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Anti-peptide antibodies sensitive to the 'active' state of the β_2-adrenergic receptor

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Abstract Antibodies directed against a peptide corresponding to the second loop of the human β_2-adrenergic receptor were induced in rabbits by immunisation with the free peptide in complete Freund's adjuvant. The resulting antibodies were affinity-purified and shown to be monospecific for the target receptor. They were able to stimulate the L-type Ca^{2+} channels in whole-cell patch-clamp experiments on isolated adult guinea-pig cardiomyocytes. This effect was similar to that obtained by the specific β_2-adrenergic agonist zinterol. The antibody effects could be blocked with the specific β_2-adrenergic inverse agonist ICI118,551 but not with the neutral antagonist alprenolol. These results suggest that the antibodies recognise the active conformer of the β_2-adrenergic receptor.

Key words: G protein-coupled receptor; Conformer; β_2-adrenergic receptor; Antibody; Peptide; Calcium current

1. Introduction

Increasing evidence suggests that G protein-coupled receptors can be found in two different conformational states: a 'resting' and an 'active' state both of which are in equilibrium. This hypothesis, based on the existence of 'constitutively active' mutated receptors [1] and on the pharmacological properties of receptors, hyperexpressed in transfected cells [2,3] or in transgenic animals [4], could explain the activation of the effector systems of these receptors in the 'unliganded' state [5–8]. Several observations, on the other hand, suggest that antibodies or autoantibodies directed against the second extracellular loop of G protein-coupled receptors are able to induce 'agonist-like' activities by an until now unknown mechanism [9,10]. We here report the stimulation of L-type Ca^{2+} channels in isolated guinea-pig cardiomyocytes by anti-peptide antibodies against the second extracellular loop of the β_2-adrenergic receptor. The pharmacological properties of these antibodies suggest that they act by stabilising the 'active' state of the receptor.

2. Materials and methods

2.1. Antibody production, purification and characterisation

Rabbits were immunised with the C-terminal cysteinylated H26Q peptide (see Table 1) derived from the human β_2-adrenergic receptor as free peptide and the anti-peptide antibodies affinity purified as previously described [11]. The concentration of the purified antibodies was calculated from the absorbance at 280 nm (1.45 corresponding to 1 mg/ml). The enzyme immunoassay consisted of a double-sandwich method in which the second antibody is a biotinylated goat anti-rabbit IgG(H+L) from Jackson Laboratories and the revelator a streptavidin-peroxydase conjugate. The substrate was H_2O_2-ABTS [11]. The specificity of the response was assayed using the H26Q and H26R peptide derived from the human β_1-adrenergic receptor (Table 1).

2.2. Immunoblotting of the receptor

Murine LB cells transfected with the human β_2-adrenoceptor gene (a kind gift of Dr. M. Viguier, Institut Cochin de Génétique Moléculaire, Paris) were grown under standard conditions in the presence of 600 μg/ml geneticin. The level of expression corresponded to 300–400 fmol/mg membrane protein. Membranes were prepared after resuspension of serum-starved cells in 50 mM TRIS-HCl and 2.5 mM $MgCl_2$ at pH 7.4 and disruption by homogenation in a Polytron. Debris was centrifuged for 15 min at $3000\times g$ and the membranes precipitated by centrifugation for 45 min at $22000\times g$. The pellet was resuspended in the disruption buffer.

Membrane proteins were separated by electrophoresis in a 10% polyacrylamide gel after denaturation and reduction in sodium dodecyl sulfate and mercaptoethanol according to Laemmli [12]. The proteins were transferred on nitrocellulose under standard conditions [13]. After saturation of the nitrocellulose with phosphate-buffered saline (PBS), pH 7.4, containing 5% skimmed milk powder, 0.1% Tween-20 overnight at 4°C, strips were incubated for 2 h at 37°C with 3 nM purified anti-peptide antibody pre-incubated, or not, for 1 h with 6 μM peptide. Strips were washed, incubated for 1 h with donkey anti-rabbit $F(ab)_2$ antibody-peroxidase conjugate (Jackson Laboratories, USA) diluted 1000-fold in blocking buffer. The strips were washed 3 times in PBS containing 0.1% Tween-20 and immunocomplexes revealed using the enhanced chemiluminescence procedure (ECL on Hyperfilm, Amersham, UK).

