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β_1-adrenoceptor antibodies induce positive inotropic response in isolated cardiomyocytes

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Abstract

β_1-adrenoceptor autoantibodies are present in approximately 30% of patients suffering from dilated cardiomyopathy. The inotropic effects mediated by these antibodies remain to be studied. Monoclonal antibodies were raised against a peptide corresponding to the second extracellular loop of the human β_1-adrenoceptor in balb/C mouse ($n = 6$), and were characterized by enzyme immunoassay after purification by protein A. Purified immunoglobulin G from non-immunized animals (controls) did not influence Ca^{2+} transient and cell shortening of rat cardiomyocytes measured by confocal-laser-scanning-microscopy. β_1-adrenoceptor antibodies caused a dose-related increase in Ca^{2+} transient (dilution 1:2: $+35.3 \pm 5.1\%$), and in cell shortening (dilution 1:2: $+40.5 \pm 6.3\%$) ($P < 0.01$ vs. controls). The effect of the β_1-adrenoceptor antibodies was blocked by the antigenic peptide and by the antagonist metoprolol. In addition, β_1-adrenoceptor antibodies induced a dose-dependent increase of the cyclic adenosine monophosphate. The inotropic response induced by isoproterenol was attenuated by the β_1-adrenoceptor antibody. β_1-adrenoceptor antibodies as partial agonists induce a specific positive inotropic effect via the protein-kinase-A-cascade.

Keywords: β_1-Adrenoceptor; Antibody; Inotropic effect; Cardiomyocyte

1. Introduction

Circulating autoantibodies against the β_1-adrenoceptor are detected by enzyme-linked immunosorbent assay (ELISA) in approximately 30% of patients suffering from dilated cardiomyopathy (Magnusson et al., 1990; Fu et al., 1994). These autoantibodies specifically recognize epitopes on the first or second extracellular loop of this G-protein coupled receptor (Wallukat et al., 1995a). Analysis of β_1-adrenoceptor autoantibodies may provide markers for autoimmunological reactions occurring in dilated cardiomyopathy (Felix et al., 2000; Dorffel et al., 1997).

The pathological relevance of this autoantibody was assessed by in-vivo investigation. Active immunization of rabbits was performed, with a peptide corresponding to the second extracellular loop. One-year immunization induced histopathological changes in the hearts of the immunized animals, with phenomena comparable to those found in dilated cardiomyopathy (Matsui et al., 1997).

Functional studies of the β_1-adrenoceptor antibodies disclosed a positive chronotropic effect in cultured, spontaneously beating neonatal rat cardiomyocytes. The agonistic effect of the antibodies was selectively blocked by the β_1-adrenoceptor antagonists (Wallukat et al., 1995b). The action of the antibodies was also neutralized by peptides corresponding to the second extracellular loop of the β_1-adrenoceptor. The antibodies apparently induce their agonist-like effect via the adrenoceptor adenylate cyclase protein kinase A cascade, since the antibodies bind to the receptor and stimulate the enzyme adenylate cyclase and the cyclic adenosine monophosphate (AMP)-dependent protein kinase A (Wallukat et al., 1995b).

However, the inotropic effects of the antibodies against β_1-adrenoceptor on cardiomyocytes remain to be eluci-

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dated. The purpose of the present study was therefore to investigate whether the antibodies raised against the second extracellular loop of the β_1-adrenoceptor have an effect on the contractility and calcium metabolism of isolated ventricular cardiomyocytes. In view of the heterogeneity of the human autoantibodies and their relative scarcity in sera, it was decided to raise monoclonal antibodies in mice using a peptide corresponding to the second extracellular loop of the β_1-adrenoceptor as immunogen.

