scFv Single Chain Antibody Variable Fragment as Inverse Agonist of the β₂-Adrenergic Receptor

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Antibodies directed against the second extracellular loop of G protein-coupled receptors were shown to possess functional activities. Using a functional monomolecular antibody against the human β₂-adrenergic receptor, a scFv fragment with high affinity for the target epitope was constructed and produced. The fragment recognized the β₂-adrenergic receptors on A431 cells, blocked cAMP accumulation induced by the β₂-agonist clenbuterol, and decreased basal cAMP accumulation in the same cells. Their in vitro activity was tested on neonatal rat cardiomyocytes. The antibody fragments blocked the chronotropic activity induced by the β₂-agonist clenbuterol. They also decreased the in vivo heart beating frequency of mice pretreated with bisoprolol (a β₁-adrenergic receptor antagonist) for 4 min after injection. The immunological approach presented here may serve as a strategy for the synthesis of a new class of allosteric modulators for G protein-coupled receptors.

The G protein-coupled receptor family is one of the main targets of currently used drugs (1). Most of the structural insights into this family of receptors were obtained from studies of the β₂-adrenergic receptor, the first of this family of neurotransmitter receptors to be cloned and sequenced. This receptor is an integral membrane protein consisting of seven membrane spanning α-helices, which form a pharmacophore pocket, linked together by extracellular and intracellular loops (2). 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 synthesize 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. 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 structure of this domain (3). Lebesgue et al. (4) reported the selection of monoclonal antibodies directed against a synthetic peptide whose sequence was derived from the second extracellular loop of G protein-coupled receptors. The selected monoclonal antibody against the β₂-adrenergic receptor had partial agonist activity as a dimer and antagonist activity as a monovalent Fab fragment (4, 5). This antibody was used to construct a scFv fragment (single chain variable fragment), which was cloned, sequenced, and expressed in Escherichia coli. In this study, we describe the sequence, the immunological, pharmacological, and physiological properties of this scFv fragment. These results open the way for the development of new strategies to synthesize molecules, which are highly specific for one of the subtypes of a particular G protein-coupled receptor family and can allosterically modulate their activity.

EXPERIMENTAL PROCEDURES

Peptides

β₂H19C (HWYRATHQEAINCYANETC), corresponding to the second extracellular loop (residues 172–190) of the human β₂-adrenergic receptor, were synthesized using Fmoc (N,N-diisopropylcarbodiimide) chemistry with an automated peptide synthesizer (6). The β₂H19C peptide was biotinylated as described in Ref. 5. The peptide was purified by HPLC and its integrity was 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 chain of the monoclonal antibody 6H8 (4) was prepared as described in Ref. 7.

scFv 6H8 was created by joining the 6H8 VH and VL genes together by PCR splicing with overlap extensions using oligonucleotides that encoded a 15-amino acid linker (G₄S₃), between the C-terminal of the VH and the N-terminal of the VL gene. The ends of the variable gene were modified by PCR using as primers, VHRev (5′-GGT GCA GCT GGT TGA-3′), which encodes the N-terminal wild type sequence of the VH, containing a PeiB site, and VHFor, containing a XhoI site which encodes 6 His residues (5′-GCC GAC TGT GGT GAT-3′), which encodes the C terminus of the VH and a part of the linker, VLRev (5′-GGC GGA GAC TGT CCT GGT GGA GAC-3′) and VLFor, containing a XhoI site which encodes 6 His residues (5′-GCC GAC TGT GGT GGA GAC-3′), were used to amplify and modify the VL domain. The scFv gene was inserted in frame with sequence PeiB of the expression vector pSW1 (8) between the PeiB and XhoI sites. The constructed vector pSW1-6H8 Hisω was cloned in HB 2116 E. coli strain.

Bacterial Expression of scFv-6H8

The bacterial expression of the recombinant scFv protein and extraction of soluble periplasmic protein are described in Ref. 7. The periplas-
mic extract was centrifuged at 10,000 × g, and the supernatant was filtered on 0.45-μm membrane (Millipore) and extensively dialyzed against PBS (50 mM Na₂HPO₄, 300 mM NaCl, 20 mM imidazole).

