Alterations in Potassium Channel Gene Expression in Atria of Patients With Persistent and Paroxysmal Atrial Fibrillation: Differential Regulation of Protein and mRNA Levels for K^+ Channels

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OBJECTIVES Our purpose was to determine whether patients with persistent atrial fibrillation (AF) and patients with paroxysmal AF show alterations in potassium channel expression.

BACKGROUND Persistent AF is associated with a sustained shortening of the atrial action potential duration and atrial refractory period. Underlying molecular changes have not been studied in humans. We investigated whether a changed gene expression of specific potassium channels is associated with these changes in patients with persistent AF and in patients with paroxysmal AF.

METHODS Right atrial appendages were obtained from 8 patients with paroxysmal AF, 10 with persistent AF and 18 matched controls in sinus rhythm. All controls underwent coronary artery bypass surgery, whereas most AF patients underwent Cox's MAZE surgery (atrial arrhythmia surgery to cure AF) (n = 12). All patients had normal left ventricular function. mRNA (ribonucleic acid) levels were measured by semiquantitative polymerase chain reaction and protein content by Western blotting.

RESULTS mRNA levels of transient outward channel (Kv4.3), acetylcholine-dependent potassium channel (Kir3.4) and ATP-dependent potassium channel (Kir6.2) were reduced in patients with persistent AF (−35%, −47% and −36%, respectively, $p < 0.05$), whereas only Kv4.3 mRNA level was reduced in patients with paroxysmal AF (−29%, $p = 0.03$). No changes were found for Kv1.5 and HERG mRNA levels in either group. Protein levels of Kv4.3, Kv1.5 and Kir3.1 were reduced both in patients with persistent AF (−39%, −84% and −47%, respectively, $p < 0.05$) and in those with paroxysmal AF (−57%, −64%, and −40%, respectively, $p < 0.05$).

CONCLUSIONS Persistent AF is accompanied by reductions in mRNA and protein levels of several potassium channels. In patients with paroxysmal AF these reductions were observed predominantly at the protein level and not at the mRNA level, suggesting a post-transcriptional regulation. (J Am Coll Cardiol 2001;37:926–32) © 2001 by the American College of Cardiology

Atrial fibrillation (AF) is a common cardiac arrhythmia affecting millions of people worldwide (1). Atrial fibrillation has the tendency to become more persistent and increasingly difficult to treat over time. During recent years, experimental studies showed that shortening of the atrial effective refractory period was one important factor contributing to the persistence of AF (2,3). This shortening has been confirmed in patients suffering from AF and atrial flutter (4,5). Experimental and human data revealed that AF- or tachycardia-induced shortening of atrial effective refractory period and action potential duration were associated with a

reduction of I_{CaL}, I_{To1} and I_{Na} currents because of reduced mRNA (ribonucleic acid) expression of these channels (6–9). Previously we have demonstrated that mRNA and protein expression of the L-type calcium channel in patients with persistent AF and more severe underlying heart disease (10) and in the present patient population (11) were significantly reduced. No alterations, however, were observed in patients with either paroxysmal or short-term persistent AF.

Theoretically, action potential duration and atrial effective refractory period can be shortened by 1) an increase in K^+ channel gene expression and activity, 2) a decrease in L-type Ca^{2+} channel (L-type Ca^{2+}) gene expression and activity or 3) a combination of both. The present study was undertaken to evaluate the impact of both persistent AF and paroxysmal AF on gene expression of potassium channels in human right atrial appendages. Therefore, the mRNA and protein expression of Kv4.3 (gene underlying the calcium independent transient outward current I_{To1}) (12), HERG (gene encoding the rapid component of the delayed recti-

