An Antibody Directed against Residues 100–119 within the α-Helical Domain of Goα Defines a Novel Contact Site for β-Adrenergic Receptors* (Received for publication, March 12, 1999, and in revised form, June 24, 1999)

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A polyclonal antiserum that recognizes residues 100–119 within the α-helical domain of Goα (K-20) caused a dissociation of Go into its component subunits and activated a chola toxin-sensitive high affinity GTPase. Consistently, the antibody mimicked the stimulatory effects of the β-adrenergic agonist, isoproterenol, on adenylyl cyclase, which is mediated by Goα, and its inhibitory action on NADPH-dependent H2O2 generation, a Gβγ-mediated response. A peptide corresponding to the target sequence of K-20 not only neutralized the receptor-mimetic effects of the antibody but inhibited the whole spectrum of isoproterenol action as well, including its antagonistic effects on adenylyl cyclase and NADPH-dependent H2O2 generation. By contrast, COOH-terminal anti-Goα selectively inhibited the stimulatory effect of isoproterenol on cAMP formation without affecting its inhibitory effect on NADPH-dependent H2O2 generation. The data are consistent with the concept that β-adrenergic receptors interact with multiple sites on Goα each playing a distinct role, and strongly suggest that antibody K-20 defines a novel contact site for β-adrenergic receptors that localizes to the α-helical domain and is essential for eliciting the complete spectrum of β-adrenergic responses.

Heterotrimeric G proteins composed of α- and βγ-subunits transduce signals from cell surface receptors to downstream effectors and regulate intracellular membrane transport events (1–6). Interaction of ligand-occupied receptors with heterotrimeric G proteins triggers the exchange of GTP for GDP on the α-subunit, leading to a sequential release of Go-GTP and the stable βγ-complex from the receptor. The released G protein subunits are then able to interact with distinct effector enzymes and ion channels. G protein activation is terminated by hydrolysis of GTP by the intrinsic GTPase activity of the α-subunit, leading to reassociation of Goa and Gβγ. The cycle is then complete, and the heterotrimeric G protein is able to be activated again.

The structural determinations for several members of the family of heterotrimeric G proteins have shown that their α-subunits are composed of two domains (1–6). The core domain contains regions with sequence similarity to other GT-Pases and has a structure very similar to Ras and elongation factor Tu (1–6). The α-helical domain is unique to α-subunits of heterotrimeric proteins and not present in other GTPases. It is therefore tempting to assume that the latter domain may be important for specific functions of heterotrimeric G proteins that are not shared by other members of the GTPase superfamily, such as coupling to heptahelical receptors. Surprisingly, current modeling suggests that receptor-G protein coupling is exclusively by the core domain of Go and segments of the β- and γ-subunits, however (1–6). The function of the α-helical domain is still under investigation. Evidence has been presented to suggest that it may influence the spontaneous rate of GDP release (7), and it has been proposed that it may function as a GTPase-activating protein (8), or may be involved in effector regulation (9).

A screening of different Goα antibodies for their applicability in studying insulin receptor-NADPH oxidase coupling revealed that one of the commercially available antibodies (K-20), which recognizes residues 100–119 within the α-helical domain of Goα, mimicked the effects of inhibitory ligands, such as the β-adrenergic agonist isoproterenol, on NADPH-dependent H2O2 generation that are transduced by Go. We therefore explored whether the epitope recognized by K-20 may define a new contact site for activated β-adrenergic receptors or be involved in an alternative route of Go activation that may be utilized for a receptor-independent activation of Go by intracellular pseudoreceptors or accessory proteins, for example (10–15).

EXPERIMENTAL PROCEDURES

Materials

The characteristics and sources of antibodies and Gα-derived peptides used in the current experiments are listed in Table I. Forskolin, 7β-deacetyl-7β-(γ-morpholino)butylrylhydrochloride, was from Research Biochemicals International and cholera toxin (A-subunit) from Calbiochem.