**Purification of the scFv Construction**

The periplasmic extract was incubated for 1 h at 4 °C with 500 μl of nickel-nitriolatriceric acid-agarose beads (Qiagen) and washed with PBS buffer, and the recombinant protein was eluted with 1 ml of PBS supplemented with 500 mM imidazole 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-blott system (Bio-Rad) in transfer buffer (25 mM Tris-HCl, 190 mM glycine, 20% methanol, pH 8.3). The membranes were soaked 1 h in PBS-T (20 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 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 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, Saclay, France).

**Immunochromes Characterization of the scFv Fragment**

The instrument BIACORE 3000 and all the reagents for analysis were obtained from BIACORE (Upsalla, Sweden). The low carboxylated dextran matrix (B1) was activated with 50 μl at 5 μl/min of a mixture 0.2 μ M N-ethyl-N'-dimethylaminopropyl carbodiimide and 0.05 μ M N-hydroxysuccinimide. Streptavidin was immobilized with the standard BIACORE protocol at a density of 0.05 pmol/mm². The biotinylated peptide (0.1 mg/ml in PBS, pH 7.6) was then immobilized on the streptavidin at a flow rate of 5 μl/min for 7 min. Kinetic studies were performed as described in Ref. 5.

**Protein Concentration Determination**

The total protein concentration of the purified scFv (active + nonactive proteins) was determined using the BCA kit (Pierce) and by measuring absorbance at 280 nm. The extinction coefficient was determined using the BCA kit (Pierce) and by measuring absorbance at 280 nm. The extinction coefficient was determined using the BCA kit (Pierce).

**Pharmacological Characterization of the scFv Fragment**

AcA43 cells were fixed 5 min with 2% paraformaldehyde and permeabilized with PBS Triton X-100 1.0% for 1 min. Slides were saturated with PBS supplemented with nonfat dry milk 5%. ScFv 6H8 and scFv 6C2 (0) (control) were incubated overnight at 4 °C. After three washes with PBS, anti-His tag antibody was added and incubated for 1 h at room temperature. Rabbit anti mouse IgG (H + L) antibody Alexa conjugated (1/200, 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 AcA43 Cells Treated in Vitro with scFv—The biochemical effects of scFv 6H8 on the β₂-adrenergic receptor were assessed by measuring the intracellular cAMP concentration of AcA43 cells (10). Cells were seeded in 6-well culture plates 24 h before stimulation and then washed and incubated with 1 ml of Hanks’ balanced medium buffered with 10 mM HEPES, containing 100 μM 2-oxoglutarate and 0.8% NaCl. The culture was aspirated and reperfused 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 BCA kit (Pierce). The protein concentration of cAMP was reported on the protein concentration, related to the number of cells/well; the results were expressed as pmol of cAMP/mg of protein. The results were normalized using cAMP content of untreated cells as 100%. Results are from duplicates of three independent experiments.

**Concluding Remarks**

The bioconjugation of scFv 6H8 with the antigenic peptide was performed through the chemical effects of scFv 6H8 on the β₂-adrenergic receptor were assayed by measuring the intracellular cAMP concentration of AcA43 cells (10). Cells were seeded in 6-well culture plates 24 h before stimulation and then washed and incubated with 1 ml of Hanks’ balanced medium buffered with 10 mM HEPES, containing 100 μM 2-oxoglutarate and 0.8% NaCl. The culture was aspirated and reperfused 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 BCA kit (Pierce). The protein concentrations of the samples were determined using BCA kit (Pierce). The protein concentration of cAMP was reported on the protein concentration, related to the number of cells/well; the results were expressed as pmol of cAMP/mg of protein. The results were normalized using cAMP content of untreated cells as 100%. Results are from duplicates of three independent experiments.
active concentration showed that only 5% of the purified recombinant protein was able to interact with the antigenic peptide H19C; this is mainly due to the bacterial expression system that has limited capacity to correctly fold the polypeptidic chain and to the natural instability of this chimeric construct (see Ref. 17 for review). The calculated concentration allowed

