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From Agonist To Antagonist: Fab Fragments of an Agonist-Like Monoclonal Anti-β_2-Adrenoceptor Antibody Behave as Antagonists

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ABSTRACT

We previously demonstrated that the monoclonal antibody Mab6H8 raised against the second extracellular loop of the β_2-adrenoceptor (β_2-AR) had an agonist-like activity, mediated by the activation of L-type Ca^{2+} channels by protein kinase A through the adenylyl cyclase pathway. We suggested that this Mab acts by stabilizing an active dimeric conformation of the β_2-AR. To substantiate this hypothesis, we prepared monomeric Fab fragments of Mab6H8. Comparison of the physicochemical parameters of antigen interaction with both the Mab and its Fab fragments were determined by surface plasmon resonance, showing a 5- to 10-fold lower affinity of the fragments compared with the bivalent antibody. We determined the biological activity of antibody and Fab fragments in two systems: spontaneous beating neonatal rat cardiomyocytes to study the chronotropic effects and isolated guinea pig cardiomyocytes to study L-type Ca^{2+} channel activation. Fab fragments as such had no "agonist-like" effects in both systems but inhibited receptor activation with the β_2-specific agonist clenbuterol. Addition of a cross-linking rabbit anti-mouse IgG restored the agonist-like effect of the Fab fragments. These results suggest that Fab fragments induce a conformational change in the receptor, inhibiting the accessibility of the pharmacophore pocket to clenbuterol. Dimerization of this receptor conformation induces an agonist-like effect. Antireceptor antibodies can thus act both as agonist in the dimeric state and as antagonist in the monomeric state.

The β_2-adrenoreceptor (β_2-AR) is one of the most studied G protein-coupled receptors. The modulation of cardiac contractility is mediated by β_2-AR and its activation might have chronic effects on cellular metabolism, on cell growth, or on excitability (Xiao et al., 1999). These receptors seem to be involved in several cardiomyopathies with an autoimmune component like Chagas' disease and cardiac electrical disturbances because receptor-activating autoantibodies were detected in patients with such cardiomyopathies (Sterin-Borda et al., 1988; Chiale et al., 1995; Hoebeke, 1995; Elies et al., 1996). Polyclonal rabbit antibodies produced against a peptide corresponding to the second extracellular loop of the human β_2-AR, which is a target of the autoantibodies, had agonist-like effects. Such antibodies were able to stimulate the β_2-AR, to increase the chronotropic effect in rat neonatal cardiomyocytes, and to induce calcium influx in guinea pig cardiomyocytes via L-type calcium channels. This activation was blocked by a specific β_2-inverse agonist ICI 118,551 but not with the neutral antagonist alprenolol (Mijares et al., 1996). More recently, a monoclonal antibody, sharing the agonist-like effects of the rabbit antibodies, was produced and immunochemically and pharmacologically characterized (Lebesgue et al., 1998). This antibody could be an exquisite tool to study the activation mechanism of the β_2-AR and especially the hypothesis that dimerization is a requisite for receptor activation.

Several observations indeed point to such a mechanism. Studying coexpression of chimeric muscarinic/adrenergic receptors, Maggio et al. (1993) came to the conclusion that only dimers could explain restoration of receptor function in cells

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ABBREVIATIONS: β_2-AR, β_2-adrenoreceptor; ICI 118,551, β_2-antagonist (±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3[(1-methylethyl)amino]-2-butanol hydrochloride; Mab6H8, monoclonal antibody against a peptide corresponding to the second extracellular loop of the human β_2-AR; Fab, monomeric fragments of antibody; β_2-H19C, peptide corresponding to the second extracellular loop of the human β_2-AR (amino acids 172-190); Fura-AM, Fura 2 acetoxymethyl ester; R_{eq}, relative response bound at equilibrium; K_A, association constant; k_{obs}, association rate; k_{on}, association rate constant; k_{off}, dissociation rate constant; K_I, inhibition constant; PKA, protein kinase A.

cotransfected with two complementary chimeras. Hebert et al. (1996) showed that agonist stimulation stabilized the dimeric state of the receptor and that inhibition of dimerization by a synthetic peptide led to inhibition of isoproterenol activation of the receptor. Similarly, Hebert et al. (1998) used wild-type and mutant β_2-AR expressed in Sf9 cells and demonstrated that the intermolecular interactions between receptors might play a role in G protein-mediated signaling. Gouldson et al. (1997, 1998) carried out a fusion of theoretical and experimental data to validate the hypothesis that G protein-coupled receptor activation involves receptor dimers.

This study was undertaken to evaluate the pharmacological properties of the dimeric Mab6H8 and its monomeric Fab fragments. The physicochemical parameters of the interaction between the Mab and its fragments with the target peptide were compared before their physiological effects were analyzed in two in vitro setups: spontaneously beating neonatal rat cardiomyocytes, which have been found to be a sensitive model for analysis of functional antibodies (Wallukat and Wollenberger, 1987); and single cell L-type Ca^{2+} channel activation, monitored by a fluorescent Ca^{2+} chelator in isolated guinea pig cardiomyocytes (Grynkiewicz et al., 1985).

