

## Modulation of the M2 Muscarinic Acetylcholine Receptor Activity with Monoclonal Anti-M2 Receptor Antibody Fragments\*

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Jean-Christophe Peter‡§, Gerd Wallukat¶, Jean Tugler||, Damien Maurice||,  
Jean-Christophe Roegel||, Jean-Paul Briand‡, and Johan Hoebeke‡\*\*

From the ‡CNRS, Unité Propre de Recherche 9021, Institut de Biologie Moléculaire et Cellulaire, Laboratory of Therapeutical Chemistry and Immunology, F-67084 Strasbourg, France, ¶Max Delbrück Centrum for Molecular Medicine, D-13092 Berlin, Germany, and ||Forenap Therapeutic Discovery, Institute of Pharmacology, School of Medicine, F-67000 Strasbourg, France

**Antibodies directed against the second extracellular loop of G protein-coupled receptors are known to have functional activities. From a partial agonist monoclonal antibody directed against the M2 muscarinic receptor, we constructed and produced a single chain variable fragment with high affinity for its target epitope. The fragment is able to recognize its receptor on Chinese hamster ovary cells transfected with the M2 muscarinic acetylcholine receptor to block the effect of carbachol on this receptor and to exert an inverse agonist activity on the basal activity of the receptor. The antibody fragment is also able to increase the basal rhythm of cultured neonatal rat cardiomyocytes and to inhibit in a non-competitive manner the negative chronotropic effect of carbachol. This antibody fragment is able to exert its inverse agonist activity *in vivo* on mouse heart activity. The immunological strategy presented here could be useful to develop specific allosteric inverse agonist reagents for G protein-coupled receptors.**

The G protein-coupled receptor family is one of the main targets of currently used drugs (1). The M2 muscarinic acetylcholine receptor (M2ACh-R)<sup>1</sup> belongs to this receptor family. This receptor is an integral membrane protein consisting of seven membrane spanning  $\alpha$ -helices linked together by extra- and intracellular loops that form a pharmacophore pocket. One of the pharmacological challenges posed by this family of receptors is the presence of multiple subtypes, all recognizing the same endogenous ligands. This suggests a high conservation of the pharmacophore for a particular family of receptors, thus explaining the difficulty to develop drugs (agonists or antagonists) specific for one of these subtypes. Autoantibodies directed against cardiovascular G protein-coupled receptors functionally interfering with the target have been described in

several cardiovascular diseases (2–6). Most of these autoantibodies are directed against the second extracellular loop and are exquisitely specific for one of the receptor subtypes in view of the highly variable sequence of this domain (7). Elies *et al.* (8) have produced a monoclonal antibody directed against a peptide derived from the N-terminal part of the second extracellular loop of the M2ACh-R. This monoclonal antibody (IgG 2a) displays a partial agonist activity on its target receptor because of its natural bivalency. It is able to stabilize constitutive active receptor dimers and paradoxically induces a small decrease in carbachol affinity for the M2ACh-R.

In this study, this antibody was used to construct an scFv fragment (single chain variable fragment), which was cloned, sequenced and expressed in *Escherichia coli*. We describe the immunochemical, pharmacological, and physiological properties of this scFv fragment. As reported in a previous study with an inverse agonist scFv-directed against the  $\beta_2$  adrenergic receptor (9) this may open the way to the design of allosteric inverse agonist reagents that are specific for one subtype of receptor.

### EXPERIMENTAL PROCEDURES

**Peptides**—M2-G19K (GVRTVEDGECYIQFFSNA(K)) peptide and its truncated version V9C (VRTVEDGEC) corresponding to the second extracellular loop (residues 167–184) of the human M2ACh-R were synthesized using *N*-(9-fluorenyl)methoxycarbonyl (Fmoc) chemistry with an automated peptide synthesizer (10). The peptides were purified by high pressure liquid chromatography and their integrity assessed by matrix-assisted laser desorption ionization time-of-flight spectrometry.

**Construction of the Single-chain Antibody Gene**—The single-chain antibody gene fragment encoding the heavy and light variable region of the monoclonal antibody B8E5 (8) was prepared as described in Ref. 9.

scFv B8E5 was created by joining the B8E5 V<sub>H</sub> and V<sub>L</sub> genes together by PCR splicing with overlap extensions using oligonucleotides that encoded a 15-amino-acid linker (G<sub>4</sub>S)<sub>3</sub> between the C terminus of the V<sub>H</sub> and the N terminus of the V<sub>L</sub> gene. The ends of the variable gene were modified by PCR using as primers, V<sub>H</sub>Rev (5'-GGT GCA GCT GCA GCA GTC TGG GGG AGA CT-3'), which encodes the N-terminal wild type sequence of the V<sub>H</sub> containing a PstI site, and V<sub>H</sub>For (5'-ACC ACC GGA TCC GCC TCC GCC TGA GGA GAC TGT GAG CGT-3'), which encodes the C terminus of the VH and a part of the linker. V<sub>L</sub>Rev (5'-GGA GGC GGA TCC GGT GGT GGC GGA TCT GGA GGT GGC GGA AGC GAT ATT GTG ATG GCC CA-3') and V<sub>L</sub>For, containing a XhoI site that encodes His<sub>6</sub> residues (5'-GCA ATT CCT CGA GTT AGT GAT GGT GAT GGT GAT GTT TGA TCT CCA GCT TGG TGC C-3'), were used to amplify and modify the V<sub>L</sub> domain. The scFv gene was inserted in frame with the PelB sequence on the expression vector pSW1 (11) between the PstI and XhoI sites. The constructed vector pSW1-6H8 His<sub>6</sub> was cloned in HB 2151 *E. coli* strain.

**Bacterial Expression of scFv-B8E5**—The bacterial expression of the recombinant scFv protein and extraction of soluble periplasmic protein are described in Ref. 12. The periplasmic extract was centrifuged at 10,000  $\times$  g, and the supernatant was filtered on a 0.45- $\mu$ m membrane (Millipore) and extensively dialyzed against PBS (20 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, 2.7 mM KCl, pH 7.4).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ746180.

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\*\* To whom correspondence should be addressed: UPR 9021 CNRS "Immunologie et Chimie Thérapeutiques," IBMC, 15, rue René Descartes, F-67084 Strasbourg, France. Tel.: 33-3-88-41-70-24; Fax: 33-3-88-61-06-80; E-mail: j.hoebeke@ibmc.u-strasbg.fr.

<sup>1</sup> The abbreviations used are: M2ACh-R, M2 muscarinic acetylcholine receptor; scFv, single chain variable fragment; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; ECG, electrocardiogram.