Fig. 1. Nucleotide (light gray) and amino acid (black) deduced sequences of the scFv 6H8. The two restriction sites PstI and XhoI used for cloning are represented in gray bold characters. The CDR of the 6H8 antibody, according to the Kabat numbering, are underlined, and the (G4-S)3 linker between the heavy and light variable domain is represented in italic bold characters (GenBank™/EBI accession number AJ574851).
us to determine the kinetic parameters of the scFv 6H8 using a BIACORE system and the BIAeval 3.1 software. A Langmuir binding model gave us the following kinetic parameters: the $k_{\text{on}} = 1.0 \times 10^{6} \text{ M}^{-1} \text{ s}^{-1}$, the $k_{\text{off}} = 4.8 \times 10^{-3} \text{ s}^{-1}$, and the equilibrium constant $K_{A} = 2.1 \times 10^{8} \text{ M}^{-1}$. These parameters are similar to those calculated under the same conditions for the 6H8 proteolytic Fab fragment ($k_{\text{on}} = 0.89 \times 10^{6} \text{ M}^{-1} \text{ s}^{-1}$, $k_{\text{off}} = 6.93 \times 10^{-3} \text{ s}^{-1}$) (5).

To assess the ability of the scFv to interact with the receptor, immunocytochemical experiments were performed on A431 cells that express at their surface the $\beta_2$-adrenergic receptor. Fig. 2b shows the presence of a specific vesicular and membrane labeling when using the scFv 6H8 fragment.

**Pharmacological Characterization of the scFv Fragment**—We next determined whether the scFv construction had pharmacological properties on the $\beta_2$-adrenergic receptors. A431 cells stimulated with salbutamol (10 nM) were treated with different scFv concentrations. Fig. 3 shows that scFv 6H8 was able to inhibit the activation of the $\beta_2$-adrenergic receptor in a dose-dependent manner. Interestingly, it was able to significantly decrease the basal accumulation of cAMP. This is the main characteristic of inverse agonists (see Ref. 18 for review). These results were confirmed in vitro by measuring the beating rate of neonatal rat cardiomyocytes stimulated by clenbuterol (a specific $\beta_2$-agonist) (Fig. 4). The basal beating rate of the cardiomyocytes as well as the clenbuterol dose-response curve were decreased. In view of the fact that the clenbuterol dose-response curve was not shifted to the right but only showed a decrease in the maximal response, we conclude that the scFv acts in a noncompetitive manner.
Physiological Characterization of the scFv Fragment—To investigate the possible in vivo effects of scFv on the heart, we injected the scFv construction in mice. Before scFv injection, mice were pretreated for 10 min with bisoprolol hemifumarate (a specific $\beta_1$-antagonist) to limit the regulatory activity of the $\beta_1$-adrenergic receptor on the heart. Intravenous injection of
100 μl of 110 nm active scFv gave a statistically significant decrease of the beating rate (about 14%), 2 min after the injection (Fig. 5). NaCl or control scFv did not significantly alter the beating rate. The scFv control was injected at the same protein concentration as the 6H8 scFv. The intravenous injection of the inverse agonist ICI 118,551 at 10 mg/kg decreased the beating rate to a larger extent (26%).

**DISCUSSION**

G protein-coupled receptors are one of the main targets of cardiovascular and neurological drugs, since most of the neurotransmitter receptors belong to this family. The pharmacological existence of subfamilies of these receptors was first assessed by showing that the same neurotransmitters had different effects on different tissues. The molecular basis of this diversity was confirmed by cloning, sequencing, and expressing molecules coded by different genes but recognizing the same neurotransmitters. The putative structure of these proteins suggests that the neurotransmitters are localized in an intramembrane pocket, which only allows a limited amount of variability to embed different agonist and antagonists. The difficulty to synthesize molecules with an exquisite specificity for one of the receptor subtypes could be explained by the constancy of the pharmacophore pocket belonging to members of the same family. In contrast, the extracellular domains of receptors of the same family can vary more widely, which suggests the possibility to raise antibodies possessing the exquisite specificity sought for by the pharmacologists.