2. Materials and methods

2.1. Peptides and production of monoclonal antibodies

A peptide (H26R, H-W-W-R-A-E-S-D-E-A-R-R-C-Y-N-D-P-K-C-C-D-F-V-T-N-R) corresponding to the sequences of the second extracellular loop of the human β_1-adrenoceptor was commercially synthesized by Vitrogen of Ontario, Canada. The production of monoclonal antibodies was performed as previously described (Mobini et al., 2000). Purified immunoglobulin G (IgG) from non-immunized animals was used for control purposes. The antibodies were dialyzed (molecular weight cut off, MWCO: 100 kDa, 1:100,000) against experimental buffer [in mmol/l]: 117 NaCl, 2.8 KCl, 0.6 $MgCl_2$, 1.2 KH_2PO_4, 1.2 $CaCl_2$, 20 glucose, and 10 HEPES, pH = 7.3) for 30 h to remove molecules < 100 kDa.

2.2. ELISA

ELISA was used to check the specificity of antibodies before their application. Nunc (Roskilde, Denmark) microtiter plates were coated with solutions of 10 μg/ml peptide in 0.1 M Na_2CO_3/1% β-mercaptoethanol for 1 h at room temperature. After saturation of the wells with 3% Phosphor Milk Tween (PMT) buffer (3% skimmed milk/0.1% Tween 20 in phosphate-buffered saline, pH = 7.4), the monoclonal antibodies were added to the plates and incubated for 2 h at 37 °C. The antibodies were revealed by successive incubations for 1 h at 37 °C with biotinylated rabbit anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories, San Diego, CA), diluted 1:1000 in 3% PMT, and with a streptavidin-peroxidase conjugate (Jackson Immuno Research Laboratories) at 1:1000 dilution in the same buffer. After washing the wells in phosphate-buffered saline, H_2O_2-2,2′-azino-di-3-ethylbenzthiazoline sulphonate (Boehringer Mannheim, Germany) substrate was added and the absorbance was read at 405 nm in a Titertek (Flow, Irvine, UK) ELISA reader after 1 h.

2.3. Cell isolation

Adult rat cardiac myocytes were isolated as recently described (Kubin et al., 1999). Briefly, hearts were perfused for 3 min with oxygenated Krebs–Henseleit buffer (37 °C, pH = 7.4) containing the following (in mmol/l): 110 NaCl, 2.6 KCl, 1.2 KH_2PO_4, 1.2 $CaCl_2$, 20 glucose, and 10 HEPES, pH = 7.3. Typically, about 2×10^6 cells per rat heart were obtained, most of which (95%) showed the typical rod-shaped morphology with no blebs or granulations. The cells were then plated on 4-well chamber-glass slides (Nunc, Naperville, IL, USA) which had been coated with 10 μg/ml Laminin. After an attachment period of 30 min, the buffer was exchanged for a staining solution containing 0.1% dimethyl sulfoxide (DMSO), 0.025% Pluronic F-127, 0.2% bovine serum albumin, and 5 μM cell-permeable Fluo-3 (F6142 Sigma, Deisenhofen, Germany). Cells were incubated at room temperature for 45 min on an orbital shaker oscillating at 40 rev/min. After incubation, the loading solution was replaced with fresh buffer, and incubation continued for an additional 30 min. The cells were incubated on a chamber slide with a volume of 500 μl.

2.4. Measurement of contractility and Ca^{2+} transient

Single cardiomyocytes were field-stimulated (1 Hz, 5 ms) and superfused continuously with experimental buffer (2 ml/min). Ca^{2+}-dependent fluorescence—expressed by relative fluorescence units (rfu)—and cell length were simultaneously measured (488 nm, 120 images/s) in the cardiomyocytes by confocal laser scanning microscopy (Odyssey XL, Noran Instruments, Middleton, WI, USA) (Felix et al., 2001). Cardiomyocytes were stained with the $[Ca^{2+}]$-sensitive probe Fluo-3. In the range considered in the present study, the fluorescence of Fluo-3 is a linear function of $[Ca^{2+}]$. Changes in the Ca^{2+} transient were recorded without calibration because of uncertain subcellular compartmentalization (Spurgeon et al., 1990). The Ca^{2+} transient is calculated as peak systolic rfu minus diastolic rfu. Under control conditions, the cardiomyocytes ($n = 90$) shortened during stimulation by 8.6 ± 0.4%, and Ca^{2+}-dependent fluorescence increased from diastolic 31.1 ± 0.7 to peak systolic 82.9 ± 3.8 rfu. After equilibration for 2 min, control data were recorded and different substances were superfused. The measured parameters were evaluated when the effect reached a stable steady state. Each cardiomyocyte was used for only a single test, and measurements were performed in blinded fashion.

