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Interaction of the Xanthine Nucleotide Binding Goα Mutant with G Protein-coupled Receptors*

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We constructed a double mutant version of the α subunit of Go that was regulated by xanthine nucleotides instead of guanine nucleotides (GoαX). We investigated the interaction between GoαX and G protein-coupled receptors *in vitro*. First, we found that the activated m2 muscarinic cholinergic receptor (MAChR) could facilitate the exchange of XTPγS for XDP in the GoαX$\beta\gamma$ heterotrimer. Second, the GoαX$\beta\gamma$ complex was able to induce the high affinity ligand-binding state in the *N*-formyl peptide receptor (NFPR). These experiments demonstrated that GoαX was able to interact effectively with G protein-coupled receptors. Third, we found that the empty form of GoαX, lacking a bound nucleotide and $\beta\gamma$, formed a stable complex with the m2 muscarinic cholingeric receptor associated with the plasma membrane. Finally, we investigated the interaction of GoαX with receptor in COS-7 cells. The empty form of GoαX bound tightly to the receptor and was not activated because XTP was not available intracellularly. We tested the ability of GoαX to inhibit the activities of several different G protein-coupled receptors in transfected COS-7 cells and found that GoαX specifically inhibited Go-coupled receptors. Thus the modified G proteins may act as dominant-negative mutants to trap and inactivate specific subsets of receptors.

Hundreds of seven-transmembrane receptors activate heterotrimeric G proteins and transduce signals across cell membranes in eukaryotic cells. The stimulated receptors catalyze the exchange of GTP for GDP bound to G protein α subunits. Activated GTP-bound α subunits and free $\beta\gamma$ subunits regulate a variety of cellular effectors including enzymes and ion-channels (1–3). Signaling is normally initiated by the binding of agonist to receptor, which stabilizes the receptor in an active conformation. Receptors function to stimulate the dissociation of GDP bound to the G protein α subunits. The subsequent binding of GTP to the empty α subunit promotes the conformational change of Gα and dissociation of the $\beta\gamma$ subunits. The G protein α subunit in the nucleotide-free state appears to be an important intermediate in the activation. From studies of rhodopsin and transducin, it has been postulated that the empty G protein (nucleotide-free) forms a stable complex with the receptor (4). Both empty forms of Gi and Go α subunits have been purified under harsh conditions (1 M $(NH_4)_2SO_4$ and 20% glycerol), and they were unstable (5).

We recently reported that a mutant version of Goα, GoαX (GoαD273N/Q205L), was regulated by xanthine nucleotides, not by guanine nucleotides (6). GoαX bound XDP[1] and XTP instead of GDP or GTP. GoαX bound G protein $\beta\gamma$ subunits only in the presence of XDP, and XTP stimulated dissociation of the GoαX$\beta\gamma$ heterotrimer. XTP-bound GoαX underwent a conformational change similar to the activated wild-type Goα. In the present study, we investigated the interaction between GoαX and G protein-coupled receptors. We found that GoαX mutant proteins retained the receptor binding specificity of the wild-type Goα and were able to interact with Go-coupled receptors, such as the m2 muscarinic cholinergic receptor (MAChR), *N*-formyl peptide receptor (NFPR), and thrombin receptor, but not with m1 MAChR or thyrotropin-releasing hormone (TRH) receptor which do not couple to wild-type Go. Because the concentrations of XDP and XTP are relatively low *in vivo* (7), GoαX mutant proteins are essentially nucleotide-free unless exogenous xanthine nucleotides are provided. Thus, GoαX provides an excellent model to study the receptor interaction of empty G protein α subunits. Consistent with the previously reported studies on the empty form of transducin (4), our data are most readily interpreted as showing that "empty" GoαX can form a stable complex with appropriate receptors on the membrane.

EXPERIMENTAL PROCEDURES

Materials—Purified bovine retinal transducin $\beta\gamma$ were generous gifts from Dr. O. Nakanishi (Division of Biology, Caltech). Xanthine and guanine nucleotides were from Sigma. All the radioactive ligands including [^{35}S]ATPγS, [^{35}S]GTPγS, [^{3}H]QNB, and fML[^{3}H]P were from NEN Life Science Products.

Expression and Purification of His$_6$-tagged Goα—Both wild-type Goα and mutant GoαX were subcloned into the *Escherichia coli* expression vector pET-15b (Novagen) with His_6 tag at the N terminus. These clones were used to transform the *E. coli* strain BL21(DE3), and proteins were expressed. Expression and purification of these proteins was described previously (6, 8). After harvesting the culture, cell extracts were resuspended in the binding buffer (5 mM imidazole, 0.5 M NaCl, 160 mM Tris-HCl, pH 7.9, and 1 mM βMe). The His_6-tagged proteins were purified from Ni^{2+}-NTA column according to the protocol provided by Novagen. Purified proteins were stored in TED buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM dithiothreitol) with 0.1 mM $MgCl_2$ and 0.1 mM nucleotide diphosphate (GDP or XDP as appropriate).