Abbreviations and Acronyms

AF	=	atrial fibrillation
CABG	=	coronary artery bypass surgery
DNA	=	deoxyribonucleic acid
GAPDH	=	glyceraldehyde-3-phosphate dehydrogenase
HERG	=	gene encoding rapid component of the delayed rectifier I_{Kr}
Kir3.1	=	gene encoding part of the I_{KACh}, together with $I_{Kir3.4}$
Kir3.4	=	gene encoding part of the I_{KACh}, together with $I_{Kir3.1}$
Kir6.2	=	gene encoding part of the I_{KATP}
Kv1.5	=	gene encoding ultra rapid component of the delayed rectifier I_{Kur}
Kv4.3	=	gene underlying calcium independent transient outward current I_{To1}
LV	=	left ventricular, left ventricle
NYHA	=	New York Heart Association
PCR	=	polymerase chain reaction
RNA	=	ribonucleic acid
SR	=	sinus rhythm

fier) (13), Kv1.5 (gene encoding the ultra-rapid delayed rectifier, I_{Kur}) (14,15), Kir3.1/Kir3.4 (heterotetrameric complex of these two genes forms the acetylcholine dependent K^+ current, I_{KACh}) (16) and Kir6.2 (gene encoding the inward rectifier K^+ current, forming I_{KATP} with the sulfonylurea receptor) (17) were examined in patients with persistent AF and in those with paroxysmal AF who were undergoing cardiac surgery. Patients with lone AF (AF without underlying disease) or patients with AF scheduled for coronary artery bypass surgery (CABG) were matched with patients in sinus rhythm without history of AF and undergoing CABG.

MATERIALS AND METHODS

Patient selection and atrial tissue collecting. The day before surgery, one investigator (AET) assessed the clinical characteristics of the patient. Patients' history and previous electrocardiograms were used to establish type and duration of AF. In addition, medication use and exercise tolerance (according to the New York Heart Association [NYHA]

classification) were determined. Echocardiography data were obtained within three months before surgery. Right atrial appendages were obtained from 10 patients with persistent AF and from 8 patients with paroxysmal AF. All patients were euthyroid. The AF patients were matched for age, gender and degree of heart failure with 18 clinically stable patients in sinus rhythm undergoing CABG. The Institutional Review Board approved the study, and all patients gave written informed consent. Immediately after excision, the right atrial appendages were snap-frozen in liquid nitrogen and stored at $-85°C$.

RNA isolation and cDNA synthesis. Total ribonucleic acid was isolated and processed as described previously (11). Briefly, first strand cDNA (deoxyribose nucleic acid) was synthesized by incubation of 1 μg of total RNA in reverse transcription 10× buffer, 200 ng of random hexamers with 200 units of Moloney murine leukemia virus reverse transcriptase, 1mM of each dNTP and 1 unit of RNase inhibitor (Promega, The Netherlands) in 20 μl. Synthesis reaction was performed for 10 min at 20°C, 20 min at 42°C, 5 min at 99°C and 5 min at 4°C. All the products were checked for contaminating DNA.

Semiquantitative polymerase chain reaction (PCR) analyses. We have previously described and validated these methods (11). In short, the cDNA of interest and the cDNA of the ubiquitously expressed housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were coamplified in a single PCR. Primers (Eurogentec, Seraing, Belgium) were designed for Kv4.3, HERG, Kv1.5, Kir3.4, Kir6.2 and the housekeeping gene GAPDH (Table 1).

The PCR products were separated on agarose gel by electrophoresis and stained with ethidium bromide. The density of the PCR products was quantified by densitometry. Linearity for the PCR was established by making a correlation between the number of cycles and the density of gene of interest and GAPDH (data not shown).

Protein preparation and Western blotting. Frozen right atrial appendages of five patients in sinus rhythm, five patients with paroxysmal AF and five patients with persistent AF were homogenized in RIPA buffer as previously

Table 1. The Sequence for the Primers

Protein	Sequence	Cycles	Annealing Temp. (°C)
GAPDH	F 5′-CCC ATC ACC ATC TTC CAG GAG CG-3′, R 5′-GGC AGG GAT GAT GTT CTG GAG AGC C-3′.	Var.	Var.
To1 Kv4.3	F 5′-CAG CAA GTT CAC AAG CAT CC-3′ R 5′-AGC TGG CAG GTT AGA ATT GG-3′.	31	52
Kr HERG	F 5′-GTC AAT GCC AAC GAG GAG GT-3′, R 5′-CTG GTG GAA GCG GAT GAA CT-3′	27	58
Kur Kv1.5	F 5′-AAC GAG TCC CAG CGC CAG GT-3′ R 5′-AGG CGG ATG ACT CGG AGG AT-3′	32	64
KACh Kir3.4	F 5′-CAC CCT GGT GGA CCT CAA GTG GCG C-3′ R 5′-AGC TCC GGG CTT GGC AGG TCA TGC-3′	30	56
KATP Kir6.2	F 5′-CAC GCT GGT GGA CCT CAA GT-3′ R 5′-CAC GAT GAG GCT CAG GAT GG-3′	29	59

