

Alterations in Cardiac Sarcoplasmic Reticulum Ca^{2+} Regulatory Proteins in the Atrial Tissue of Patients With Chronic Atrial Fibrillation

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- OBJECTIVES** Our purpose was to determine whether atrial fibrillation (AF) patients have alterations in sarcoplasmic reticulum (SR) Ca^{2+} regulatory proteins in the atrial myocardium.
- BACKGROUND** Clinically, AF is the most frequently encountered arrhythmia. Recent studies indicate that an inability to maintain intracellular Ca^{2+} homeostasis with a consequent increase in membrane-triggered activity could be the primary initiating factor in some circumstances, and that cytosolic Ca^{2+} abnormalities are an important mediator of sustained AF.
- METHODS** We measured the maximum number of [^3H]ryanodine binding sites (Bmax) and the expression levels of ryanodine receptor (RyR) mRNA and calcium-adenosine triphosphatase (Ca^{2+} -ATPase) mRNA in atrial myocardial tissue from 13 patients with AF due to mitral valvular disease (MVD) and 9 patients with normal sinus rhythm (NSR).
- RESULTS** In AF patients, 1) Bmax was significantly lower in each atrium (0.21 ± 0.03 pmol/mg [right], 0.16 ± 0.04 pmol/mg [left]) than in the right atrium (0.26 ± 0.08 pmol/mg) of NSR patients; 2) Bmax was significantly lower in the left atrium than in the right atrium; 3) Bmax in the left atrium was significantly lower at higher levels of pulmonary capillary wedge pressure; 4) the expression level of RyR mRNA was significantly lower in both the left ($1.24 \times 10^{-2} \pm 1.28 \times 10^{-2}$) and right ($1.70 \times 10^{-2} \pm 1.78 \times 10^{-2}$) atrium than in the right atrium of NSR patients ($6.11 \times 10^{-2} \pm 2.79 \times 10^{-2}$); and 5) the expression level of Ca^{2+} -ATPase mRNA was significantly lower in both the left ($5.67 \times 10^{-2} \pm 4.01 \times 10^{-2}$) and right ($7.71 \times 10^{-2} \pm 3.56 \times 10^{-2}$) atrium than in the right atrium ($12.60 \times 10^{-2} \pm 3.92 \times 10^{-2}$) of NSR patients.
- CONCLUSIONS** These results provide the first direct evidence of abnormalities in the Ca^{2+} regulatory proteins of the atrial myocardium in chronic AF patients. Conceivably, such abnormalities may be involved in the initiation and/or perpetuation of AF. (J Am Coll Cardiol 1999;34:255-63)
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Atrial fibrillation (AF) is the most frequently encountered arrhythmia in the clinical setting. Several epidemiological observations suggest that AF is self-perpetuating; that is, the tachyarrhythmia itself may produce electrophysiological changes that exacerbate or maintain the problem (1-3). The main electrophysiological mechanism underlying AF appears to be a progressive decrease in atrial refractoriness, a phenomenon that has been termed "electrical remodeling" (3). Although this phenomenon is thought to be of impor-

tance in the self-perpetuation of AF, the cellular and molecular mechanisms that might initiate and/or maintain AF remain unclear.

Ca^{2+} is a ubiquitous intracellular signal mediator, and the cardiac sarcoplasmic reticulum (SR) has the ability to sequester Ca^{2+} . An action potential (AP) induces Ca^{2+} release from the SR via Ca^{2+} -induced Ca^{2+} release. Myocardial contraction is triggered by Ca^{2+} release from the SR through a Ca^{2+} release channel referred to as the ryanodine receptor (RyR), and myocardial relaxation depends on ATP-supported SR Ca^{2+} -ATPase (calcium-adenosine triphosphatase), which mediates Ca^{2+} uptake into the SR. Moreover, Ca^{2+} , once released from the SR, interacts with sarcolemmal binding sites to produce an inward current that affects the repolarization of the AP. The notion that a disordered regulation of cytosolic Ca^{2+} plays a causal role in

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Abbreviations and Acronyms

AF	= atrial fibrillation
Ca ²⁺ -ATPase	= calcium-adenosine triphosphatase
mRNA	= messenger ribonucleic acid
MVD	= mitral valvular disease
NSR	= normal sinus rhythm
RT-PCR	= reverse transcription-polymerase chain reaction
RyR	= ryanodine receptor
SR	= sarcoplasmic reticulum

ventricular fibrillation is suggested by several studies of ventricular fibrillation *in vitro*, which have shown intracellular Ca²⁺ overload (4-8). Furthermore, recent studies have indicated that a failure of intracellular Ca²⁺ homeostasis, with a consequent increase in membrane-triggered activity (9), could be the primary initiating factor in AF in some circumstances. Increases in intracellular Ca²⁺ concentration have been shown to produce a negative feedback effect on L-type Ca²⁺ channel activity with a decrease in the plateau phase of the AP (10).