2.5. Measurement of cyclic AMP

Cyclic AMP accumulation in the cell cultures ($3.7 \times 10^4 \pm 1.1 \times 10^4$ cells) was assessed after superfusion for 5 min with experimental buffer, control antibodies (1:5, 1:2), β_1-adrenoceptor antibodies (1:5, 1:2), and isoproterenol (0.1 μmol/l, 1.0 μmol/l), respectively. The samples were extracted by homogenisation in buffer containing 4 mM ethylenediaminetetraacetic acid (EDTA) (to prevent enzy-

matic degradation of cyclic AMP), followed by heating for several min in a boiling-water bath to coagulate protein. After centrifugation in the supernatant, the cyclic AMP was assessed by the Cyclic AMP [^{3}H] Assay System (Amersham, UK). The BCA Protein Assay Kit (Pierce, IL, USA) was used to assay the total cell protein concentration.

2.6. Statistical analysis of data

Results are expressed as mean values ± S.E.M. for n calculations. Effects of the dilutions of antibodies were analysed using nonparametric repeated-measures analysis of variance with data alignment. Post-hoc analyses were performed (Mann–Whitney U-tests) after overall testing.

3. Results

ELISA applied only in the plasma of immunized animals revealed antibodies against β_1-adrenoceptor with a titre over 1/10,000. Tests were performed to determine the influence of different dilutions on calcium metabolism and systolic cell shortening (e.g., IgG, 1:5 = 0.41 μmol/l) for the monoclonal antibody against β_1-adrenoceptor, and for controls. Superfusion of the rat cardiomyocytes with control antibodies ($n = 6$) obtained from non-immunized animals did not influence Ca^{2+} transient or cell contraction. However, when the cells were superfused with antibodies against the second extracellular loop of β_1-adrenoceptor ($n = 6$), Ca^{2+} transient and cell shortening demonstrated parallel increase. The increase of systolic cell shortening and Ca^{2+} transient depended on the dilution of the β_1-adrenoceptor antibody with experimental buffer (Fig. 1). When antibodies against the β_1-adrenoceptor were diluted to 1:2, Ca^{2+} transient increased by +35.3 ± 5.1% ($P < 0.01$ vs. controls) and systolic cell shortening increased by +40.5 ± 6.3% ($P < 0.01$ vs. controls). The effect produced by the antibodies occurred within 5 min and remained unchanged after superfusion with fresh experimental buffer. Diastolic Ca^{2+}-dependent fluorescence did not change significantly. The increase in Ca^{2+} transient was consequently due primarily to increase in systolic Ca^{2+}-dependent fluorescence. The agonist isoproterenol also induced a dose-dependent and pronounced increase of Ca^{2+} transient and of systolic cell shortening (Fig. 1).

The effects of the β_1-adrenoceptor antibody (1:5) were completely eliminated in the presence of the β_1-adrenocep-