2.3. Electrophysiological measurements

Cardiomyocytes were isolated using a collagenase/protease digestion technique described elsewhere [14]. Dissociated cells were placed in a 1 ml chamber on the stage of an inverted microscope (Nikon, Diaphot 300, Japan). The chamber was continuously perfused at a rate of 1 ml/min with Tyrode's normal solution. The composition of this solution was (in mM): NaCl, 140; KCl, 5.4; *N*-2-hydroxy-ethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES), 10; $CaCl_2$, 1.8; $MgCl_2$, 1: NaH_2PO_4, 0.33; and glucose, 11. The pH was 7.3.

Whole-cell voltage-clamp experiments were done in these isolated single cells at room temperature (22–25°C). During the experiments, cells were locally superfused with an extracellular solution containing (in mM): tetraethylammonium chloride (TEACl), 140; CsCl, 6; HEPES, 10; $CaCl_2$, 1.8; $MgCl_2$, 1; and glucose, 10. The pH was adjusted to 7.3 with TEAOH. This solution was superfused at a rate of 1 ml/min. All drug and antibody solutions were prepared daily and diluted as desired in the extracellular solution. The patch pipettes (2–4 MΩ) were fabricated with borosilicate glass. Pipettes were filled with (in mM): CsCl, 130; HEPES, 11; EGTA ethylene glycol-bis (β-aminoethylether)-*N,N,N',N'*-tetraacetic acid), 10; TRIS-GTP, 1; and Mg^{2+}-ATP, 5. The pH was adjusted to 7.3 with CsOH. After the formation of a gigaseal (4–10 GΩ) between the cell membrane and the pipette and rupturing of the membrane patch, whole-cell L-type Ca^{2+} currents (I_{Ca}) were elicited by 500 ms voltage steps applied from a holding potential of −80 mV to +10 mV every 7 s.

Cell capacity (proportional to cell size) was measured by integration of the current elicited by a 10 mV, 20 ms pulse and this value was

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used to evaluate I_{Ca} density (pA/pF). Currents were recorded using a RK300 Patch and Cell Clamp amplifier (Biologic, Meylan, France) connected to a PC 486 computer, which was equipped with pClamp software (version 6.0.2, Axon Instruments). The currents were stored in the computer for subsequent analysis with pClamp software. The calcium currents were measured as the difference between inward peak current and the current at the end of the depolarisation pulse.

3. Results and discussion

Taking advantage of the existence of a T-cell epitope in the sequence corresponding to the second extracellular loop of the human β_2-adrenergic receptors [15], we were able to obtain high titers of anti-peptide antibodies in rabbits and to affinity-purify these antibodies on a CNBr-Sepharose activated peptide column as previously described [11]. The antibodies were monospecific for the β_2-adrenergic receptor peptide since no cross-reaction could be shown on the correponding sequence of the β_1-adrenergic receptor (Fig. 1). As shown previously [11], the antibodies were able to detect the receptor by immunoblotting in a specific manner as assessed by inhibition experiments with the antigenic peptide (Fig. 2). In view of the high conservation of this domain in the rodent species, whose receptor sequence is known (Table 1), it was considered that antibodies cross-reactive against the guinea-pig receptor, whose sequence is as yet unknown, was very likely.

To study the stimulating activity of the anti-peptide antibodies on the β_2-adrenergic receptor, the whole-cell patch-clamp technique on guinea-pig cardiomyocytes was used to assess the influx of Ca^{2+} ions by L-type calcium channels (I_{Ca}), which is known to be stimulated by cAMP dependent mechanisms, induced by stimulation of the β-adrenergic receptors [16]. Since, the β-adrenergic system of guinea-pig cardiomyocytes is primarily of the β_1-subtype [17], the activation of the Ca^{2+} channel by a specific β_2-selective partial agonist, zinterol [18] was assayed to quantify the contribution of the β_2-adrenergic receptors. The increase in I_{Ca} was 14% of that obtained with the non-subtype specific agonist isoproterenol (Fig. 3 and Table 2). This result corresponds to that known about the β_2-adrenergic component in guinea-pig cardiomyocytes [17].