Membrane Preparation from Baculovirus-infected Sf9 Cells—Sf9 cells were grown and infected with recombinant baculoviruses encoding either m2 MAChR or NFPR (9–11). Infected cells were centrifuged and resuspended at $<10^7$ cells/ml in HME/PI buffer (20 mM NaHepes, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 10 μg/ml leupeptin). The cell suspension was homogenized by 10 strokes in a glass homogenizer followed by passing through a 27 gauge hypodermic needle several times. The homogenate was briefly centrifuged at 3,000 $\times$ *g* for 10 min, and then the supernatant was collected and centrifuged at 30,000 $\times$ *g* for 30 min. The pellet was washed once with HME/PI, and the final pellet was resuspended in HME/PI at a protein concentration of 5 mg/ml.

Synthesis of XTPγS—XTPγS was synthesized from XDP and ATPγS

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[1] The abbreviations used are: XDP, xanthine diphosphate; XTP, xanthine triphosphate; MAChR, muscarinic cholinergic receptor; NFPR, *N*-formyl peptide receptor; TRH, thyrotropin-releasing hormone; NDK, nucleotide diphosphate kinase; IP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); XTPγS, xanthine 5′-*O*-(3-thiotriphosphate); [^{3}H]QNB, quinuclidinylbenzilate; fML[^{3}H]P, formyl-methionyl-leucyl-phenylalanine.

with nucleotide diphosphate kinase (NDK) as described previously (12). To produce ^{35}S-labeled XTPγS, the reaction contained 10 μM XDP, 1 μM [^{35}S]ATPγS, and 10 units of NDK (Sigma) in 100 μl of NDK buffer (1 mM $MgCl_2$, 5 mM dithiothreitol, and 20 mM Tris-HCl, pH 8.0). The mixture was incubated at room temperature for 2 h. The resulting concentration of [^{35}S]XTPγS was about 1 μM (1 μCi/pmol). The radiochemical purity of XTPγS was monitored by TLC on Avicel/PEAE plates (Analtech) in 0.07 N HCl.

Nucleotide Binding of Purified Goα—Binding of [^{35}S]GTPγS and [^{35}S]XTPγS to the recombinant Goα or the mutant proteins was performed as described (6). The binding reaction contained 0.5 μg of purified protein in TED buffer, with 0.1 mM $MgCl_2$, 1 μM ATP, and 0.1 μM GTPγS or XTPγS (20,000 cpm/pmol). For the time course experiments, 20-μl aliquots were withdrawn from a 200-μl reaction, diluted 10-fold with ice-cold TED buffer containing 0.1 mM $MgCl_2$, filtered through 45-μm nitrocellulose, washed, and dried. The amount of bound radioactivity was determined by scintillation counting.

Radioligand Binding of Receptors—The ligand binding assays of membrane-bound receptors were performed as described (9–11). The total concentration of m2 MAChR and the affinities of NFPR were determined by incubating membranes with 2 nM [^{3}H]QNB or various concentrations of fML[^{3}H]P for 1 h in 20 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, and 1 mM EDTA at 30 °C in a final volume of 0.5 ml. Nonspecific binding was defined as binding that was not displaced by 10 μM atropine for m2 MAChR or 10 μM fMLP for NFPR. Unbound ligands were removed by filtration through Whatman GF/C filters and washing four times using ice-cold binding buffer. The amount of bound radioactivity was determined by scintillation counting.

Binding of Goα on Sf9 Cell Membranes—0.2 μg of purified Goα or Goα mutant proteins were incubated with 100 μg of Sf9 cell membranes in TED buffer of a final volume of 100 μl at room temperature for 1 h. The membranes were centrifuged and subjected to Western blot using antibodies against Goα.

Cell Culture and Transfection—COS-7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. 1×10^5 cells/well were seeded in 12-well plates 1 day before transfection. All transfection assays contained a total amount of 1 μg of DNA, and pCIS encoding β-galactosidase was used to maintain a constant amount of DNA. To each well, 1 μg of DNA was mixed with 5 μl of LipofectAMINE (Life Technologies, Inc.) in 0.5 ml of Opti-MEM (Life Technologies, Inc.), and 5 h later, 0.5 ml of 20% fetal calf serum in Dulbecco's modified Eagle's medium was added to the medium. After 48 h, cells were assayed for inositol phosphate levels as described previously (13, 14).

