A Novel Fluorescence Method for the Rapid Detection of Functional β_1-Adrenergic Receptor Autoantibodies in Heart Failure

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Objectives   This study sought to develop a rapid method for the detection of activating autoantibodies directed against the β_1-adrenoceptor (anti-β_1-Abs) in patients with heart failure.

Background   The anti-β_1-Abs are supposed to play a pathophysiological role in heart failure. However, there is no reliable method for their detection. With a complex screening strategy (enzyme-linked immunosorbent assay, immuno-fluorescence, cyclic adenosine monophosphate [cAMP]–radioimmunoassay) we have previously identified antibodies targeting the second extracellular β_1-receptor loop (anti-β_1-EC$_{II}$) in 13% of patients with ischemic cardio-myopathy (ICM) and in 26% with dilated cardiomyopathy (DCM).

Methods   To detect anti-β_1-Abs, we measured β_1-receptor–mediated increases in intracellular cAMP by fluorescence reso-nance energy transfer using a highly sensitive cAMP sensor (Epac1-based fluorescent cAMP sensor).

Results   The immunoglobulin G (IgG) prepared from 77 previously antibody-typed patients (22 ICM/55 DCM) and 50 matched control patients was analyzed. The IgG from all 22 previously anti-β_1-EC$_{II}$–positive patients (5 ICM/17 DCM) induced a marked cAMP increase, indicating receptor activation (49.8 $\pm$ 4.2% of maximal isoproterenol-induced signal). The IgG from control patients and 32 previously anti-β_1-EC$_{II}$–negative patients (17 ICM/15 DCM) did not significantly affect cAMP. Surprisingly, our technology detected anti-β_1-Abs in 23 DCM patients formerly judged antibody-negative, but their cAMP signals were generally lower (31.3 $\pm$ 6.8%) than in the previous group. "Low"-activator anti-β_1-Abs were blocked preferentially by peptides corresponding to the first, and "high"-activator anti-β_1-Abs by peptides correspond-ing to the second β_1-extracellular loop. Beta-blockers alone failed to fully prevent anti-β_1-EC$_{II}$–induced receptor activa-tion, which could be achieved, however, by the addition of β_1-EC$_{II}$ peptides.

Conclusions   Our novel method of detecting anti-β_1-Abs proved to be fast and highly sensitive. It also revealed an insuffi-cient ability of beta-blockers to prevent anti-β_1-EC$_{II}$–induced receptor activation, which opens new venues for the research on anti-β_1-Abs and eventual treatment options in heart failure.   (J Am Coll Cardiol 2007; 50:423–31) © 2007 by the American College of Cardiology Foundation

Over the past 2 decades, evidence has accumulated from both animal-based and patient-based studies that functionally active autoantibodies targeting the human β_1-adrenergic receptor (anti-β_1-Abs) may play an important role in the development and clinical course of progressive cardiac dilatation and failure (1,2). The anti-β_1-Abs were initially described in up to 50% of patients with Chagas' cardiomyopathy (3,4). Later, they also were detected in patients with myocarditis (5), and in patients with chronic heart failure caused by ischemic cardiomyopathy (ICM) or dilated cardiomyopathy (DCM) (5–7). In DCM patients, anti-β_1-Abs positivity has been linked to a more depressed left ventricular function (6), a higher prevalence of serious ventricular arrhythmias (8,9), and a higher incidence of sudden cardiac death (9). The definition of antibody positivity, however, depended on highly divergent screening methods used in these studies (i.e., enzyme-linked immunosorbent assay [ELISA] with receptor peptides, Western blotting of heart tissues, functional assays with neonatal rat cardiomyocytes, or detection by surface plasmon resonance [6,7,9–13]). Until now, this issue has not been solved satisfactorily.

**Abbreviations
and Acronyms**

anti-β_1-Abs = activating
autoantibodies against the
β_1-adrenergic receptor

β_1-AR = β_1-adrenergic
receptor/β_1-adrenoceptor

β_1-EC$_I$ = the first
extracellular loop of the
β_1-adrenergic receptor

β_1-EC$_{II}$ = the second
extracellular loop of the
β_1-adrenergic receptor

cAMP = cyclic adenosine
monophosphate

CFP = cyan fluorescent
protein

DCM = dilated
cardiomyopathy

Epac1-camps = Epac1-
based fluorescent cAMP
sensor

FRET = fluorescence
resonance energy transfer

GST = glutathione-S-
transferase

ICM = ischemic
cardiomyopathy

iso$_{max}$ = maximal signal
induced by isoproterenol

PKA = protein kinase A

YFP = yellow fluorescent
protein

Previously, using a 3-step screening procedure composed of an ELISA with synthetic receptor peptides as well as immuno-fluorescence and cyclic adenosine monophosphate (cAMP) responses in cells expressing native human β-adrenergic receptors (β-ARs), have screened 104 heart failure patients and 108 matched healthy control patients for functionally active anti-β_1-Abs. According to this screening procedure, the prevalence of stimulating anti-β_1-Abs was <1% in healthy patients, about 10% in ICM patients, and 26% in DCM patients (6). Interestingly, the prospective follow-up of these patients over more than 10 years showed an almost 3-fold increased cardiovascular and all-cause mortality risk in anti-β_1-Abs–positive compared with antibody–negative DCM patients (14). By contrast, no stimulating anti-β_1-Abs were detected in patients with hypertensive or valvular heart disease (15).

