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Anti-M_2 muscarinic receptor antibodies inhibit β-adrenoceptor-mediated inotropic response in rat myocardium

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Abstract

The modulation of the inotropic effect by affinity-purified antibodies against a synthetic peptide corresponding to the second extracellular loop of the human muscarinic M_2 receptors was studied in adult rat ventricular myocardium. These anti-muscarinic M_2 receptor antibodies shifted the dose–response relationship of the β-adrenoceptor agonist isoproterenol to higher concentrations whereas preimmune rabbit immunoglobulin G (IgG) or antibodies against the N-terminus of the β_1-adrenoceptor had no effect. This effect of anti-muscarinic M_2 receptor antibodies was fully blocked after preincubation with the antigenic peptide. No significant change of maximal inotropic response to isoproterenol was observed in the presence of anti-muscarinic M_2 receptor antibodies. The anti-muscarinic M_2 receptor antibodies did apparently not hamper the access of the muscarinic receptor agonist carbachol. The muscarinic receptor antagonist atropine attenuated the effect of the anti-muscarinic M_2 receptor antibodies. The present study demonstrates for the first time in intact adult ventricular myocardium a specific stimulatory muscarinic activity of antibodies raised against a part of the muscarinic M_2 receptor protein. © 1997 Elsevier Science B.V.

Keywords: Muscarinic M_2 receptor; Antibody; Peptide; Inotropic effect; Papillary muscle

1. Introduction

Circulating autoantibodies against muscarinic M_2 acetylcholine receptors, α_1- and β_1-adrenoceptors have been demonstrated in patients with idiopathic dilated cardiopathy and malignant hypertension (Magnusson et al., 1990; Fu et al., 1993, 1994a,b). These autoantibodies were found to be directed against the second extracellular loop of these G-protein coupled receptors. Functional studies of the antibodies have revealed that they were able to display stimulatory activity of receptor-mediated events, indicating that they may be involved in the pathogenesis of the respective cardiovascular diseases. However, such functional characterization is hampered by limited amount available of human circulating autoantibodies. To explore further biological effects of anti-muscarinic receptor autoantibodies found in patients with dilated cardiomyopathy (Fu et al., 1993, 1994b), we raised anti-peptide antibodies against the same autoimmune epitope on the heart muscarinic M_2 acetylcholine receptor (Fu et al., 1994d, 1995). These antibodies have been shown to specifically recognize the muscarinic receptor protein in both rat and human myocardium and to exert stimulating muscarinic activity as demonstrated by inhibition of isoproterenol-stimulated cAMP accumulation and by negative chronotropic effect on cultured rat neonatal cardiomyocytes (Fu et al., 1994c,d, 1995; Schulze et al., 1995). The latter was believed to represent primarily an atrial function. However, an effect of the antibodies against muscarinic M_2 receptors on the function of ventricular myocardium remained to be studied.

The purpose of the present study was to investigate whether the antibodies raised against the second extracellular loop of the heart muscarinic M_2 receptor, have an effect on the function of intact ventricular myocardium. It is known that neither stimulation nor blockade of muscarinic M_2 receptors in mammalian ventricular my-

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ocardium exerts prominent effects per se, in contrast to the inhibitory effects on atrial myocardium. When the β-adrenoceptors of the ventricle are stimulated, however, a simultaneous stimulation of the muscarinic M_2 receptors exerts a marked attenuation (Levi, 1971; Christiansen et al., 1987). Thus, in order to explore whether these antibodies exhibit stimulatory muscarinic activity in the ventricle, their influence on β-adrenoceptor-stimulated inotropic effect had to be studied. The effect of the specific antibody was also compared with that of the muscarinic receptor agonist carbachol and the muscarinic receptor antagonist atropine.

We report here that anti-muscarinic M_2 receptor antibodies raised in rabbits were able to decrease the potency of the β-adrenoceptor agonist isoproterenol as an inotropic agent in isolated rat heart papillary muscles and thus to mimic in part the effects of a typical cholinergic agonist as carbachol in ventricular myocardium.

2. Materials and methods

2.1. Synthetic M_2-receptor peptide and anti-peptide antibodies

A peptide corresponding to the sequence (residues 169–193: V–R–T–V–E–D–G–E–C–Y–I–Q–F–F–S–N–A–A–V–T–F–G–T–A–I) of the second extracellular loop of the human muscarinic M_2 receptor (Peralta et al., 1987) was synthesized commercially by Vetrogen (London, Canada). Anti-peptide antibodies were raised in rabbits and characterized by enzyme immunoassay, immunoblotting, immunocytochemistry, ligand binding and chronotropism as previously described (Fu et al., 1995).