Moreover, the finding of a blockade of electrical remodeling by verapamil and its enhancement by hypercalcemia suggests that cytosolic Ca²⁺ overload may be an important mediator of AF (11). To judge from such evidence, there is a possibility that alterations in SR Ca²⁺ regulatory proteins, which play an important role in the regulation of intracellular Ca²⁺, may occur in the atrial myocardium of patients with chronic AF.

The purpose of our present study was to determine whether patients with chronic AF have alterations in the SR Ca²⁺ regulatory proteins (RyR and Ca²⁺-ATPase) in their atrial tissue. We studied RyR binding and the expression levels of RyR mRNA (messenger ribonucleic acid) and Ca²⁺-ATPase mRNA in the right and/or left atrium after its removal during cardiac surgery from patients with AF and from patients with normal sinus rhythm (NSR).

METHODS

Selection of patients. We examined right atrial tissue (atrial appendage and/or atrial free wall) from 13 patients in whom atrial fibrillation (AF) had been sustained for more than six months; this tissue was obtained during cardiac surgery for mitral valvular disease (MVD). Right atrial tissue (atrial appendage) from nine patients with a normal sinus rhythm (NSR), obtained during cardiac surgery for coronary artery bypass, thoracic aortic aneurysm, or MVD, was used as control material. We also examined left atrial tissue (atrial free wall) obtained from 10 AF patients with MVD. All the patients were evaluated by the Second Department of Internal Medicine, Yamaguchi University School of Medicine, and they underwent surgery at the Yamaguchi University Hospital. Informed consent was obtained from each patient. The protocols were in accord with guidelines laid down by the Institutional Review Board, Yamaguchi University Hospital. Demographic data, including age, sex, diagnosis, and hemodynamic data, are shown in Table 1. In addition to their usual medication, all the patients received preoperative sedation and perioperative anesthesia. Hemodynamic and echocardiographic data were obtained by reviewing data from preoperative cardiac catheterizations and echocardiograms.

Myocardial tissue samples. Right and/or left atrial tissues were frozen immediately and stored at -80°C until needed. They were used to prepare a crude homogenate for the assay of ryanodine receptor binding and to produce RNA for reverse transcription-polymerase chain reaction (RT-PCR) amplification.

Atrial crude homogenate preparation. A crude homogenate for the [³H]ryanodine binding assay was prepared as previously described, with some modifications (12-14). Atrial tissue (~100 mg) was homogenized twice for 20 s each time, using a Brinkmann Polytron, in 20 mmol/liter Tris-maleate containing 0.3 mol/liter sucrose, 0.1 mol/liter KCl, 5 mg/liter leupeptin, and 0.1 mmol/liter phenylmethylsulfonyl fluoride (PMSF), at pH 7.0. The homogenate

Table 1. Clinical Characteristics of Patients With NSR

Patient No.	Diagnosis	Age/Gender	EF (%)	RAP (mm Hg)	mPAP (mm Hg)	PCWP (mm Hg)	LAd (mm)
1	IHD	64/M	66	8	15	11	38
2	IHD	63/M	63	3	14	9	41
3	IHD	67/M	55	4	12	8	31
4	IHD	60/M	64	3	11	5	43
5	TAA	74/M	68	-	-	-	41
6	TAA	71/F	63	-	-	-	43
7	TAA	72/M	72	2	14	4	36
8	TAA	67/M	62	2	14	4	34
9	MVD	65/F	65	0	14	7	43

NSR = normal sinus rhythm; EF = left ventricular ejection fraction; RAP = right atrial pressure; mPAP = mean pulmonary arterial pressure; PCWP = pulmonary capillary wedge pressure; LAd = left atrial diameter; IHD = ischemic heart disease; TAA = thoracic aortic aneurysm; MVD = mitral valvular disease.

was filtered through two layers of cheesecloth. Protein concentration was determined by the method of Lowry et al. (15), using bovine serum albumin as standard. Aliquots of homogenate were frozen in liquid nitrogen and stored at -80°C until used.

RNA preparation. Total cellular RNA was isolated from each frozen tissue sample (~50 mg) by the method of acid guanidinium thiocyanate/phenol/chloroform extraction (16), then stored at -80°C.

Assay of [³H]ryanodine binding. [³H]Ryanodine-binding assays were carried out according to previously described methods (12-14). Briefly, crude homogenate (0.3 mg/ml) was incubated for 90 min at 37°C in 25 mmol/liter imidazole (pH 7.4), 1.0 mol/liter KCl, 1.103 mmol/liter CaCl₂, and 0.95 mmol/liter EGTA (20 μmol/liter free Ca²⁺), in each case with a concentration of [³H]ryanodine from within the range 0.6 to 20 nmol/liter. The reaction was terminated by rapid filtration of 1 ml of the incubation mixture through a glass fiber filter (Whatman GF/C, Maidstone, United Kingdom) under reduced pressure. To minimize the nonspecific binding component, each filter was immediately washed with 5 ml of ice-cold buffer (25 mmol/liter imidazole, 1.0 mol/liter KCl, 1.103 mmol/liter CaCl₂, 0.95 mmol/liter EGTA, at pH 7.4) and removed while under vacuum. After the addition of 5 ml of scintillation fluid, the radioactivity was counted in a scintillation counter (LSC-5100, Aloka, Tokyo, Japan). Nonspecific binding was determined in the presence of 2 μmol/liter unlabeled ryanodine.