In our studies, the definition of functionally active anti-β_1-Abs was based mainly on their effects on receptor-mediated signaling (i.e., their effects on cellular cAMP levels and on the activity of the cAMP-dependent protein kinase [PKA]). Cyclic AMP is a universal second messenger of many G protein–coupled receptors, including the β-adrenergic receptor family. It exerts its effects via PKA, cAMP-gated ion channels, phosphodiesterases, and exchange proteins directly activated by cAMP (Epac1 and 2) (16). In recent years, several fluorescence methods have been developed to measure cAMP in intact cells. Fluorescence resonance energy transfer (FRET) between green fluorescent protein variants fused to the regulatory and catalytic subunits of PKA has been described to study the spatio-temporal dynamics of cAMP in neurons (17) or cardiac myocytes (18). More recently, we have reported the development of novel single-chain fluorescence indicators by fusing enhanced cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) directly to the cAMP binding domain of Epac proteins, which allowed achievement of a higher sensitivity and better temporal resolution of the cAMP measurements (19,20). Here, we decided to take advantage of this novel tool to analyze the prevalence of functionally active anti-β_1-Abs in a cohort of previously antibody-typed patients with heart failure caused by DCM or ICM, and to

study the ability of β-blockers to inhibit anti-β_1-Abs–induced receptor activation.

Methods

cAMP measurements by Epac-FRET. To establish a novel cell-based method for the detection of functional anti-β_1-Abs, human embryonic kidney HEK293 cells stably expressing human β_1-AR (0.4 pmol/mg membrane protein) were transiently transfected with Epac1-based fluorescent cAMP sensor (Epac1-camps) using calcium phosphate precipitation. Twenty-four to 48 h after transfection, cAMP measurements were performed microscopically as described (19). The cells were maintained in buffer A (144 mmol/l NaCl, 5 mmol/l KCl, 2 mmol/l CaCl$_2$, 1 mmol/l MgCl$_2$, 20 mmol/l HEPES, pH = 7.3), supplemented with 50 nmol/l ICI 118551 (Sigma, Deisenhofen, Germany) to avoid cross-reactivity with the endogenous β_2-AR (approximately 0.1 pmol/mg according to binding assays, data not shown). The immunoglobulin G (IgG) preparations were diluted in the same buffer and added to the cells at 0.13 μg/μl protein concentration. (-)-Isoproterenol (Sigma) was used to determine the maximal cAMP response.

Blocking experiments. To determine relevant epitopes for the anti-β_1-Abs, we incubated them with peptides (0.5 μg/μl) derived from the human β_1-EC$_I$ (AGRWEYGS-FFCELQ) and β_1-EC$_{II}$ sequences (ARAESDEAR-RCYNDPKCCDFVTNRE) (21) for 6 h at 4° C. A peptide corresponding to the β_2-EC$_{II}$ sequence (ATHQEAIN-CYANETCCDFFTNRE) (22) served to control for specificity. Cyclic AMP signals were then measured by Epac-FRET and compared with those of unblocked IgG preparations. To inhibit receptor signaling by functional anti-β_1-Abs, we used the clinically relevant β-AR antagonists alprenolol, metoprolol (Sigma), bisoprolol (Merck, Darmstadt, Germany), and carvedilol (provided by Dr. S. Engelhardt).

Patients and samples. The IgG preparations available from 77 previously analyzed patients with heart failure caused by DCM (n = 55) or ICM (n = 22) (6,15) were included in the present study. Diagnosis of DCM had been based on a left ventricular end-diastolic volume >110 ml/m^2 and an ejection fraction <55% (determined by ventriculography) together with an exclusion of coronary heart disease (by angiography) (6). No exposure to cardio-toxic substances, myocarditis, or other systemic heart disease was evident from medical history in these patients. At the time of sample acquisition, all patients had been in stable hemodynamic condition and none of them had received β-blocking agents or sympathomimetic substances. Table 1 summarizes the clinical and hemodynamic data of the patients (DCM, n = 55; ICM, n = 22) whose IgG preparations were analyzed. Control subjects (n = 50) were selected from healthy blood donors, and matched for gender and age. All assays were carried out with IgG fractions isolated by caprylic acid precipitation and normalized to

Table 1	Clinical and Hemodynamic Data of Patients With DCM and ICM	
Parameter	DCM (n = 55)	ICM (n = 22)
Age, yrs	56 (51–65)	58 (54–65)
Female	8 (14.5)	3 (13.6)
New York Heart Association functional class, II/III/IV	14 (25)/23 (42)/18 (33)	8 (36)/10 (46)/4 (18)
Heart rate, beats/min	79 (71–92)	87 (78–95)
PVR, dyne $\times$ s/cm^5	151 (105–179)	140 (99–168)
SVR, dyne $\times$ s/cm^5	1,425 (1,185–1,726)	1,460 (1,224–1,628)
Ejection fraction, %	36 (28–44)	36 (23–49)
Cardiac index, l/min $\times$ m^2	2.61 (2.11–3.10)	2.55 (2.10–3.18)
Contractility, mm Hg/s	1,180 (980–1,420)	1,487 (1,110–1,837)
Relaxation, mm Hg/s	1,040 (880–1,240)	1,064 (902–1,328)

Data are median (interquartile range) or n (%).

DCM = dilated cardiomyopathy; ICM = ischemic cardiomyopathy; PVR = peripheral vascular resistance; SVR = systemic vascular resistance.

total IgG concentration (800 μg/ml), as described previously (6,15,23). For the assays, IgG prepared from anti-β_1-EC$_{II}$–positive Lewis/CrlBR rats with dilated immune-cardiomyopathy (generated by immunization against a β_1-EC$_{II}$/glutathione-S-transferase [GST] fusion protein), anti-β_1-EC$_I$ antibodies prepared from Lewis rats immunized with a β_1-EC$_I$/GST fusion protein, and IgG from corresponding isogenic anti-β_1-Abs–negative rats served as positive or negative controls, respectively (24). The study was approved by the Medical Ethics Committee of the University of Würzburg.