2.2. Other synthetic peptides

Peptides corresponding to the sequences of the extracellular loops of the human β_1-adrenoceptor (residues 197–222: 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), α_1-adrenoceptor (residues 192–218: G–W–K–E–P–V–P–P–D–E–R–F–C–G–I–T–E–E–A–G–Y–A–V–F–S–S–V) and angiotensin AT_1 receptor (residues 165–191: I–H–R–N–V–F–F–I–E–N–T–N–I–T–V–C–A–F–H–Y–E–S–Q–N–S–T–L) were synthesized commercially by Vetrogen (London, Ontario, Canada).

2.3. Enzyme-linked immunoabsorbent assay (ELISA)

ELISA was performed basically as previously described (Fu et al., 1995) with the following modifications: 5 μg/ml of peptides was coated; serial dilutions (from 1:100) of purified antibodies against muscarinic M_2 receptor peptide were added to the coated microtiter plates.

2.4. Blocking of the specific binding sites of the anti-M_2 receptor antibody by the antigenic peptide

In order to test the specificity of the anti-muscarinic M_2 receptor antibodies they were incubated with the antigenic peptide for 24 h at 4°C in order to block the specific antigenic binding sites. The concentration in the incubation mixture was about 6 μmol/l of the antibody and about 180 μmol/l of the antigenic peptide. In order to run corresponding control experiments, the antibody against the N-terminus of the β_1-adrenoceptors was also preincubated with the antigenic peptide under corresponding conditions.

2.5. Preparation of rat papillary muscle

Rat ventricular myocardium was used as an experimental model to study effects of receptor antibodies against human acetylcholine M_2 receptors since the antigenic peptide used in the present study corresponds to a sequence which is totally conserved in human and rat species (Peralta et al., 1987; Venter et al., 1988). Rat heart papillary muscles were isolated as described previously with minor modifications (Skomedal and Osnes, 1983). During pentobarbital anesthesia hearts were removed and quickly mounted in a perfusion apparatus. During coronary perfusion with the physiological salt solution mentioned below, left ventricular papillary muscles were excised and mounted in an organ bath with 10 ml of a slightly different physiological salt solution. During coronary perfusion the solution contained the following (mmol/l): 120.5, NaCl; 3.0, KCl; 0.5, $CaCl_2$; 1.2, $MgSO_4$; 2.4, KH_2PO4; 24.9, $NaHCO_3$ and 10.0, glucose, continuously gassed with 95% O_2/5% CO_2 at 31°C (pH 7.4). During incubation in the organ bath the salt solution was identical except for NaCl which was 119.0 mmol/l and $CaCl_2$ which was 2.0 mmol/l. The direct current signals from the amplifiers were analog-to-digital converted by a National Instruments NB-MIO-16X board mounted in an Apple Macintosh computer, model Quadra 700. The logged data were stored in files as time stamped and event marked unfiltered binary clusters by software developed for the purpose in the visual programming language LabVIEW®. The software could later open the files for analysis and compute appropriately low-pass filtered trend curves for developed tension versus time for each contraction–relaxation cycle. Areas representative for the control (basal) period and the periods with β-adrenergic stimulation could be selected to calculate averaged contraction–relaxation cycles which were representative for these periods. These cycles were then used to determine values for typical descriptive parameters like maximal developed tension (T_{max}) and maximal development of tension (T'_{max}).

2.6. Experimental design

The papillary muscles were allowed to equilibrate for 60 min before the experiments started. The salt solution