Materials and Methods

Peptides. Peptide β_2-H19C (His-Trp-Try-Arg-Ala-Thr-His-Gln-Glu-Ala-Ile-Asn-Cys-Tyr-Ala-Asn-Glu-Thr-Cys) corresponds to the part of the second extracellular loop (residues 172–190) of the human receptor (Emorine et al., 1987; Kobilka et al., 1987). The peptide was synthesized with the 9-fluorenylmethyloxycarbonyl procedure with an automated Applied Biosystems 431A peptide synthesizer. The peptide was purified by HPLC and checked by mass spectrometry.

Purification of Mab6H8. Mab6H8, recognizing an epitope corresponding to amino acids 173 to 180 of the human β_2-AR (Lebesgue et al., 1998), was purified from ascitic fluids on an affinity column made by coupling β_2-H19C peptide to CNBr-activated Sepharose by a standard procedure (Pharmacia, Uppsala, Sweden): The ascitic fluids (2 ml) were diluted 1:20 in PBS, and the antibody, adsorbed on the affinity column in PBS, was eluted with 3 M $MgCl_2$ and immediately dialyzed against PBS. The concentration of the purified antibodies was calculated initially from the absorbance at 280 nm (absorbance of 1.45 corresponding to 1 mg/ml for a 1-cm optical pathway). Purified monoclonal antibodies were aliquoted and stored at −80°C.

Fab Fragments. Fab fragments were obtained by papain (Sigma, St. Louis, MO) digestion of the affinity-purified Mab6H8. The Fab fragments were separated from the Fc fragments on a Hi-Trap protein A column with a Gradi-Frac system (Pharmacia). Purity was checked by SDS-polyacrylamide gel electrophoresis. No unhydrolyzed IgG could be detected.

Surface Plasmon Resonance. Surface plasmon resonance allows the analysis of antigen-antibody interactions in real time (Van Regenmortel et al., 1998). It also allows determination of the active concentration of analytes in solution (Christensen, 1997). The instrument BIAcore 2000 and the reagents for analysis were obtained from Pharmacia. The carboxylated dextran matrix (CM5) was activated with 50 μl at 10 μl/min of a mixture 0.2 M *N*-ethyl-*N*′-dimethylaminopropyl carbodiimide and 0.05 M *N*-hydroxysuccinimide. Streptavidin was immobilized with the standard Pharmacia protocol (Pharmacia Biosensor AB, 1994) at a density of 0.06 pmol/mm^2. The β_2-H19C peptide was biotinylated by reacting for 2 h at room temperature with a 5-fold molar excess in a 0.1 M $NaHCO_3$ buffer at pH 8.5 of sulfosuccinimidyl-6-(biotinamido) hexanoate (Sigma) and separated from the free biotinylating reagent on a deslating PD10 column (Pharmacia). The ligand (biotinylated β_2-H19C peptide, 1 mg/ml phosphate sodium buffer, pH 6) was immobilized on the streptavidin at a flow rate of 10 μl/min for 2 min.

Analytes (Mab6H8 and its Fab fragments at 30 nM as assessed by absorbance at 280 nm) obtained after purification were perfused at different flows, ranking from 3 to 90 μl/min, to calculate their active concentration with the mass transport limitation theory (Christensen, 1997; Richalet-Sécordel et al., 1997).

Kinetic and equilibrium parameters of the antibody-peptide interaction were studied in the following way. Increasing concentrations of Mab and Fab diluted in 150 mM NaCl, buffered with 10 mM HEPES and supplemented with 3.4 mM EDTA and 0.001% Surfactant P20 were allowed to react with the peptide during 5 min (flow of 5 μl/min). The dissociation-running buffer was allowed for 5 min at the same flow rate. The matrix CM5 was regenerated during 5 min in 3 M $MgCl_2$. Blanks were determined by studying the binding of Mab6H8 and its Fab fragments on an irrelevant biotinylated peptide immobilized on the same streptavidin sensor chip.

To study the influence of peptide immobilization on the interaction, competition studies were performed. The biotinylated peptide β_2-H19C was immobilized on the sensor surface as described in previous paragraphs. Mab6H8 (30 nM) and Fab fragments (300 nM) were mixed before injection with different concentration of free β_2-H19C. The initial linear rate of the interaction (k_0) was taken as a measure of the remaining combining sites. Percentage inhibition was calculated as $k_{0i}/k_{0a} \times 100$ in which k_{0a} was the initial rate in the absence of inhibitor and k_{0i} the initial rate in the presence of inhibitor.

Cardiomyocyte Stimulation. Rat neonatal cardiomyocytes were prepared from ventricles of 1- to 2-day-old Wistar rats by a modified method according to Halle and Wollenberger (1970). The cells were cultured as monolayers for 4 days at 37°C in SM 20-1 medium supplemented with 10% heat-inactivated calf serum and 2 μM fluorodeoxyuridine and exhibited a spontaneous basal pulsation rate of about 160 beats/min. The cardiomyocyte cultures were washed with fresh medium containing serum and incubated for 30 min at 37°C with the same medium containing 10^{-8} M arachidonic acid (Wallukat et al., 1994). The flasks were transferred to the heatable stage of an inverted microscope, and the basal beating rate was determined. Ten small circular fields (0.8 mm^2, 10 mm apart) were inspected through the perforation of a metal template: Single beating cells or clusters of synchronously beating cardiomyocytes in each of the 10 fields were selected, and the number of contractions counted for 15 s. This procedure was repeated for two to five identically treated culture flasks. The compounds to be tested were dissolved in the same medium used for the determination of the basal beating rate and incubated with the monolayers for the times indicated.