TABLE I

Plan of intravenous treatment of 12 mice for the study of the scFv activity on their heart function via the M2ACh-R

12 mice were treated with six different treatments on seven days (J1–J7). Three groups of four mice were treated every day at the same hour. They were treated with 100  $\mu$ L of scFv B8E5 (10  $\mu$ M) alone or in combination with carbachol (0.1 mg/kg), isoproterenol (2 mg/kg), or with 100  $\mu$ L of carbachol, isoproterenol, and 0.8% NaCl.

mouse n°1	mouse n°2	mouse n°3	mouse n°4	
Carbachol	scFv + Isoproterenol	scFv	Isoproterenol	J1
NaCl	Carbachol	scFv + Isoproterenol	scFv	J2
scFv + Carbachol	NaCl	Carbachol	scFv + Isoproterenol	J3
	scFv + Carbachol	NaCl	Carbachol	J4
Isoproterenol		scFv + Carbachol	NaCl	J5
scFv	Isoproterenol		scFv + Carbachol	J6
scFv + Isoproterenol	scFv	Isoproterenol		J7

mouse n°5	mouse n°6	mouse n°7	mouse n°8	
	scFv + Carbachol	NaCl	Carbachol	J1
Isoproterenol		scFv + Carbachol	NaCl	J2
scFv	Isoproterenol		scFv + Carbachol	J3
scFv + Isoproterenol	scFv	Isoproterenol		J4
Carbachol	scFv + Isoproterenol	scFv	Isoproterenol	J5
NaCl	Carbachol	scFv + Isoproterenol	scFv	J6
scFv + Carbachol	NaCl	Carbachol	scFv + Isoproterenol	J7

mouse n°9	mouse n°10	mouse n°11	mouse n°12	
scFv + Isoproterenol	scFv	Isoproterenol		J1
Carbachol	scFv + Isoproterenol	scFv	Isoproterenol	J2
NaCl	Carbachol	scFv + Isoproterenol	scFv	J3
scFv + Carbachol	NaCl	Carbachol	scFv + Isoproterenol	J4
	scFv + Carbachol	NaCl	Carbachol	J5
Isoproterenol		scFv + Carbachol	NaCl	J6
scFv	Isoproterenol		scFv + Carbachol	J7

**Production and Purification of Fab Fragments**—Monoclonal antibody B8E5 (8) was purified from ascitic fluids on an affinity column made by coupling G19K peptide to CNBr-activated Sepharose by a standard procedure (Amersham Biosciences); the ascitic fluids (2 ml) were diluted 1:20 in PBS, and the antibody, adsorbed on the affinity column in PBS, was eluted with 0.2 M glycine, pH 2.7, and immediately dialyzed against PBS.

Fab fragments were obtained by papain (Sigma) digestion (13) of the affinity purified monoclonal antibody B8E5. The Fab fragments were separated from the Fc fragments on a Hi-Trap protein A column with a Gradi-Frac system (Amersham Biosciences) followed by a second affinity chromatography purification. Purity was checked by SDS-polyacrylamide gel electrophoresis. No unhydrolyzed IgG could be detected.

**Purification of the scFv Construction**—The scFv recombinant antibody fragment was purified from periplasmic extract on a protein-L immobilized Sepharose (Pierce) 1-ml column, and the adsorbed recombinant proteins were eluted with 0.2 M glycine, pH 2.7, and immediately dialyzed against PBS.

**Gel Electrophoresis and Western Blot Analysis**—SDS-PAGE analysis was performed as a standard procedure using 12.5% acrylamide gels followed by staining with Coomassie Brilliant Blue (Serva) or immunoblotting. For Western blot analysis, the proteins were transferred from

the gels onto a PROTRAN Nitrocellulose Transfer membrane (Schleicher & Schuell) using a mini trans-blot system (Bio-Rad) in transfer buffer (25 mM Tris-HCl, 190 mM glycine, 20% methanol, pH 8.3). The membranes were soaked for 1 h in PBS-T (20 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, 2.7 mM KCl, pH 7.4) supplemented with 5% nonfat milk powder and 0.1% Tween 20. This was followed by a 1-h incubation with anti-His tag antibody conjugated to horseradish peroxidase (HRP 1/2000) (Sigma). The antibody was diluted in the blocking solution PBS-T milk. The proteins on the membranes were revealed by the classical procedure of the ECL reagents (Amersham Biosciences).

**Immunochemical Characterization of the scFv Fragment**—The instrument Biacore 3000 and all the reagents for analysis were obtained from Biacore (Uppsala, Sweden). The carboxylated dextran matrix (CM5) was activated with 50  $\mu$ l at 5  $\mu$ l/min of a mixture 0.2 M *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide and 0.05 M *N*-hydroxysuccinimide. For kinetic analysis the G19K peptide was immobilized with the standard Biacore protocol. Kinetic studies were performed as described (14).

Inhibition assays were performed using Biacore technology; the same concentration of scFv B8E5 was preincubated with or without different concentration of V9C or G19K peptides for 30 min and then injected on the sensor chip with high density of V9C peptides immobilized by its C terminus end with standard Biacore procedure. Under this mass trans-

PstI

1 CAG GTG CAG CTG CAG CAG TCT GGG GGA GAC TTA GTG AAG CCT GGA GGG TCC 51  
 Q V Q L Q Q S G G D L V K P G G S

52 CTG AAA GTC TCC TGT GCA GGC TCT GGA TTC ACT TTC AGT AGC TAT GGC ATG 102  
 L K V S C A A S G F T F S S Y G M  
 CDR H1

103 TCT TGG GTT CGC CAG ACT CCA GAC AAG AGG CTG GAG TGG GTC GCA ACC ATT 153  
S W V R Q T P D K R L E W V A T I

154 ACT AGT GGT GGT AGT TAC ACC TAC TAT CCA GAC AGT GTG AAG GGG CGA TTC 204  
T S G G S Y T Y Y P D S V K G R F  
 CDR H2

205 ACC ATC TCC AGA GAC AAT GCC AAG AAC ACC CTG TAT CTG CAA ATG AGC AGT 255  
 T I S R D N A K N T L Y L Q M S S

256 CTG AAG TCT GAG GAC ACA GGC ATG TAT TAC TGT GCA AGA CAC ATC AAC TAT 306  
 L K S E D T A M Y Y C A R H I N Y

307 AGG TAT GAC GGG GCC TTT GAC TAC TGG GGC CAA GGC ACC ACG CTC ACA GTC 357  
R Y D G A F D Y W G Q G T T L T V  
 CDR H3

358 TCC TCA GGC GGA GGC GGA TCC GGT GTG GCG GAT CTG GAG GTG GCG GAA GCG 408  
 S S G G G G S G G G G S G G G S

409 ATA TTG TGA TGG CGC AGT CTC CAT CCT CCC TGA GTG TGT CAG CAG GAG AGA 459  
 D I V M A Q S P S S L S V S A G E

460 AGG TCA TTA TGA GCT GCA AGT CCA GTC AGA GTC TGT TAA ACA GTA GAA ATC 510  
 K V I M S C K S S Q S L L N S R N  
 CDR L1

511 AAA AGA ACT ACT TGG CCT GGT ACC AGC AGA AAC CAG GGC AGT CTC CTA AAC 561  
Q K N Y L A W Y Q Q K P G Q S P K

562 TGT TGA TCT ACG GGG CAT CCA CTA GGG AAT CTG GGG TCC CTG ATC GCT TCA 612  
 L L I Y G A S T R E S G V P D R F  
 CDR L2