When the affinity-purified anti-peptide antibodies were added to the incubation medium of the cardiomyocytes, an increase in I_{Ca} similar to that induced by zinterol was observed. Presaturating the antibody with the immunogenic peptide completely blocked this response, confirming that the antibody activity was mediated by the antibody combining sites. The β-adrenergic antagonist, propranolol, added at the same time as the antibody completely blocked its effect, confirming that the enhancement of I_{Ca} was mediated by the β-adrenergic receptor. The subtype specificity of the anti-peptide

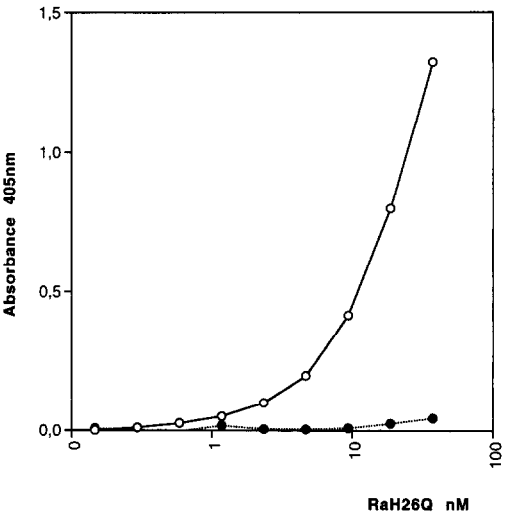

Fig. 1. Immunochemical specificity of the affinity-purified antibodies against the second extracellular loop of the human β_2-adrenergic receptor (RaH26Q). Absorbance values are given for the enzyme immunoassay against the peptide corresponding to the β_2-adrenergic receptor (amino acids 172–197) [28] (○–○) and that corresponding to the β_1-adrenergic receptor (amino acids 197-222) [32] (●–●) as shown in Table 1.

antibody was shown by the blocking effect of the β_2-selective inverse agonist, ICI118,551 [2]. When the non-subtype specific neutral antagonist alprenolol [2,3] was added under the same conditions, the stimulation of I_{Ca} by the anti-peptide antibodies was not inhibited but increased significantly (Fig. 4 and Table 2). This is not due to the different dissociation rates of the antagonists, since propranolol and alprenolol have similar binding kinetics [19]. Neither the addition of the β_2-selective partial agonist zinterol had an effect after activation of the receptor by the anti-peptide antibodies nor the anti-peptide antibody after activation by the β_2-specific partial agonist, zinterol.

These results can be explained by the hypothesis that the anti-peptide antibodies recognise an epitope which is present on the 'activated' state of the receptor. Since the two-state receptor hypothesis states that the receptor in the unliganded state is in equilibrium between the 'resting' and 'active' conformers, the stimulating effect of the antibodies are to be interpreted as a recognition of the unliganded 'active' conformer. The existence of unliganded 'active' receptor conformers in guinea-pig and human cardiomyocytes is suggested by the existence of calcium current activation by empty β-adrenoceptors [7]. The β_2-selective inverse agonist, shifting the receptor to the 'resting' conformation, makes the epitope recognised by the anti-peptide antibody inaccessible and the functional effect

Table 1
Comparison of the sequence of the second extracellular loop of the human β_2-adrenoceptor to show the high homology conserved over the different species and the differences with the human β_1-adrenoceptor

Species	Subtype	Sequence
Human [28]	β_2-	H-W-Y-R-A-T-H-Q-E-A-I-N-C-Y-A-N-E-T-C-C-D-F-F-T-N-Q
Mouse [29]	β_2-	H-W-Y-R-A-T-H-**K**-**K**-A-I-D-C-Y-T-E-E-T-C-C-D-F-F-T-N-Q
Hamster [30]	β_2-	H-W-Y-R-A-T-H-**Q**-**K**-A-I-**D**-C-Y-**H**-**K**-E-T-C-C-D-F-F-T-N-Q
Rat [31]	β_2-	H-W-Y-R-A-T-H-**K**-**Q**-A-I-**D**-C-Y-**A**-**K**-E-T-C-C-D-F-F-T-N-Q
Human [32]	β_1-	H-W-**W**-R-A-**E**-**S**-**D**-**E**-A-**R**-**R**-C-Y-**N**-**D**-**P**-**K**-C-C-D-F-**V**-T-N-**R**

Conserved mutations are underlined, non-conserved mutations are bold and underlined.
The numbers in brackets correspond to the references.