Table 2. Baseline Characteristics of Patients With Paroxysmal AF, Persistent AF and Matched Control Patients in Sinus Rhythm of Both Groups at the Moment of Surgery

	PAF	SR (PAF)	CAF	SR (CAF)
	8	8	10	10
M/F (n)	6/2	6/2	6/4	6/4
Age	51 ± 7	56 ±11	63 ± 11	65 ±17
Duration of AF: median, range (months)			18 (8–64)	
Duration SR before surgery: median, range (days)	1.5 (0–30)			
Underlying heart disease (n)				
Coronary artery disease	2*	8	3*	10
Hypertension	1	1	3	2
Lone AF	6*	0	5*	0
Surgical procedure				
CABG	2*	8	4*	10
MAZE	6*	0	6*	0
New York Heart Association for exercise tolerance				
Class I	7	5	6	5
Class II	1	3	4	5
Medication				
Beta-blockers	1*	5	3	6
Calcium antagonist	0	3	3	3
Digitalis	0	1	5	3
ACE inhibitor	0	1	4	2

*p value < 0.05 compared with the control group. Values are presented as mean value ± SD or number of patients.

ACE = angiotensin converting enzyme; AF = atrial fibrillation; CABG = coronary artery bypass grafting; CAF = chronic persistent atrial fibrillation; M/F = male/female; PAF = paroxysmal atrial fibrillation; SR (CAF) = matched controls in sinus rhythm of patients with persistent AF; SR (PAF) = matched controls in sinus rhythm of patients with paroxysmal AF.

described (11). Protein concentration was determined according to the Bradford method (Sigma, Zwyndrecht, The Netherlands), with bovine albumin as a standard. Protein expression was determined by Western blot analysis and expressed as the ratio to levels of GAPDH. Therefore, denatured protein (10 μg) was separated by SDS-PAGE, transferred to nitrocellulose membranes (Stratagene, Amsterdam, The Netherlands) and incubated with primary antibodies against GAPDH (Affinity Reagents, Golden, Colorado), anti Kir3.1, anti Kv4.3 and anti Kv1.5 (Alomone Labs, Jerusalem, Israel). Anti-mouse IgG (Santa Cruz Biotechnology, Heerhugowaard, The Netherlands) was used as secondary antibody. Signals were detected by the ECL detection method (Amersham, Roosendaal, The Netherlands) and quantified by densitometry. The specificity of the band was tested by pre-incubation of the antibody with the antigen. The band densities were evaluated by densitometric scanning using a Snap Scan 600 (Agfa, Ryswyk, The Netherlands). There was a linear relation between protein amounts on the membrane and immunoreactive signals of Kir3.1, Kv4.3, Kv1.5 and GAPDH (data not shown).

Definitions

Persistent AF. "Persistent AF" is defined as the continuous presence of AF until the moment of cardiac surgery (i.e., at least two consecutive electrocardiograms of AF more than one week apart and without intercurrent sinus rhythm). Persistent AF has a nonspontaneously converting character. Previously, this type of AF was classified as "chronic AF" (18).

Paroxysmal AF. "Paroxysmal AF" typically occurs in episodes with a duration shorter than 24 h (but longer lasting paroxysms are not unusual) with intermittent sinus rhythm. Paroxysmal AF is either spontaneously converting or is terminated with intravenously administered anti-arrhythmic drugs. The presence of paroxysmal AF at the moment of cardiac surgery cannot be controlled (18).

Statistical Analysis

All PCR and SDS-PAGE procedures were performed in duplicate series, and mean values were used for statistical analysis. For determination of correlations the Spearman correlation test was used. One-way analysis of variance was used for all group comparisons. All p values are two-sided; a p value <0.05 was considered statistically significant. SPSS version 8.0 was used for all statistical evaluations.