613 CAG GCA GTG GAT CTG GAA CCG ATT TCA CTC TTA CCA TCA GCA GTG TGC AGG 663  
 T G S G S G T D F T L T I S S V Q

664 CTG AAG ACC TGG CAG TTT ATT ACT GTC AGA ATG ATC ATA GTT ATC CGC TCA 714  
 A E D L A V Y Y C Q N D H S Y P L  
 CDR L3

715 CGT TCG GTG CTG GGA CCA AGC TGG AGA TCA AAC ATC ACC ATC ACC ATC ACT 765  
T F G A G T K L E I K H H H H H

Xho I

766 TAA CTC GAG  
 \*

FIG. 1. Nucleotide (*light gray*) and amino acid (*black*) deduced sequences of the scFv B8E5. The two restriction sites PstI and XhoI used for cloning are represented in *gray bold characters*. The complementary determining region of the B8E5 antibody according to the Kabat numbering are *underlined*, and the (G<sub>4</sub>S)<sub>3</sub> linker between the heavy and light variable domain is represented in *italic bold characters*. (GenBank™ accession number AJ746180).

fer condition, the slope of the curves is directly correlated with the active concentration of scFv (15).

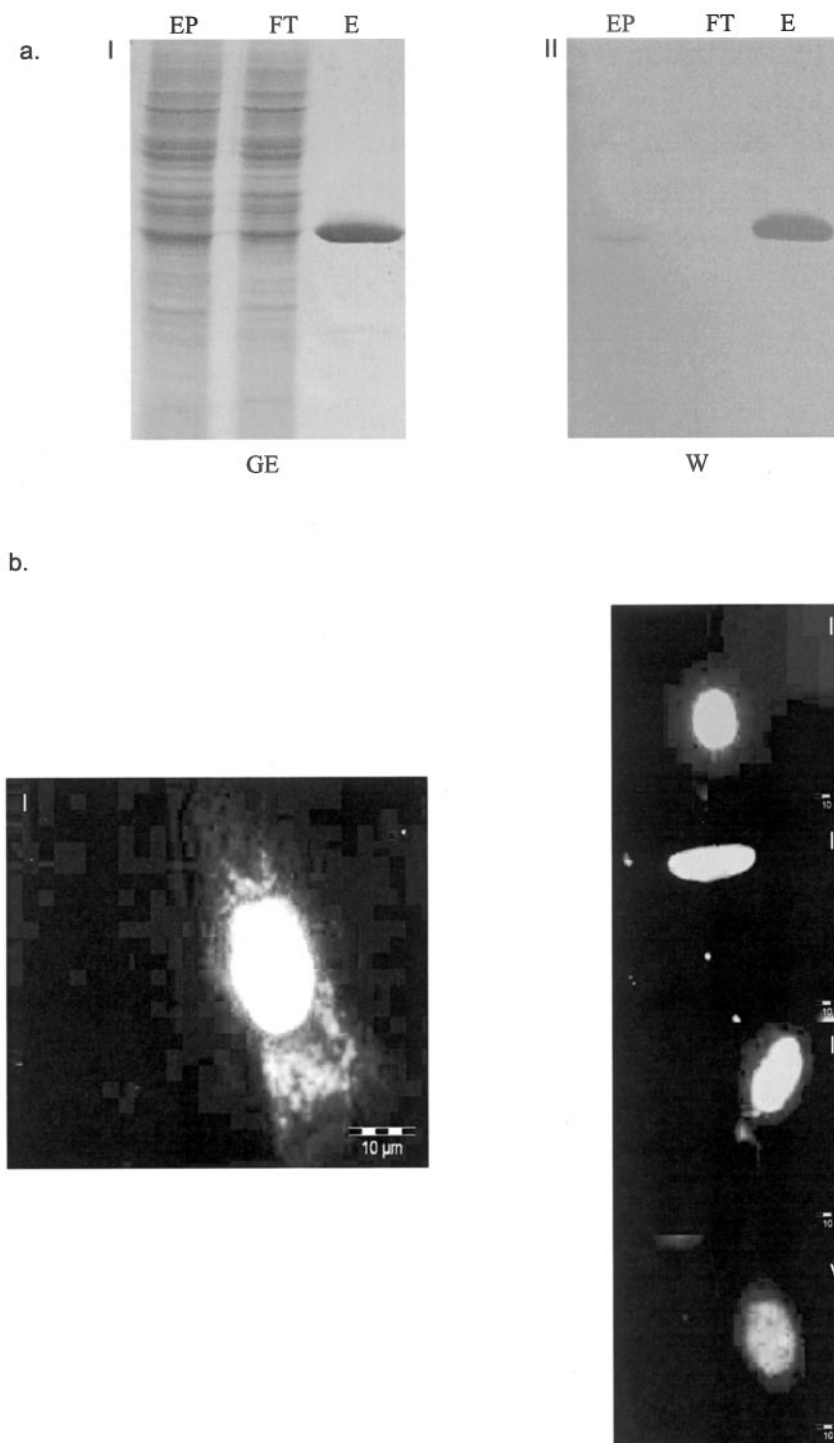
**Protein Concentration Determination**—The total protein concentration of the purified scFv (active + non-active proteins) was determined using the BCA kit (Pierce) and by measuring absorbance at 280 nm. The extinction coefficient was determined using the ExPASy program tool available on the Web.<sup>2</sup>

**Immunocytochemistry**—CHO human M2ACh-R, M1ACh-R, and M3ACh-R transfected cells (16) (kindly provided by Dr. M Waelbroeck) were fixed for 5 min with 2% paraformaldehyde and permeabilized with PBS and 0.1% Triton X-100 for 1 min. Slides were saturated with PBS supplemented with 5% nonfat dry milk. ScFv B8E5 and scFv 9C2 (17) (control) were incubated for 1 h at room temperature 4 °C. After three washes with PBS, biotinylated protein-L (1/500, Pierce) was added and incubated for 1 h at room temperature. Alexa-conjugated Streptavidin (1/500, Molecular Probes, Junction City, OR) was allowed to react with

the fixed primary antibody for 1 h at room temperature. 4',6-Diamidino-2-phenylindole (1 μg/ml, Sigma) was used for nuclear staining.

