH adjusted to 7.4 with NaOH) that is known to suppress I_{Kr} and enhance I_{Ks} (Sanguinetti and Jurkiewicz, 1992). Nisoldipine (3 μM) and E-4031 (10 μM), drugs that selectively block $I_{Ca,L}$ and I_{Kr} channels, were added to bath solution. The standard pipette solution contained 110 mM aspartic acid, 30 mM KCl, 5.0 mM magnesium-ATP, 5.0 mM creatine phosphate dipotassium salt, and 5.0 mM HEPES (pH adjusted to 7.25 with KOH). Amphotericin B (Sigma-Aldrich, St. Louis, MO) was used in pipette solution to achieve patch perforation. Amphotericin B was prepared as a 600 mg/ml stock solution in dimethyl sulfoxide (DMSO) and diluted to 600 μg/ml in the pipette solution. We front-filled patch pipettes by dipping them into pipette solution and then back-filled with pipette solution containing amphotericin B (600 μg/ml). The averaged membrane capacitance in 119 cells was 150 ± 13 pF.

Immunoblot Analysis. Immunoblot analysis was performed as described previously (Zheng et al., 2002). In brief, cardiomyocytes isolated from adult guinea pig ventricles were maintained in culture medium without serum or growth factors for 1 h and were incubated with culture medium to which ginsenoside Re (10 μM) with or without various blockers was added for 15 min. Cell lysates were prepared from approximately 1×10^8 cardiomyocytes; those with 20 μg of total proteins were electrophoresed on SDS/acrylamide gels and subjected to immunoblot analysis by incubation with a 1:1000-diluted anti-phosphoAkt (^{473}Ser) antibody (Cell Signaling, Danvers, MA) or a 1:1000-diluted anti-Akt antibody (Cell Signaling), followed by incubation with a 1:40,000-diluted horseradish peroxidase-conjugated anti-rabbit IgG (Dako Japan Co. Ltd., Kyoto, Japan). Proteins were detected using an advanced enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL).

Proliferation Assay of MCF-7 and LNCaP. MCF-7 cells were obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University (Sendai, Japan), and LNCaP cells from American Type Culture Collection (Manassas, VA). They were maintained in 1:1 Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (F12) medium (DMEM/F12) with 10% fetal bovine serum at 37°C in a moist environment. Cells were seeded in triplicate at a density of 1.6×10^5 cells/ml in phenol-red-free DMEM/F12 with 10% charcoal-treated fetal bovine serum. Five days after cells had been incubated in the presence of 17β-estradiol (E_2; 10 nM), 5α-dihydrotestosterone (DHT; 10 nM), or ginsenoside Re (10 μM), they were collected and cell numbers were counted.

Receptor-Binding Assay. Binding of ginsenoside Re to the androgen receptor (AR), estrogen receptor-α (ERα), and progesterone receptor (PR) was analyzed with the receptor competitor assay (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In brief, fluorescently tagged receptor ligands that are bound to the ligand-binding domain (LBD) of the human AR, ERα, and PR show high fluorescent polarization; displacement of fluorescently tagged ligands by unlabeled ligands decrease fluorescent polarization (Boyer et al., 2000). In this system, the change in polarization reflects displacement of fluorescently tagged ligands (Boyer et al., 2000); therefore, we measured the change in fluorescent polarization with a polarizer-attached fluorescent spectrometer (FP-6500; JASCO Corporation, Tokyo, Japan).

A Fluorescence Resonance Energy Transfer-Based Coactivator Recruitment Assay. Recruitment of coactivator upon agonist binding to ERα receptor was assayed using a FRET indicator, *SCCoR* (single cell coactivator recruitment), as described previously (Awais et al., 2004, 2006). In brief, an intramolecular FRET-based indicator was constructed to visualize the ligand-dependent recruitment of a coactivator peptide containing a LXXLL motif to the ERα-LBD connected via a short flexible linker. This fusion protein was sandwiched between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) in such a way that excitation and emission spectra of these fluorescent proteins are suitable for FRET in single living cells. The indicator was designated as ER-*SCCoR*. An agonist promotes interaction between a receptor and a coactivator within *SCCoR*, which results in an increase in FRET from CFP to YFP. In contrast, an antagonist inhibits receptor/coactivator interaction. To construct AR-*SCCoR* and PR-*SCCoR*, the LBD of ER in the ER-SCCoR was replaced with the LBD of AR and PR, respectively (Awais et al., 2004, 2006). CHO-K1 cells (ATCC) were transfected with indicators for AR-*SCCoR*, ERα-*SCCoR*, or PR-*SCCoR* in the presence of LipofectAMINE 2000 reagent (Invitrogen) in glass-bottomed dishes. Twelve to 24 h after transfection, cells were imaged at room temperature using a microscope (Axiovert 135; Carl Zeiss, Jena, Germany) with a cooled charged-coupled device camera MicroMAX (Roper Scientific Inc., Tucson, AZ), controlled by MetaFluor (Molecular Devices). Cells were excited at 440 ± 10 nm for 100 ms, and fluorescence images were obtained using filters at 480 ± 15 and 535 ± 12.5 nm in a microscope with a 40× oil immersion objective.