RESULTS

Stimulation of XTPγS Binding of GoαX by Activated M2 Muscarinic Receptor—To test if GoαX could interact with G protein-coupled receptors, we investigated the receptor-stimulated nucleotide binding of GoαX. Activated G protein-coupled receptors are known to facilitate the binding of GTPγS to G protein α subunits. It has been reported that recombinant m2 MAChR from Sf9 cells stimulated the binding of GTPγS to wild-type Goα 2–3-fold in response to muscarinic agonists (9, 10). We infected Sf9 cells with recombinant baculoviruses encoding m2 MAChR and prepared membranes. The concentration of receptor was about 20 pmol/mg of membrane protein, determined from [^{3}H]QNB binding. We have previously shown that GoαX mutant proteins bind only xanthine nucleotides, but not guanine nucleotides. In this experiment, we reconstituted purified GoαX with Sf9 cell membranes containing m2 MAChR in the presence of XDP and G protein βγ subunits purified from bovine retina, and followed agonist-dependent stimulation of XTPγS binding to GoαX. We found that carbachol accelerated the XTPγS binding of GoαX, in a fashion similar to the acceleration of GTPγS binding observed with wild-type Goα (Fig. 1*a*). In control experiments using wild-type Sf9 cell membranes, both atropine and carbachol had no effect on the XTPγS binding of GoαX (Fig. 1*a*). βγ subunits were required for the carbachol-dependent stimulation of nucleotide binding (Fig. 1*b*), suggesting that only the trimeric complex of GoαX with βγ can be activated to exchange XDP for XTPγS by the ligand-bound receptors.

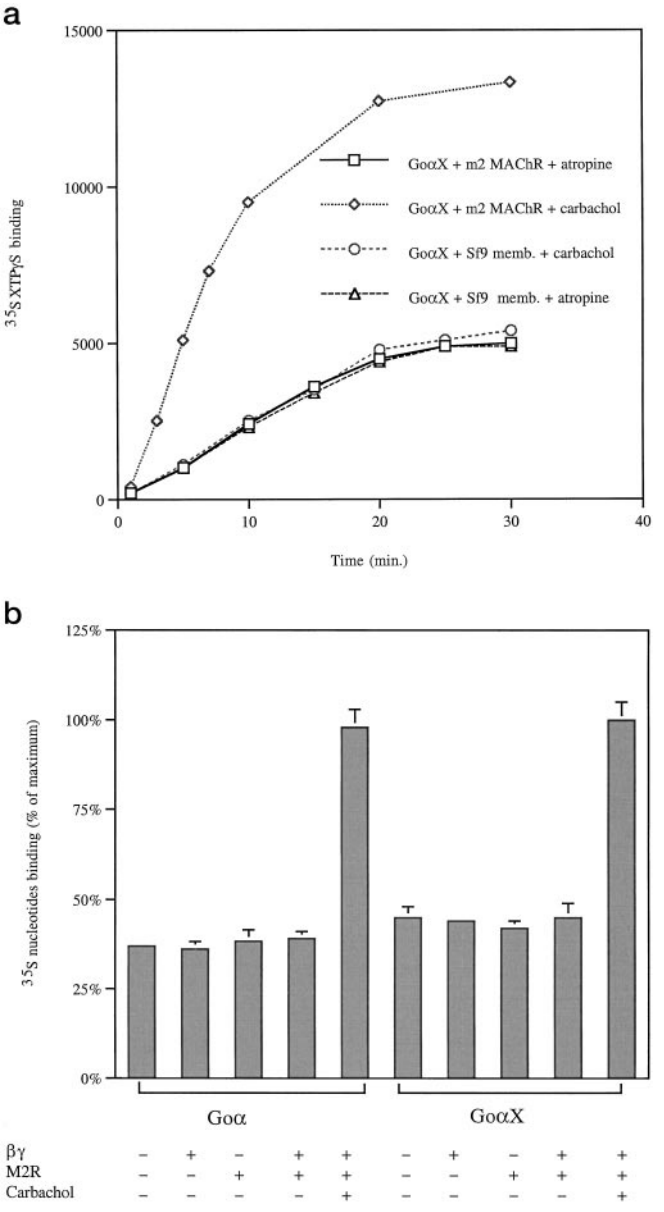

FIG. 1. **M2 MAChR stimulated XTPγS binding of GoαX.** *a*, 0.5 μg of purified GoαX was incubated with 1 μg of βγ, 100 μg of m2 MAChR membranes, or control Sf9 cell membranes and with 10 μM XDP in TEDM buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM $MgCl_2$) for 30 min at room temperature, and then the mixture was diluted 10-fold with TEDM buffer containing 0.1 μM [^{35}S]XTPγS (20,000 cpm/pmol) and 100 μM carbachol or 2 μM atropine at time 0. 20-μl aliquots were withdrawn and assayed for the bound nucleotides at the indicated times. *b*, 0.5 μg of purified wild-type Goα or GoαX were subjected to the similar nucleotide binding assay as in *panel a* with GTPγS or XTPγS under indicated conditions. Only data at 20 min were shown as the percentage of maximum binding.