was changed after 45 min of equilibration. The salt solution then contained ascorbate (10^{-4} mol/l) and the α_1-adrenoceptor antagonist prazosin (10^{-7} mol/l) and when used also the muscarinic receptor antagonist atropine (10^{-6} mol/l). The concentrations of antagonist(s) was kept constant during the experimental period. The muscles were then exposed either to antibodies (100 μl of the stock solution giving about 70–100 nmol/l) or preimmune IgG or to the vehicle for 90 min. The presence of immunoglobulins did not apparently influence the contractility of the papillary muscles when compared to the vehicle at the end of the equilibration period. All the muscles in the different experimental groups were then exposed to cumulatively increasing concentrations of the β-adrenoceptor agonist isoproterenol until the maximal response was obtained in order to evaluate the dose–response relationship to β-adrenoceptor stimulation during the various conditions. Corresponding experiments with the antibodies prereacted with the antigenic M_2-peptide were also performed. Isoproterenol was added directly to the organ bath in volumes of 25–75 μl to give the appropriate final concentrations. Each muscle was used for one experiment only to avoid problems related to possible changes in responsiveness. As a reference and for comparison with the antibodies, the muscarinic receptor agonist carbachol was used. The effect of carbachol upon the β-adrenoceptor mediated inotropic response was evaluated in two ways: carbachol was either added to the muscles 15 min before isoproterenol (2.0×10^{-7}, 1.5×10^{-6} or 2.5×10^{-5} mol/l carbachol) or added after the maximal response to isoproterenol was achieved (2.5×10^{-5} mol/l carbachol).

In order to compare the accessibility of carbachol and atropine to their receptor sites in the presence and absence of specific anti-M_2 receptor antibodies, some experiments were performed by adding specific rabbit anti-M_2 receptor antibodies 5 min after a maximal stimulation by 4.5×10^{-6} mol/l isoproterenol and by adding 2.5×10^{-5} mol/l carbachol after a further 90 min. 10^{-5} mol/l atropine was added 10 min after carbachol. Corresponding controls without antibodies were run in parallel.

2.7. Definitions

T_{max} is the maximal developed tension in each contraction–relaxation cycle.

$T'_{max} = (dT/dt)_{max}$ is the maximal rate of tension development, i.e. rise of contraction.

$pD_2 = -\log EC_{50}$ (EC_{50} is the concentration giving half maximal effect).

Changes in contractile force were expressed as changes in the maximal development of tension ($T'_{max} = (dT/dt)_{max}$). Horizontal positioning of the dose–response curves was expressed by pD_2 values.

2.8. Calculations and statistics

The values after responses to increasing concentrations of agonist were generally calculated as percent of control values (100%). Dose–response curves were first constructed according to Ariëns and Simonis (1964) by estimating centiles (EC_{10} to EC_{100}) for each single experiment and calculating the corresponding means. This calculation provides mean curves that express the response as fractional response or percent of maximum and display horizontal positioning and the mean slope of the curves. The response figures were also recalculated and expressed as percent of control in order to yield curves that display differences in maxima as well. The significance levels of differences were expressed by calculating P according to Students one-sample test or two-sample test as appropriate (Minitab®). A value of P less than or equal to 0.05 was considered to reflect significant differences.

2.9. Drugs

(−)-Isoproterenol hemisulphate, prazosin hydrochloride, carbamylcholine chloride (carbachol), atropine sulphate and ascorbic acid were purchased from Sigma (St. Louis, MO, USA). Stock solutions were prepared in double distilled water and kept at −20°C to avoid oxidation. Further dilutions of the drugs were made fresh daily and kept cool (0–4°C) and dark. Repetitive experiments showed that drug solutions treated in this way were stable.

3. Results

3.1. Characterization and crossreactivity of the antibodies

By use of ELISA, both rabbits were shown to produce antibodies with a titre over 1:10 000. The affinity purification of antiserum yielded 2.05 mg of antibody from 10 ml of serum, corresponding to approximately 1.1% of the total immunoglobulin fraction. No crossreactivity with antigenic peptides corresponding to the second extracellular loop of human α_1-adrenoceptors, β_1-adrenoceptors or angiotensin AT_1 receptors was observed.

3.2. Horizontal positioning of the dose–response relationship to β-adrenoceptor stimulation

3.2.1. Influence of atropine and carbachol

Isoproterenol produced a dose-dependent increase in contractility in the rat papillary muscles with a pD_2 value of 7.04 ± 0.11 (mean ± S.E.M., $n = 13$, Fig. 1). The muscarinic receptor antagonist atropine (10^{-6} mol/l) shifted the dose–response relationship to lower concentrations of isoproterenol with a pD_2 value of 7.34 ± 0.06 (mean ± S.E.M., $n = 16$, $P < 0.02$, Fig. 1).