Methods

Subjects, Preparation of Fat Cells, and Fat Cell Ghosts—Experimental details have been described in detail elsewhere (16). Briefly, adipose tissue was from nonobese subjects undergoing elective abdominal or cosmetic breast surgery. The specimens were cut into small pieces, and fat cells were isolated in a HEPES-buffered Krebs-Henseleit solution, pH 7.4, containing 20 mM HEPES, 10 mM NaHCO3, 5 mM glucose, 20 gliter albumin, and 1 mg/ml collagenase (CLS, Worthington). After 30 min, fat cells were washed and resuspended in 10 volumes of an ice-cold lysing medium containing 20 mM MES, pH 6.0, 2 mM MgCl2, 1 mM CaCl2, 5 mM KCl, and 100 mg/liter soybean trypsin inhibitor. Cell lysis was completed by mechanical shaking, and fat cell ghosts were collected by low speed centrifugation (1,000 × g, 4 °C, 20 min).

Receptor-mediated Modulation of NADPH-dependent H2O2 Generation—A two-step procedure was used, as reported elsewhere (16). Plasma membranes from adipocytes were first exposed to hormones

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1 The abbreviations used are: MES, 2-(N-morpholino)ethanesulfonic acid; GTPγS, guanosine-5′-O-(3-thiotriphosphate); MOPS, 3-(N-morpholino)propanesulfonic acid; AMP(PNP), adenylyl imidodiphosphate; PBS, phosphate-buffered saline; CTX, cholera toxin; NEM, N-ethylmaleimide.
and were then assayed for NADPH oxidase activity. The activation step was carried out in 30 mM MOPS, pH 7.5, containing 120 mM NaCl, 1.4 mM CaCl₂, 2.5 mM MgCl₂, 10 mM NaHCO₃, and 0.1% human albumin. Membranes were first incubated with 5 mM insulin in absence or presence of 5 μM isoprotroenol for 5 min to allow receptor occupation. Thereafter, 50 μM GTP-γ-S was added. After 20 min, ghosts were collected by centrifugation, washed, and then resuspended in 30 mM MES, pH 5.8, containing 120 mM NaCl, 4 mM MgCl₂, 1.2 mM KH₂PO₄, 1 mM NaNO₂, 250 μM NADPH, and 10 μM FAD for determination of NADPH-dependent H₂O₂ generation.

To assess the effects of G protein antibodies and peptides corresponding to their target sequences on NADPH-dependent H₂O₂ generation, membranes were exposed to both types of agents at 4 °C for 45 min, as indicated in the legends to figures, and were then subjected to the two-step procedure described above.

**Determination of Adenyl Cyclase Activity**—Adenyl cyclase activity of human fat cell plasma membranes was determined in 30 mM Tris-HCl, pH 7.5, containing 1 mM ATP, 2.5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM 3-isobutyl-1-methylxanthine, 10 μM GTP, 10 μM creatine phosphate, 0.1 μg of creatine kinase, and 0.1% bovine serum albumin in a final volume of 100 μl. Reactions were initiated by addition of 5–8 μg of membrane protein and were continued for 15 min at 37 °C in the absence or presence of isoprotroenol, as indicated. Reactions were terminated by 100 μl of ice-cold perchloric acid (5%). CAMP was determined by radioimmunoassay (Amersham Pharmacia Biotech) after neutralization. To assess the effect of Goα-derived peptides and antibody K-20 on adenylyl cyclase activity, membranes were pretreated with the K-20 antibody or peptides, as described above.

**Cholera Toxin Treatment**—For cholera toxin (CTX) labeling, membranes (500 μg) were incubated for 45 min at 37 °C in 1 ml of 30 mM MOPS, pH 7.5, containing 2.5 mM MgCl₂, 1.4 mM CaCl₂, 120 mM NaCl, 1 mM thymidine, 10 mM arginine, 10 mM NAD, 10 μM NADPH, and 100 μg of cholera toxin A subunit. Membranes were washed, and then subjected to GTase assay.