Reverse transcription (RT) and polymerase chain reaction (PCR) amplification (RT-PCR). The cDNA was prepared using a Takara RNA PCR Kit (Takara, Tokyo, Japan) in a buffer containing 10 mmol/liter Tris-HCl, pH 8.3, 50 mmol/liter KCl, 5 mmol/liter MgCl₂, and 1 mmol/liter each of dCTP, dGTP, dTTP, and dATP, with 20 U of recombinant ribonuclease inhibitor, 2.5 μmol/liter random 9 mers, 1.3 μg of total RNA, 5 U of avian myeloblastosis virus reverse transcriptase, all in a volume of 20 μl. This reaction mixture was incubated for 10 min at 30°C followed by 30 min at 42°C to initiate synthesis of cDNAs. Reverse transcriptase was inactivated at 99°C for 5 min, and this mixture was then used for the amplification of specific cDNAs by PCR. The PCR was performed as follows: to 20 μl of the RT reaction mixture were added 2 μl of 0.1 mol/liter forward primer, 2 μl of 0.1 mol/liter reverse primer, 8 μl of 10 × amplification buffer (100 mmol/liter Tris-HCl, pH 8.3, 500 mmol/liter KCl), 12 μl of 25 mmol/liter MgCl₂, 55 μl of H₂O, 0.5 μl of [α-³²P]dCTP (Amersham), and 0.5 μl (2.5 U/100 μl) of *Taq* polymerase. The primers for the amplification of the cardiac RyR, Ca²⁺-ATPase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed from published sequences. For RyR they were based on rabbit sequences (17): at positions 1577-1597 (*sense primer*, 5'-GGA AAT CCA

TTC TGA ATT CTC-3') and 2043-2064 (*antisense primer*, 5'-GCA GTC GTC ACA AAC GGC TCG GTG-3') (predicted length of the PCR product, 487 bp). For Ca²⁺-ATPase they were based on human cardiac sequences (18): at positions 2748-2771 (*sense primer*, 5'-CGA AAA CCA GTC CTT GCT GAG GAT-3') and 2970-2993 (*antisense primer*, 5'-TAC TCC AGT ATT GGC ATG CCG AGA-3') (predicted length of the PCR product, 296 bp). For GAPDH they were based on human sequences (19): at positions 102-125 (*sense primer*, 5'-CTT CAT TGA CCT CAA CTA CAT GGT-3') and 805-828 (*antisense primer*, 5'-CTC AGT GTA GCC CAG GAT GCC CTT-3') (predicted length of the PCR product, 726 bp).

Next, 100 μl of reaction mixture was overlaid with 20 μl mineral oil, and cycling was performed 22 times by means of a thermal cycler (Perkin-Elmer/Cetus, San Diego, California) using the following parameters: denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min, followed by a final incubation at 72°C for 7 min.

RT-PCR cloning and sequencing of the cardiac ryanodine receptor cDNA. Heart total RNA was reverse-transcribed into single-strand cDNA and amplified under the same conditions as above. The amplified products were separated on a 1% agarose gel (Doujin, Kumamoto, Japan), then isolated from the gel and directly ligated into a pCRII vector (Invitrogen, San Diego, California). Both strands of the insert into the plasmid were completely sequenced by the dideoxy chain termination method (20) using the dsDNA Cycle Sequencing System (GIBCO BRL, Gaithersburg, Maryland).

Quantitation of PCR products. The optimal number of amplification cycles needed to allow quantitation of cardiac RyR, Ca²⁺-ATPase, and GAPDH gene PCR products was determined. The PCR products for each cycle were subjected to 5% polyacrylamide gel electrophoresis (PAGE) and autoradiography, and the associated radioactivity was measured using an imaging analyzer (model BAS-2000; Fuji Photo Film, Tokyo, Japan). The optimal number of cycles for cardiac RyR, Ca²⁺-ATPase, and GAPDH was found to be 22 in each case (21).

Assessment of expression of cardiac ryanodine receptor and Ca²⁺-ATPase mRNA. The relative radioactivity associated with cardiac RyR or Ca²⁺-ATPase PCR products in each sample was calculated by dividing the radioactivity associated with the RyR or Ca²⁺-ATPase PCR products by the radioactivity associated with the GAPDH gene product (internal control; amplified simultaneously). Each level of RT-PCR product was the average of duplicate data. We used GAPDH as an internal control because the densitometric scores for the mRNAs did not differ among the groups of patients. Furthermore, this enzyme of the glycolytic pathway is constitutively expressed in most tissues and

Table 2. Clinical Characteristics of Patients With AF

Patient No.	Diagnosis	Age/Gender	EF (%)	RAP (mm Hg)	mPAP (mm Hg)	PCWP (mm Hg)	LAd (mm)
1	MVD	69/F	72	9	25	18	84
2	MVD	50/F	52	7	42	23	50
3	MVD	47/M	54	6	34	23	56
4	MVD	63/F	72	9	22	12	75
5	MVD	64/M	61	8	30	21	49
6	MVD	68/F	67	2	33	15	54
7	MVD	55/F	54	4	11	10	66
8	MVD	66/F	64	3	18	10	59
9	MVD	68/M	43	1	18	12	52
10	MVD	63/F	55	9	22	19	58
11	MVD	62/M	64	9	22	19	58
12	MVD	73/F	55	9	20	14	62
13	MVD	47/F	48	5	19	15	64

AF = atrial fibrillation; EF = left ventricular ejection fraction; RAP = right atrial pressure; mPAP = mean pulmonary arterial pressure; PCWP = pulmonary capillary wedge pressure; LAd = left atrial diameter; MVD = mitral valvular disease.

is the most widely accepted internal control in the molecular biology literature (22).