Statistics. Data are presented as median (interquartile range) or absolute numbers (percent), as appropriate. In figures, data are shown as mean (SEM). Continuous data were analyzed by 1-way analysis of variance (blocking experiments) and the Student t test. Statistical analysis was performed using SPSS 13.0.1 (SPSS Inc., Chicago, Illinois). After confirming normal distribution of the measured FRET signals (by applying the Kolmogorov-Smirnov test), Gaussian distribution curves per subgroup were generated using Origin 6.1 (OriginLab Corp., Northampton, Massachusetts). All p values are reported 2-sided. A p value <0.05 was considered statistically significant.

Results

Detection of functionally active anti-β_1-Abs with a fluorescent Epac-sensor. To determine the anti-β_1-Abs via receptor-induced cAMP accumulation in β_1-AR expressing HEK293 cells, we used a recently developed fluorescent cAMP sensor (Epac1-camps). The sensor detects intracellular cAMP with high spatial and temporal resolution by changes in FRET between CFP and YFP fused to the cAMP binding site of Epac1 (Fig. 1A). Compared with conventional radioimmunoassay (RIA) cAMP detection systems, the new FRET assay has an about 10-fold higher sensitivity, rendering this method suitable for the detection of small changes in intracellular cAMP levels (Fig. 1B). To test whether this technology can be used

to detect antibodies activating the β_1-AR, we used IgG fractions from anti-β_1-EC$_{II}$–positive Lewis/CrlBR rats with dilated immune cardiomyopathy and isogenic control
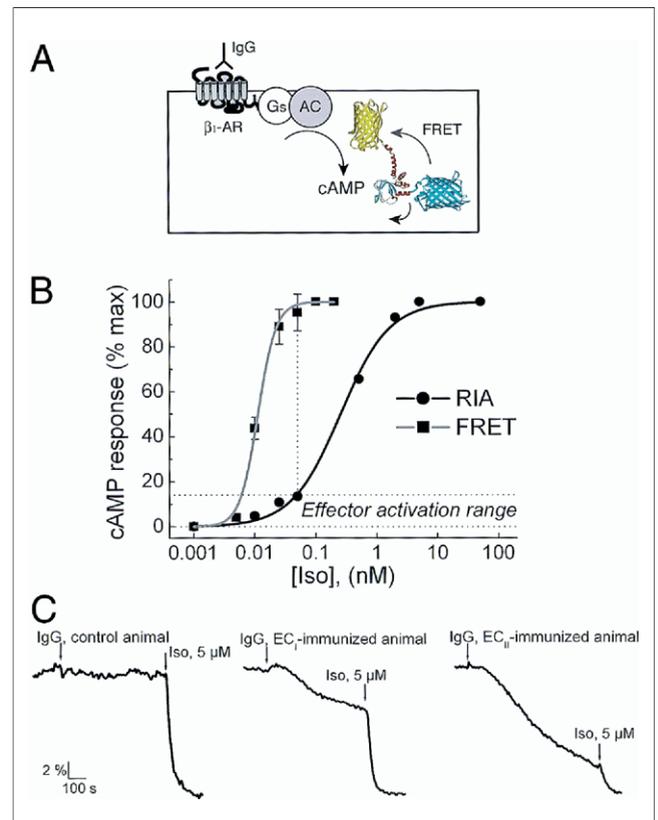

| Figure 1 | Detection of Functional Rat Anti-β_1-Abs by a Novel FRET-Based Assay |

Cell-based approach for the detection of functional activating autoantibodies against the β_1-adrenergic receptor (anti-β_1-Abs). **(A)** Receptor activation induced by binding of antibodies to its accessible extracellular loops leads to an increase in cyclic adenosine monophosphate (cAMP) through sequential activation of G$_s$ proteins and adenylyl cyclase (AC). The cAMP is detected by fluorescence resonance energy transfer (FRET) **(grey arrow)** between cyan fluorescent proteins (CFP) and yellow fluorescent proteins (YFP) fused to the cAMP-binding domain of Epac1. The sensor (Epac1-based fluorescent cAMP sensor [Epac1-camps]) changes its conformation upon cAMP binding **(black arrow)**, resulting in a decrease in FRET. **(B)** Measuring cAMP levels in isoproterenol (Iso)-stimulated human embryonic kidney HEK293 cells stably expressing human β_1-AR by conventional radioimmunoassay (RIA) (Amersham, Freiburg, Germany) or Epac-FRET. One of 3 independent RIA concentration-response curves (EC$_{50}$ = 0.53 $\pm$ 0.19 nmol/l) is shown. The cAMP range that can be monitored by Epac-FRET until it gets saturated is presented by 2 horizontal dotted lines. At 0.05 nmol/l isoproterenol, Epac1-camps becomes saturated, indicating an intracellular cAMP concentration of approximately 20 μmol/l (according to in vitro measurements in cell lysates with pure cAMP [19]). This extremely sensitive sensor is characterized by a high dynamic range at physiologically relevant cAMP concentrations 0.1 to 20 μmol/l (which are covered by only 15% of the maximal cAMP-RIA signal), making minor cAMP changes previously undetected by conventional assays well detectable by Epac-FRET. **(C)** The immunoglobulin (Ig)G prepared from rats immunized with glutathione-S-transferase (GST) fusion proteins containing the first (EC$_I$-immunized, **center**) or second extracellular loop of the human β_1-AR (EC$_{II}$-immunized, **right**) were assayed for activity using human embryonic kidney HEK293 cells stably expressing human β_1-AR transfected with Epac1-camps, and compared with nonimmunized animals (control animals, **left**) to assess the reliability of the method. Representative FRET ratio traces of 1 of 6 independent experiments are presented (% corresponds to the relative change in YFP/CFP intensity ratio). The decrease in FRET reflects an increase in intracellular cAMP.

rats (24). None of the IgG preparations from the control rats significantly affected cAMP levels in β_1-AR expressing HEK293 cells, whereas the IgG preparations from immunized rats containing functionally active anti-β_1-EC_{II} antibodies produced clear-cut signals and induced a rapid and pronounced loss in Epac-FRET (Fig. 1C, right). The FRET signal produced by these antibodies corresponded to about 75% of the maximal signal induced by isoproterenol (iso$_{max}$). Next, we tested the functional effects of rat antibodies generated by active immunization against the first β_1-EC loop (human) fused to bacterial GST. Interestingly, these rat anti-β_1-EC_I antibodies also stimulated cAMP production, but the FRET signals induced were significantly lower than those induced by rat anti-β_1-EC_{II}, yielding only approximately 40% of iso$_{max}$ (Fig. 1C, center). The onset of both the anti-β_1-EC_{II}–provoked and the anti-β_1-EC_I–provoked responses occurred with an initial delay of 100 to 150 s compatible with slow binding kinetics of the antibodies and the resultant conformational rearrangement of the receptor molecule. These experiments with anti-β_1-Abs generated in rats suggested that our novel method also might be suitable for the detection of anti-β_1-Abs in patients.