**GTase Activity**—GTP hydrolysis was determined essentially as described by Jakobs and Aktories (20). Untreated or CTX-treated membranes were preincubated (20 min, 4 °C) in 20 mM Tris-HCl, pH 7.6, containing 2.5 mM MgCl₂, 0.5 mM EDTA, and 100 μM N-ethylmaleimide (NEM) to inactivate Go. After washing, NEM-treated membranes were exposed to antibody K-20 or the peptide corresponding to its target sequence for 40 min at 0 °C, as indicated. For determination of GTase activity, membranes (5–10 μg of protein) were incubated in 0.1 ml Tris-HCl (20 mM, pH 7.6) containing 0.2 μM GTP, 0.5 mM ATP, 0.5 mM AMP/VP/NP, 2.5 mM MgCl₂, 1 mM EDTA, 1 mM diithiothreitol, 5 mM creatine phosphate, 0.025 μg of creatine kinase, and [γ-³²P]GTP (0.1–0.2 μM) at 37 °C for 10 min. Reactions were terminated by addition of 25% (w/v) activated charcoal. Release of [³²P]P was determined by counting aliquots of the supernatants for radioactivity.

Low affinity GTase activity was determined by measuring the rate of GTP hydrolysis at 50 μM GTP. Less than 16% of total GTP hydrolysis was due to low affinity GTPases under the conditions used.

**Immunoprecipitation**—After treatment with K-20, membranes were pelleted and solubilized in 1% Triton X-100 for 30 min at 0 °C. Goα was immunoprecipitated by COOH-terminal anti-Goα (RM/1), which does not discriminate between heterotrimeric and monomeric Goα (17, 18). Immunoprecipitations were carried out in 10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, proteinase inhibitors (0.4 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, 2 μM pepstatin, 1 unit/ml aprotinin), and antibody RM/1 at a dilution of 1:80 and 50 μM GTP·S. The samples (30 μg of membrane protein/100 μl of precipitation buffer) were incubated overnight at 4 °C. The immune complexes were captured with protein A-agarose beads (Amersham Pharmacia Biotech) and washed three times with 1 ml of washing buffer (20 mM Tris, pH 7.4, containing 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, and the protease inhibitors). The beads were boiled in SDS-sample buffer.

Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to Hybond polyvinylidene difluoride membranes. Western blotting was performed using anti-Goα (RM/1) and anti-Gβ (SW1 from NEN Life Science Products) antibodies. Bands were visualized by chemiluminescence using the ECL kit from Amersham Pharmacia Biotech.
is mediated by βγ-subunits (16). At maximal concentrations, both isoproterenol and K-20 inhibited the stimulatory effect of insulin on NADPH-dependent H₂O₂ generation completely, and their effects could be reversed by Gβ-antibodies or agents that specifically bind Gβγ. Thus, K-20 mimicked the action of activated β-adrenergic receptors in every aspect studied, suggesting that the epitope recognized by K-20 may either define a new contact site for β-adrenergic receptors on the α-helical domain of Gs or be involved in an alternative route of Gs activation that may be utilized for a receptor-independent activation of Gs.

The stimulatory effect of K-20 on GTP hydrolysis could be neutralized by a peptide corresponding to its target sequence, as expected (Fig. 4A). Surprisingly, the peptide not only neutralized the effect of the antibody on GTPase activity but inhibited isoproterenol action on GTP hydrolysis as well (Fig. 4B). At a concentration of 2.5 μM, the peptide suppressed the stimulatory effect of isoproterenol over the whole range of concentrations tested, suggesting that the epitope recognized by K-20 may in fact be essential for interaction of activated β-adrenergic receptors with Gs (Fig. 4B).

Fig. 5 compares the effects of peptide 100–119 and of a COOH-terminal decapeptide derived from Gbgα (Table I) on isoproterenol-stimulated rates of cAMP formation and its inhibitory effect on NADPH-dependent H₂O₂ generation. At the concentrations used, both peptides decreased isoproterenol induced cAMP production to basal levels (Fig. 5A). The inhibitory effect of isoproterenol on NADPH-dependent H₂O₂ generation was also reversed by peptide 100–119 (Fig. 5B). Surprisingly, the COOH-terminal decapeptide was only effective in inhibiting cAMP formation, a Gbgα-mediated response, but failed to influence the inhibitory effect of isoproterenol on NADPH-dependent H₂O₂ generation, which is mediated by βγ-subunits (Fig. 5B). The effect of peptide 100–119 was specific, inasmuch as this fragment had no effect on forskolin-stimulated rates of cAMP production (Fig. 6).