Statistical analysis. All data are presented as mean \pm SD. Comparisons between data were performed by a two-way analysis of variance (ANOVA) followed by the Scheffé test. Differences were taken to be significant at $p < 0.05$.

RESULTS

Clinical characteristics and hemodynamic data. The preoperative hemodynamic and echocardiographic data for the two groups are shown in Tables 1 and 2. The right atrial pressure (RAP; 6.2 ± 3.0 mm Hg), pulmonary capillary wedge pressure (PCWP; 15.7 ± 4.7 mm Hg), and mean pulmonary arterial pressure (mPAP; 24.2 ± 8.4 mm Hg) in patients with AF due to MVD were significantly higher than the corresponding values in patients with NSR ($3.1 \pm$

2.5 , 6.9 ± 2.7 , 13.4 ± 1.4 mm Hg, respectively, $p < 0.05$). Moreover, the left atrial diameter (LAd; 62.8 ± 12.5 mm) measured by echocardiography in patients with AF due to MVD was significantly larger ($p < 0.05$) than the atrial diameter (38.9 ± 4.4 mm) measured in patients with NSR. The left ventricular ejection fraction was similar in the two groups (AF vs. NSR, $59 \pm 9\%$ vs. $64 \pm 5\%$).

Assay of [³H]ryanodine binding. In patients with AF due to MVD, there was a significant depression in the maximum number of binding sites (Bmax) in each atrium (0.21 ± 0.03 pmol/mg in the right atrium, 0.16 ± 0.04 pmol/mg in the left atrium) by comparison with the level in the right atrium (0.26 ± 0.08 pmol/mg) of patients with NSR (Fig. 1). In patients with AF, the Bmax in the left atrium was significantly lower than that in the right atrium. In contrast, the dissociation constant (Kd) of [³H]ryanodine

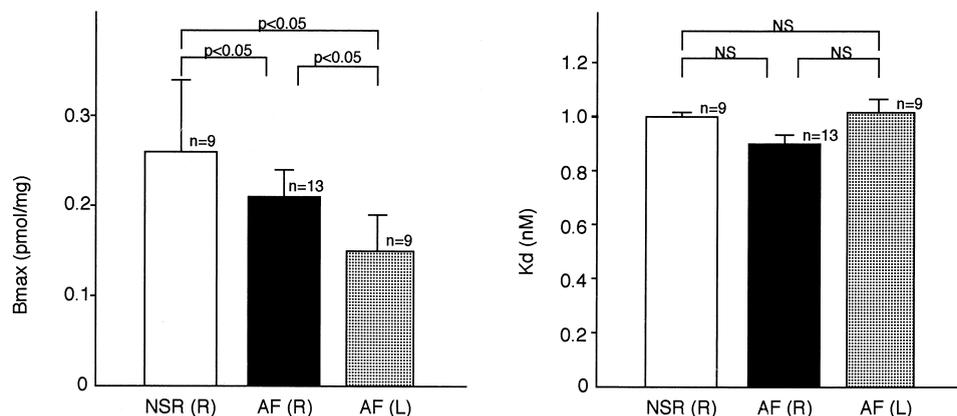


Figure 1. Characteristics of [³H]ryanodine binding to atrial tissue in patients with either atrial fibrillation (AF) or normal sinus rhythm (NSR). In patients with AF, there was a significant depression in the maximum number of binding sites (Bmax) in each atrium (by comparison with the value for the right atrium in patients with NSR). The dissociation constant (Kd) did not differ significantly among the three groups. R, right atrium; L, left atrium. Data are mean \pm SD.