The muscarinic receptor agonist carbachol dose-dependantly shifted the dose–response relationship of isoproterenol to higher concentrations. In the presence of 2.5×10^{-5} mol/l carbachol, the pD_2 value for isoproterenol was 6.52 ± 0.09 (mean ± S.E.M., $n = 16$, $P < 0.01$, Fig. 1).

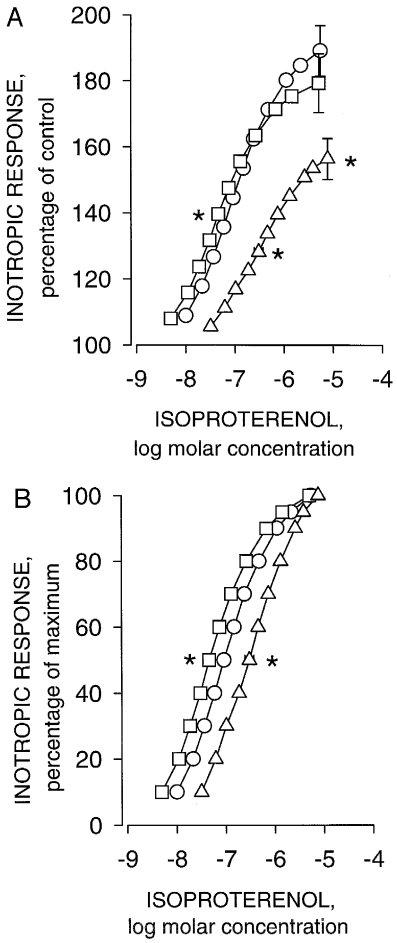

Fig. 1. Inotropic response of rat heart papillary muscle to β-adrenoceptor stimulation in the absence and presence of the muscarinic receptor antagonist atropine and the muscarinic receptor agonist carbachol: (A) expressed as percent of basal values, (B) expressed as percent of individual maxima (fractional response). Isoproterenol alone (○, $n=13$), isoproterenol in the presence of 10^{-6} mol/l atropine (□, $n=16$) and isoproterenol in the presence of 2.5×10^{-5} mol/l carbachol (△, $n=16$). * Indicates significant differences from corresponding values in the presence of isoproterenol alone. Abscissa: logarithm of molar concentration of isoproterenol. Ordinate: (A) inotropic response (increase of T'_{max}) expressed in percent of basal values (100%) before addition of isoproterenol, (B) inotropic response (increase of T'_{max}) expressed in percent of individual maxima. S.E.M. of pD_2 values are indicated by horizontal bars and of maximal response by vertical bars where they exceed the width of the symbols.

Table 1
Effects of antibodies upon the dose–response relationship to isoproterenol in rat papillary muscle

	pD_2 value	Max. increase (% of control)	No. of exp.
Anti-M_2 antibody			
Antibody alone	6.67 ± 0.16	134.0 ± 24.9	9
Antibody + atropine	$7.19^{a}\pm0.12$	87.2 ± 18.1	5
Preimmune rabbit IgG			
Preimmune IgG alone	7.44 ± 0.08	103.0 ± 17.4	9
Preimmune IgG + atropine	7.41 ± 0.09	111.7 ± 14.3	9
M_2-receptor ligands			
Atropine	$7.34^{b}\pm0.06$	79.4 ± 8.9	16
Carbachol	$6.52^{b}\pm0.09$	$56.4^{b}\pm6.2$	16

Values are given as mean ± S.E.M.
[a] Significantly different from antibody alone ($P=0.01$).
[b] Significantly different from isoproterenol alone ($P<0.01$).

The dose–response relationship in the presence of atropine reflected the condition without any muscarinic stimulation. Thus, this condition was used as reference for possible influences of the antibodies upon the effect of isoproterenol.

3.2.2. Effect of anti-muscarinic M_2 receptor antibodies

The stock solutions of these antibodies were added to the organ bath giving a final concentration of about 70–100 nmol/l. Anti-acetylcholine M_2 receptor antibodies shifted the dose–response relationship to higher concentrations of isoproterenol with a pD_2 value of 6.67 ± 0.16 (mean ± S.E.M., $n=9$, $P<0.01$, Tables 1 and 2, Fig. 2). In contrast, preimmune rabbit IgG or antibodies against the N-terminus of the β_1-adrenoceptor (Magnusson et al., 1994) did not shift the positioning of the dose–response curve to isoproterenol compared to the presence of atropine (Tables 1 and 3). These curves thus served as controls for the dose–response curves in the presence of antibodies against the muscarinic M_2 receptors.