**DISCUSSION**

This report demonstrates that polyclonal antibodies directed against a segment of the α-helical domain encompassing residues 100–119 of Gbgα caused a dissociation of Gbgα into its component subunits, stimulated a cholera toxin-sensitive high affinity GTPase, and were as efficient in stimulating adenylyl cyclase activity as the β-adrenergic agonist isoproterenol, suggesting that this antibody binds to and stimulates Gbgα in manner similar to that of ligand-occupied receptors. Consistently, the antibody not only stimulated adenylate cyclase but mimicked the inhibitory action of isoproterenol on NADPH-dependent H₂O₂ generation as well, which is mediated by Gβγ, indicating that activation of a single G protein, Gbgα, can provide enough Gβγ for eliciting a Gβγ-mediated response, which has been questioned (21). With one possible exception (22), K-20 is the first example of an antibody exhibiting receptor-mimetic effects, a property that should be extremely useful in elucidating the role of Gbgα in processes where receptors have not yet been identified, such as membrane traffic (11, 23) or complex cellular responses, including cell differentiation (24).

The antigenic site recognized by antibody K-20 encompasses the distal end of helix A and the beginning of the following loop,
a region of general sequence diversity among Gb-subunits that is freely accessible and seems to be poorly ordered in crystals of Gb1–GTP·Y3 (25). Interestingly, in Gb12, an adjacent region, e.g. the helix B-helix C segment, undergoes substantial structural changes upon GTP hydrolysis resulting in an opening of the nucleotide binding pocket (26). However, current modeling suggests that the antigenic site recognized by K-20 may be too distant from the plasma membrane (>35 Å) to be involved in direct physical contact with activated Gb-adrenergic receptors (5, 6), raising the interesting possibility that helix A and the beginning of the following loop may be utilized for a receptor-independent activation of Gb. Indeed, it is well established that the activity of G proteins may be directly modulated by a diverse group of proteins, including terminal complement complexes (13), presenilin (15), neuromodulin (11), tubulin (27), caveolins (28, 29), not yet identified proteins (12), or amphiphilic small molecular weight compounds, such as mastoporans (10), carbolins (30), and taste substances (14). Surprisingly, the peptide corresponding to the antigenic site of K-20 (residues 100–119) not only neutralized the action of the antibody but also impaired 100–119 not only neutralized the action of the antibody but also impaired 100–119 not only neutralized the action of the antibody but also impaired action by peptide 100–119. K-20 (dilution 1:10,000) was preincubated in the absence or presence of 2.5 μM peptide 100–119 for 1 h at 4 °C, and then assayed for its effect on high affinity GTPase activity, as described. B, reversal of the stimulatory effect of isoproterenol on high affinity GTPase activity. Membranes (5 μg of protein) were preincubated in the absence (■) or presence (▲) of 2.5 μM peptide 100–119 for 1 h at 4 °C, and then assayed for isoproterenol-stimulated GTPase activity, as described. Values are means ± S.D. of three separate experiments for each condition.

**Fig. 4.** Reversal of the stimulatory effect of isoproterenol on GTP hydrolysis by a peptide corresponding to the target sequence of K-20 (residues 100–119 of Gb). A, neutralization of K-20 action by peptide 100–119. K-20 (dilution 1:10,000) was preincubated in the absence or presence of 2.5 μM peptide 100–119 for 1 h at 4 °C, and then assayed for its effect on high affinity GTPase activity, as described. B, reversal of the stimulatory effect of isoproterenol on high affinity GTPase activity. Membranes (5 μg of protein) were preincubated in the absence (■) or presence (▲) of 2.5 μM peptide 100–119 for 1 h at 4 °C, and then assayed for isoproterenol-stimulated GTPase activity, as described. Values are means ± S.D. of three separate experiments for each condition.