**Pharmacological Characterization of the scFv Fragment; cAMP Response by M2ACh-R CHO Transfected Cells Treated in Vitro with scFv**—The biochemical effects of scFv B8E5 on the M2ACh-R were assessed by measuring the intracellular cAMP concentration of M2ACh-R CHO transfected cells. Cells were seeded in 6-well culture plates 24 h before stimulation and then washed and incubated with 1 ml of Hank's balanced medium buffered with 10 mM HEPES containing 100 μM of isobutylmethylxanthine to block cAMP hydrolysis. After 30 min, scFv was added at different concentrations, and 30 min after the first treatment forskolin and carbachol were added to a final concentration of 1 μM and 30 nM, respectively. Stimulation of the cells was performed during the first 15 min, and then the supernatant was aspirated and the reaction was stopped by adding 1 ml of boiling water. The cAMP content was determined using a competitive immunoenzymatic assay (Biotrak cAMP, Amersham Biosciences). The protein concentrations of the samples were determined using a BCA kit (Pierce). The concentra-

<sup>2</sup> Personal communication, [www.expasy.org/tools/protparam.html](http://www.expasy.org/tools/protparam.html).



**FIG. 2. Immunological characterization of scFv B8E5.** *a*, purification of the scFv B8E5 by protein L affinity chromatography. *GE* corresponds to a SDS-PAGE electrophoresis stained with Coomassie Blue, and *W* corresponds to the Western blot (see "Experimental Procedures") of the periplasmic extract (*EP*), the flow through (*FT*), and the elution (*E*) of the purified scFv protein. *b*, immunocytochemistry on CHO cells transfected or not with different subtypes of MACH-R. The panel shows a nuclear 4,6-diamidino-2-phenylindole labeling (*white*) merged with an immune labeling of the same cells (*gray*). A specific membranar and cytoplasmic fluorescence of the CHO M2ACh-R transfected cells incubated with the scFv B8E5 can be seen (*I*). No labeling was observed with untransfected CHO cells (*II*), the CHO transfected by M1ACh-R (*III*), the CHO with the M3ACh-R (*IV*), and with CHO M2ACh-R transfected cells treated with a control scFv (*V*).

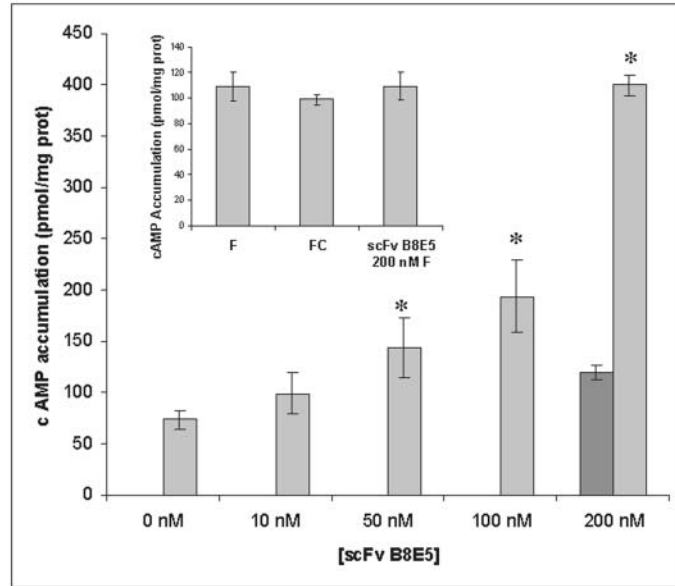
tion of cAMP was reported on the protein concentration related to the number of cells/well; the results were expressed as pmol cAMP/mg protein. Results are from duplicates of three independent experiments.

**Beating Frequency of Neonatal Rat Cardiomyocytes in Culture**—Rat neonatal cardiomyocytes were prepared from ventricles of 1–2 day-old Sprague-Dawley rats using a modified method (18). The cells were cultured as monolayers for 8 days at 37 °C in SM 20–1 medium (32) supplemented with 10% heat-inactivated calf serum and 2 μM fluorodeoxyuridine and exhibited a spontaneous basal pulsation rate of 160 ± 20 beats/min. The cardiomyocyte cultures were washed with fresh medium containing serum and incubated for 2 h at 37 °C with the same medium under stationary conditions. The flasks were transferred to the heatable stage of an inverted microscope, reagent was added, and the increase of the beating rate was determined for at least 10 observations for each experimental point in three independent experiments.

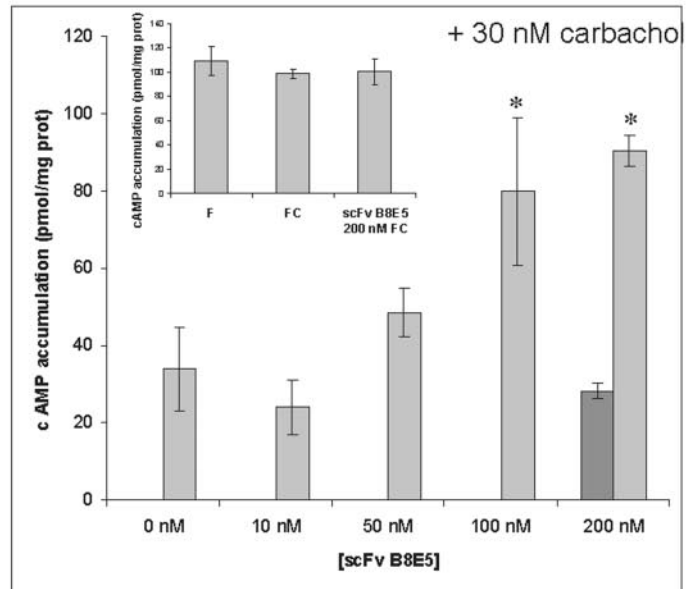
**Physiological Characterization of the scFv Fragment in Vivo on Mice**—Twelve male BALB/c mice (20 g each) were given tail intravenous injections while being kept in individual mouse ECG boxes as described in Ref. 19 for four continuous weeks. The ECG boxes (Rodent Restrainers for ECG, Sesams, Logelbach, France) have four conductive surfaces on their bottom to position the four limbs of the conscious animal. These surfaces are coated with contact gel and connected to analogue amplifiers for signal amplification and filtering (0.5–30 Hz cutoff frequencies; Bessel type filter, order 2; 50 Hz notch filter (–30 db); Sesams, Logelbach, France). The amplified and filtered signals are digitized at 256 Hz on a signal processor board (64 channels, 16-bit resolution) and stored in digital form on a personal computer using the HEM recording software (Notocord, Croissy sur Seine, France).