High Affinity Ligand Binding of N-formyl Peptide Receptor Induced by GoαX—Another well documented indication of receptor-G protein interaction is that GTP or GTPγS inhibits the high affinity binding of G protein-coupled receptors to their agonists. NFPR receptors expressed in Sf9 cells are known to be in the low ligand affinity state (~60 nM fMLP) (11), presumably because of the lack of mammalian Gi-like G proteins in Sf9 cells, whereas NFPR in neutrophils and NFPR expressed in mouse L cells exhibited high affinity ligand binding (0.5–3 nM fMLP) (15, 16). Therefore, we reconstituted GoαX with NFPR from Sf9 cells and investigated whether GoαX could induce the high affinity ligand binding state in NFPR receptors. Sf9 cell membranes containing NFPR receptors were prepared as de-

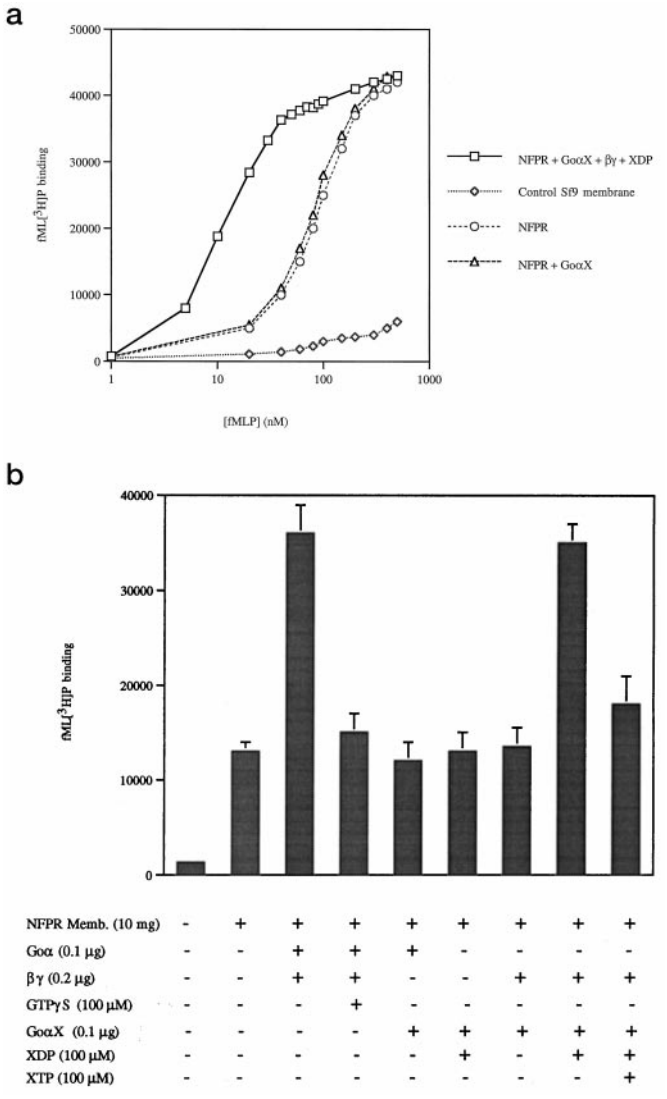

FIG. 2. **High affinity ligand binding of NFPR induced by GoαX.** *a*, 10 μg of NFPR membranes or wild-type Sf9 cell membranes were incubated with various concentrations of fML[^{3}H]P for 1 h in 20 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, and 1 mM EDTA at 30 °C in a final volume of 0.5 ml, in the presence of 0.1 μg of GoαX, 0.2 μg of βγ, and 100 μM XDP, or GoαX alone. The amount of bound radioligand was then determined. Nonspecific binding was defined as binding in the presence of 10 μM cold fMLP, which was less than 10% of total ligand binding, and was subtracted before analyzing. *b*, NFPR was incubated with 50 nM fML[^{3}H]P and various reagents under the same conditions as *panel a*.

scribed under "Experimental Procedures." The expression level of NFPR was about 20 pmol/mg of membrane protein, determined by fML[^{3}H]P binding. We incubated the NFPR with wild-type Goα or mutant GoαX in the presence of βγ and varieties of nucleotides and determined their affinities for the agonist fMLP. As expected, NFPR expressed in Sf9 cells showed low ligand affinity binding of fMLP (~100 nM), and GoαX alone did not affect ligand binding (Fig. 2*a*). More interestingly, NFPR exhibited high affinity ligand binding (~10 nM) when GoαX, βγ, and XDP were present (Fig. 2*a*). Both βγ and XDP were required to induce the high ligand affinity state of NFPR, and XTP inhibited the fMLP binding of the receptors (Fig. 2*b*). In the control experiments, wild-type Go αβγ heterotrimer was also found to convert the NFPR to the high affinity ligand binding state, which was inhibited by GTPγS (Fig. 2*b*). These experiments demonstrated that the heterotrimeric complex of GoαXβγ can interact efficiently with NFPR.