RESULTS

Patients. Ten patients with persistent AF and eight patients with paroxysmal AF were included. These two groups were compared with two groups of controls in sinus rhythm, which were matched for gender, age and left ventricular (LV) function (Table 2). Six of the eight patients with paroxysmal AF suffered from intractable paroxysmal AF without any underlying heart disease and were scheduled for Cox's MAZE surgery. The median duration of sinus rhythm before surgery was 1.5 days. The median frequency of paroxysms was once a day, with a median duration of 3 h. Three patients with paroxysmal AF were in AF at the moment of surgery and harvesting of the right atrial appendage. Control right atrial appendages were obtained

Table 3. Comparison of mRNA and Protein Expression for Patients With Persistent and Paroxysmal AF With their Matched Controls in Sinus Rhythm

	mRNA Expression				Correlation mRNA and Protein
	SR1	CAF	SR2	PAF	
Kv4.3	1.12 ± 0.1	0.73 ± 0.09*	1.08 ± 0.11	0.77 ± 0.1†	0.75
Kv1.5	1.35 ± 0.12	1.28 ± 0.11	1.37 ± 0.14	1.56 ± 0.14	0.31
HERG	0.43 ± 0.04	0.42 ± 0.03	0.52 ± 0.04	0.62 ± 0.03	n.a.
Kir3.4/Kir3.1	1.9 ± 0.1	1.03 ± 0.1*	1.92 ± 0.11	1.59 ± 0.13	0.74*
Kir6.2	1.04 ± 0.07	0.67 ± 0.07*	1.1 ± 0.07	0.96 ± 0.07	n.a.

*$p < 0.005$; †$p < 0.05$.

AF = atrial fibrillation; CAF = chronic persistent atrial fibrillation; PAF = paroxysmal atrial fibrillation; SR = sinus rhythm.

from clinically stable patients in sinus rhythm who were scheduled for CABG. Although the AF groups and their controls in sinus rhythm differed with respect to the underlying heart disease, all had a normal LV function and were in functional NYHA class I or II for exercise tolerance. Also, atrial and LV dimensions were similar among groups (data not shown).

Alterations in mRNA levels in persistent and paroxysmal AF. Changes in transcription of the gene of interest were determined by comparison of gene-of-interest/GAPDH ratios between patients with persistent or paroxysmal AF and their matched controls in sinus rhythm. No differences in GAPDH densities were found between the groups (data not shown).

Patients with persistent AF showed significant reductions of mRNA contents for Kv4.3 (-35%, $p = 0.02$), Kir3.4 (-47%, $p = 0.0003$) and Kir6.2 (-36%, $p = 0.03$) (Table 3). Patients with paroxysmal AF showed only reduction of the Kv4.3 mRNA level (-29%, $p = 0.03$, Table 3). No differences in mRNA contents for Kv1.5 and HERG were found between patients with persistent AF and those with paroxysmal AF, compared with patients in sinus rhythm (Table 3). Although the group samples are small, the mRNA levels of Kv4.3, Kv1.5 and Kir3.4 in both patients with persistent AF and those with paroxysmal AF seemed not to be influenced by any drug (data not shown).

Alterations in protein levels in persistent and paroxysmal AF. From the total patient group there were five patients with persistent AF, five with paroxysmal AF and five patients in sinus rhythm with enough right atrial appendage tissue to isolate proteins. Changes in protein expression were studied for Kv4.3, Kv1.5 and Kir3.1 in relation to protein levels of GAPDH. The protein expression of Kv1.5/GAPDH and Kir3.1/GAPDH was markedly reduced in patients with persistent AF compared with patients in sinus rhythm (-84%, $p = 0.001$ and -47%, $p = 0.002$, respectively) and patients with paroxysmal AF (-64%, $p = 0.005$ and -40%, $p = 0.007$, respectively, Figs. 1B and C). Similar results were obtained for Kv4.3/GAPDH protein content (i.e., a reduction both in patients with persistent AF [-39%, $p = 0.04$] and in those with paroxysmal AF [-57%, $p = 0.001$, Fig. 1A]). A positive correlation could be demonstrated between mRNA levels and protein levels of Kv4.3 and Kir3.1, but not of Kv1.5, for

patients with paroxysmal AF, persistent AF and sinus rhythm (Table 3). Although the group samples are small, the protein ratio of Kv4.3, Kv1.5 and Kir3.1 seemed not to be influenced by any drug (data not shown).