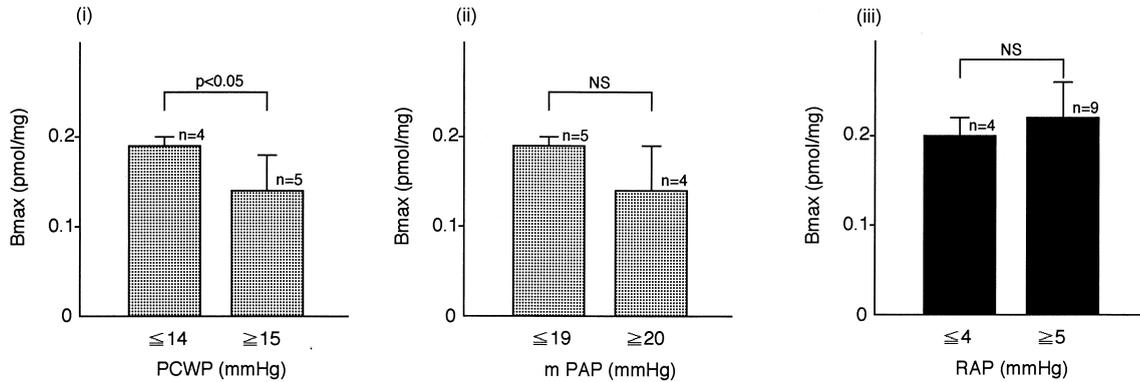


Figure 2. Relation between the maximum number of [³H]ryanodine binding sites (Bmax) in the atrium of patients with atrial fibrillation (AF) and magnitude of pulmonary capillary wedge pressure (PCWP) (i), mean pulmonary arterial pressure (mPAP) (ii), and right atrial pressure (RAP) (iii). The Bmax for RyR was smaller at higher levels of PCWP. There was no significant difference between the Bmax values obtained for the two levels of mPAP or the two levels of RAP. Data are mean ± SD.

binding did not differ significantly among the three groups (Fig. 1).

Next, the Bmax data for AF patients were broken down according to the patient's PCWP, mPAP, or RAP. Figure 2 (i) shows the relationship between the Bmax for RyR in the left atrium and PCWP. The Bmax was 0.19 ± 0.01 pmol/mg in the group with the lower levels of PCWP (≤14 mm Hg) and 0.14 ± 0.04 pmol/mg in the group with the higher PCWP (≥15 mm Hg), and there was a significant difference between the two groups. For mPAP (ii in Fig. 2), the Bmax in the left atrium was 0.19 ± 0.01 pmol/mg in the group with the lower levels (≤19 mm Hg) and 0.14 ± 0.05 pmol/mg in the group with the higher levels (≥20 mm Hg). To judge from these values, Bmax tended to be smaller, though not significantly (p = 0.09),

at higher levels of mPAP. No correlation was found between RAP and the Bmax for RyR in the right atrium (iii in Fig. 2).

Analysis of expression levels of mRNA for the cardiac ryanodine receptor and Ca²⁺-ATPase. The expression levels of the mRNAs encoding cardiac RyR and Ca²⁺-ATPase were examined, using total RNA extracted from atrial tissues taken from patients with AF or NSR. Figure 3 shows the levels of these two mRNAs in patients with AF or NSR, and representative mRNA levels for RyR and Ca²⁺-ATPase. The expression level of RyR mRNA in homogenates prepared from the right (1.70 × 10⁻² ± 1.78 × 10⁻²) and the left (1.24 × 10⁻² ± 1.28 × 10⁻²) atrium of patients with AF was significantly lower than that

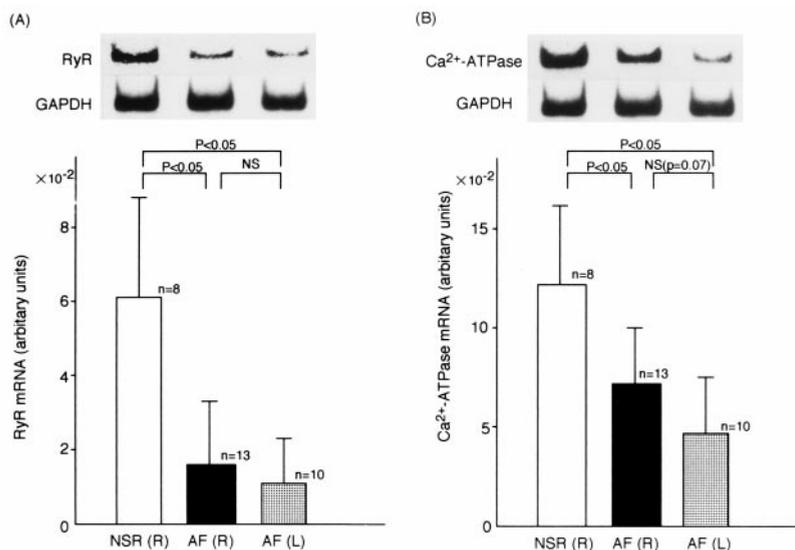


Figure 3. Expression levels of the mRNAs encoding cardiac RyR (A) and Ca²⁺-ATPase (B) in the atrial tissue of patients with either atrial fibrillation (AF) or normal sinus rhythm (NSR), and representative data showing the levels of the two mRNAs. In patients with AF, there was a significant depression in the expression levels of RyR mRNA (A) and Ca²⁺-ATPase mRNA (B) in each atrium (by comparison with the levels in the right atrium of patients with NSR). R, right atrium; L, left atrium. Data are mean ± SD.