High prevalence of anti-β_1-Abs in DCM patients. We then analyzed IgG preparations that were available from previously antibody-typed DCM patients (n = 55) (6) and healthy control patients (n = 50) matched for gender and age. None of the IgG preparations from healthy control patients significantly altered Epac-FRET signals (Fig. 2A, left). In contrast, IgG preparations from all 17 DCM patients previously judged anti-β_1-Abs–positive induced a marked and rapid loss of FRET signals, indicating an increase in intracellular cAMP caused by β_1-AR activation (49.5 ± 3.9% of iso$_{max}$; Abs+) (Fig. 2A, right), similar to the responses obtained with activating rat anti-EC_{II}-Abs. Surprisingly, we detected anti-β_1-Abs also in 23 of 38 (60%) DCM patients formerly judged antibody–negative (Abs–) (Fig. 2A, center), but the cAMP signals induced by these antibodies were significantly lower than in the previous group (31.3 ± 6.8%, p < 0.001 [Student *t* test]) (Fig. 2A, center). At first sight, their effects on cAMP production seemed to be comparable with those of low-activating rat anti-β_1-Abs, generated against the human β_1-EC_I loop.

To perform a systematic analysis of the different (human) anti-β_1-Abs–induced cAMP responses, we plotted distribution histograms of the amplitude of the FRET signals for DCM patients and for healthy control patients, respectively. In the latter, we observed a single peak Gaussian distribution of the FRET signals ranging from −13.0% to +18.6% of iso$_{max}$, with a mean value of 5.0 ± 6.2%. Positivity was thus defined as a FRET signal above 2 SD of the mean value of healthy control patients (i.e., >17.4% [5.0% + 2 × 6.2%]). In contrast to the single peak Gaussian distribution of the healthy control patients, the histogram for DCM patients showed a multiple peak distribution pattern. Sub-
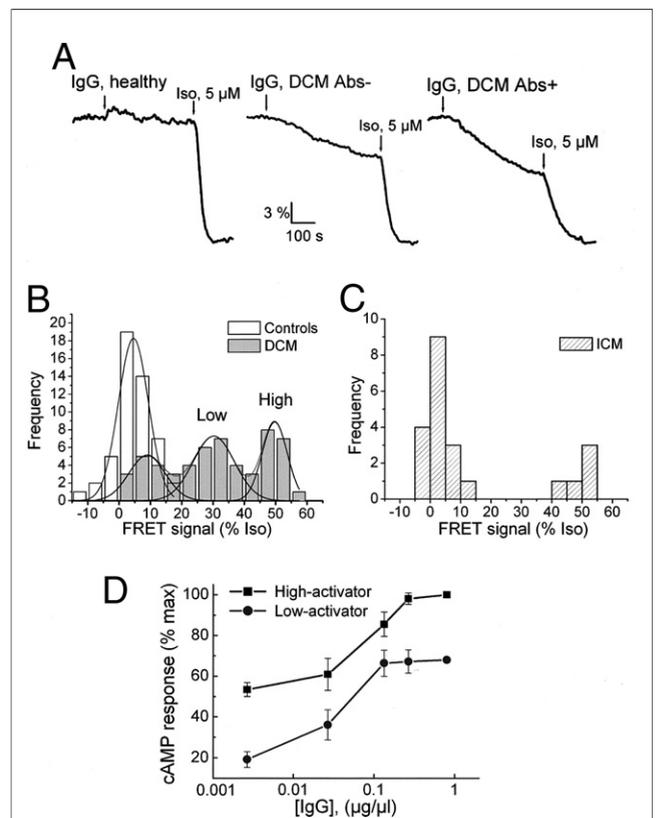

Figure 2   FRET Detection of Functional Anti-β_1-Abs in Patients

Measuring cAMP by Epac-FRET detects functional anti-β_1-Abs in dilated cardiomyopathy (DCM) patients. **(A)** None of the IgG prepared from healthy control patients (n = 50) induced a significant cAMP response in living cells **(left)**. The IgG from DCM patients previously judged anti-β_1-Abs-positive (15) (Abs+) elicited marked cAMP responses (49.5 ± 3.8% of maximal Iso signal, **right**). The IgG from 23 of 38 (60%) previously anti-β_1-Abs-negative judged patients (6) (Abs−) showed a robust but significantly smaller increase in cAMP (31.3 ± 6.8%, p < 0.01 according to the Student *t* test, center). Representative experiments of at least 3 different cells for each IgG preparation are shown. **(B)** Histogram with Gaussian distribution curves based on the strength of the FRET signal in healthy control patients versus DCM patients (R^2 = 0.98 for a 3-peak Gaussian distribution). The antibody induced FRET signals allow discrimination of 3 groups of activity: no activity (n = 15), low activity (n = 22), high activity (n = 18); grouping was performed as described in the text. **(C)** Frequency and distribution of FRET signals obtained with IgG preparations from n = 22 previously antibody-typed ischemic cardiomyopathy (ICM) patients. Five of them showed high FRET activity, and the remainder was classified antibody-negative. **(D)** Concentration-response relationship between high-activator and low-activator IgG showing significantly different activation capacities over a wide range of antibody concentrations (p < 0.05; Student *t* test). The percent ± SEM of maximal Iso-induced cAMP responses is presented (normalized to maximal changes in FRET induced by high-activator anti-β_1-Abs; representative experiments, n = 4). Abbreviations as in Figure 1.