Measurement of Change of Cytosolic Calcium Ion Concentration in Isolated Guinea Pig Cardiomyocytes. Guinea pig cardiomyocytes were isolated with a collagenase/protease digestion technique described by Le Guennec et al. (1993). Isolated cardiomyocytes were incubated with 4×10^{-6} M Fura 2 acetoxymethyl ester (Fura-AM; Molecular Probes, Eugene, OR) for 1 h at room temperature in Tyrode's normal solution. The composition of this solution was 140 mM NaCl, 5.4 mM KCl, 10 mM HEPES, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.33 mM NaH_2PO_4, and 1.1 mM glucose. The pH was 7.4.

Fluorescent signals were recorded with a dual-excitation fluorescence imaging system (ION OPTIX Corporation, Milton, MA). The Fura 2-AM-loaded cells were transferred into a chamber mounted on the stage of an inverted epifluorescence microscope (model Eclipse TE300; Nikon Corporation, Tokyo, Japan). The experiments were done in these isolated loaded cells at room temperature (22–25°C). During the experiments, cells were locally superfused with Tyrode's normal solution. Excitation ultraviolet light wavelengths (340 and 380 nm) were selected with interference filters (Omega Optical, Brattleboro, VT) and a dichroic mirror, and the emitted light was filtered at 510 nm. Fluorescent signals obtained at 340 and 380 nm

were measured with a fluorescence system interface and stored in a personal computer Pentium II, 333 MHz for data processing and analysis. The analysis was done with a software video acquisition, version 4.3 (ION OPTIX Corporation). The cytosolic calcium ion concentration can theoretically be calculated from the fluorescence ratio of the two-excitation wavelengths, according to the method of Grynkiewicz et al. (1985).

To observe the activity of L-type Ca^{2+} channels all compounds, Mab6H8, and Fab were dissolved in an external solution containing 140 mM tetraethylammonium chloride, 6 mM CsCl, 10 mM HEPES, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 11 mM glucose. The pH was adjusted to 7.4 with tetraethylammonium hydroxide. These solutions were applied with a perfusion system, DAD-12 (ALA Scientific Instruments Inc., Westbury, NY). This system was equipped with temperature regulation and pressure control. All drugs, Mab6H8, and Fab were prepared daily and diluted as desired in this extracellular solution. Rabbit anti-mouse IgG from cross-links the Fab fragments was purchased from Sigma.

Results

Physicochemical Characterization of Mab6H8 and Fab Fragments. The active concentration, i.e., the concentration of antibody analytes (30 nM as assessed with the absorbance at 280 nm) able to react with the target peptide was evaluated with the Christensen method to analyze the surface plasmon resonance sensorgrams (1997). The purified MabH68 had an effective concentration of 27.8 ± 1.8 nM, and the Fab fragments of 30.3 ± 1.9 nM, confirming the purity of the reagents. The kinetic equilibrium constants were determined with the biotinylated peptide β_2-H19C immobilized on a streptavidin sensor CM5 chip in a BIAcore instrument. Concentrations from 2 to 60 nM antibody or Fab fragments were tested and evaluated with the BIAevaluation 3 module with as model Langmuir binding with mass transfer. From R_{eq} as a function of the concentration an equilibrium constant (K_A) was calculated of $1.57 \pm 0.02 \times 10^9$ M^{-1} and $1.29 \pm 0.00 \times 10^8$ M^{-1} for the Mab6H8 and the Fab fragments, respectively.

From k_{obs} as a function of the concentration with $k_{obs} = k_{on}$ [Analyte] + k_{off} for a bimolecular reaction, the k_{on} and k_{off} were calculated as 2.93×10^{-6} M^{-1} s^{-1} and $1.87 \pm 0.01 \times 10^{-3}$ s^{-1} for the 6H8 Mab and 0.89×10^6 M^{-1} s^{-1} and $6.93 \pm 0.01 \times 10^{-3}$ s^{-1} for the Fab fragments, respectively. The equilibrium constants K_A calculated as k_{on}/k_{off} were 1.6×10^9 M^{-1} and 1.3×10^8 M^{-1} for Mab6H8 and the Fab fragments, respectively.

Competition experiments with the unbiotinylated β_2-H19C peptide were performed to assess the importance of the N-terminal biotinyl function in the interaction with the formula of Cheng and Prusoff (1973):

$$K_I = (1 + K_A[A])/IC_{50} \quad (1)$$

in which K_I is the inhibition constant (M^{-1}), K_A the association constant of the analyte (Mab6H8 or Fab fragments), A the molar concentration of analyte used, and IC_{50} the molar concentration of competitor needed to inhibit at 50% the maximal response. A K_I of 3.2×10^8 M^{-1} and of 0.48×10^8 M^{-1} was calculated for the Mab 6H8 and the Fab fragments, respectively.