Importantly, in patients with paroxysmal AF the mean protein expression of Kv1.5 appeared to be related to the duration of sinus rhythm after the last episode of AF. Patients in AF at the moment of surgery showed the lowest protein expression, comparable to patients with persistent AF. Patients in sinus rhythm at the moment of surgery showed the highest protein expression (Fig. 2).

DISCUSSION

Both experimental (2,3,19,20) and human (4,5,21,22) AF are accompanied by shortening of the action potential duration and effective refractory period. This shortening can be mediated by either an increase in K^+ channel gene products and/or activity, or a decrease in L-type Ca^{2+} channel gene products and/or activity. Previously, we demonstrated a reduced mRNA and protein expression of the L-type calcium channel in patients with long-standing AF but not in those with paroxysmal AF (10,11). The present study shows that in patients with long-standing *persistent* AF, the mRNA and protein expression of almost all investigated potassium channel genes were reduced. In *paroxysmal* AF patients, reduction in mRNA levels was confined to Kv4.3, whereas the investigated protein levels (Kv4.3, Kv1.5 and Kir3.1) were all decreased. Finally, there was a significant positive correlation between the duration of sinus rhythm after the last episode of paroxysmal AF and content of protein expression of Kv1.5, suggesting a protective effect of high protein contents or a normalization of protein content after a longer duration of sinus rhythm.

Differences in mRNA and protein expression. We determined mRNA and protein levels of genes encoding a number of potassium channels. Unfortunately, no antibodies against all the potassium channels have yet been generated. Therefore, we could study only protein expression of Kv4.3, Kv1.5 and Kir 3.1. Nevertheless, this study reports profound changes in protein expression in both persistent and paroxysmal AF. In contrast, reduction of mRNA contents seems almost an exclusive feature for persistent AF. The observed reduction in Kv4.3 mRNA expression
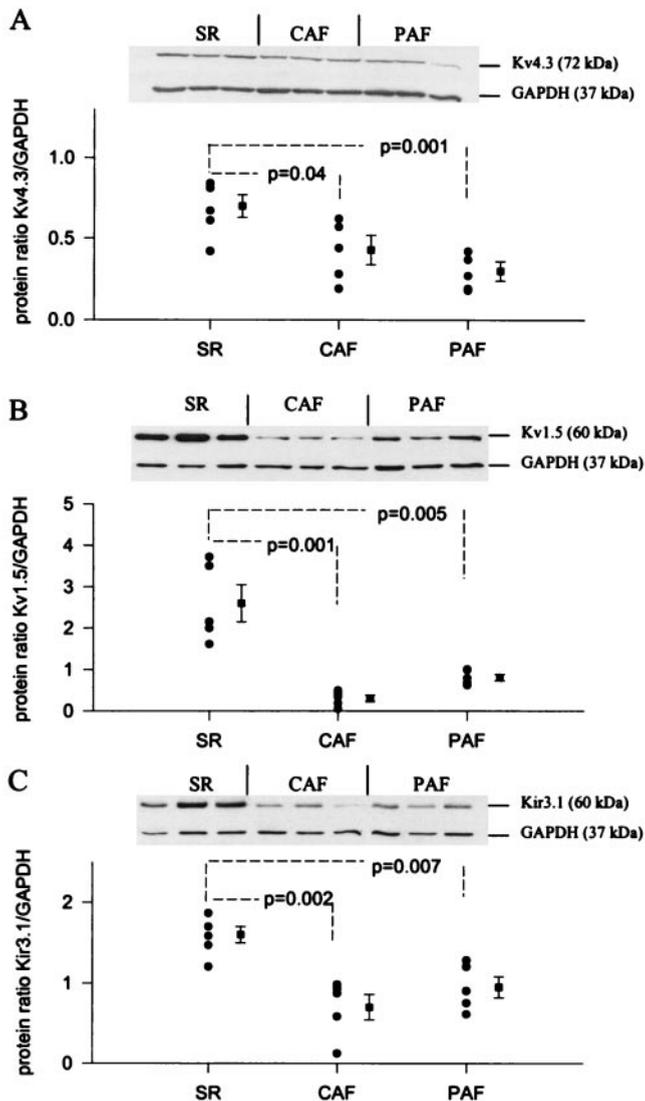
Figure 1. The top of each panel shows a typical Western blot analysis of 10 μg of protein homogenates of three patients in sinus rhythm (SR), three patients with persistent (chronic) atrial fibrillation (CAF) and three patients with paroxysmal AF (PAF). The immunoblots were done for anti-Kv4.3 (**A**), anti-Kv1.5 (**B**), and anti-Kir3.1 (**C**) with glyceraldehyde-3-phosphate dehydrogenase (37 kD) as an internal control. All data are presented as density units/density units. Values are mean ± SEM.