Three-lead electrocardiograms (DI, DII, DIII derivations) were recorded from 12 conscious mice during 60 min. Mice were not anesthe-

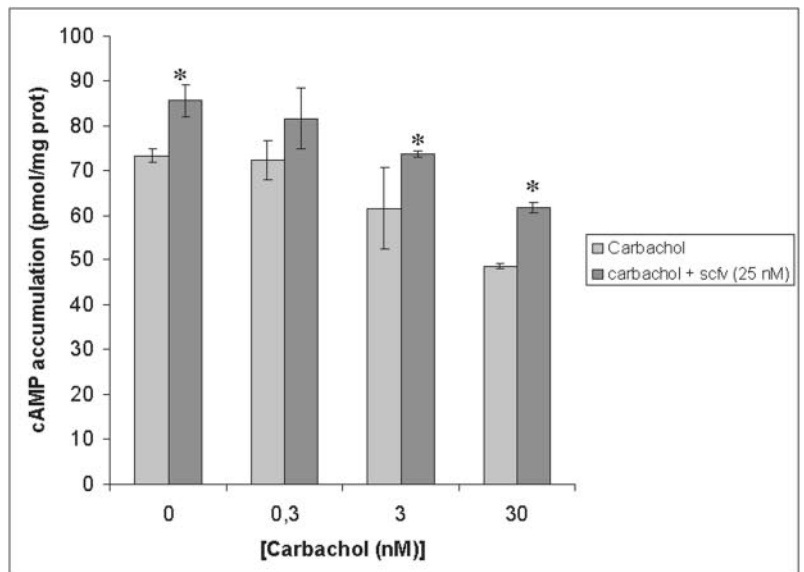
a



b



c



**FIG. 3. Biochemical effects of scFv B8E5 on the M2ACh receptor.** cAMP accumulation was measured in CHO M2ACh-R transfected cells treated with forskolin (1  $\mu$ M) and scFv B8E5 in the absence (a) and presence (b) of carbachol (30 nM) or (c) increasing concentration of carbachol (0.3–30 nM). Results are expressed in pmol/mg protein. scFv is able to increase intracellular cAMP upon forskolin stimulation and blocks cAMP decrease in carbachol/forskolin-stimulated cells in a dose-dependent manner. Control scFv 9C2 (dark gray) did not have any significant effects on cAMP accumulation with or without carbachol. The inserted histograms in a and b show stimulation of untransfected CHO cells with 1  $\mu$ M forskolin (F) and 30 nM carbachol (FC) with or without scFv. The antibody fragment exerts no effect on the cAMP level on CHO untransfected cells. Asterisks show results statistically different from experiments without scFv at  $p < 0.05$  in a Student's  $t$  test.

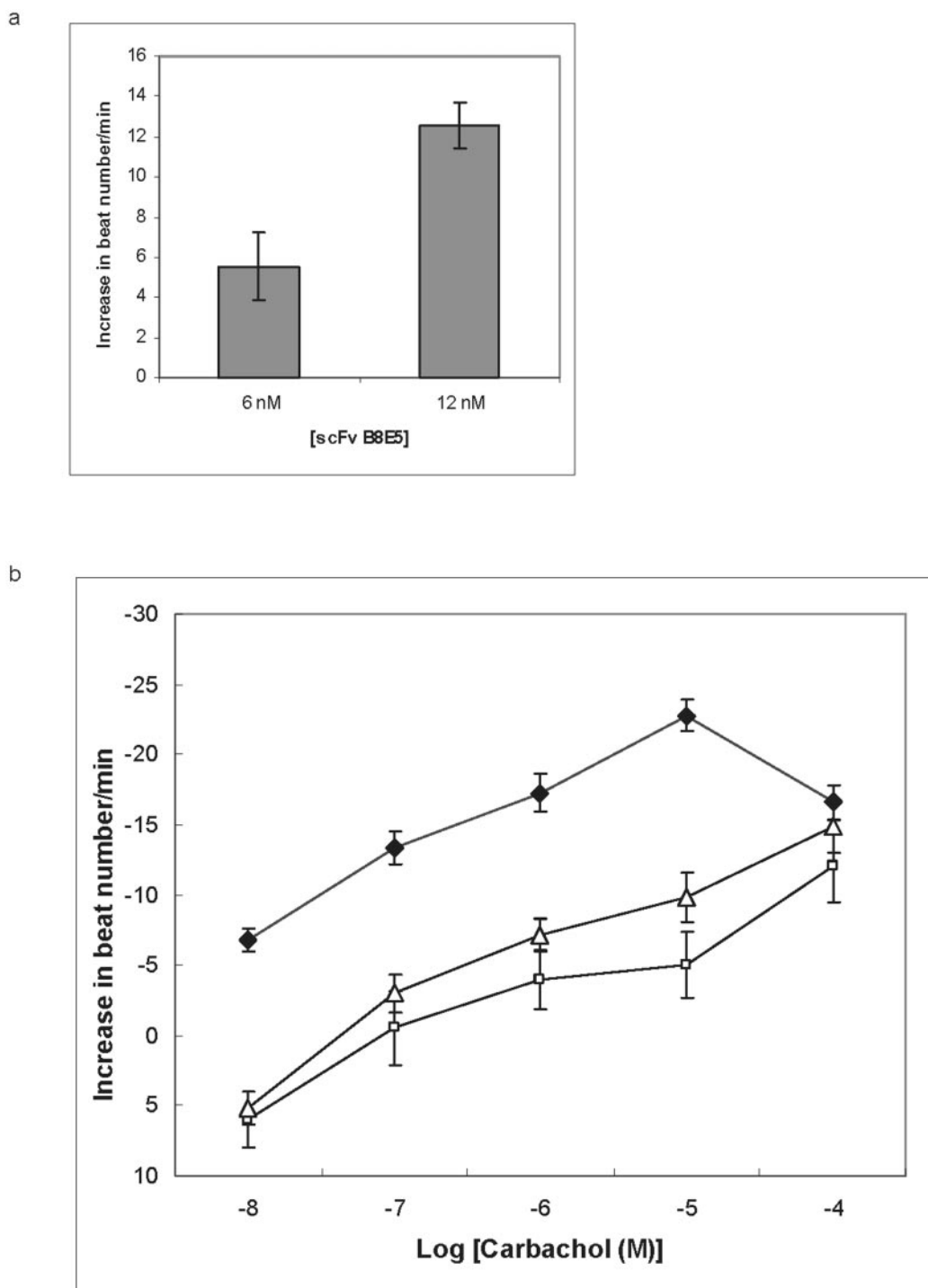


FIG. 4. Pharmacological effects of scFv B8E5 on neonatal rat cardiomyocytes *in vitro*. scFv B8E5 increased spontaneous beating (a) and inhibited the carbachol stimulation of neonatal rat cardiomyocytes in a non-competitive manner (b); the decrease in beat number/min is represented by increasing concentration of carbachol in the presence of scFv B8E5 (6 nM (□) and 12 nM (△) or without co-treatment (◆).

tized to prevent ECG modifications (20). After 20 min in the ECG box, each treatment in a 100- $\mu$ l volume was injected intravenously (Table I). The plan of the intravenous injection was designed to have a period of 1 day between two injections of carbachol.

The ECG signal was analyzed off line. The QRS complexes were identified, and artifact, ectopic beats, and normal beats were recognized and annotated (ECG-Auto, EMKA Technologies, Paris, France). The heart rate (60/mean interval between the R wave peaks of adjacent normal QRS complexes) (bpm) was calculated based on the beat per beat data.

**Statistics**—Statistical analysis was performed using Minitab software. To compare the percent of variation of the cardiac rhythm with

the rhythm at  $t = 0$ , we used a Student's paired match  $t$  test to compare the integral of each curve upon the different treatments.