Cardiomyocyte Stimulation. As previously described (Lebesgue et al., 1998), Mab6H8 was able to increase the beating frequency of neonatal rat cardiomyocytes in culture. The maximal response was obtained at 15 nM. Figure 4A shows the activity of the antibody at 24 nM, which is completely abolished in the presence of the β_2-specific inverse agonist ICI 118,551. Interestingly, the maximal response obtained with the β_2-specific agonist clenbuterol is never attained with the antibody. Moreover, as illustrated in Fig. 1A, addition of clenbuterol after the antibody results in a decrease of the maximally obtained increase in beating frequency obtained with the same concentration of clenbuterol alone.

The Fab fragments as such did not show any "agonist-like" effect (Fig. 1B). When, however, they were cross-linked with a rabbit anti-mouse IgG, they showed the full capacity of the original antibody to increase the beating frequency of the cardiomyocytes. This increase was specific for the β_2-AR because it could be completely blocked by the β_2-specific inverse agonist ICI 118,551. Moreover rabbit anti-mouse IgG had no intrinsic effect.

When clenbuterol was added after the Fab fragments, the resulting increase in beating frequency was only 50% of that obtained in the absence of Fab fragments. The inhibitory activity of Mab6H8 toward the agonist activity, which was suggested in Fig. 1A, is clearly confirmed in Fig. 1B with the Fab fragments.

Figure 2A shows that the specific full agonist induces the desensitization phenomenon of the β_2-AR after 60 min. The beating frequency was practically abolished at 125 min. After the washing, the beating frequency activity was recovered only 12%. Finally, this effect was blocked by the specific β_2-antagonist.

Because it was previously shown that agonist-like autoan-

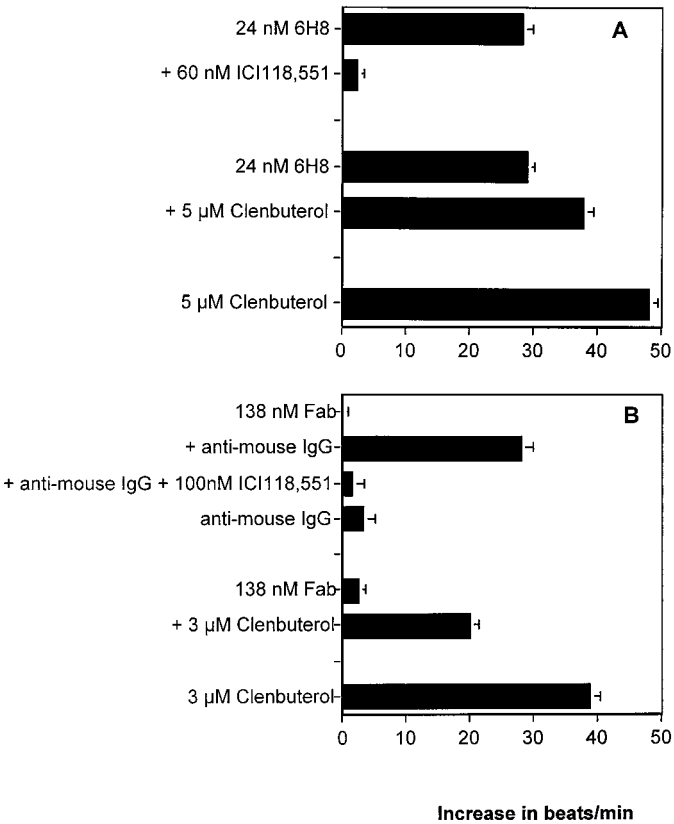

Fig. 1. Chronotropic effect of cardiomyocytes in culture in presence of Mab6H8 (A) or its Fab fragments (B) (n = 30 for every measurement).

tibodies against the β_1-AR did not induce a desensitization phenomenon (Magnusson et al., 1994), the effect of Mab6H8 on the desensitization was studied on neonatal rat cardiomyocytes. The effect of the antibody lasted for 4 h, even after renewing the medium, suggesting that the receptor-antibody complex remained stable and was able to continue to activate the transduction mechanism. Only when the inverse agonist was added could the receptor activation be inhibited, pointing toward the dissociation of the receptor-antibody complex.