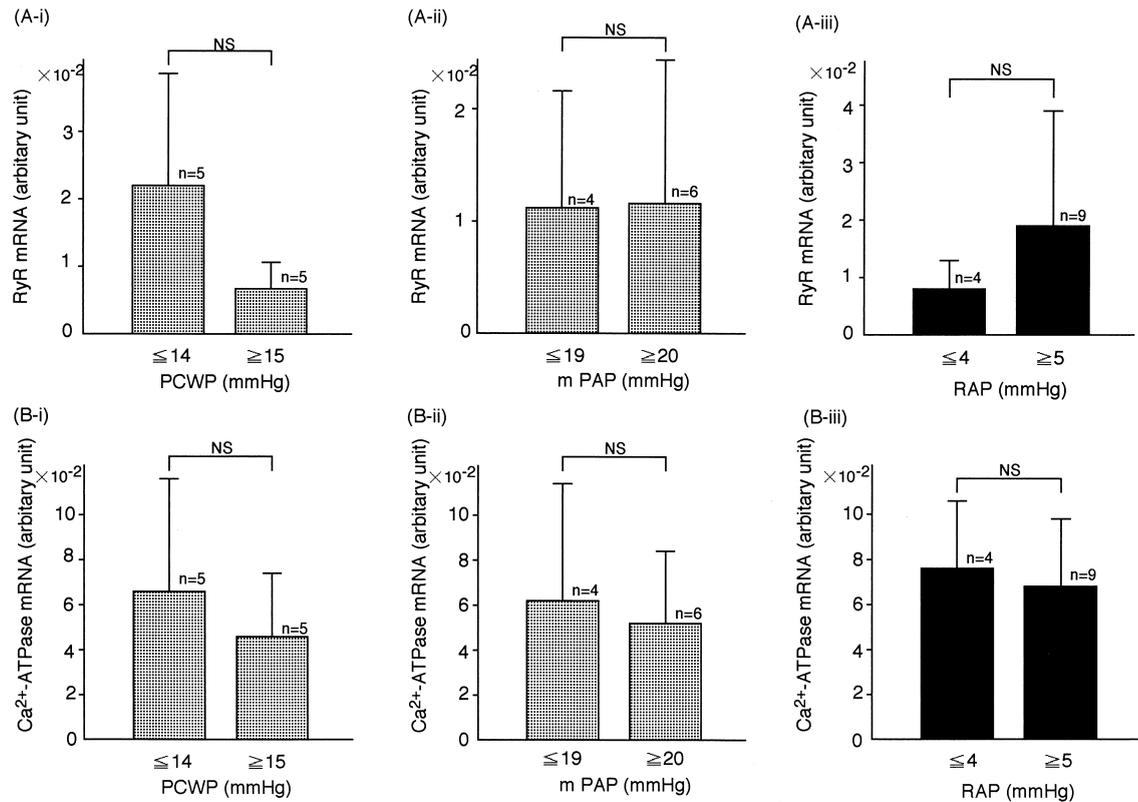


Figure 4. Relation between the expression level of RyR mRNA (A) or Ca²⁺-ATPase mRNA (B) in the atrium of patients with atrial fibrillation (AF) and magnitude of pulmonary capillary wedge pressure (PCWP) (A-i, B-i), mean pulmonary arterial pressure (mPAP) (A-ii, B-ii), and right atrial pressure (RAP) (A-iii, B-iii). Data are mean ± SD.

in the right atrium ($6.11 \times 10^{-2} \pm 2.79 \times 10^{-2}$) of patients with NSR (Fig. 3,A). The expression level of Ca²⁺-ATPase mRNA in the right ($7.71 \times 10^{-2} \pm 3.56 \times 10^{-2}$) and the left ($5.67 \times 10^{-2} \pm 4.01 \times 10^{-2}$) atrium of patients with AF was also significantly lower than that in the right atrium ($12.60 \times 10^{-2} \pm 3.92 \times 10^{-2}$) of patients with NSR (Fig. 3B). There were no significant differences in the expression level of these two mRNAs between the right and left atrium of patients with AF (Fig. 3A and B).

Next, the data for the expression levels of RyR mRNA and Ca²⁺-ATPase mRNA in the left atrium of patients with AF were broken down according to the patient's PCWP and mPAP, and the expression levels of these two mRNAs in the right atrium of patients with AF were broken down according to their RAP. In fact, there was no significant difference in the RyR mRNA expression levels between groups with low or high values for any of these pressure parameters (Fig. 4A-i to A-iii). Furthermore, there were no significant differences in the Ca²⁺-ATPase mRNA expression levels between groups with low or high values for any of the pressure parameters (Fig. 4B-i to B-iii).

DISCUSSION

The main findings of the present study were that in patients with atrial fibrillation (AF) due to mitral valvular disease

(MVD): 1) there were significant decreases in the B_{max} for RyR and the expression levels of RyR mRNA and Ca²⁺-ATPase mRNA in both atria, and the B_{max} for RyR was smaller in the left atrium than in the right; and 2) the B_{max} in the left atrium was smaller at higher levels of PCWP. Conversely, the expression levels of RyR mRNA and Ca²⁺-ATPase mRNA in the left atrium were not significantly affected by the levels of PCWP or mPAP, and no correlation was found between RAP and any of these parameters in the right atrium. To our knowledge, although there have been many investigations of sarcolemmal electrophysiological characteristics (11,23-27), this is the first investigation in which alterations in SR Ca²⁺ regulatory proteins (RyR and Ca²⁺-ATPase) in atrial myocardial tissue have been evaluated in patients with chronic AF due to MVD. These alterations might act to sustain abnormal intracellular Ca²⁺ handling and changes in the electrophysiologic properties of atrial tissue, and thus be responsible for the maintenance of AF. However, such changes could also be a consequence of AF. We will elaborate on this point later in the Discussion.