**Molecular Modeling**—ScFv B8E5 model was built *in silico* using the Biopolymer, Homology, and Discover software of Accelrys (San Diego, CA). The  $V_H$  and  $V_L$  chains of the scFv B8E5 were built from the Protein Data Bank accession codes 1IFH and 2MCP, respectively. The sequences of the templates (see Fig. 6) were changed to those of the scFv B8E5, and the modified chain was submitted to a steepest descent energy minimization of 2000 steps until a root mean square derivative of 0.001 kcal/mol  $\text{\AA}$  was reached. The assembly was again submitted to a conjugate gradient minimization procedure of 2000 steps with the backbone fixed.

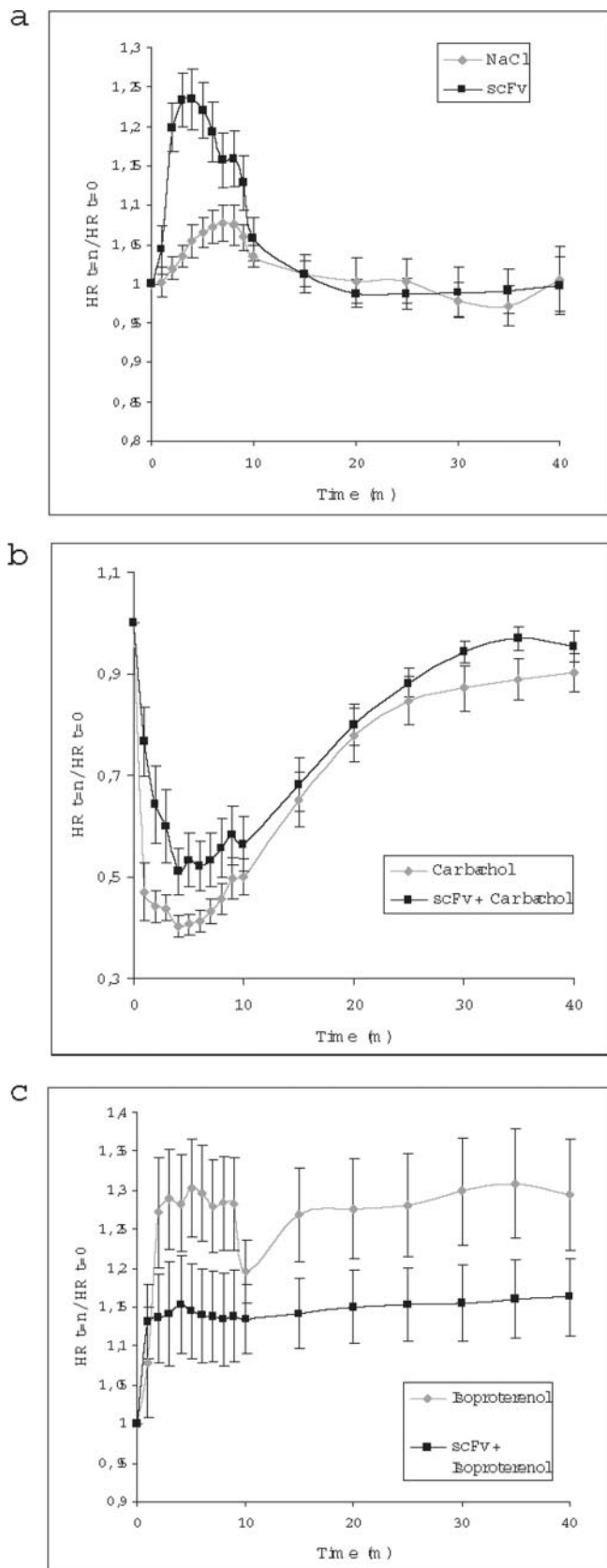


FIG. 5. Physiological effects of the scFv B8E5 on mouse heart, *in vivo* cardiac physiology measurements. Mean ( $\pm$ S.E.) of the variation of the cardiac rhythm at different time after intravenous injection of scFv control and scFv B8E5 alone (a), carbachol (0.1 mg/kg) with and without scFv B8E5 (b), and isoproterenol (2 mg/kg) alone or in combination with scFv B8E5 (c) reported on the cardiac rhythm normalized at  $t = 0$ . The integrals of each graph were compared at  $p < 0.05$  in a paired matched Student's  $t$  test.

## RESULTS

*Cloning, Sequencing, Expression, and Immunochemical Characterization of the scFv B8E5 Fragment*—The N-terminal part of the  $V_H$  and  $V_L$  of the B8E5 antibody was sequenced by Edman degradation to design the primers used for the amplification of the two domains. The original sequence of the  $V_H$ , EVQLVE and the  $V_L$ , DIVMAQ, helped us to design the  $V_H$ rev and  $V_L$ Rev primer. The scFv-encoding gene derived from the variable regions ( $V_H$  and  $V_L$  linked together via a short linker ( $G_4S_3$ ) of the IgG2a B8E5 monoclonal antibody (8) with addition of a C-terminal His<sub>6</sub> tag encoding sequence, was inserted in frame with the PelB sequence into the pSW1 expression vector. The sequence of the single-chain construction is represented in Fig. 1. We confirmed that the cloned  $V_L$  gene did not correspond to the aberrant  $\kappa$  transcript of the sp2/0 hybridomas (21). The plasmid pSW1-scFv B8E5 was cloned into the HB2151 *E. coli* strain, and the recombinant protein was expressed and exported to the bacterial periplasm by its leader sequence PelB (22). The scFv B8E5 was easily purified and concentrated by affinity chromatography (Fig. 2).

Surface plasmon resonance technology allows the analysis of scFv/antigenic peptide interaction in real time. It also allows the determination of the active concentration of analytes in solution (15). The active concentration showed that 100% of the purified recombinant protein was able to interact with the antigenic peptides V9C. The calculated concentration allowed us to determine the kinetic parameters of the scFv B8E5 using a Biacore System and the BIAeval 3.1 software. A Langmuir binding model gave us the following kinetic parameters: the  $k_{on} = 2.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ , the  $k_{off} = 2.15 \times 10^{-3} \text{ s}^{-1}$ , and the equilibrium constant  $K_A = 1.1 \times 10^8 \text{ M}^{-1}$ . These parameters are similar to those calculated under the same conditions for the B8E5 proteolytic Fab fragment ( $k_{on} = 7.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ,  $k_{off} = 4.7 \times 10^{-3} \text{ s}^{-1}$ ). The inhibition constants of this scFv fragment were determined as described in under "Experimental Procedures."  $K_i$  values were determined as  $5.7 \times 10^{-8} \text{ M}$  and  $4.7 \times 10^{-5} \text{ M}$  for the short V9C peptide and for the extended G19K peptide, respectively.

To assess the ability of the scFv to interact with the receptor, immunocytochemical experiments were performed on CHO M2ACh-R transfected cells. Fig. 2b shows the presence of a specific membrane and cytoplasmic labeling when using the scFv B8E5 fragment. No specific labeling could be observed on CHO untransfected cells, CHO transfected with M1ACh-R and M3ACh-R, and on CHO transfected with the M2ACh-R treated with an scFv control.