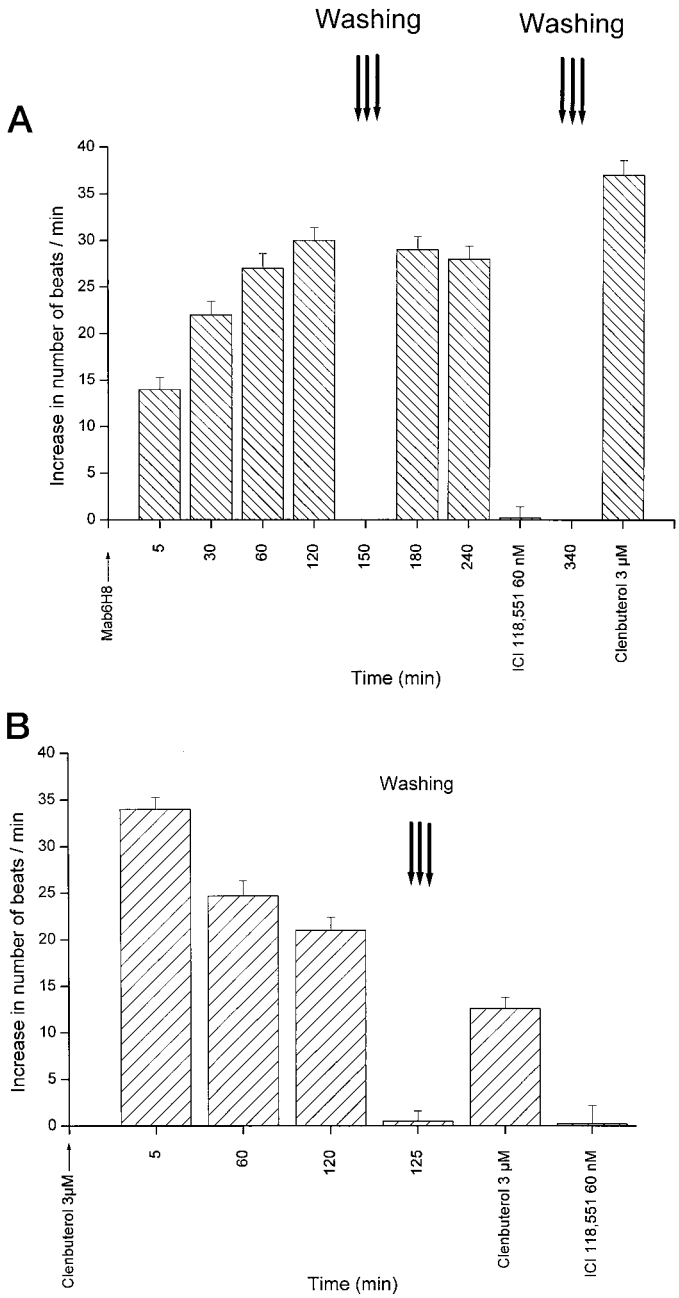

Fig. 2. A, desensitization effect of clenbuterol (3 μM) was observed after 60 min. The cells recovered partially their activity after washing. B, lack of receptor desensitization after activation with 24 nM Mab6H8. After 150 min of presence of antibody, cells were washed with new medium. The activity remained until the ICI 118,551 was added. Washing the cells and adding 1 μM clenbuterol resulted in maximal stimulation. The arrows indicate three times of washing with fresh medium at 37°C. Values are mean ± S.E. In A, $n = 30$ in each experimental condition and in B, $n = 38$, only when ICI 118,551 was applied, $n = 18$.

Finally, addition of the full agonist clenbuterol led to a maximally stimulatory effect confirming that the receptor was not desensitized (Fig. 2B).

Change of Cytosolic Calcium Ion Concentration in Guinea Pig Cardiomyocytes. To study the biological effect of Mab6H8, Fab, and specific agonists, loaded Fura-AM isolated guinea pig cardiomyocytes were used in a dual-excitation fluorescence imaging system. As shown in Table 1 and illustrated in Fig. 3A, clenbuterol (10 nM) induced a change in the cytosolic calcium ion concentration, about 30 nM from basal level. A comparable increase was observed with Mab6H8 (16 nM; Fig. 3, A and B). The perfusion with the Fab fragments (300 nM) did not modify the basal calcium ion concentration (Fig. 4 and Table 1). However, Fab application prevents Ca^{2+} channel activation by Mab6H8 and clenbuterol, as shown in Fig. 4, A and B. However, if the Fab fragments (300 nM) were perfused during 5 min and a rabbit anti-mouse IgG (at 1.5 μM) was applied subsequently, an elevation of the cytosolic calcium ion concentration could be determined (Fig. 4C). The increase in Ca^{2+} concentration was similar to that found in presence of clenbuterol or Mab6H8 (Table 1). Higher doses of clenbuterol (3 μM) could partially restore Ca^{2+} channel activation (Fig. 4D and Table 1). However, this activation is not comparable when we applied the higher doses of clenbuterol without previous perfusion of Fab (Table 1).

Using a paired two-tailed t test, the basal calcium ion increase of clenbuterol 10 nM (30 ± 11, $n = 7$) versus clenbuterol 3 μM (31 ± 9, $n = 5$) and clenbuterol 10 nM versus Mab6H8 16 nM is not significantly different ($P > .05$). When we compared clenbuterol 10 nM (30 ± 11, $n = 7$) versus Fab 300 nM + clenbuterol 10 nM (-2 ± 1, $n = 4$); clenbuterol 3 μM (31 ± 9, $n = 5$) versus Fab 300 nM + clenbuterol 3 μM (11 ± 6, $n = 6$); Mab6H8 16 nM (23 ± 11) versus Fab 300 nM (-2 ± 2, $n = 5$) + Mab6H8 16 nM (-3 ± 3, $n = 4$); and Fab 300 nM (-2 ± 2, $n = 5$) versus Fab 300 nM (-2 ± 2, $n = 5$) + IgG 1.5 μM (47 ± 17, $n = 5$), they were statistically different with the values of $P = .0112$, .0393, .0025, .002, and .004, respectively.