sequent iteration of the respective FRET values assuming a multipeak Gaussian model produced the best fit for a 3-peak Gaussian distribution, yielding a R^2 = 0.98 (R^2 = 0.47 or 0.77 for the 1- or 2-peak Gaussian distribution, respectively). The 55 DCM patients tested were then grouped at the respective intersection points of the 3 calculated Gaussian curves (Fig. 2B). Fifteen of 55 (28%) patients showed FRET signals below the lower intersection

(cutoff) point of 18.2% FRET response and were thus grouped negative (Abs−); all those patients also had been correctly grouped Abs− using the former screening algorithm. The remaining n = 40 of 55 (72%) patients were grouped at the upper intersection (cutoff) point of 41.9% FRET response as low (n = 22 of 55, 40%) or high activators (n = 18 of 55, 33%), respectively (Fig. 2B). All (n = 17) previously identified anti-β_1-Abs–positive DCM patients and 1 patient unidentified with the former methodology were classified high activators, whereas all (n = 22) low activators (18.2% < FRET signal <41.9%) had been judged Abs− with the former screening algorithm.

In summary, the analysis of Epac-FRET signals from the first patient collective analyzed showed an anti-β_1-Abs prevalence of more than 70% (n = 40 of 55) in DCM, which is higher than obtained by the ELISA/immunofluorescence/cAMP-RIA screening procedure (6). It seems that the Abs+ group can be separated into 2 subgroups on the basis of the FRET data, which were classified as low activators (FRET amplitude 18.2% to 41.9% of iso$_{max}$) and high activators (FRET amplitude ≥42% of iso$_{max}$).

Next, we analyzed n = 22 still available IgG preparations from a previously antibody-typed ICM collective (6,15). Five previously antibody-positive judged patients had anti-β_1-Abs producing strong FRET signals in a range of those classified high activators (Fig. 2C). In contrast, IgG preparations from the other 17 ICM patients (previously judged antibody-negative) did not produce any significant FRET signals, indicating that low-activator anti-β_1-Abs probably are not a typical feature of ICM.

As a consequence, the 2 newly identified FRET-positive DCM populations were studied in more detail. Analyzing the concentration response relationship, we found that low-activator IgG, even at higher concentrations, did not induce cAMP levels of a similar order of magnitude as high-activator IgG (Fig. 2D). This finding renders lower titers of anti-β_1-Abs in sera of low activators an unlikely explanation for the lower cAMP response, but rather suggests a different mechanism of action of this type of anti-β_1-Abs at the receptor level.

High or low activating capacity defines anti-β_1-Abs against different epitopes. As suggested by the data obtained with our rat anti-β_1-Abs, one reason for qualitative differences between antibodies in terms of FRET activity might be differences in the receptor epitopes targeted by different anti-β_1-Abs. Previously, the occurrence of functional anti-β_1-Abs against β_1-EC$_{II}$ and β_1-EC$_{I}$, but not β_1-EC$_{III}$, has been reported in DCM patients (11). In addition, functional anti-β_1-Abs against the β_1-EC$_{II}$ and β_1-EC$_{I}$ epitopes have also been generated successfully in animals by immunization, suggesting a certain antigenicity of these 2 epitopes (25). To analyze whether different targeted epitopes might account for high or low FRET activating capacities of the anti-β_1-Abs detected in our DCM patients, we incubated them with synthetic peptides

corresponding to β_1-EC$_{II}$ or to β_1-EC$_{I}$ and analyzed the blocking effect of each of these peptides. These experiments showed that FRET signals of high-activator IgG could be blocked specifically by peptides corresponding to β_1-EC$_{II}$,
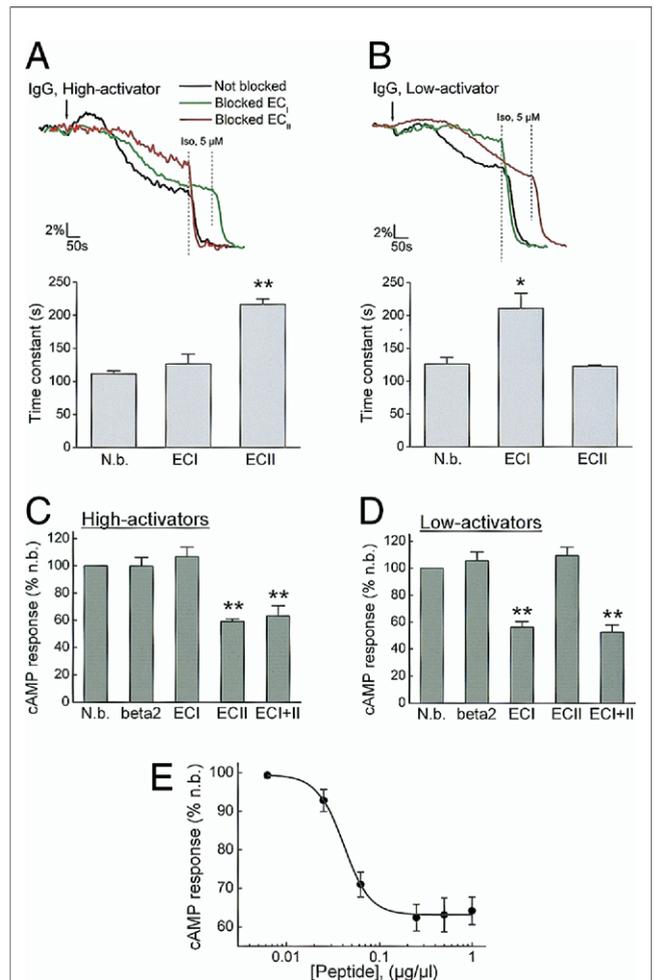