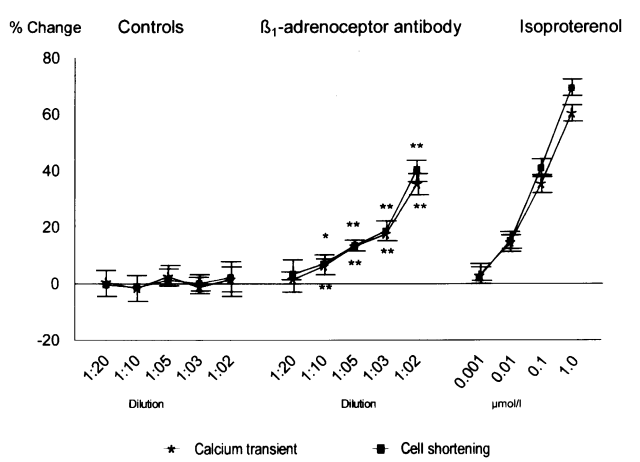

Fig. 1. Effects of various dilutions of antibodies (controls/β_1-adrenoceptor antibodies) on isolated field-stimulated rat cardiomyocytes. Changes of Ca^{2+} transient (peak systolic rfu − diastolic rfu) and systolic cell shortening during superfusion of control antibodies (controls, $n = 6$) (left plot), of β_1-adrenoceptor antibodies ($n = 6$) (middle plot) and of different concentrations of isoproterenol (μmol/l, $n = 6$) (right plot). The values (% changes from baseline) are means ± S.E.M. for $n = 6$ different myocytes. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. controls.

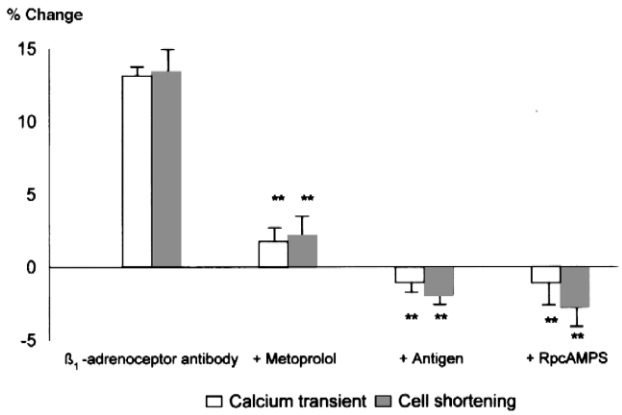

Fig. 2. Interaction of different substances (metoprolol 2.8 μmol/l, antigen 12.3 μmol/l, RpcAMPS 50 μmol/l), with β_1-adrenoceptor antibodies (1:5) on isolated field-stimulated rat cardiomyocytes. Changes of Ca^{2+} transient (peak systolic rfu − diastolic rfu; open bars, $n = 6$) and of systolic cell shortening during superfusion (filled bars, $n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. β_1-adrenoceptor antibody.

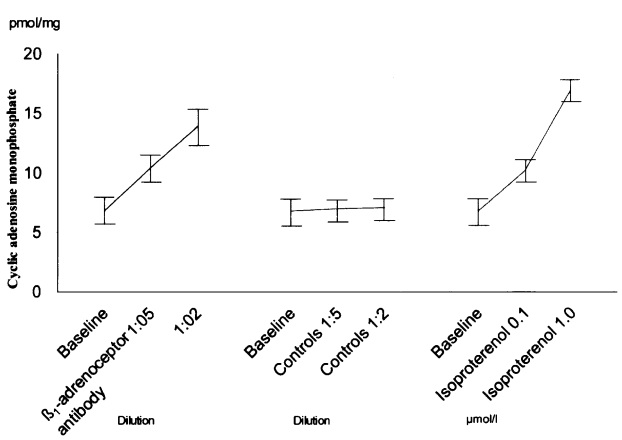

Fig. 3. Effects of various dilutions of antibodies (controls/β_1-adrenoceptor antibodies—1:5, 1:2) and isoproterenol (0.1 μmol/l, 1.0 μmol/l) on synthesis of cyclic adenosine monophosphate (pmol/mg protein) of isolated rat cardiomyocytes ($n = 3$).

tor-selective antagonist metoprolol (2.8 μmol/l, $n = 6$) ($P < 0.01$ vs. β_1-adrenoceptor antibody 1:5) (Fig. 2). In contrast, the β_2-adrenoceptor-selective antagonist 1-[(2,3-Dihydro-7-methyl-1*H*-inden-4-yl) oxy]-3-[1-methylethyl)-amino]-2-butanol hydrochloride (ICI 118,551, 0.1 μmol/l) did not attenuate the inotropic effects induced by the β_1-adrenoceptor antibody (1:5) (data not shown).