Also, some cells were perfused with lower concentrations of Fab (25 or 50 nM, $n = 4$, Table 2); we cannot observe fluorescence increase when Mab6H8 was applied sequentially. These concentrations were able to inhibit the antibody fixation. The rise of calcium ion increase induced by Mab6H8 was not abolished by an application of a concentration of 5 nM fragments ($n = 3$, Table 2).

We applied the same statistical test to the results obtained in the dose-response of the Fab applications. When we compared Mab6H8 16 nM (23 ± 11, $n = 7$) versus Fab 300 nM

TABLE 1
Statistical analysis of fluorescence increase: effects of the applications of Mab6H8, Fab, clenbuterol, and rabbit anti-mouse IgG
The data presented are mean and S.D.

Experimental Condition	ΔF	n
Clenbuterol 10 nM	30 ± 11	7
Clenbuterol 3 μM	31 ± 9	5
Mab6H8 16 nM	23 ± 11	7
Fab 300 nM	−2 ± 2	5
Fab 300 nM + Mab6H8 16 nM	−3 ± 3	4
Fab 300 nM + clenbuterol 10 nM	−2 ± 1	4
Fab 300 nM + clenbuterol 3 μM	11 ± 6	6
Fab 300 nM + IgG 1.5 μM	47 ± 17	5

(-2 ± 4, $n = 4$) + Mab6H8 16 nM (-2 ± 4, $n = 4$); Mab6H8 16 nM (23 ± 11, $n = 7$) versus Fab 100 nM (-3 ± 3, $n = 4$) + Mab6H8 16 nM (-3 ± 3, $n = 4$); Mab6H8 16 nM (23 ± 11, $n = 7$) versus Fab 50 nM (-1 ± 2, $n = 4$) + Mab6H8 16 nM (-1 ± 2, $n = 4$); and Mab6H8 16 nM (23 ± 11, $n = 7$) versus Fab 25 nM (-2 ± 2, $n = 5$) + Mab6H8 16 nM (-3 ± 5, $n = 4$), they are statistically different with P values of .004, .007, .0093, and .0196, respectively. However, when we examined Mab6H8 16 nM (23 ± 11, $n = 7$) versus Fab 5 nM (20 ± 6, $n = 3$) + Mab6H8 16 nM (-3 ± 3, $n = 4$), they were not significantly different ($P > .05$).

Discussion

Antibodies are excellent reagents to localize receptor proteins at the microscopic and the electron microscopic levels. They are however of little use as pharmacological reagents for several reasons: 1) most of the antibodies raised against the receptor protein or against peptide sequences do not interfere with the function of the receptors; 2) polyclonal antibodies, even with functional properties are, by definition, directed against different epitopes on the target protein and interact with different affinities; and 3) even monoclonal antibodies, although monospecific generally recognize conformational epitopes, making identification of the target domains on the receptor difficult. Because we have obtained a monoclonal antibody with "agonist-like" activity, specifically directed toward a sequential epitope (amino acids 173–180 Trp-Tyr-Arg-Ala-Thr-His-Gln-Glu) of the β_2-AR (Lebesgue et al., 1998), it was worthwhile to investigate this antibody as a pharmacological tool and, more specifically, to investigate the importance of dimerization for receptor activity. The sequence 173 to 179 is conserved in the β_2-AR of all species studied, thus the antibody could be used in in vitro models, with cardiomyocytes from different species.

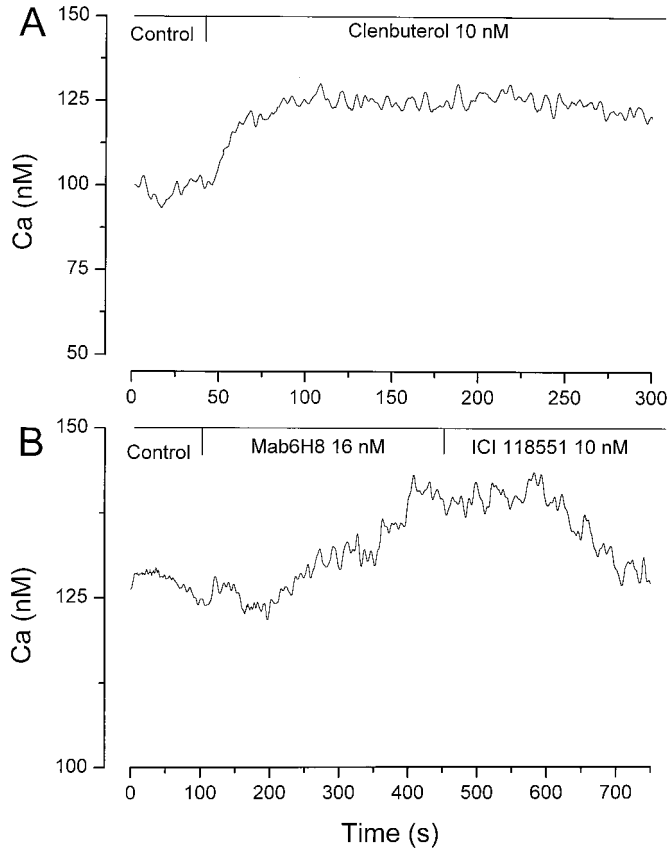

Fig. 3. β_2-Adrenergic response of cardiac cells. A, time course of calcium ion concentration in guinea pig cardiomyocytes under control conditions and after the application of clenbuterol (10 nM). B, time course of calcium ion concentration in cardiac cells under control conditions and after successive applications of affinity purified monoclonal antibody Mab6H8 (16 10 nM) and the inverse agonist ICI 118,551 (10 nM).