Figure 3 **Functional Anti-β_1-Abs Target Distinct Receptor Epitopes**

Blocking experiments for different classes of functional anti-β_1-Abs. **(A)** The cAMP generation induced by high-activator IgG is attenuated only by specific peptides derived from the second extracellular β_1-AR loop (β_1-EC$_{II}$). **(B)** Low-activator signals could be blocked in all instances by peptides corresponding to the first extracellular β_1-AR loop (β_1-EC$_{I}$) but not by β_1-EC$_{II}$ peptides (representative experiments from at least 3 cells per condition are shown). Analysis of the kinetics of the different FRET responses is presented in the lower panels of **(A)** and **(B)**. Ratio traces were fitted with a mono-exponential function, and the time constant of the decay of the respective FRET signals was determined. In both cases, the effects of EC$_{I}$ and EC$_{II}$ peptides differ significantly (*$p < 0.05$, **$p < 0.01$; one-way analysis of variance [ANOVA]). **(C)** Columns represent mean ± SEM **(error bars)** of cAMP responses induced by (n = 7) representative high-activator IgG, or **(D)** (n = 10) representative low-activator IgG prepared from DCM patients in the absence (n.b. = not blocked) or presence of peptides corresponding to the first (EC$_{I}$) and/or second extracellular β_1-AR loop (EC$_{II}$), or to EC$_{II}$ of the β_2-AR. Statistical significance was tested by one-way ANOVA (**$p < 0.01$ compared with the n.b. group). **(E)** Dose-response curve showing the effect of increasing concentrations of the β_1-EC$_{II}$ peptide on the inhibition of high-activator-induced cAMP responses (representative experiments, n = 5). The curve was fitted to the data using Origin 6.1 (OriginLab Corp., Northampton, Massachusetts) assuming a single binding site. Abbreviations as in Figures 1 and 2.

but not by peptides corresponding to β_1-EC$_I$ or by a control peptide derived from EC$_{II}$ of the β_2-AR (7 patients tested) (Figs. 3A and 3C). Concentration-response analysis of the β_1-EC$_{II}$ peptide showed that the peptide concentrations used in our experiments (0.5 $\mu g/\mu l$) were able to produce a maximal inhibitory effect (Fig. 3E). Although we could achieve a partial, about 50%, inhibition of high-activator IgG by β_1-EC$_{II}$ peptides alone, simultaneous blocking with EC$_I$ and EC$_{II}$ peptides did not achieve a further decrease in cAMP signals (Figs. 3C and 3D). In contrast, low-activator IgG was inhibited only by β_1-EC$_I$ peptides (10 patients tested) (Figs. 3B and 3D) and again, a combination of EC$_I$ and EC$_{II}$ peptides did not enhance inhibition (Figs. 3C and 3D). All sera from DCM patients with functional anti-β_1-Abs yielding cAMP signals near the cut-off value between no and low as well as low and high activators were studied in detail by such blocking experiments to ascertain the accuracy of our classification. The only patient previously judged Abs− with IgG eliciting strong Epac-FRET signals (45.4% FRET signal) was confirmed as having high-activator IgG autoantibodies blockable by β_1-EC$_{II}$ peptides. In addition, 2 patients with IgG yielding about 40% FRET response (39.9% and 40.1%, respectively; classified low activators according to the upper intersection point of 41.9%) were equally inhibited by the β_1-EC$_{II}$ peptide, whereas all the other low-activator IgG tested (n = 20 of 55, Epac-FRET responses between 20% to 39%) were inhibited only by the β_1-EC$_I$ peptide. These data are consistent with the hypothesis that the differences in cAMP production of high and low activators most likely reflect the different epitopes targeted and different active receptor conformations induced. However, to address the question of whether the differences in type, target domain, and activating capacity of anti-β_1-Abs might be prognostically (and thus clinically) relevant in the course of heart failure, a systematic screening of larger patient populations with the novel FRET method is needed.