Comparison between the right and left atrium in chronic AF in terms of the B_{max} for RyR, and the expression levels of RyR mRNA and Ca²⁺-ATPase mRNA. The present study shows a decreased ryanodine binding to atrial

tissue (implying a decreased RyR density) and decreased expression levels of RyR mRNA and Ca²⁺-ATPase mRNA in both atria in MVD patients with AF (by comparison with the values for patients with NSR). Interestingly, in our AF patients the Bmax for RyR was smaller in the left atrium than in the right, and the expression levels of the two mRNAs tended to be lower in the left atrium than in the right. We have previously reported a decrease in SR Ca²⁺ release and uptake functions, as well as a decrease in the number of RyR, and abnormal Ca²⁺ handling in the myocardium during the development of volume-overloaded heart failure (28). On this basis, in MVD patients with AF, direct mechanical overload of the left atrium might result in abnormal Ca²⁺ handling and in alterations in SR Ca²⁺ regulatory proteins.

In contrast, Ausma *et al.* (29) reported that sustained AF leads to marked structural changes in the atrium (such as loss of myofibrils, accumulation of glycogen, changes in mitochondrial shape and size, fragmentation of the SR, and dispersion of nuclear chromatin), and these findings are characteristics of intracellular Ca²⁺ overload. While the present paper was in preparation, Lai *et al.* (30) reported that the mRNAs of the L-type Ca²⁺ channel and of Ca²⁺-ATPase were downregulated in patients with AF; such changes will further contribute to the shortening of action potential duration, to cytosolic Ca²⁺ overload, and significantly to the perpetuation of AF. The observations of Ausma *et al.* (29) and Lai *et al.* (30) indicate that AF itself could cause a depression in the expression levels of Ca²⁺ regulatory proteins. Thus, the results mentioned above could be interpreted as suggesting that changes in the expression levels of Ca²⁺ regulatory proteins in patients with MVD may cause AF, or be a consequence of AF, or indeed be both a cause and a consequence in a self-perpetuating process that leads to the establishment of chronic AF.

Bmax for RyR, expression levels of RyR mRNA and Ca²⁺-ATPase mRNA and their correlation with clinical hemodynamic parameters in chronic AF. The expression levels of RyR mRNA and Ca²⁺-ATPase mRNA in the left atrium of chronic AF patients showed no significant correlation with PCWP or mPAP. Moreover, no correlation was found between RAP and the Bmax for RyR or the expression levels of RyR mRNA and Ca²⁺-ATPase mRNA in the right atrium of patients with AF. In patients with chronic AF, these gene expression changes would not improve even if normal cardiac hemodynamics could be maintained with the aid of medication.

In addition, these results suggest that the sustained decreases in the levels of gene expression were related to the AF itself (rather than being mediated by the hemodynamic deterioration). Our data is consistent with the very recent study by Lai *et al.* (30), which reported that a downregulation of genes for the L-type Ca²⁺ channel and SR Ca²⁺-ATPase is a consequence of AF. However, in their study,

the level of the mRNA for RyR showed no significant change in AF patients. We believe this inconsistency may be related to the duration of AF and/or the different levels of hemodynamic parameters. We examined patients in whom chronic AF had persisted for more than six months, while Lai *et al.* studied patients with chronic AF lasting for more than three months. Moreover, the RAP, PCWP, and mPAP in our patients with AF due to MVD were significantly higher than the corresponding values in patients with NSR. Interestingly, the Bmax for RyR in the left atrium in chronic AF was smaller at higher levels of PCWP. This result suggests that despite a markedly decreased Bmax for RyR in the left atrium in chronic AF, the compensation mechanism responding to changes in pressure may be preserved at the level of protein expression, at least to some extent.

We tried to investigate the possible correlation between left atrial diameter (LAd) and the Bmax for RyR and the expression levels of RyR mRNA and Ca²⁺-ATPase mRNA in the left atrium of patients with AF. In a report by Henry *et al.* (31), the rate of AF was 3% when LAd was <4.0 cm, but it increased to 54% when LAd was >4.0 cm. This suggests that there might be an LAd threshold for the initiation and maintenance of AF. In our study, the values of LAd for all patients with AF were >4.0 cm (Table 2). This may be why we could find no correlation between the expression level of the mRNAs for Ca²⁺ regulatory proteins and LAd (data not shown).

Alterations in SR Ca²⁺ regulatory proteins and their possible relation to the mechanisms causing chronic AF.