Binding of GoαX with M2 Muscarinic Receptor on Sf9 Cell

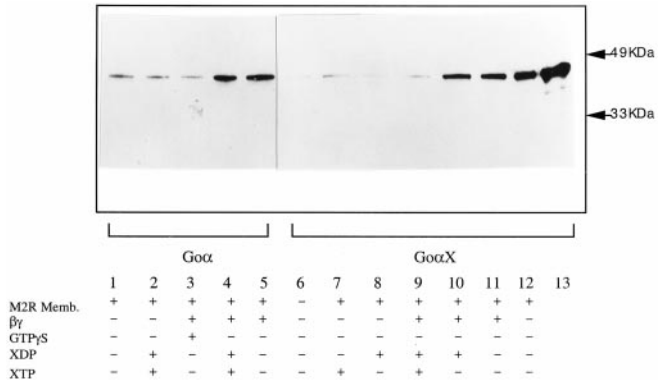

FIG. 3. **Binding of GoαX to m2 MAChR on Sf9 cell membranes.** 0.2 μg of wild-type Goα (with 100 μM GDP) or GoαX were incubated with 100 μg of m2 MAChR membranes in TED buffer of a final volume of 100 μl at room temperature for 1 h with indicated reagents. The membrane then was centrifuged and subjected to Western blot using antibodies against Goα. All nucleotide concentrations were 100 μM, and the amount of βγ was 0.5 μg. *Lane 13* shows the total amount of Goα used in each assay.

Membranes—The previous two experiments showed that GoαXβγ heterotrimer could interact with the G protein-coupled receptors efficiently and that the interaction was similar to the interaction between wild-type Go and receptors. To investigate receptor interaction of GoαX more directly, we studied binding of GoαX to receptor containing Sf9 cell membranes. Purified wild-type Goα or GoαX were incubated with Sf9 cell membranes containing m2 MAChR in the presence of different reagents. The membranes were then pelleted and subjected to Western blotting using antibodies against Goα to see if Goα remained bound to the membrane. In the control experiments using wild-type Sf9 cell membranes without m2 MAChR, both wild-type Goα and GoαX did not remain associated with the membrane. However, wild-type Goα was bound to membrane when it was coincubated with βγ. Similarly, GoαX stayed on the membrane when in complex with βγ in the presence of XDP (data not shown). These experiments using wild-type Sf9 cell membranes showed that Goα bound to the membranes only in the αβγ complex form, presumably because βγ facilitates membrane association. In the experiments using membranes containing m2 MAChR, we found somewhat surprisingly that GoαX bound to receptor-containing membranes even in the absence of carbachol and without βγ (Fig. 3, *lane 12*), whereas wild-type Goα did not (Fig. 3, *lane 1*), suggesting GoαX alone was able to bind to receptor. Interestingly, both XDP and XTP abolished the interaction between GoαX and m2 MAChR-containing membranes and released GoαX from the membrane fraction (Fig. 3, *lanes 7* and *8*), whereas GDP or GTP had no effect (data not shown), suggesting that the nucleotide-free form of GoαX can recognize and bind to Go-mediated receptor. As expected, GoαX stayed on the membrane when both XDP and βγ were present (Fig. 3, *lane 10*), and XTP promoted dissociation of the GoαXβγ complex (Fig. 3, *lane 9*). In the case of wild-type Goα, the binding pattern was the same between membranes with or without the receptors, and XDP or XTP had no effect on binding (Fig. 3, *lanes 1*-5). In a titration experiment, quantitation of GoαX revealed that the amount of GoαX bound to the membrane increased linearly until it reached saturation, and the level of saturation was proportional to the amount of receptor incubated in the reaction (Fig. 4, *a–c*). Furthermore, similar experiments using Sf9 cell membranes containing NFPR were also performed, and the results were similar (data not shown). These experiments indicated that the empty form of GoαX, without a bound nucleotide and βγ, could form a stable complex with receptor. In summary, these data