Reagents. E-4031 was purchased from Eisai Co. Ltd. (Tokyo, Japan); ginsenoside Re, DHT, mifepristone, and progesterone (P_4) were purchased from Wako (Osaka, Japan); nisoldipine, *S*-methyl-L-thiocitrulline (SMTC), L-N_5-(l-iminoethyl)ornithine (L-NIO), wortmannin, and E_2 were purchased from Sigma-Aldrich; SH-6 and PP2 were purchased from Merck (Darmstadt, Germany), and ICI182,780 and nilutamide were purchased from Tocris (Ellisville, MO). Stock solutions of E_2 (5 mM), DHT (5 mM), and mifepristone (10 mM) were prepared in ethanol; those for E-4031 (5 mM), SMTC (5 mM), and L-NIO (1 mM) in distilled water; and those for nisoldipine (10 mM), SH-6 (20 mM), PP2 (20 mM), wortmannin (5 mM), ICI182,780 (5 mM), and nilutamide (5 mM) in DMSO. They were diluted in the

bath solution to achieve the desired concentrations. The final concentrations of ethanol [<0.01% (v/v)] and DMSO [<0.05% (v/v)] did not affect K^+ channel activity, shape and proliferation of cells, or a FRET signal.

Data Analysis. All values are presented as mean ± S.E. Statistical significance was examined by repeated-measures nonparametric Friedman test for experiments of time course of I_{Ks}, multiple comparison with Kruskal-Wallis test followed by Dunn's multiple comparison test for immunoblot analysis and cell proliferation assay, and analysis of variance followed by paired Student's *t* test for FRET experiments. A *p* value less than 0.05 was considered to be significant.

Results

Both Panaxadiols and Panaxatriols Activate I_{Ks}. Ginseng root contains more than 30 types of ginsenosides divided into two major groups based on their chemical structure; panaxadiols with sugar moieties at the C-3 and C-21 positions of the sterol structure, and panaxatriols with sugar moieties at positions C-6 and C-21 (Kaku et al., 1975). We have previously reported that ginsenoside Re enhanced I_{Ks} in cardiac myocytes via a NO-dependent manner (Bai et al., 2004). To examine whether activation of I_{Ks} was specific to ginsenoside Re, we tested five ginsenosides that are commercially available: three panaxadiols (Rb1, Rc, Rd) and two panaxatriols (Re, Rg1) (Fig. 1A). Both panaxatriols (Re and Rg1) and panaxadiols (Rb1 and Rc) activated I_{Ks} (Fig. 1B). EC_{50} values were similar among Rb1, Rc, Re, and Rg1, but their maximum responses differed. The maximum extent of I_{Ks} activation was greatest for Re followed by Rc, Rg1, and Rb1, whereas Rd did not activate I_{Ks} (Fig. 1B). Because ginsenoside Re is the most potent among the five ginsenosides, in the following experiments, we used ginsenoside Re-induced I_{Ks} enhancement to examine the mechanism by which ginsenoside Re produces NO.

Ginsenoside Re Releases NO via eNOS Activation. The current-voltage curves showed that enhancement of I_{Ks} by ginsenoside Re (3 μM) was voltage-independent (Fig. 2A), which agrees with our previous report (Bai et al., 2003); thus, test potential at a single voltage (+50 mV) was used to analyze statistical significant changes. Ginsenoside Re-induced I_{Ks} enhancement started to occur within approximately 5 min and reached a pseudo-steady state between 10 and 15 min after its application, implying a role of constitutive NOS, neuronal NOS (nNOS), or eNOS, rather than inducible NOS. Application of SMTC at 3 μM, a concentration that inhibits nNOS but not eNOS (Narayanan and Griffith, 1994), did not alter enhancement of I_{Ks} amplitude by ginsenoside Re (Fig. 2B), whereas L-NIO at 1 μM, a concentration that inhibits eNOS but not nNOS (McCall et al., 1991), decreased I_{Ks} to the initial levels observed before ginsenoside Re application (Fig. 2C), indicating that ginsenoside Re produces NO via eNOS activation.