Drug discovery based on immunological reagents was already forwarded more than 20 years ago, but only recently, due to 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 (19, 20). 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 6H8 monoclonal antibody in comparison with the corresponding germ line showed 19.4 and 52.6% 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 access number 1cic (Fab/anti Fab complex) (21), while the sequence of the light chain corresponded to 90% of that of the Protein Data Bank access number 1jrh (Fab anti Nter part of the interferon-γ receptor) (22). The high similarity of the scFv fragment with antibody combining sites

![Model of the scFv combining site.](image)

Fig. 6. Model of the scFv combining site. a, sequences are compared with the germ line sequences and with the sequences of the template antibodies used for molecular modeling. b, model of the scFv 6H8. The CDRs are represented in different colors: CDR L1 in light blue, L2 in blue, L3 in dark blue, H1 in orange, H2 in red, and H3 in brown. The amino acids, which differ from the template models, are represented in stick and balls. The hypervariable loop amino acids, which differ from the two pdb template models, are represented in bold characters and the CDRs are highlighted.
of the known three-dimensional structure allowed us to construct a realistic model of the scFv fragment in silico. Ten amino acids of the CDR of the scFv 6H8 are different from the sequence of the Protein Data Bank templates (Fig. 6). These amino acids seem to form a small groove at the VH-VL interface in this flat paratope where the essential epitopic Trp-173 of the H19C peptide or the second extracellular loop of the β2-adrenergic receptor could fit in.

The physicochemical parameters of the epitope-scFv interaction as measured by the BIACORE system allowed us to confirm that the scFv fragment had similar binding properties as the Fab fragment of the 6H8 antibody. The amount of actively binding scFv fragments was, however, only 5% of the total purified amount of protein. The total amount of purified scFv was determined using a BCA kit and absorbance reading at 280 nm. The measured and calculated extinction coefficients are quite similar (respectively, 1.964 and 2.123), which indicates that the measured protein concentration corresponds to the scFv. The active concentration of scFv was taken into account to study the pharmacological and physiological properties of the scFv fragments. Cytochemistry experiment showed a specific labeling of membrane, membrane invagination, and cytoplasmic vesicles of the β2-adrenergic receptor as described in Ref. 23. This distribution corresponded to diffuse membrane receptors, activated receptors concentrated in invaginations, and submembranar vesicles and new synthesized receptors transported by cytoplasmic vesicles toward the membrane.

The Fab fragments of the antibody were shown to block the activation of L-type Ca2+ channels by the β2-selective agonist clenbuterol, suggesting that the combining site-receptor complex blocked Gs protein coupling, adenylyl cyclase activation, and protein kinase A phosphorylation, the normal biochemical cascade induced by β-agonists. To directly check the mechanism of blocking, we studied the accumulation of cAMP in A431 cells, which have been shown to possess a large amount of β2-adrenergic receptors (10). The scFv fragments indeed blocked the cAMP accumulation induced by the β2-agonist salbutamol, but moreover they blocked the basal cAMP accumulation in the cells, ascribed to the existence of spontaneous active receptors (24). 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 → resting conformational equilibrium to the right. We functionally confirmed the inverse agonist properties of the scFv fragment both in vitro and in vivo.

The scFv fragment was able to decrease the spontaneous beating rate of neonatal rat cardiomyocytes in culture. It also blocked the effect on the same cells of the β2-agonist clenbuterol. This effect did not shift the clenbuterol dose-response curve to higher agonist concentrations but decreased the maximal obtained response. These results suggest that the scFv fragment blocks the accessibility of the pharmacophore pocket in a noncompetitive manner.

A conformational change induced by the scFv on the extracellular loop could thus close the pharmacophore pocket for the agonist. The scFv was also able to decrease the beating frequency in vivo of the heart of conscious mice pretreated with the β2-agonist bisoprolol. Although the decrease only stayed for 4 min, probably due to the rapid filtration of the scFv by the kidneys (25–27), it was similar to that observed by the β2-specific inverse agonist ICI 118,551, physiologically confirming its biochemical inverse agonist activity. Recently it was shown that the inverse agonist ICI 118,551 could induce a conformation with high affinity for the G protein (28). The decrease in beating frequency could thus not only be explained by closing of the pharmacophore pocket by the scFv but also by induction of the same receptor conformation as that induced by ICI 118,551.

To summarize, we have completely characterized a polypeptide with a specific inverse agonist activity on the β2-adrenergic receptor. It is the first 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 synthesize shorter peptide fragments, which could share the same properties.

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