**Fig. 5.** Reversal of the antagonistic effects of isoproterenol on adenylyl cyclase and NADPH oxidase activities by peptide 100–119: comparison with the effects of a COOH-terminal peptide corresponding to residues 385–394 of Gb. Plasma membranes were preincubated for 45 min at 4 °C in the absence (■) or presence of 7.5 μM peptide corresponding to residues 385–394 (▲) or of 2.5 μM peptide corresponding to residues 100–119 (▲). A, suppression of the stimulatory effects of isoproterenol on cAMP production by both peptides. Membranes were assayed for adenylyl cyclase activity in the presence of increasing concentrations of isoproterenol, as described under “Methods.” Addition of vehicle had no effect on isoproterenol stimulated adenylyl cyclase activity. B, selective reversal of the isoproterenol-induced inhibition of NADPH-dependent H2O2 generation by peptide 100–119. Membranes were incubated with 5 nM insulin and 50 μM GTP·Y3 in absence or presence of 5 μM isoproterenol, and then assayed for NADPH oxidase activity. Addition of vehicle had no effect on isoproterenol induced inhibition of H2O2 generation. Values are means ± S.D. of four separate experiments for each condition.
signal transducing complex that could include receptors, G proteins, effectors, and even intracellular targets of the second messengers generated (28, 29). It has been reported that G proteins bind to chief structural proteins of these organelles, the caveolins, via a sequence that lies between the switch-I and switch-II regions of the α-subunit (28, 29). An incorporation of this latter site into structural models of the receptor/G protein complex changes the predicted orientation of the G protein in a way that brings the α-helical domain in close proximity to the membrane, which is consistent with the present data predicting a direct contact of activated β-adrenergic receptors with the distal end of helix A and the beginning of the following loop.

Overall, it thus appears that antibody K-20 defines an as yet unrecognized contact site for β-adrenergic receptors on Ga, that localizes to the α-helical domain. This latter segment is the first example of a receptor binding region outside the core GTPase domain of Ga and is essential for eliciting the responses transmitted by both Ga and Gβγ.

As yet, the most precisely defined site of receptor contact involves the extreme carboxyl terminus, which is thought to be of fundamental importance for receptor recognition and G protein activation (1–6, 32–35). Indeed, COOH-terminal anti-Ga and the peptide corresponding to its target sequence were as efficacious as peptide 100–119 in blocking the isoproterenol-induced suppression of NADPH-dependent H2O2 generation, a Gβγ-mediated response. Thus, a contact of activated β-adrenergic receptors with the extreme carboxyl terminus of Ga appears to be required solely for receptor signaling via Ga, but is apparently not critical for receptor recognition and dissociation of the Gα heterotrimer. The latter finding is difficult to reconcile with current models of G protein activation but is consistent with the observations of others, indicating that COOH-terminal Ga antibodies act as reliable and specific inhibitors of receptor signaling via Ga-subunits only. By contrast, their effects on Gβγ-mediated responses are variable and may depend on the G protein, receptors, or cell type studied. Thus, antibodies directed against the COOH termini of Ga and Ga12 have been shown to block signaling by m3 muscarinic and AT1A angiotensin II receptors via both types of component subunits of Gα and Gα12, while anti-Ga119, and anti-Gα2 or anti-Gα3 had no effect on Gβγ-mediated responses elicited by sotr 3-somatostatin receptor stimulation or during transcytosis, respectively (23, 36–38).

Together with the essential role of the epitope recognized by antibody K-20, the observation that COOH-terminal anti-Ga selectively impaired β-adrenergic receptor signaling via Ga without affecting the Gβγ-mediated response is consistent with the concept that β-adrenergic receptors interact with multiple sites on Ga, each playing a distinct role, as has also been proposed by others (39). More importantly, this latter finding implies that the activation and/or release of Gβγ by ligand-occupied β-adrenergic receptors may not require the simultaneous activation of Ga, which is in marked contrast to current concepts of G protein regulation. A subunit-specific activation of a G protein has in fact been described for the insulin-like growth factor II receptor, which may selectively turn on Gaβγ-mediated pathways (40). However, this latter type of selectivity is achieved through sequestration of free βγ-subunits by the activated receptors, which is not comparable with the mechanism outlined above. Whether receptors exist that selectively activate Ga without affecting the function of Gβγ or vice versa remains to be established.

In conclusion, the current findings show that a commercially available polyclonal antiserum directed against residues 100–119 within the α-helical domain of Ga (K-20) acts as a β-adrenergic receptor-mimetic agent, and defines a novel contact site for activated β-adrenergic receptors that localizes to the α-helical domain. In contrast to the COOH terminus of Ga, which seems to be critical for β-adrenergic receptor signaling via Ga only, the epitope recognized by antibody K-20 appears to be essential for eliciting the complete spectrum of β-adrenergic responses mediated by both Gaα as well as Gβγ.

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