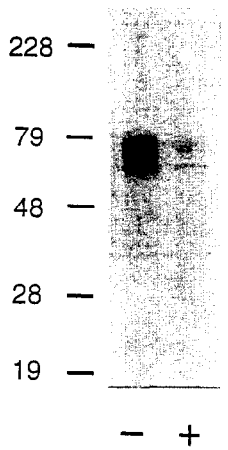

Fig. 2. Immunoblot of the human receptor from membranes of mouse LB cells transfected with the human receptor gene. (−) Incubation with 3 nM purified anti-H26Q antibody; (+) incubation with 3 nM purified anti-H26Q antibody in the presence of 6 μM H26Q peptide.

of the latter disappears. It is worthwhile to note in this context that human autoantibodies directed against the second extracellular loop of the β_1-adrenergic receptor do not immunoprecipitate the antagonist–receptor complex [20]. The neutral antagonist, alprenolol, does not change the receptor conformational equilibrium enabling the anti-peptide antibodies to recognise the active 'conformer' and exert their activating effect on the Ca^{2+} channels. The increase in I_{Ca} induced by the anti-peptide antibodies in the presence of alprenolol compared to that induced by the antibodies alone, could be ascribed to an intrinsic agonist effect of the latter. The antibodies, by stabilising the 'active' conformation, can shift the equilibrium towards the 'active' state.

Finally, the total inhibition by propranolol of the anti-peptide antibody induced increase in I_{Ca} suggests that this agent has an inverse agonist activity. Conflicting results have been obtained concerning the 'inverse agonist' potency of propranolol [2,3]. The results presented here suggest that, for a cell expressing the receptor under normal condtions, it can be considered an inverse agonist.

The 'two-state' receptor model has been hypothesized from results obtained in mutated 'constitutively' active receptors [1], and from studies on agonist-independent stimulation of receptors [5–8]. The first immunological evidence for this model in normal cardiomyocytes is presented here. It emphasizes the importance of the second extracellular loop in the equilibrium between the two states of G protein-coupled receptors as was already inferred from point mutation studies [21] and recently confirmed by the production of a constitutively active thrombin receptor after chimeric substitutions in this region of the receptor [22]. Immunological studies confirm that the main epitopic target of functional autoantibodies on G protein-coupled receptors are also localised at the second extracellular loop [9], suggesting that these antibodies act by influencing the 'conformer' equilibrium. The accumulating evidence that oligomers of G protein-coupled receptors could reflect the active state of these receptors [23] suggests that antibodies directed against specific extracellular epitopes could stabilise at least the dimeric form.

If these autoantibodies are pathologically important, inverse agonists should be the drugs of choice to block their pathogenic effects. The beneficial effects of some β_1-selective antagonists in idiopathic cardiomyopathy [24], a disease in which autoantibodies directed against the second extracellular loop of the β_1-adrenergic receptor have been observed [25–27], could be due to the inverse agonist properties of the drugs used.

Finally, anti-peptide antibodies 'sensitive' to one of the conformers are new pharmacological tools to distinguish between

Table 2
Statistical analysis of patch-clamp data: effects of the direct application of different drugs and anti-peptide antibody upon I_{Ca}

Drug	I_{Ca} (% of control)[a]	n[b]
Isoproterenol (100 nM)	129 ± 51	3
Zinterol (10 nM)	18 ± 4	5
RaH26Q (3 nM)	20 ± 3	5
RaH26Q (3 nM)+H26Q (15 μM)	− 10 ± 5	3
RaH26Q (3 nM)+Propranolol (5 μM)	− 8 ± 3	4
RaH26Q (3 nM)+ICI118,551 (10 nM)	− 11 ± 1	3
RaH26Q (3 nM)+Alprenolol (10 μM)	28 ± 3	3
Zinterol (10 nM) after RaH26Q (3 nM)	21 ± 2	3
RaH26Q (3 nM) after Zinterol (10 nM)	26 ± 8	3

[a]Mean ± SD.
[b]Number of cells tested.