β-blockers do not fully abolish high-activator-induced cAMP signals. Blockade of sympathetic hormone receptors by β-receptor antagonists has proven beneficial in heart failure (26). Thus, one possible strategy to combat functional anti-β_1-Abs in antibody-positive patients might be the pharmacological neutralization of autoantibody-mediated stimulatory effects (2). Therefore, we analyzed the effects of a panel of clinically relevant β-receptor antagonists on anti-β_1-Abs–mediated cAMP increases (i.e., antibody-induced Epac-FRET signals) in our transfected cell system. Both β_1-selective (bisoprolol, metoprolol) and nonselective (alprenolol, carvedilol) beta-blockers largely diminished the anti-β_1-EC$_{II}$–induced cAMP production. The effect of beta-blockers had a fast onset and led to a rapid reduction of cAMP levels in cells stimulated with anti-β_1-EC$_{II}$ antibodies (Figs. 4A and 4C). However, even at saturating concentrations, all β-receptor antagonists tested blocked the anti-β_1-EC$_{II}$–induced effects only partially, resulting in a 50% (alprenolol), 60% (bisoprolol), or up to
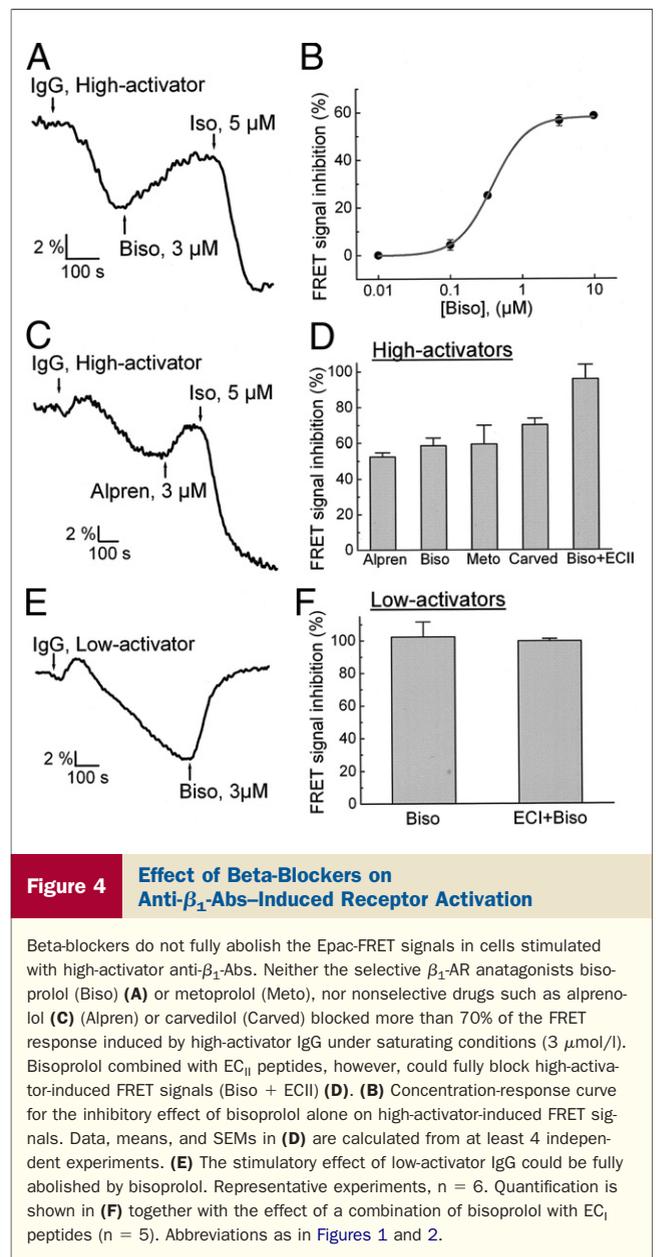

Figure 4

Effect of Beta-Blockers on Anti-β_1-Abs–Induced Receptor Activation

Beta-blockers do not fully abolish the Epac-FRET signals in cells stimulated with high-activator anti-β_1-Abs. Neither the selective β_1-AR antagonists bisoprolol (Biso) **(A)** or metoprolol (Meto), nor nonselective drugs such as alprenolol **(C)** (Alpren) or carvedilol (Carved) blocked more than 70% of the FRET response induced by high-activator IgG under saturating conditions (3 μmol/l). Bisoprolol combined with EC$_{II}$ peptides, however, could fully block high-activator-induced FRET signals (Biso + ECII) **(D)**. **(B)** Concentration-response curve for the inhibitory effect of bisoprolol alone on high-activator-induced FRET signals. Data, means, and SEMs in **(D)** are calculated from at least 4 independent experiments. **(E)** The stimulatory effect of low-activator IgG could be fully abolished by bisoprolol. Representative experiments, n = 6. Quantification is shown in **(F)** together with the effect of a combination of bisoprolol with EC$_I$ peptides (n = 5). Abbreviations as in Figures 1 and 2.

70% (carvedilol) reduction of the antibody-induced Epac-FRET signals (Figs. 4B and 4D). In contrast, bisoprolol was able to fully revert cAMP signals induced by low-activating anti-β_1-EC$_I$ antibodies (Figs. 4E and 4F). These data suggest that the clinically available beta-blocking agents can only partially shield from the harmful high-activator antibody-induced cAMP production in anti-β_1-EC$_{II}$–positive patients, illustrating the need for novel, more specifically antibody-directed therapeutic strategies in heart failure.

Interestingly, when bisoprolol was added to cells pretreated with EC$_{II}$ peptides, the stimulatory effect of high-activator anti-β_1-EC$_{II}$ antibodies on cAMP production was fully blocked (Fig. 4D), indicating a synergistic effect between receptor peptides and β-blockers. Such a combi-

nation strategy might, in the future, represent a clinically promising therapeutic approach.

Discussion

Detection method and mechanisms of anti-β_1-Abs–induced receptor activation. Here we established a novel FRET-based method for the detection of functional anti-β_1-Abs by measuring cAMP signals induced via β_1-AR in intact cells. This method seems to be highly specific and sensitive. The high sensitivity originates from 2 important biological amplification mechanisms. First, signals from receptors to second messengers are often considerably amplified, making cAMP a sensitive indicator of β_1-receptor activation. Second, the high sensitivity of Epac-camps for cAMP makes it particularly capable of detecting changes at lower cytosolic cAMP levels, while, like physiological cAMP effectors, it becomes saturated at higher levels of cAMP. Our novel single-step fluorescent method detected functionally active anti-β_1-Abs both in specifically immunized rats and in patients with heart failure. It confirmed earlier data on the presence of activating anti-β_1-Abs targeting the second extracellular β_1-receptor loop (anti-β_1-EC$_{II}$) in about one-third of the patients with DCM, and in a smaller fraction of patients with ICM (10% to 20%). Moreover, the novel method unveiled a further class of functional anti-β_1-Abs directed against the first extracellular β_1-receptor loop (anti-β_1-EC$_I$) in about another third (36%) of DCM patients undetected by previous screening methods.

It is not yet clear how antibodies reacting with the extracellular loops of the β_1-AR affect receptor activity. In the β_2-AR, amino acids in the transmembrane helices III, V, and VI have been assigned an anchoring function for agonists (27). The second extracellular loop of the receptor is predicted to form a β-hairpin, which partly dips down into the ligand binding site, and thus might influence receptor–ligand interactions to some extent (28). The conformation of this hairpin is thought to be stabilized by cysteines situated in EC$_I$ and EC$_{II}$. In the β_2-AR, it has been shown that reduction or mutation of one or several of these cysteines results indeed in a significant reduction of agonist and antagonist affinities (29). Thus, in β-AR correct folding of the EC$_I$ and EC$_{II}$ loops may be essential for correct formation of the ligand binding pocket and might explain why antibodies directed against these loops can interfere with ligand binding, modulate receptor conformation, and thereby also modulate receptor activity (2,30).