Ginsenoside Re Activates eNOS via a Phosphoinositide 3-Kinase/Akt-Dependent Pathway. eNOS is activated at least through two mechanisms: a Ca^{2+}-dependent mechanism involving the Ca^{2+}-binding protein calmodulin (Kone, 2000; Goligorsky et al., 2002) and a phosphorylation-dependent mechanism involving the serine/threonine kinase Akt (Kone, 2000; Goligorsky et al., 2002). SH-6 (10 μM), an Akt inhibitor, completely reversed enhancement of I_{Ks} by ginsenoside Re (Fig. 3A). c-Src and PI3-kinase are key upstream signaling molecules of Akt. Ginsenoside Re-induced I_{Ks} activation was inhibited by the c-Src inhibitor PP2 (Fig. 3B) and the PI3-kinase inhibitor wortmannin (Fig. 3C), indicating that ginsenoside Re activates eNOS via a c-Src/PI3-kinase/Akt-dependent mechanism.

As a complementary experiment, we examined effects of preincubation of various blockers on ginsenoside-induced I_{Ks} enhancement. The fractional enhancement of I_{Ks} was obtained as I_{Ks} tail currents averaged from five consecutive traces in the steady state after drug application divided by control I_{Ks} tail currents averaged from five consecutive traces just before drug application. We first examined effects of SMTC, L-NIO, SH-6, PP2, and wortmannin on I_{Ks} in the control condition; I_{Ks} density was slightly decreased (Fig. 4, B–G). However, the magnitude of I_{Ks} reduction was not significantly different from the value at the corresponding time (30 min) in the time-control experiments without addition of any reagents (Fig. 4, A and G), suggesting that the observed I_{Ks} reduction is time-dependent run-down of I_{Ks}, rather than specific effects of blockers. Then, we found that preincubation with L-NIO, SH-6, PP2, and wortmannin abolished enhancement of I_{Ks} by ginsenoside Re, whereas preincubation with SMTC did not affect enhancement of I_{Ks} by ginsenoside Re