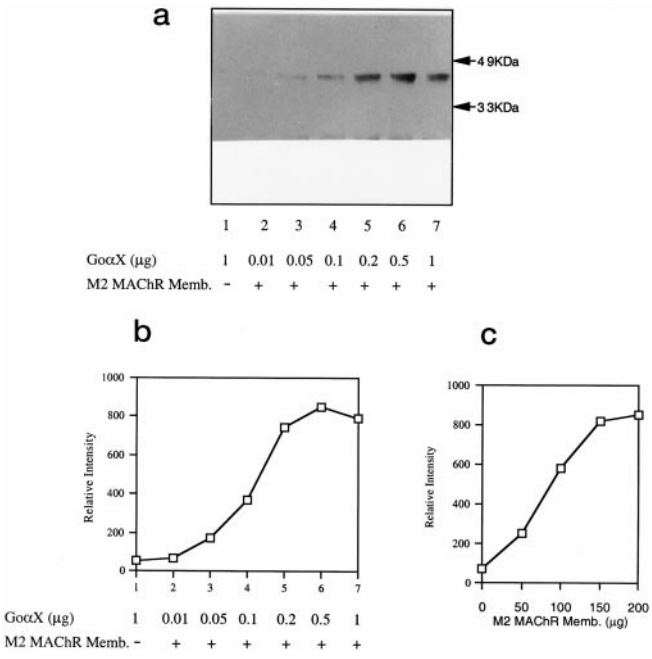

FIG. 4. **Titration of GoαX bound to m2 MAChR on Sf9 cell membranes.** The binding assays were done under the same conditions described in Fig. 3 legend. *a*, indicated amount of GoαX was incubated with 100 μg of m2 MAChR membranes in TED buffer. *b*, the relative intensities of bands in *panel a* were quantitated. *c*, quantitated binding of 0.2 μg of GoαX with indicated amount of m2 MAChR membranes. Wild-type Sf9 cell membranes were used to maintain a constant amount of membranes of 200 μg in each binding reaction.

suggest that GoαX with XDP bound and βγ bind to membranes, whereas the XTP form is found to be cytoplasmic. The nucleotide-free form is able to bind to Go-mediated receptors.

Dominant-negative Effect of GoαX on Receptor Activation in COS-7 Cells—Because our experiments suggested that empty GoαX was able to bind to the receptor *in vitro*, we went on to test for this interaction in intact cells. Indeed, we found that GoαX was able to interact with receptors and inhibit their activities in COS-7 cells consistent with the observation that GoαX did not dissociate from the receptors without xanthine nucleotides. Thrombin receptors are known to couple with G proteins from both the Gi and Gq families (17). In COS-7 cells transfected with the thrombin receptor, endogenous Gq is activated by the addition of thrombin and stimulates PLCβ isoforms to elevate cellular IP_3 concentration. Inhibition of receptor activation in transfected cells by wild-type G proteins was observed before (18). Thus if cells are cotransfected with both the thrombin receptor and wild-type Goα subunit, the activation of Gq is inhibited because of the competition of Goα for the receptor or endogenous G protein βγ subunits (Fig. 5*a*). We cotransfected thrombin receptor and GoαX to determine whether GoαX could compete with endogenous Gq for the receptors. Indeed, we found that GoαX inhibited Gq activity stimulated by thrombin, and the inhibition was proportional to the amount of GoαX cDNA used in the transfection (Fig. 5*a*). Because GoαX in the absence of XDP does not interact with βγ and does not affect the βγ-stimulated PLCβ activity in COS-7 cells (6), the inhibition by GoαX of Gq activation stimulated by the thrombin receptor must come from the competitive binding of GoαX to the receptor. Similar experiments were performed with m1 MAChR and TRH receptor which were known to couple only to the Gq family of Gα proteins and not to the Goα family (10, 19, 20). GoαX had no effect on the activation of m1 MAChR or TRH receptor (Fig. 5, *b* and *c*). On the other hand, wild-type Goα, which can compete for endogenous βγ, inhibited both m1 MAChR and TRH receptor stimulation as expected. In COS-7 cells, the activation of thrombin receptor, m1 MAChR, and TRH receptor share the same Gq pathway downstream of the receptor. Because GoαX inhibited only the thrombin receptor activity, but had no effect on m1 MAChR or TRH receptor, we concluded that GoαX inhibited thrombin receptor stimulation by competitive binding to the receptor.

To test if GoαX could bind to other Go-coupled receptors in cells, we looked into the interaction between GoαX and m2 MAChR. Because m2 MAChR couples only to the Gi family of Gα proteins and not to the Gq family (9, 21), we could not assay their interaction in the same way as the thrombin receptor by monitoring PLC activities in COS-7 cells transfected with the receptor and GoαX. Therefore, we constructed an artificial pathway by cotransfecting both m2 MAChR and G15α into COS-7 cells. G15α is known as a promiscuous G protein that can be activated by all kinds of G protein-coupled receptors, and G15α also activates PLCβ isoforms (21). In cells cotransfected with both m2 MAChR and G15α, we were able to activate endogenous PLCβ isoforms by the addition of the muscarinic agonist carbachol. We found that this m2 MAChR stimulation pathway could also be inhibited by GoαX (Fig. 5*d*). All these experiments suggested that GoαX was able to interact with G protein-coupled receptors in cells and retained the receptor specificity of wild-type Goα; it coupled with thrombin receptor and m2 MAChR, but not with m1 MAChR or TRH receptor. Furthermore, GoαX exhibited dominant-negative inhibitory effects against these receptors in cells.