In the presence of 10^{-6} mol/l atropine, the effect of the anti-M_2 antibodies was attenuated and the pD_2 value in this situation was 7.19 ± 0.12 (mean ± S.E.M., $n=5$, $P=0.01$, Table 1).

Table 2
Statistical evaluations (Student's two-sample test) of effects of antibodies upon the dose–response relationship to isoproterenol in rat papillary muscle

	ΔpD_2 values (mean ± S.E.M.)
Anti-M_2 antibody versus preimmune rabbit IgG	0.77 ± 0.18, $P<0.01$ (95% CI: 0.38; 1.17)
Anti-M_2 antibody versus anti-β_1 antibody [a]	0.68 ± 0.16, $P<0.01$ (95% CI: 0.31; 1.06)
Anti-β_1 antibody [a] versus preimmune rabbit IgG	0.09 ± 0.09, ns (95% CI: −0.10; 0.29)
Anti-M_2 antibody + peptide [b] versus anti-β_1 antibody [a] + peptide [b]	0.01 ± 0.20, ns (95% CI: −0.50; 0.52)

ns, not significant.
[a] Antibody against the N-terminus of β_1-adrenoceptors.
[b] The antigenic peptide with the same composition as the second extracellular loop of the muscarinic M_2 receptor.

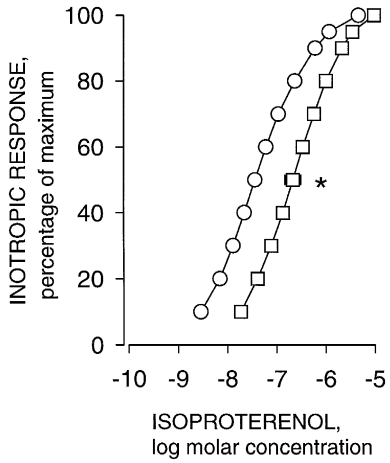

Fig. 2. Inotropic response of rat heart papillary muscle to β-adrenoceptor stimulation in the presence of anti-muscarinic M_2 receptor antibodies. The antibodies were introduced 90 min before exposure to isoproterenol added cumulatively at increasing concentrations. Isoproterenol in the presence of preimmune rabbit IgG (○, $n=9$), isoproterenol in the presence of antibodies raised in rabbits against the second extracellular loop of the muscarinic M_2 receptor (□, $n=9$). * Indicates siginificant difference from corresponding value in the presence of isoproterenol alone. Abscissa: logarithm of molar concentration of isoproterenol. Ordinate: inotropic response (increase of T'_{max}) expressed in per cent of the individual maxima. S.E.M. of pD_2 values are indicated by horizontal bars where they exceed the width of the symbols.

After preincubation of the specific muscarinic M_2 receptor antibody for 24 h with a 30-fold surplus molar amount of the antigenic peptide, the influence of this antibody upon the horizontal positioning of the dose–response curve to isoproterenol was no longer observed (Tables 2 and 3). Thus, blocking of the anti-muscarinic M_2 receptor antibody with the corresponding peptide eliminated the effect of this antibody. The presence of peptide did, however, apparently slightly shift the dose–response curve towards higher concentrations of isoproterenol (Tables 2 and 3).

3.3. Maximal inotropic response to isoproterenol

3.3.1. Influence of atropine and carbachol

Atropine (10^{-6} mol/l) did not significantly influence the maximal response to isoproterenol (Fig. 1a). Carbachol dose-dependently, however, gave the expected reduction in maximal response. When 2.5×10^{-5} mol/l carbachol was added before isoproterenol, its maximal inotropic response was reduced to $63.0 \pm 6.9\%$ compared to the absence of carbachol ($P < 0.01$, Fig. 1a). When 2.5×10^{-5} mol/l carbachol was added after the maximal inotropic response to isoproterenol was achieved, carbachol reduced the response to $38.5 \pm 4.5\%$ compared to the response to isoproterenol in the absence of carbachol ($P < 0.01$). The reduction in maximal inotropic response parallelled the rightward shift of the dose–response curve with respect to concentration of carbachol (correlation coefficient $r^2 = 0.9960$).

3.3.2. Effect of anti-muscarinic M_2 receptor antibodies

Overall there was no significant change in the maximal responses to isoproterenol in the presence of any antibody. Thus, unlike the effect of carbachol, there was no reduction in maximal β-adrenoceptor-mediated effect in the presence of the specific antibodies against the second extracellular loop of the M_2 receptor (Table 1).