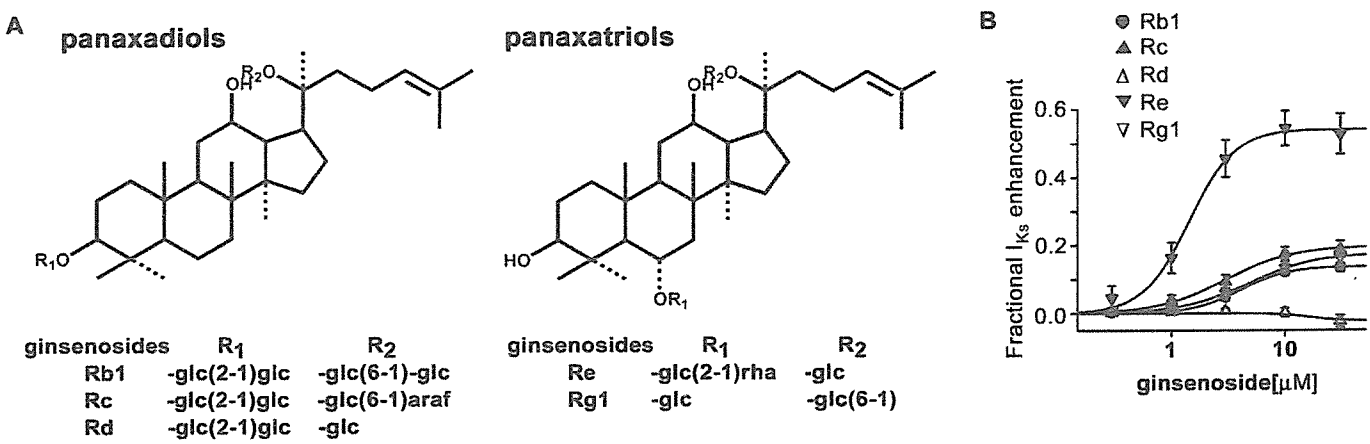

Fig. 1. Both panaxadiols and panaxatriols activate I_{Ks}. A, chemical structures of panaxadiols and panaxatriols used. B, dose-response curves for I_{Ks} activation by five ginsenosides. Continuous lines are results of fitting to the Hill equation. The EC_{50} values were 4.1 ± 0.5 μM for Rb1, 3.0 ± 0.5 μM for Rc, 1.4 ± 0.4 μM for Re, and 4.8 ± 0.7 μM for Rg1; the maximum responses were 13.7 ± 2.3, 19.8 ± 3.1, 54.4 ± 7.1, and 17.7 ± 4.1%, respectively. Ginsenoside Rd did not significantly enhance I_{Ks}.

(Fig. 4, B–G), further supporting that ginsenoside Re activates eNOS via a c-Src/PI-3kinase/Akt-dependent mechanism.

Ginsenoside Re Activates I_{Ks} via Sex-Hormone Receptors. Receptor-type tyrosine kinases and receptors that link to nonreceptor type tyrosine kinases, such as c-Src, activate PI3-kinase (Porter and Vaillancourt, 1998; Wymann and Pirola, 1998). Those include growth factor receptors, an insulin receptor, and gonadal steroid receptors (Porter and Vaillancourt, 1998; Wymann and Pirola, 1998). Gonadal steroids such as testosterone and E_2 exert some biological effects that are too rapid (seconds to minutes) to be compatible with the conventional transcriptional mechanism ("nontranscriptional mechanism") (Weiss and Gurpide, 1998; Baron et al., 2004). We have recently demonstrated that testosterone and E_2 enhance I_{Ks} via the nontranscriptional pathway involving Akt-dependent eNOS activation in cardiomyocytes (Bai et al., 2005a). Because ginsenoside has a four-ring steroid-like structure (Fig. 1A) (Kaku et al., 1975) and exhibits estrogenic activities (Kim et al., 2004), we tested the hypothesis of whether ginsenoside Re exhibited its action via activation of gonadal steroid receptors using inhibitors of sex steroid receptors. Nilutamide (1 μM), ICI-182,780 (5 μM), and mifepristone (1 μM), inhibitors of AR, ERα, and PR, respectively, partially inhibited ginsenoside Re-induced I_{Ks} enhancement in cardiac myocytes (Fig. 5, A–C). A simultaneous application of three inhibitors completely inhibited I_{Ks} enhancement by ginsenoside Re (Fig. 5D).

Ginsenoside Re Induces Akt Phosphorylation. Phosphorylation of Akt at ^{473}Ser occurs when Akt is activated via a PI3-kinase-dependent pathway (Kohn et al., 1996). We confirmed that ginsenoside Re induced phosphorylation of Akt in cardiomyocytes in a concentration-dependent manner (Fig. 6, A and B). Phosphorylation of Akt in cardiac myocytes was inhibited by PP2, wortmannin, and SH-6 (Fig. 6, C and D). It was partially inhibited by ICI182,780, nilutamide, or mifepristone and was completely inhibited by a combination of ICI182,780, nilutamide, and mifepristone (Fig. 6, C and D). These biochemical data further confirm that ginsenoside Re activates Akt via the nongenomic pathway of gonadal steroid receptors.