DISCUSSION

GoαX (GoαD273N/Q205L) was the first reported mutant of heterotrimeric G protein α subunits that bound xanthine nucleotides, not guanine nucleotides (6). It bound βγ only in the presence of XDP and could be activated by XTP. We continued to study the interaction of GoαX with G protein-coupled receptors in this report. The interaction of G proteins and their receptors is best demonstrated in two experiments: agonist-stimulated GTPγS binding of G protein α subunits and inhibition of high affinity ligand binding of the receptors by GTPγS. To test if GoαX can interact with G protein-coupled receptors and be activated by their agonists, we reconstituted purified GoαX with Sf9 cell membranes containing m2 MAChR or NFPR. First, we found that binding of XTPγS to GoαX was stimulated by the muscarinic agonist carbachol in the presence of m2 MAChR. In similar experiments using wild-type Goα, GTPγS binding was also stimulated by carbachol. In both cases, βγ was required for the carbachol-dependent nucleotide binding, suggesting that only GoαXβγ heterotrimer could interact with the receptors effectively. Second, we tested GoαX to determine whether it could induce the high affinity state in NFPR receptors expressed in Sf9 cells. The NFPR expressed in these cells is known to be in the low affinity state probably because of lack of mammalian Gi-like proteins in Sf9 cells (11). In our experiments, we found that GoαX could convert NFPR into the high affinity state in the presence of βγ and XDP, and this effect was inhibited by XTP. These two experiments demonstrated that GoαX, when in complex with βγ and XDP, could interact with G protein-coupled receptors effectively and be activated by the agonists.

Because cells lack xanthine nucleotides, GoαX provides an excellent model to study empty G protein α subunits. The empty form of Gα is an important intermediate in receptor activation and has long been proposed to form a stable complex with activated receptors. However stable interaction between empty G proteins and their receptors was only reported in the transducin-rhodopsin system. Empty transducin apparently formed a stable complex with light-activated rhodopsin and