*Pharmacological Characterization of the scFv Fragment*—Next we determined if the scFv construction had pharmacological properties on the M2ACh-R. Activation of the M2ACh-R induces a decrease of intracellular cAMP mediated by the  $G_i$  protein (23). CHO M2ACh-R transfected cells were pretreated with 1  $\mu\text{M}$  of forskolin, which activates adenylyl cyclase. The decrease of intracellular measured cAMP thus will be directly correlated with activation of the M2ACh-R. CHO M2ACh-R transfected cells stimulated with forskolin were treated with different scFv concentrations in the presence or absence of carbachol (30 nM). Interestingly, the B8E5 scFv fragments were able to significantly increase the forskolin-induced accumulation of cAMP in a dose-dependent manner (Fig. 3a). This is the main characteristic of inverse agonists (see Ref. 24 for review). Moreover, Fig. 3b shows that scFv B8E5 was able to inhibit the activation of the M2 muscarinic receptor by carbachol in a dose-dependent manner. Also, a fixed concentration of 25 nM scFv seems to inhibit in a non-competitive manner the effect of carbachol (Fig. 3c). These results were confirmed *in vitro* by measuring the beating rate of neonatal rat cardiomyocytes stimulated by

## A.

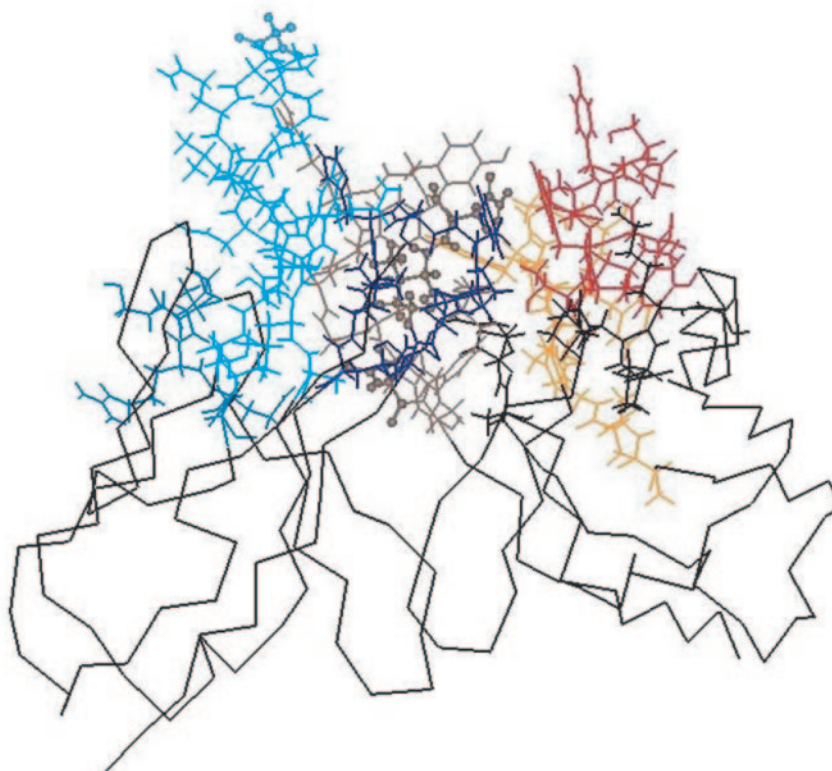
**VH B8E5** QVQLQQSGGDLVKPGGSLKVSCAASGFTFSSYGMSWVRQTPDKRLEWVATI--TSGGSYTYYPDSVKGRFTIS  
**B21C** QVQLQQPGAE~~LVKPGASVKLSCKASGYTFTSY~~MMHWVKQRPGRGLEWIGRIDPNSGG--TKYNEKFKSKATLT  
**11h** EVQLVESGGDLVKPGGSLKLSCAASGFSSSYGMSWVRQTPDKRLEWVATI--**S**NGGGYTYYPDSVKGRFTIS

**VH B8E5** RDNAKNTLYLQMSLKSSEDTAMYYCARHINRYD-GAFDYWGQGTTLTVSS  
**B21C** VDKPSSTAYMQLSSLTSEDSAVYYCAR  
**11h** RDNAKNTLYLQMSLKSSEDSAMYYCARR--ERYD**EN**GFAYWGQGTTLTVSA

**VL B8E5** DIVMAQSPSSLSVSAGEKVMSCSSQSLNLSRNQKNYLAWYQQKPGQSPKLLIYGASTRESGVPDRFTGSGSGT  
**Ke112** NIVLTQSPASLAVSLGQRATISCRASESVDSYGN--SFMHWYQQKPGQPPKLLIYLASNLESGVPARFSGSGSRT  
**2 mcp** DIVMTQSPSSLSVSAGERVTMSCKSSQSLNSGNQKNFLAWYQQKPGQPPKLLIYGASTRESGVPDRFTGSGSGT

**VL B8E5** DFTLTISSVQAEDLAVYYCQNDHSYPLTFGAGTKLEIK  
**Ke112** DFTLTI DPVEADDAATYYCQNNEDP  
**2 mcp** DFTLTISSVQAEDLAVYYCQNDHSYPLTFGAGTKLEIK

## B.



**FIG. 6. Model of the scFv combining site.** A, sequences are compared with the germ line sequences and with the structure templates used for molecular modeling. B, model of the scFv B8E5. The complementary determining regions are represented in different colors: complementary determining region L1 in light blue, L2 in blue, L3 in dark blue, H1 in orange, H2 in red, and H3 in brown. The amino acids that differ from the template models are represented in stick and balls. The hypervariable loop amino acids that differ from the two Protein Data Bank template models are represented in bold characters, and the complementary determining regions are highlighted.

TABLE II

Alignments of the second extracellular loop of the five subtypes of muscarinic acetylcholine receptors

The numbers correspond to the sequence number of the first amino acid. The epitope recognized by the monoclonal B8E5 is underlined.

<i>M2ACh-R</i>	163	QFIVGVRTVEDGECYIQFFSNA
<i>M1ACh-R</i>	165	QYLVGERTVLAGQCYIQFLSQP
<i>M3ACh-R</i>	208	QYFVGKRTVPPGECFIQFLSEP
<i>M4ACh-R</i>	172	QFVVGKRTVDPNQCFIQFLSNP
<i>M5ACh-R</i>	170	QYLVGKRTVPLDECCIQFLSEP

carbachol (Fig. 4). The basal beating rate of the cardiomyocytes as well as the carbachol dose-response curve were increased. In view of the fact that the carbachol dose-response curve in presence of scFv only showed a decrease in the maximal response not present with the 9C2 scFv control (data not shown), it typically

represents a non-competitive inhibition of the carbachol effect. We thus conclude through two different experiments that the scFv acts in a non-competitive manner.