Figure 2. Correlation between the mean protein expression of Kv4.3 (**triangle**, r = 0.67, p > 0.05), Kv1.5 (**circle**, r = 0.97, p = 0.02) and Kir3.1 (**square**, r = 0.56, p > 0.05) in patients with paroxysmal atrial fibrillation and the duration of sinus rhythm before surgery.

(gene encoding the calcium independent transient outward current) both in patients with persistent AF and in those with paroxysmal AF is in accordance with experimental and human studies (3,8). In dogs subjected to rapid atrial pacing (400 beats/min), the transient outward current was reduced by 70% after six weeks (3), with a concomitant reduction of mRNA and protein expression (6).

In the heart, Kir3.1 and Kir3.4 gene products appear to be responsible for the acetylcholine-activated K$^+$ current (16), representing an important atrial inwardly rectifying current. Activation of this channel (e.g., by vagal stimulation) shortens the action potential duration and refractory period. The Kir3.4 gene was used for mRNA expression determination, and a reduction in Kir3.4 mRNA expression was found in patients with persistent AF. For Western
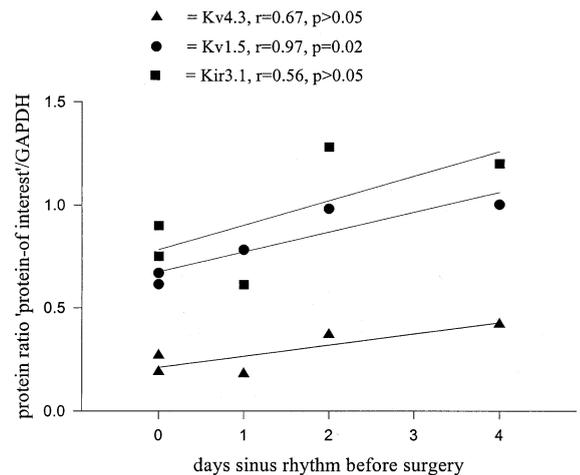
blotting Kir3.1 was analyzed. A reduction in protein level, which may occur to protect the cell against further shortening of the action potential duration during AF, was observed both in patients with persistent AF and in those with paroxysmal AF. The down-regulation observed in our study is, however, in contrast to findings by others on the electrophysiological level. In a comparable group of patients with persistent AF, an increase in inwardly rectifying currents (I_{K1} and I_{KACh}) was measured in isolated myocardial cells (23). This apparent inconsistency between protein level and current density can be explained only by assuming a change in single-channel properties—such as an increase of mean open time, an increase in channel conductance or a change in voltage dependency—in patients with persistent AF.