Diagnostic implications. Compared with the previously developed ELISA/immunofluorescence/cAMP-RIA procedure (6), the novel FRET-based method is simpler, less time consuming, and at least as precise (i.e., correctly identifying all patients previously considered anti-β_1-EC$_{II}$-positive). The new method is based on detecting antibody-induced increases in cAMP. This second messenger most likely accounts for the harmful effects provoked by activating

anti-β_1-Abs (1,31). In addition, by quantifying antibody-induced cAMP signals, the novel assay allows differentiation between functional anti-β_1-Abs classes (i.e., high and low activators), which are presumably characterized by their binding to distinct extracellular receptor domains. However, studies in larger patient populations will be required to confirm and elucidate further the relationship between epitopes recognized by autoantibodies (β_1-EC$_I$ or β_1-EC$_{II}$, respectively) and the extent of cAMP stimulation elicited by these antibodies. Furthermore, we cannot exclude in patients the occurrence of functionally relevant antibodies reacting with epitopes other than β_1-EC$_I$ or β_1-EC$_{II}$ (2,25). Such antibodies might, in fact, be less (or not at all) susceptible to the blocking peptides used in our study, and thus might have been missed by our approach. On the other hand, the novel FRET method now offers the possibility of testing a panel of different blocking peptides to identify other potential epitopes for functional receptor antibodies.

In summary, screening by Epac-FRET seems to represent a very sensitive single-step approach, allowing detection of different kinds of activating antibodies directed against the human β_1-AR.

Therapeutic implications. In contrast to bisoprolol-sensitive low-activator anti-β_1-Abs, neither β_1-selective nor nonselective beta-receptor antagonists could fully block the stimulatory effects of high-activator anti-β_1-Abs: all beta-blockers tested inhibited anti-β_1-EC$_{II}$-mediated cAMP production only partially (60% to 70%), with carvedilol appearing to be more effective than the other substances. A possible explanation for this finding might be different (allosteric) mechanisms by which antibodies or antagonists act on the receptor. By binding to the extracellular loops distant from the agonist and antagonist ligand binding sites in the transmembrane core of the receptor, anti-β_1-EC antibodies might in fact induce different types of conformational changes of the receptor molecule (1,30). In addition, we cannot exclude in patients the occurrence of functional antibodies reacting with epitopes other than the extracellular loops (2,25), thereby leading to a conformation of the receptor that prevents effective binding of beta-blocking agents.

Although the inhibitory effects of β-AR antagonists may contribute to their clinical effectiveness in anti-β_1-Abs–positive heart failure patients, the only partial inhibition of anti-β_1-EC$_{II}$ antibodies should further stimulate research in the field of specifically antibody-directed therapeutic strategies. In addition to beta-blocking agents, such strategies might intend to eliminate stimulating anti-β_1-Abs by nonselective or selective immunoadsorption (32,33), by receptor-homologous peptides (similar to those used in the present pilot study), or by direct targeting of the autoantibody-producing B-cells themselves (34). In addition, our data provide a rationale for a combination therapy with beta-blockers and receptor peptides assuming a synergistic effect of the 2 approaches because bisoprolol fully abolished the stimulatory effects of anti-β_1-EC$_{II}$ antibodies

on cAMP production in cells pretreated with the β_1-EC$_\text{II}$ peptide (Fig. 4D).

Clinical implications. Evidence accumulated during the past 20 years suggests a pathophysiological relevance of functional anti-β_1-Abs in heart failure (2,25). Recent experiments in rats even indicate that activating anti-β_1-Abs against the presumably antigenic β_1-EC$_\text{II}$ loop (100% sequence-identity between human and rat) can actually cause left ventricular dilatation and failure (1,24). However, the clinical relevance of autoantibodies against myocyte membrane antigens, including the β_1-AR, has been difficult to assess, because low titers of such antibodies also can be detected in the healthy population as a part of the immunologic repertoire (35).

Regarding activating anti-β_1-Abs, we show here that their prevalence is almost negligible in healthy subjects (2,6), even with a highly sensitive screening method. In contrast, we detected functional anti-β_1-Abs in about 20% of patients with ICM (all of them high activators), and in about 70% of patients with DCM, half of them high activators (apparently directed against β_1-EC$_\text{II}$), and about another half low activators (apparently directed against β_1-EC$_\text{I}$). It is conceivable that methodological differences in screening for functional anti-β_1-Abs (which may comprise autoantibodies directed against β_1-EC$_\text{II}$, β_1-EC$_\text{I}$, or against both epitopes) account for the wide range in anti-β_1-Abs prevalence rates reported earlier (2). The limited number of patient sera analyzed in the present pilot study precludes a valid estimation of the prognostic relevance of the newly identified anti-β_1-Abs classes. However, regarding the 12-year follow-up data of the here-analyzed 55 DCM patients, we did not find any obvious differences in survival between antibody-negative and low-activator DCM patients (not shown), whereas the presence of high-activator (anti-β_1-EC$_\text{II}$) antibodies seemed to be associated with a worse prognosis, which would corroborate our previous results (6,14). These preliminary findings on the possible clinical relevance of different functional anti-β_1-Abs classes need to be checked in large, well-characterized patient populations (study currently underway).

In conclusion, our novel fluorescence-based method for the detection of functional anti-β_1-Abs is a fast, fully functional, and reliable live cell diagnostic assay with expected clinical impact, now facilitating robust large-scale screening of heart failure patients.

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