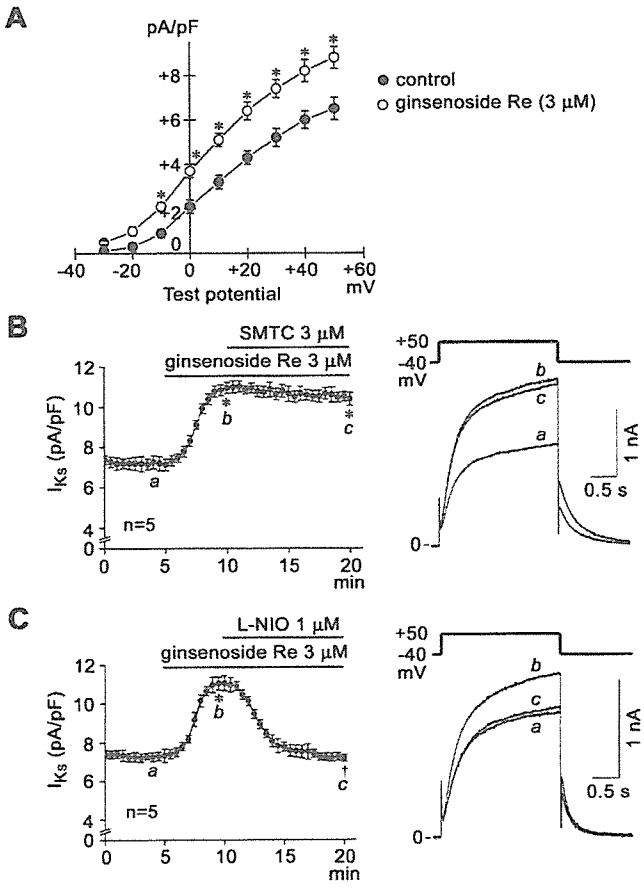

Fig. 2. NO produced by eNOS, but not nNOS, is responsible for I_{Ks} enhancement by ginsenoside Re. A, the current-voltage curves in the absence (control) and presence of ginsenoside Re (3 μM). *, $p < 0.05$ versus control. B and C, effects of SMTC (B), a nNOS inhibitor, and L-NIO (C), an eNOS inhibitor, on I_{Ks} enhancement by ginsenoside Re. Left, time course of experiments in 5 cells. x-Axis is time after start of experiments, and y-axis is averaged current density of I_{Ks}. I_{Ks} were continuously elicited by depolarizing pulses to +50 mV at 0.1 Hz. *, $p < 0.05$ versus control, †, $p < 0.05$ versus in the presence of ginsenoside Re. Right, representative superimposed current traces recorded at the timing indicated by italic lower-case alphabets.

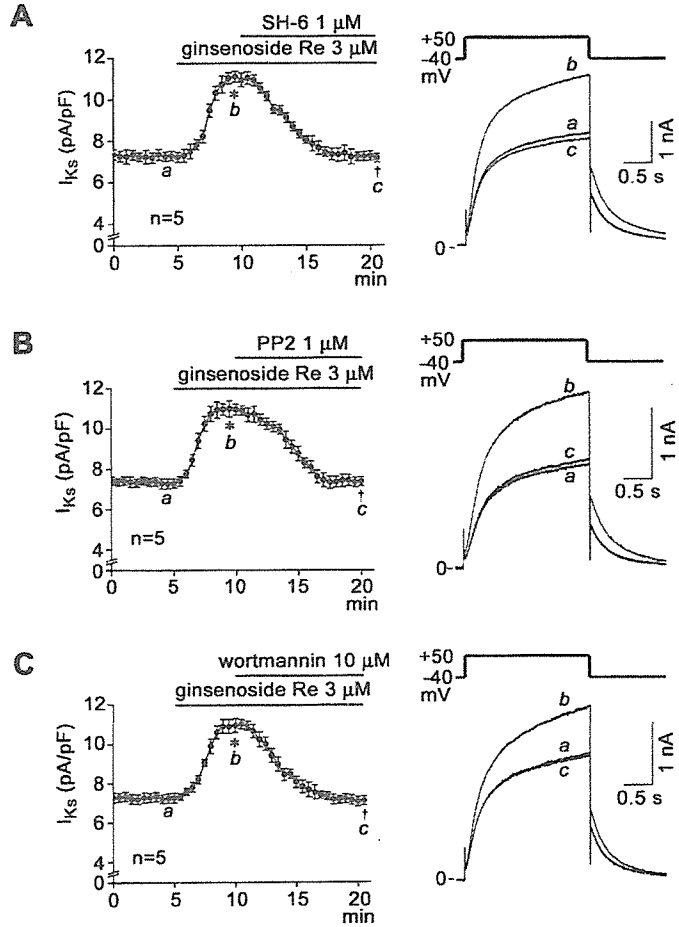

Fig. 3. Signaling cascade of I_{Ks} enhancement by ginsenoside Re. A–C, effects of SH-6 (A), an Akt inhibitor, PP2 (B), a c-Src inhibitor, and wortmannin (C), a PI-3 kinase inhibitor, on ginsenoside Re-induced I_{Ks} enhancement. Left, time course of experiments in five cells. x-Axis is time after start of experiments, and y-axis is averaged current density of I_{Ks}. I_{Ks} were continuously elicited by depolarizing pulses to +50 mV at 0.1 Hz. *, $p < 0.05$ versus control; †, $p < 0.05$ versus in the presence of ginsenoside Re. Right, representative superimposed current traces recorded at the timing indicated by italic lower-case alphabets.