3.3.3. Influence of carbachol and atropine in the presence of anti-muscarinic M_2 receptor antibodies

In the presence of the anti-muscarinic M_2 receptor antibodies, 2.5×10^{-5} mol/l carbachol was able to reduce the inotropic response to 4.5×10^{-6} mol/l isoproterenol by $57.8 \pm 20.9\%$ (mean ± S.E.M., $n=4$, $P < 0.01$) which was rather comparable to the response to carbachol in the absence of the antibody ($55.1 \pm 6.7\%$ (mean ± S.E.M., $n=6$, $P < 0.01$)). Both in the presence and absence of the antibody, 10^{-5} mol/l atropine was able to reverse the effect of carbachol by 32.0 ± 16.6 and $48.2 \pm 12.3\%$, respectively. Although the reversal responses to atropine were not statistically significantly different, the nominal difference suggests a difference in accessibility to the binding sites for atropine in the presence compared to the absence of anti-muscarinic M_2 receptor antibody in accordance with the effect of atropine on the horizontal shift of the dose–response curve of isoproterenol caused by the antibody (Table 1).

Table 3
Comparison of effects upon the dose-relationship to isoproterenol in rat papillary muscles of receptor antibodies alone and after prereaction with antigenic peptide

	pD_2 values in the absence of antigenic peptide [a]	pD_2 values after prereaction with antigenic peptide [a]
Anti-M2 receptor antibodies	6.67 ± 0.16 ($n=9$)	7.08 ± 0.20 ($n=5$)
Anti-β_1 adrenoceptor antibodies [b]	7.35 ± 0.03 ($n=12$)	7.09 ± 0.03 ($n=6$)

[a] The antigenic peptide with the same composition as the second extracellular loop of the muscarinic M_2 receptor.
[b] Antibody against the N-terminus of β_1-adrenoceptors.
Values are given as mean ± S.E.M.

4. Discussion

The present study shows for the first time stimulatory activity in intact ventricular myocardium of specific antibodies against the second extracellular loop of the heart muscarinic acetylcholine receptor protein. The effect was revealed as an ability to attenuate the inotropic effect of isoproterenol in papillary muscle.

The stimulatory effects were specific for the rabbit anti-muscarinic M_2 receptor antibodies as the effects were not shared by preimmune rabbit IgG and by the antibodies against N-terminus of β_1-adrenoceptor antibodies. The latter was previously shown to have no functional activity, being different from antibodies against the second extracellular loop of the β_1-adrenoceptors (Magnusson et al., 1994). The specificity was also tested by the blocking effect of preincubation with the antigenic peptide corresponding to the second extracellular loop of the muscarinic M_2 receptor protein. This peptide fully eliminated the stimulatory muscarinic effect induced by the rabbit anti-muscarinic M_2 receptor antibodies. Moreover, these antibodies were shown not to crossreact with other antigenic peptides corresponding to the extracellular loops of human α_1-adrenoceptors, β_1-adrenoceptors or angiotensin AT_1 receptors which are also coupled to G-proteins and have a basic structure of seven transmembrane domains as muscarinic M_2 receptors. Thus, the stimulatory activity of the anti-muscarinic M_2 receptor antibodies is specifically related to their recognition of the second extracellular loop of the muscarinic M_2 receptor proteins.

The antibodies partly mimicked the effect of the muscarinic receptor agonist carbachol. The ability of carbachol in reducing the potency of isoproterenol as an inotropic agent in ventricular myocardium was fully shared by the anti-muscarinic M_2 receptor protein antibodies (Table 1). The anti-muscarinic M_2 antibodies did not, however, reduce the maximal inotropic effect of isoproterenol. This is in striking contrast to the parallel reduction of both potency and efficacy of isoproterenol by carbachol. The lack of effect on the efficacy of isoproterenol by the antibodies indicates that interaction with the second extracellular loop of the M_2 receptor protein is different from the interaction of a small molecular ligand at the carbachol binding site. The interaction with the second extracellular loop is thus apparently not sufficient to elicit a complete muscarinic response with both reduction of potency and of efficacy of isoproterenol.