A prerequisite for the comparison of the bivalent monoclonal antibody with its monovalent Fab fragments is a complete analysis of the physicochemical parameters (active concentration, kinetic, and equilibrium constants) of the two reagents to use them at equivalent concentrations. Surface plasmon resonance is the only tool available to determine these parameters. Active concentrations for both the monoclonal antibodies and the Fab fragments were similar, approaching 100%. The affinity of the Fab fragments was however approximately 10 times less than that of the bivalent antibody. This loss in affinity is due to a 3-fold decrease in both the association rate and the dissociation rate constant and corresponds to what has previously been described for other antigen-antibody reactions (Chatellier et al., 1996). Competition experiments showed the same difference between antibody and Fab fragments but the absolute affinity was 5 times lower. This is probably due to the higher flexibility of the peptide in solution compared with the restricted structure of the biotinylated peptide immobilized on the chip. For further physiological studies, Fab fragments and monoclonal antibody were used in excess of the calculated affinity for the free peptide to saturate the receptors.

From the experiments on neonatal rat cardiomyocytes, two conclusions can be drawn. The dimeric nature of the antibody is essential for agonist-like activity because Fab fragments only induced a positive chronotropic effect when cross-linked with a rabbit anti-mouse IgG. However, both the monoclonal antibody and, even more accentuated, the Fab fragments inhibited the full agonist activity of clenbuterol. These results suggest that the antibody has a dual function: it induces the production of an active dimer, confirming the importance of such structures for transducing the signal, and it also induces a conformational change in the monomeric receptor, altering the accessibility of the pharmacophore pocket for the full agonist. Indeed, the size of the antibody and its Fab fragments make it unlikely that they compete for the pharmocophore pocket buried into the lipid bilayer. Antibodies have thus an allosteric effect on the ligand-binding activity of the agonists.

These results were confirmed at the single-cell level with activation of L-type Ca^{2+} channels by protein kinase A (PKA) as marker of receptor activation (Lebesgue et al., 1998). The monoclonal antibody induced Ca^{2+} channel activation mediated by the β_2-AR because it was inhibited by ICI 118,551. Fab fragments did not have this effect but blocked the antibody activity by competition for the same epitope. Cross-linking of Fab fragments had the same effect as the monoclonal antibody, again pointing toward dimerization as an essential process. The Fab fragments also were completely inhibiting clenbuterol (10 nM) activation of the β_2-AR, suggesting that the high-affinity binding site of the agonist is no more accessible in the pharmacophore pocket. Because clenbuterol at high concentrations partially overcame this inhibition, the conformational change in the pharmacophore

pocket seems not to be an all-or-none phenomenon, tolerating a shift toward the full agonist conformation at high clenbuterol concentrations.

Our results can be interpreted by the existence of three conformers of the β_2-AR. The first conformer corresponds to the monomeric resting state, specifically recognized by the inverse agonist in which the amino acids 173 to 179 are no longer accessible for antibody recognition. The second conformer is also in the monomeric form and is induced by recognition with the antibody-combining site. This conformer has lost the capacity to bind agonists at low concentrations as assessed by the inhibition of clenbuterol activation. When this conformer is dimerized by cross-linking with the bivalent Mab or rabbit anti-mouse IgG, it activates the transduction mechanism but never to the full extent of a real agonist. This dimer has also the property not to be sensitive to desensitization. Because the antibody activates the Ca^{2+} channels through PKA phosphorylation (Lebesgue et al., 1998), the hypothesis can be considered that the PKA phosphorylation sites on the receptor responsible for heterologuous desensitization (Clark et al., 1988) are not accessible to the enzyme in the antibody-induced conformer. Finally, full agonists induce a third conformation activating the transduction pathway with a maximal efficiency, leading to desensitization. Recognition by an antibody combining site directed to the first amino acid residues linking the fourth transmembrane domain to the fifth transmembrane domain, thus resulting in a receptor conformation was until now not suspected (Gouldson et al., 1998). The dimerization normally obtained in

TABLE 2
Statistical analysis of fluorescence increase: dose-response of Fab applications
The data presented are mean and S.D.