In order to ascertain specificity, the β_1-adrenoceptor antibodies were incubated with a 30-fold surplus molar amount of the antigenic peptide of the second extracellular loop for 24 h at 4 °C, in order to block the specific antigenic binding sites. After this pre-incubation, the mixture was tested in a dilution of 1:5 (Fig. 2). The effect of the β_1-adrenoceptor antibodies was fully blocked after pre-incubation with the antigenic peptide ($P < 0.01$ vs. β_1-adrenoceptor antibody 1:5).

To test whether the antibodies induce their agonist-like effect via stimulation of the protein kinase A cascade, the protein kinase A inhibitor Rp-Adenosine-3′,5′-cyclic monophosphothioate triethylamine (RpcAMPS) was used to specifically block this pathway. After pre-incubation (20 min at room temperature) of cardiomyocytes with RpcAMPS (50 μmol/l, $n = 6$), the monoclonal β_1-adrenoceptor antibody (1:5) was not capable of inducing an increase in Ca^{2+} transient, or systolic cell shortening of the cardiomyocytes ($P < 0.01$ vs. β_1-adrenoceptor antibody 1:5) (Fig. 2). Cyclic AMP was analysed after superfusion with controls (1:5, 1:2), antibodies against the β_1-adrenoceptor (1:5, 1:2), and isoproterenol (0.1 μmol/l, 1.0 μmol/l). In contrast to controls, monoclonal antibodies raised against the second extracellular loop of the β_1-adrenoceptor and isoproterenol increased cyclic AMP in dose-dependent fashion (Fig. 3).

Metoprolol, RpcAMPS, ICI 118,551 and antigen did not influence the Ca^{2+} transient and contractility of cardiomyocytes when these substances were separately superfused. RpcAMPS and metoprolol furthermore abolished the effects mediated by the agonist isoproterenol (0.1 μmol/l).

Additional experiments were performed to assess the interaction between the agonist isoproterenol and the β_1-adrenoceptor antibody. As shown in Figs. 1 and 2, antibodies against the β_1-adrenoceptor induced a positive inotropic effect. Isoproterenol (0.1 μmol/l) also brought about an increase in Ca^{2+} transient, as well as systolic cell shortening. However, the isoproterenol-induced increase in Ca^{2+} transient and in systolic cell shortening was attenuated in the presence of the antibodies raised against the β_1-adrenoceptor (1:5) (Fig. 4).

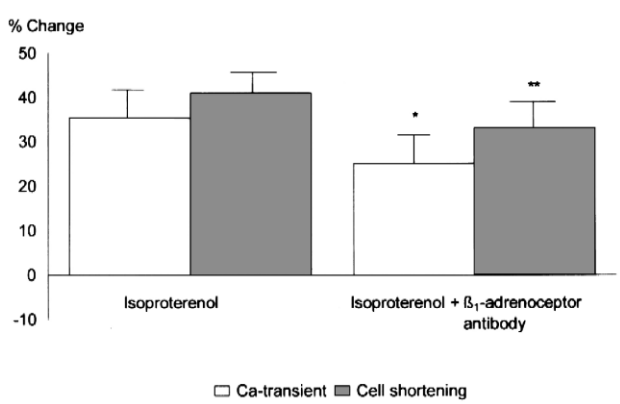

Fig. 4. Interaction between the agonist isoproterenol (0.1 μmol/l) and the β_1-adrenoceptor antibody (1:5). Isoproterenol (0.1 μmol/l) was tested separately ($n = 6$, left plot) and in presence of the monoclonal β_1-adrenoceptor antibodies (1:5, $n = 6$, right plot). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. isoproterenol (0.1 μmol/l).