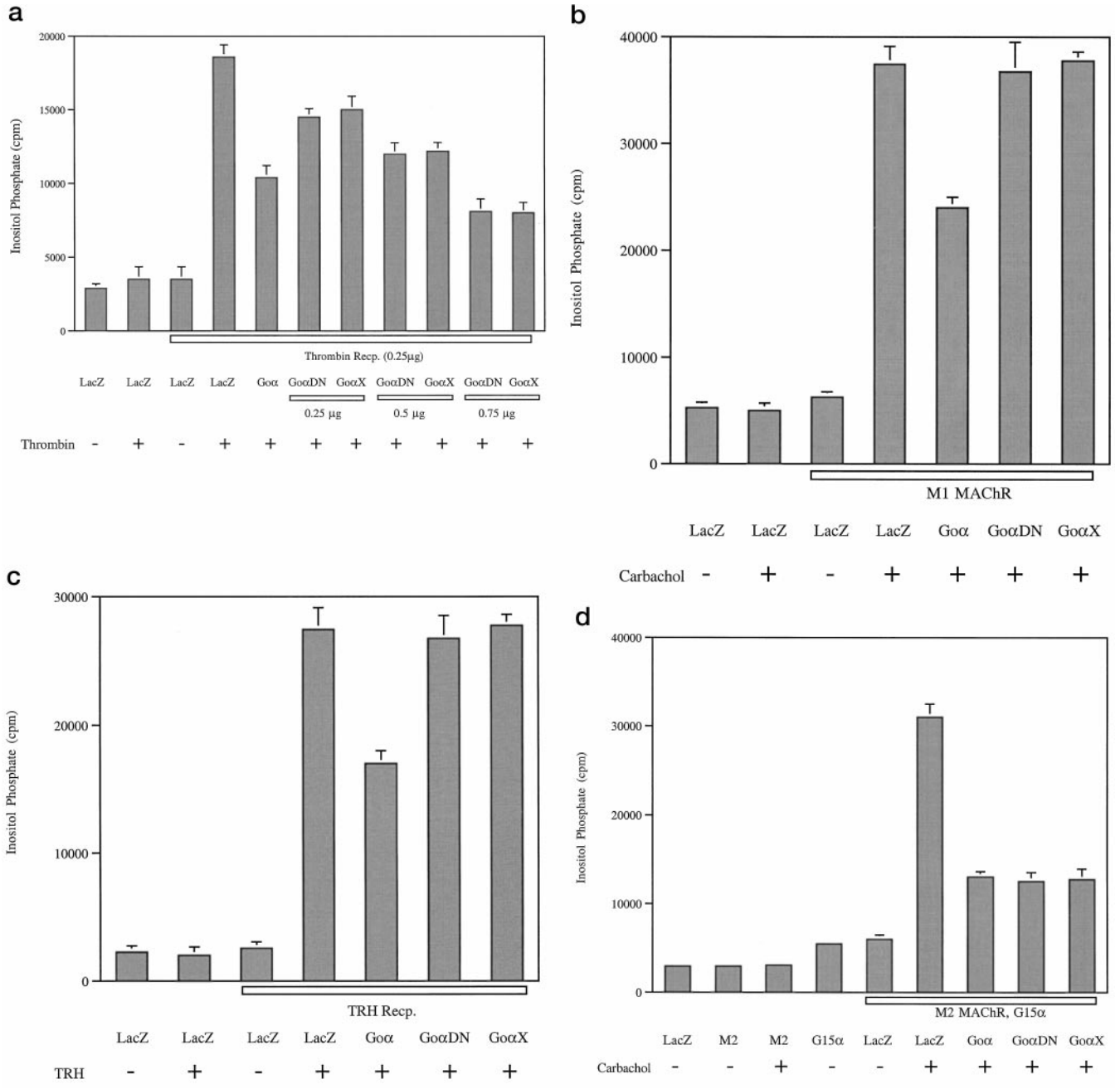

FIG. 5. **The negative inhibitory effects of GoαX on receptor-stimulated PLCβ activation in COS-7 cells.** 1×10^5 cells/well were seeded in a 12-well plate and then were transfected with cDNAs encoding the indicated G proteins (GoαDN designates GoαD273N) and thrombin receptor (*a*), m1 MAChR (*b*), TRH receptor (*c*), or m2 MAChR (*d*). In *panels a–c*, the amount of receptor cDNA used in each well was 0.25 μg, and the amount of Goα cDNA was 0.75 μg/well unless otherwise indicated. In *panel d*, the amount of both m2 MAChR and G15α cDNA was 0.2 μg/well and that of Goα was 0.6 μg/well. The total amount of cDNA for each well was adjusted to 1.0 μg by the addition of CMV-LacZ cDNA. After cells were labeled with [^{3}H]inositol overnight, they were incubated in the medium containing 0.1 unit/ml thrombin (*a*), 1 μM carbachol (*b* and *d*), or 1 μM TRH (*c*) before levels of inositol phosphates were determined.

stayed on the rod outer segment membrane. Interestingly, deactivation of the rhodopsin did not lead to the dissociation of transducin from the complex (4). In this report, we showed that empty GoαX was able to bind to the receptor on the membrane in the absence of βγ subunits and without agonists, and the interaction could be abolished by either XDP or XTP. The amount of GoαX associated on the membranes with m2 MAChR was proportional to the amount of receptor at saturation. Interestingly, binding of GoαX alone did not convert the receptor to the high ligand affinity conformation, which required the αβγ complex. Therefore, the binding of GoαX alone to the receptor is not functional in contrast with the binding in the presence of βγ and XDP.

Because GoαX appears to form a stable complex with the receptor, we tested whether GoαX could inhibit receptor activation in cells. In transfected COS-7 cells, we showed that GoαX was able to inhibit thrombin receptor or m2 MAChR stimulated PLCβ activities via the Gq or G15 pathway, but had no effect on m1 MAChR or TRH receptor stimulation. Because both thrombin receptor and m2 MAChR are known to couple with wild-type Go, and m1 MAChR and TRH receptor only couple with Gq, we interpret the data to mean that GoαX retained the receptor specificity of wild-type Go and was able to interact with Go-coupled receptors in cells. The inhibitory binding of GoαX enables us to specifically block Go-coupled receptors in certain systems. This could be a useful means to analyze

different receptor-stimulated signal transduction pathways, and could perhaps be useful in drug screening associated with G protein-coupled receptors.

In the previous report (6), we showed that the single Goα mutant, GoαD273N, lost the ability to bind either guanine nucleotides or xanthine nucleotides and could not bind βγ under any conditions. Surprisingly, GoαD273N can still bind to receptors. In transfected COS-7 cells, we found that GoαD273N inhibited thrombin receptor and m2 MAChR activation, in a fashion similar to GoαX (Fig. 5, *a* and *d*). GoαD273N also retained the same receptor specificity as wild-type Goα; *i.e.* it had no effect on m1 MAChR or TRH receptor stimulated pathways (Fig. 5, *b* and *c*). In the Sf9 cell membrane binding assay, it only bound to the m2 MAChR membranes, not to the control wild-type Sf9 cell membranes. However in contrast to GoαX, GoαD273N was not released from the m2 MAChR membranes by XDP or XTP, consistent with its inability to bind nucleotides (data not shown). The reason that GoαD273N mutant proteins do not bind xanthine nucleotides is not clear. Apparently it must have a structure similar to that of the empty Goα which enables it to bind receptors, but the structure is probably not stable locally around the nucleotide binding pocket. Nevertheless, GoαD273N may also be useful as a dominant-negative inhibitor of receptor functions.

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