The reduction of Kir6.2 mRNA levels in patients with persistent AF may be related to depletion of ATP by an increase in metabolic demand during AF. This depletion of ATP could promote opening of Kir6.2, leading to enhanced repolarization (24) and subsequently increased expression of this channel (25). When activation of Kir6.2 continues, the myocyte may eventually respond by reducing the gene expression of this channel. There is still uncertainty whether atrial ischemia indeed plays a role in triggering electrical remodeling by AF. First, in a canine model White et al. (26) demonstrated that induced AF immediately caused an increase in coronary atrial perfusion and oxygen consumption of atrial myocardium, but without induction of ischemia. On the other hand, a progressive increase in metabolic demand during persistent AF may lead to repeated episodes of atrial ischemia, contributing to activation of the ATP-dependent potassium channel. The latter is suggested by results of Ausma et al. (27), who demonstrated similarities between cellular structural changes induced by AF and those seen in hibernating myocardium.

The observed reduction in protein expression of Kv1.5 in patients with persistent AF and in those with paroxysmal

AF could be due to post-transcriptional changes because, at the mRNA level, no changes were found between the groups. The reduction in protein expression is in agreement with the previous data of Van Wagoner et al. (8) in patients with persistent AF. However, in a canine model of the group of Nattel (3), no changes could be found in the current density of I_{Kur}. It should be pointed out that the molecular species underlying canine I_{Kur}, probably Kv3.1, is to be likely different from that underlying human atrial I_{Kur}, Kv1.5 (28).

No changes in mRNA expression were found for the HERG gene, the gene encoding the rapid component of the delayed rectifier. This is in accordance with data of Yue et al. (3) and suggests that the HERG gene is less involved in repolarization at the atrial level during AF.

Finally, we observed a positive correlation between the duration of sinus rhythm before surgery and the protein levels of Kv1.5 in patients with paroxysmal AF; patients in AF at the moment of surgery had lower protein levels compared with patients in sinus rhythm. This finding may suggest that alterations in protein expression, and possibly also structural changes, occur early (most paroxysms lasted <24 h) and could be reversible.

Underlying mechanisms. The observed reduction in gene expression of three potassium currents clearly cannot explain the observed shortening of effective refractory period and action potential duration. One may hypothesize that a reduction in the potassium channels' gene expression is an adaptation mechanism that serves to prolong the initially reduced atrial effective refractory period and action potential duration.

The observed discrepancy between alterations in mRNA and protein expression in patients with paroxysmal AF may suggest the influence of a different compensatory mechanism. We hypothesize that reduction in protein channels occurs because of calcium overload (20,29) and structural changes, including atrophy (27,30), in atrial tissue during AF by an increased expression of proteolytic enzymes (31). An increased expression of the proteolytic system is observed in heart tissue during atrophy, calcium overload and stunning (32–36). Increased protein degradation in muscle atrophy and calcium overload seemed predominantly induced by activation of a non-lysosomal ATP-dependent proteolytic process. Medina et al. (31) showed that the ubiquitin proteasome-dependent pathway, a highly conserved pathway consisting of ubiquitin, ubiquitin-conjugating enzymes, deubiquitinases and proteasome, is activated in atrophying muscles of the heart during starvation. Another common cytosolic proteinase-regulating pathway in eukaryotes is the calcium-dependent pathway, which consists of a diverse group of calcium-dependent cysteine proteinases (calpains in vertebrate tissues) (37). The increase in cytosolic calcium (29,38) during AF could be an important activator of this calcium-dependent pathway by promoting activation of neutral proteases such as calpains, which once accomplished, leads to proteolysis of numerous

cytoskeletal, membrane-associated and regulatory proteins (32–35) and, in turn, leads to degeneration of the myocardial cell.

Study limitations. Drugs and differences in underlying diseases may influence gene expression of ion channels. In this study, to minimize the influence of particular clinical parameters on gene expression, we included only patients with normal LV function, and when possible, drugs were discontinued before surgery.

Because of the limited amount of tissue available, no matched controlled analysis could be performed for determination of protein levels. However, no significant changes in mRNA levels between the various control groups of patients in sinus rhythm were observed. Therefore, in our opinion, a comparison among persistent AF, paroxysmal AF and sinus rhythm patients seems to be justified.

The paroxysmal AF patients included in this study represent patients who were difficult to treat and who underwent predominantly MAZE surgery. Furthermore, it should be noted that in all groups the number of patients was small. Therefore, the present data cannot be extrapolated uncritically to all (paroxysmal) AF patients.

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