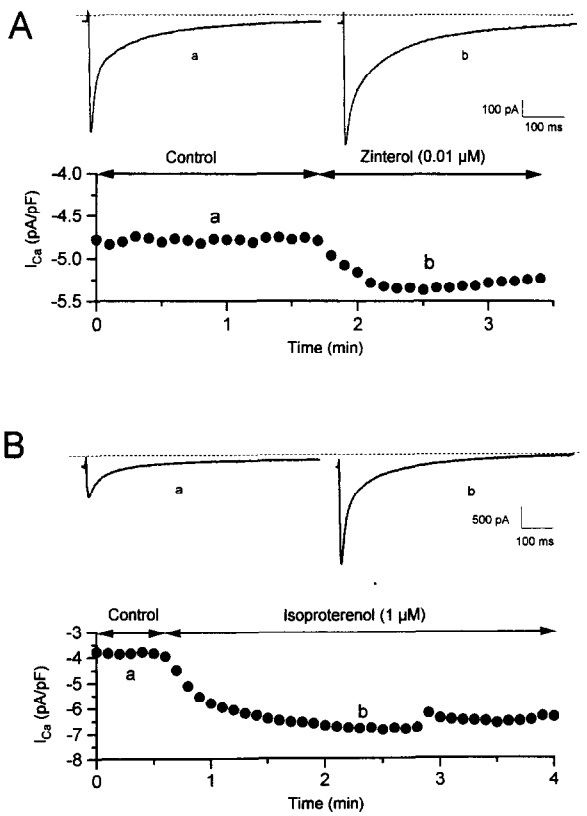

Fig. 3. Effect of agonists on calcium current (I_{Ca}) of ventricular guinea-pig cardiomyocytes. A: (upper part) Traces of calcium currents recorded from the same cell under control conditions (a) and 2.5 min after 0.01 μM zinterol application (b) dotted line: zero current. (lower part) Time course of I_{Ca} density in the same cell before and after application of zinterol (0.01 μM). B. (upper part) Traces of calcium currents recorded from the same cell under control conditions (a) and after application of isoproterenol (1 μM). (lower part) Time course of I_{Ca} density under control conditions and after application of isoproterenol (1 μM).

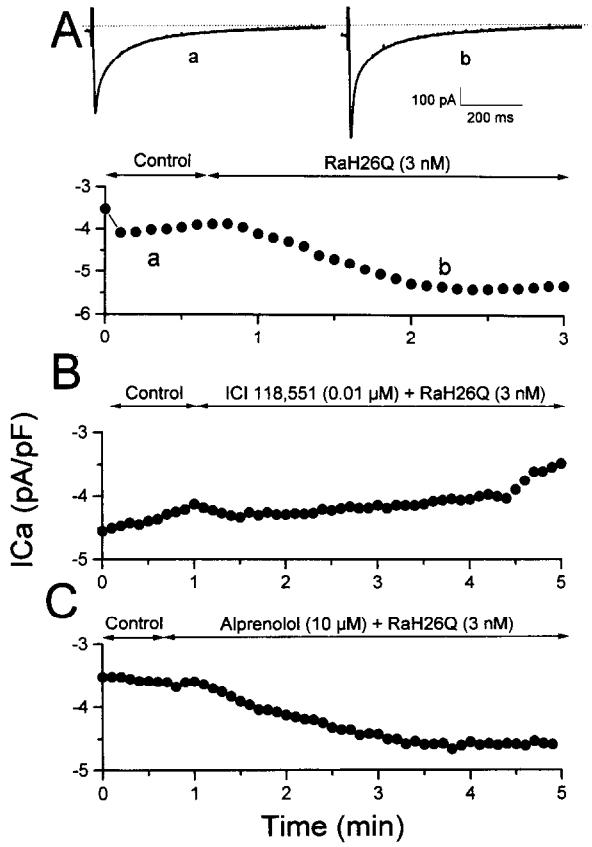

Fig. 4. Effects of anti-peptide antibody RaH26Q on calcium currents (I_{Ca}) of ventricular guinea-pig cardiomyocytes. A: (upper part) Traces of calcium currents recorded from the same cell under control conditions (a) and 2.3 min after 3 nM RaH26Q application (b); dotted line: zero current. (lower part) Time course of I_{Ca} density in the same cell before and after application of anti-peptide RaH26Q (3 nM) showing an increase of this current. B: Time course of I_{Ca} density under control conditions and after application of RaH26Q (3 nM) and ICI118,551 (10 nM). The antibody effect is completely abolished. C: Modification of the time course of I_{Ca} density by simultaneous application of RaH26Q (3 nM) and alprenolol (10 μM). The effect of the antibody is increased (for statistical analysis of the results, see Table 2).

neutral antagonists and inverse agonists for the different G protein-coupled receptors.

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