4. Discussion

Autoimmune responses to various myocardial antigens have been proposed as factors involved in the pathogenesis of patients suffering from dilated cardiomyopathy. Various findings indicate that autoimmunological reactions against the β_1-adrenoceptor may also play a pathogenic role in patients with dilated cardiomyopathy (Limas et al., 1990). The incidence of the β_1-adrenoceptor autoantibody is higher in patients with poorer left ventricular function (Jahns et al., 1999a). Autoimmune reactions against the β_1-adrenoceptor are not present in cardiomyopathies secondary to valvular or hypertensive heart disease, which argues for a pathogenetic role of this antibody in dilated cardiomyopathy (Jahns et al., 1999b). Furthermore, immunization of rabbits with a peptide corresponding to the second extracellular loop of the β_1-adrenoceptor induced histopathological changes in the hearts of the immunized animals, with phenomena comparable to those found in dilated cardiomyopathy (Matsui et al., 1997).

The inotropic effects—including their subcellular mechanisms induced by the β_1-adrenoceptor antibodies—remain to be elucidated. The present study discloses that antibodies against the second extracellular loop of the β_1-adrenoceptor induce a positive inotropic effect in adult isolated cardiomyocytes. The study further reveals an effect on intracellular Ca^{2+} transient, which induces an increase in systolic cell shortening. Inhibition of protein kinase A by RpcAMPS suppressed the positive inotropic effect produced by isoproterenol and by the antibodies raised against the second extracellular loop of the β_1-adrenoceptor. Furthermore, the β_1-adrenoceptor antibody induced an increase of intracellular cyclic AMP. These data indicate that the adenylate cyclase/protein kinase system constitutes the pathway by which the antibody raised against the β_1-adrenoceptor displays its positive inotropic effect.

The β_1-adrenoceptor antibodies attenuate the maximum positive inotropic effect induced by the pure agonist isoproterenol. Antibodies against the β_1-adrenoceptor accordingly act as partial agonists. Our experiments indicate that antibodies against the β_1-adrenoceptor can block overstimulation by the increased catecholamine levels found in

patients with heart failure. On the other hand, continuous stimulation of the receptor may induce electrophysiological imbalance, accompanied by tachycardia and arrhythmia (Chiale et al., 1995). The failing human heart is characterized by reduced responsiveness to β-adrenergic agonists (Bristow et al., 1982). In addition to catecholamines, β_1-adrenoceptor antibodies induce a downregulation of β_1-adrenoceptors (Podlowski et al., 1998). Iwata et al. (2001) were furthermore able to show that the immunization of rabbits immunized with a peptide corresponding to the second extracellular loop of β_1-adrenoceptor developed cardiac dysfunction and β_1-adrenoceptor desensitisation. β_1-adrenoceptor antagonists can block these effects of β_1-adrenoceptor antibodies. Inhibition of β_1-adrenoceptor autoantibodies may contribute to the beneficial effects of β-adrenoceptor blockade in chronic heart failure.

In the present study we analysed the effects of the antibody raised against one specific epitope of the human β_1-adrenoceptor. About 30% of patients suffering from dilated cardiomyopathy develop antibodies against this epitope. Jahns et al. have further demonstrated antibodies against different synthetic receptor peptides in 51% of patients suffering from dilated cardiomyopathy. Only the subgroup directed against the second extracellular loop also recognized native human β-adrenoceptors of the cell membrane. Antibodies from this subgroup demonstrated functional activity (Jahns et al., 1999a). However, antibodies from different species recognizing the second extracellular loop of β_1-adrenoceptor can have divergent effects on cyclic AMP production (Jahns et al., 2000). In our study, monoclonal antibodies were raised against a free peptide corresponding to the second extracellular loop of β_1-adrenoceptor. This antibody clearly demonstrated dose-dependent increase of cyclic AMP and inotropy of cardiomyocytes.

In conclusion, the β_1-adrenoceptor antibody as a partial agonist induces a specific positive inotropic effect via the protein kinase A cascade.

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