**Physiological Characterization of the scFv Fragment**—To investigate the possible *in vivo* effects of the scFv B8E5 on the heart, we injected the scFv construction in mice alone or combined with the muscarinic receptor agonist carbachol (0.1 mg/kg) or the  $\beta$ -adrenoreceptor agonist isoproterenol (2 mg/kg). The animals had a comparable heart rate before the different administrations (data not shown). ScFv injected alone induced a significant increase ( $p = 0.012$ ) of the heart rate compared with the NaCl treatment at the beginning of the recording (Fig. 5a).

Injection of carbachol (0.1 mg/kg intravenously injected) induced a marked decrease of the heart rate. This effect was

significantly blunted ( $p = 0.041$ ) in the presence of scFv B8E5 (Fig. 5b).

Isoproterenol (2 mg/kg), a  $\beta$ -agonist, induced a marked increase of the heart rate. This increase was significantly reduced ( $p = 0.042$ ) in the presence of scFv B8E5 (Fig. 5c).

#### DISCUSSION

Most of the neurotransmitter receptors belong to the family of G protein-coupled receptors representing one of the main targets of cardiovascular and neurological drugs. The pharmacological existence of subfamilies of these receptors was first assessed by showing that the same neurotransmitters had different effects on different tissues. Cloning, sequencing, and expressing molecules, coded by different genes but recognizing the same neurotransmitters, highlighted this molecular diversity. In view of the putative structure of these proteins, the localization of the neurotransmitters in an intramembrane pocket was suggested. This fact only allows a limited amount of variability to dock different agonist and antagonists. On the other hand, the constancy of the pharmacophore pocket between members of the same family could explain the difficulty to design and synthesize molecules with an exclusive specificity for one of the receptor subtypes. In contrast, the extracellular domains of receptors of the same family can vary more widely which suggests the possibility to raise antibodies possessing the single specificity intended by the pharmacologists.

Drug discovery based on immunological reagents was already forwarded more than 20 years ago, but only recently because of the expansion of biotechnological tools could it be considered as a realistic goal. Indeed, recent results have shown that such approach could lead to functional peptides derived from antibodies directed against cell receptors (25). The finding that autoantibodies against G protein-coupled receptors were present in and responsible for different cardiovascular diseases prompted us to use a similar approach for the development of antibody fragments of low molecular weight interfering with this family of receptors.

Cloning and sequencing of the variable regions of the B8E5 monoclonal antibody in comparison with the corresponding germ line showed 45% and 42.6% variability, respectively, for the heavy chain and for the light chain. The sequence of the variable part of the heavy chain corresponded for 84% to that of the Protein Data Bank accession code 1IFH (26) (IgG 2A Fab fragment complexed with a 7-mer peptide from influenza virus hemagglutinin HA1-(101–107)), whereas the sequence of the light chain corresponded for 94.7% to that of the Protein Data Bank accession code 2MCP (27) (phosphocholine binding immunoglobulin Fab fragment McPC603). The high similarity of the scFv fragment with antibody combining sites of a known three-dimensional structure allowed us to construct a realistic model of the scFv fragment *in silico* (Fig. 6). The percents of  $\phi$  and  $\psi$  angles core region occupancy are for the  $V_H$  and for the  $V_L$  of 74.6% and 73.9%, respectively.

The physicochemical parameters of the epitope-scFv interaction as measured by surface plasmon resonance allowed us to confirm that the scFv fragment had similar binding properties as the Fab fragment of the B8E5 antibody. Difference in inhibition constants for the long (G19K) and the short (V9C) peptides is certainly caused by a lesser accessibility of the epitope for the antibody fragment on the long peptide. The same observation was made previously with the monoclonal antibody (8). Active concentration calculation of purified scFv shows that 100% of the purified recombinant proteins are able to interact with their antigen. Protein L used for the purification of the scFv interacted with the VL  $\kappa$  chain (28, 29) in a conformational way. The 100% of activity of purified scFv thus can be explained by the fact that only correctly folded recombinant pro-

teins can also interact with protein L. A cytochemistry experiment showed a specific labeling of membrane and of the cytoplasm of M2ACh-R transfected cells compared with untransfected cells and cells transfected with two other subtypes of muscarinic receptors. These results are in accordance with the data presented in Table II, which show that the recognized epitope VRTVE is different in the other muscarinic receptors subtype. Indeed, the V residue at the Nter portion of the epitope, important for the interaction peptide/antibody (8), is not present in the other subtypes of muscarinic receptors. These results confirm the high specificity of this antibody fragment on the M2ACh-R.

In our previous studies, an scFv fragment of an antibody directed against the second extracellular loop of the  $\beta_2$  adrenergic receptor was shown to have an inverse agonist activity (9). We supposed that monovalency of the anti-M2ACh-R antibody fragment compared with the bivalency of the partial agonist monoclonal antibody B8E5 led to the same properties. To directly check the pharmacological activity of this recombinant protein, we studied the accumulation of cAMP in M2R-Ach CHO transfected cells in the presence of forskolin with and without carbachol. The scFv fragments not only inhibited the cAMP decrease induced by carbachol, but they also increased the cAMP accumulation induced by forskolin. This means that the scFv fragments behave as inverse agonists, *i.e.* molecules specifically recognizing the resting conformation of the receptor and shifting the active  $\rightarrow$  resting conformational equilibrium to the right. A dose-response carbachol curve in the presence of scFv suggested that carbachol inhibition was non-competitive, suggesting an allosteric modulation of the receptor.

The scFv fragment was able to increase the spontaneous beating rate of neonatal rat cardiomyocytes in culture. We previously checked the 100% identity between the sequence of the second extracellular loop of the M2ACh-R from human and rat. The scFv fragment also blocked the effect on the same cells of the muscarinic agonist carbachol. This effect did not shift the carbachol dose-response curve to higher agonist concentrations but decreased the maximally obtained response. These results confirm that the scFv fragment blocks the accessibility of the pharmacophore pocket in a non-competitive manner or allosterically prevent the conformation transition from inactive to active state of the receptor.

This antibody fragment is able to decrease parasympathetic activity via its action on the M2ACh muscarinic receptor (no difference of sequence can be observed between the second extracellular loop of rat and human) and thus to induce an increase of the heart rate from  $t = 1$  to  $t = 10$ .

The antibody fragment is also able to block the action of carbachol *in vivo*. These results were similar to that observed *in vitro*, physiologically confirming the biochemical inverse agonist activity of the fragment. Moreover, isoproterenol treatment combined with scFv showed a smaller increase of the heart rate than for isoproterenol alone. This phenomenon was observed with low doses of pirenzepine, an inverse agonist of the M2 muscarinic receptor (30), combined with isoprenaline in healthy human volunteers (31).

To summarize, we have completely characterized a polypeptide with a specific inverse agonist activity on the M2 muscarinic receptor. It is the second example of a G protein-coupled receptor inverse agonist shifting by an allosteric mechanism the receptor to its resting conformation. The structural model that we obtained from the scFv fragment could help us to design shorter peptide fragments with similar properties.

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