The muscarinic receptor agonist carbachol was apparently fully active in modulating the maximal inotropic effect of β-adrenoceptor stimulation in the presence of a M_2 receptor antibody. This observation indicates that the binding of the antibody to the second extracellular loop does not create a hindrance for small molecular muscarinic M_2 receptor agonists to reach their active site(s) at least to an extent that evokes full pharmacological effects. Thus, muscarinic receptor agonistic activity can originate from different parts of the receptor. The finding that carbachol fully reduces the maximal inotropic effect in the presence of the antibody, is an indirect support of the inability of the latter to influence the maximal inotropic response to β-adrenoceptor stimulation. The anti-muscarinic M_2 receptor antibodies are thus apparently acting as allosteric ligands relative to the carbachol recognition sites at the muscarinic M_2 receptor proteins (Tucek and Proska, 1995).

The finding that the antibodies appear to be partial agonists at the muscarinic M_2 receptor in the sense that they mimicked only some of the cholinergic effects, are in accordance with our earlier finding regarding antibodies against the second extracellular loop of the β_1-adrenoceptor (Magnusson et al., 1994). Moreover, we have shown that anti-β_2 adrenoceptor antibodies recognize and stabilize the active conformation of the receptor proteins (Mijares et al., 1996). There is also evidence that the agonist conformation of G-protein coupled receptors is in the dimeric form (Hebert et al., 1996). Although speculative, the agonist-like activity of the divalent receptor antibodies could well be due to stabilization of the receptors in the dimeric form.

Preaddition of the muscarinic antagonist atropine (10^{-6} mol/l) partly prevented the effect of the M_2 receptor antibody in dose–response studies. Thus, the potency of atropine in this respect was apparently low (Table 1). Although speculative, the binding of atropine may interfere with the access of the antibodies to the second extracellular loop of the muscarinic M_2 receptor protein. Alternatively, atropine may make the receptors less prone to conformational changes by the anti-muscarinic M_2 receptor antibodies and/or induce an inactive conformation of the muscarinic M_2 receptor proteins and thus hamper the stabilization of the receptors in an active conformation (and possibly dimeric form) by the receptor antibodies (see above).

The present findings on rabbit anti-muscarinic M_2 receptor antibodies may have clinical relevance. Autoantibodies against heart acetylcholine M_2 receptors have been found in patients with dilated cardiomyopathy (Fu et al., 1993, 1994b) and in patients with Chagas' cardiomyopathy (Elies et al., 1996). The anti-acetylcholine M_2 receptor antibodies have demonstrated stimulatory activites (Fu et al., 1994d; present study). We have not measured the concentration of anti-muscarinic M_2 antibodies in the plasma of patients with dilated cardiomyopathy directly. But from the purification studies (see Section 2), we can estimate that the plasma concentration will be in the range of 10^{-10} and 10^{-8} mol/l. The concentration of anti-muscarinic M_2 receptor antibodies in plasma from patients with Chagas' cardomyopathy is higher (Elies et al., 1996). Thus, the concentration of anti-muscarinic M_2 antibody used in the present study is apparently relevant compared to those estimated in vivo.

In conclusion, the present study demonstrates for the first time in intact ventricular myocardium a specific stim-

ulatory muscarinic activity of antibodies raised against the second extracellular loop of the muscarinic M_2 acetylcholine receptor. The effect does apparently not originate from the binding sites for small molecular muscarinic agonists. An antibody evoked attenuation of the inotropic effect to β-adrenoceptor stimulation may have clinical implications in patients with cardiomyopathy in whom autoantibodies against the same epitope on human muscarinic M_2 receptors were found.

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References

Ariëns, E.J., Simonis, A.M., 1964. Drug–receptor interactions: Interaction of one or more drugs with one receptor system. Mol. Pharmacol. 1, 119.

Christiansen, H.B., Horgmo, G.I., Skomedal, T., Osnes, J.-B., 1987. Enhancement of the α-adrenergic inotropic component of noradrenaline by simultaneous stimulation of muscarinic acetylcholine receptors in rat myocardium. Eur. J. Pharmacol. 142, 93.

Elies, R., Ferrari, I., Wallukat, G., Lebesgue, D., Chiale, P., Elizari, M., Rosenbaum, M., Hoebeke, J., 1996. Structural and functional analysis of the B-cell epitopes recognized by anti-receptor autoantibodies in patients with Chagas' disease. J. Immunol. 157, 4203.