Experimental Condition	ΔF	n
Fab 300 nM + Mab6H8 16 nM	−2 ± 4	4
Fab 100 nM + Mab6H8 16 nM	−3 ± 3	4
Fab 50 nM + Mab6H8 16 nM	−1 ± 2	4
Fab 25 nM + Mab6H8 16 nM	−3 ± 5	4
Fab 5 nM + Mab6H8 16 nM	20 ± 6	3

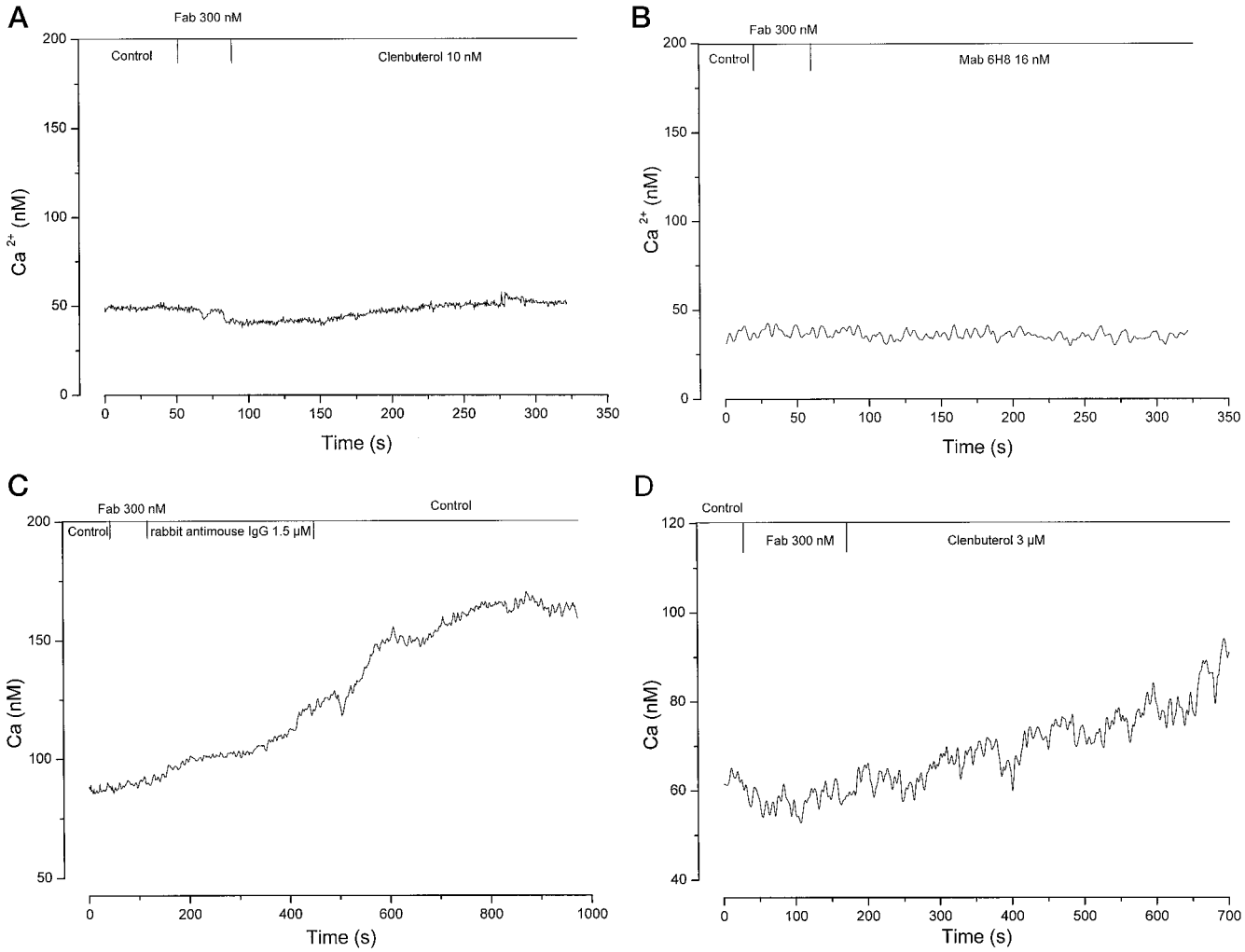

Fig. 4. Effects of Fab on the β_2-adrenergic response of cardiac cells. A, time course of calcium ion concentration in guinea pig cardiomyocytes under control conditions and after the successive applications of Fab (300 nM) and clenbuterol (10 nM). B, time course of calcium ion concentration in guinea pig cardiomyocytes under control conditions and after successive applications of Fab (300 nM) and affinity purified monoclonal antibody Mab6H8 (16 nM). C, time course of calcium ion concentration in guinea pig cardiomyocytes under control conditions and after successive applications of Fab (300 nM), IgG (1.5 μM), and finally under control conditions. D, time course of calcium ion concentration in guinea pig cardiomyocytes under control conditions and after successive applications of Fab (300 nM) and clenbuterol (3 μM). In all experiments the applications of Fab (300 nM) correspond to 5 min of perfusion.

transfected cells hyperexpressing the receptor is also important in cells that express the receptor under normal conditions, such as cardiomyocytes. The structure of the antigen-antibody complex obtained by modeling or by crystallographic analysis could thus lead to new subtype-specific peptide or peptidomimetic antagonists, with monomers, or to a new class of agonists, with such molecules in the dimeric form.

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