Some reports have described animal models that clearly show the phenomenon of AF generating (or "begetting") further AF (23,24). In contrast to the baseline state, in which there was prompt and spontaneous termination of induced AF, animals subjected to chronic, rapid atrial rates developed persistent AF. The mechanism underlying this increase in AF duration appears to involve a progressive decrease in atrial refractoriness, a phenomenon that has been termed "electrical remodeling" (3). In addition to the decreased refractoriness, factors such as atrial dilation, depressed conduction, and increased heterogeneity either in intra-atrial conduction or in the recovery of excitability are all considered to be crucially important mechanisms leading to the establishment of chronic AF (24). Although such electrical remodeling is assumed to be important in the self-perpetuation of AF, the mechanism underlying this phenomenon is unknown. In the myocardium, the inward Ca²⁺ current is thought to be produced by Ca²⁺ released from the SR (via Ca²⁺-induced Ca²⁺ release), which then interacts with sarcolemmal binding sites (32). In fact, increases in intracellular Ca²⁺ concentration have been shown to produce a negative feedback effect on L-type Ca²⁺ channel activity, with a consequent decrease in the plateau phase of the action potential (10). This being so, the inward Ca²⁺ current might be controlled in a complex way by a

variety of factors, including the L-type Ca²⁺ current-trigger for Ca²⁺ release, the Ca²⁺ load in the SR, SR function, and the physical proximity between elements of the system (L-type Ca²⁺ channels, RyRs, Ca²⁺-ATPase, and so forth).

Of the above factors, the one we should like to focus on at present is the SR Ca²⁺ regulatory proteins. Interestingly, several *in vitro* studies of ventricular fibrillation have shown intracellular Ca²⁺ overload (4-8). As mentioned above, we have previously reported a decrease in SR Ca²⁺ release and uptake functions, as well as a decrease in the number of RyR, and an abnormal Ca²⁺ handling in the myocardium during the development of volume-overloaded heart failure (28). With regard to the establishment of AF, the following is a possible scenario. The decreased expression level of Ca²⁺-ATPase mRNA in patients with MVD shown in our study could cause a reduced re-uptake of intracellular Ca²⁺, and thus result in intracellular Ca²⁺ overload in the atrium.

In addition to the decreased expression level of Ca²⁺-ATPase mRNA, a decrease in RyR density and a decrease in the expression level of RyR mRNA could both contribute to a failure of Ca²⁺ homeostasis. Consequent abnormalities in the SR Ca²⁺ regulatory proteins may cause a further intracellular Ca²⁺ abnormality leading to additional damage to the SR Ca²⁺ regulatory proteins. Such self-perpetuating changes could, in part, contribute to the maintenance of AF as AF begets further AF. However, although this seems an attractive idea to us, direct evidence of changes in Ca²⁺ handling during the initiation or maintenance of AF is still lacking, and further studies will be needed to investigate the actual changes occurring in intracellular Ca²⁺ transients in atrial myocytes during the development of chronic AF.

Study limitations. In our study, we used only the right, not the left, atrium of patients with NSR as a control. We could not obtain left atrial tissue from surgical patients except for those undergoing a left atrial plication procedure for a giant left atrium. In those patients with NSR used as controls, the hemodynamic data (obtained by reviewing the results of preoperative cardiac catheterization and echocardiography) was normal, and we decided that there was no abnormal overload in either atrium and considered them to be under the same conditions. Moreover, because of the small number of MVD patients with NSR, we could not compare data from MVD patients with AF and MVD patients with NSR. However, we could determine the B_{max} for RyR and the expression levels of RyR mRNA and Ca²⁺-ATPase mRNA in both atria in the one MVD patient with NSR (Table 1, patient no. 9). Interestingly, in this patient the B_{max} for RyR and the expression levels of RyR mRNA and Ca²⁺-ATPase mRNA in both atria were larger than the mean values for those MVD patients with chronic AF (data not shown). This patient had normal hemodynamic data except for a slightly enlarged left atrium (diameter was 43 mm). This result suggests that markedly increased chamber size and intra-atrial pressure may be important factors for the initiation of chronic AF.

Because the amount of atrial tissue obtained was small, we used crude membrane preparations for the RyR binding assay, as in a previous study using small samples (33). Although, in a study of the present type, the question of tissue normalization is an important point, we could not accurately assess at the SR vesicle content and we could not carry out a histopathological study on identical samples.

We have speculated that abnormalities in SR Ca²⁺ regulatory proteins may contribute to a sustaining of intracellular Ca²⁺ overload; however, we did not directly measure intracellular Ca²⁺ transients. Clearly, an examination of Ca²⁺ transients and SR Ca²⁺-release and -uptake functions in human atrial myocytes from patients with chronic AF would be of great interest.

Conclusions. Recently, several investigators have reported electrophysiological abnormalities of the sarcolemma and discussed intracellular Ca²⁺ overload as a possible cause of the initiation and/or maintenance of AF. However, there is a lack of direct evidence of either abnormal intracellular Ca²⁺ handling or abnormalities in the modulators of the intracellular Ca²⁺ concentration. In this study, we report direct evidence of abnormalities in SR Ca²⁺ regulatory proteins in the atrium of patients with chronic AF, and we suggest that these changes might be a cause and/or result of AF, perhaps forming part of a self-perpetuating process by which AF begets further AF, leading to the establishment of a chronic condition.

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