Fu, L.X.M., Herlitz, H., Wallukat, G., Hilme, E., Hedner, T., Hoebeke, J., Hjalmarson, Å., 1994a. A functional autoimmune epitope on α-1 adrenoceptors in patients with malignant hypertension. Lancet 344, 1660.

Fu, L.X.M., Hoebeke, J., Magnusson, Y., Matsui, S., Matoba, M., Hedner, T., Herlitz, H., Hjalmarson, Å., 1994b. Autoantibodies against cardiac G-protein coupled receptors in patients with cardiomyopathy but not with hypertension. Clin. Immunol. Immunopathol. 72, 15.

Fu, L.X.M., Schulze, W., Wolf, W.P., Hjalmarson, Å., Hoebeke, J., 1994c. Immunocytochemical localization of M2 muscarinic receptor in rat ventricles using anti-peptide antibodies. J. Histochem. Cytochem. 42, 337.

Fu, L.X.M., Wallukat, G., Hjalmarson, Å., Hoebeke, J., 1994d. Agonist-like activity of anti-peptide antibodies directed against an autoimmune epitope on the heart muscarinic receptors. Recept. Channels 2, 121.

Fu, L.X.M., Schulze, W., Wallukat, G., Hjalmarson, Å., Hoebeke, J., 1995. Functional epitope analysis of the second extracellular loop of the human heart muscarinic acetylcholine receptor. J. Mol. Cell. Cardiol. 27, 427.

Fu, L.X., Magnusson, Y., Bergh, C.H., Liljeqvist, J.Å., Waagstein, F., Hjalmarson, Å., Hoebeke, J., 1993. Localization of autoimmune epitope on the second extracellular loop of human muscarinic receptor-2 in patients with idiopathic dilated cardiomyopathy. J. Clin. Invest. 91, 1964.

Hebert, T.E., Moffett, S., Morello, J.P., Loisel, T.P., Bichet, D.G., Barret, C., Bouvier, M., 1996. A peptide derived from a β_2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. J. Biol. Chem. 271, 16384.

Levi, M.N., 1971. Sympathetic–parasympathetic interactions in the heart. Circ. Res. 29, 437.

Magnusson, Y., Marullo, S., Höyer, S., Waagstein, F., Andersson, B., Vahlne, A., Guillet, J.-G., Strosberg, A.D., Hjalmarson, Å., Hoebeke, J., 1990. Mapping of a functional autoimmune epitope on the β1-adrenergic receptor in patients with idiopathic dilated cardiomyopathy. J. Clin. Invest. 86, 1658.

Magnusson, Y., Wallukat, G., Waagstein, F., Hjalmarson, Å., Hoebeke, J., 1994. Autoimmunity in idiopathic dilated cardiomyopathy: Characterization of antibodies against the β1-adrenoceptor with positive chronotropic effect. Circulation 89, 2760.

Mijares, A., Lebesgue, D., Argibay, J., Hoebeke, J., 1996. Anti-peptide antibodies sensitive to the 'active' state of the β_2-adrenergic receptor. FEBS Lett. 399, 188.

Peralta, E.G., Winslow, J.W., Peterson, G.L., Smith, D.H., Ashkenazi, A., Ramachandran, J., Schimerlik, M.I., Capon, D.J., 1987. Primary structure and biochemical properties of an M2 muscarinic receptor. Science 230, 600.

Schulze, W., Fu, L.X.M., Hoebeke, J., Hjalmarson, Å., 1995. Localization of muscarinic receptors in heart biopsies from patients with heart disease using rabbit antibodies against an autoimmune epitope on human M2 muscarinic receptors. J. Mol. Cell. Cardiol. 27, 1757.

Skomedal, T., Osnes, J.-B., 1983. Qualitative differences between the inotropic response in rat papillary muscles to α-adrenoceptor and β-adrenoceptor stimulation by both noradrenaline and adrenaline. Acta Pharmacol. Toxicol. 52, 57.

Tucek, S., Proska, J., 1995. Allosteric modulation of muscarinic acetylcholine receptors. Trends Pharmacol. Sci. 16, 205.

Venter, J.C., Fraser, C.M., Chung, F.Z., Kerlavage, A.R., Robinson, D.A., Gocayne, J.D., Fitzgerald, M.G., 1988. Cloning and expression of adrenergic and muscarinic cholinergic receptor genes. Adv. Exp. Med. Biol. 236, 173.