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Reconstitution of rat brain μ opioid receptors with purified guanine nucleotide-binding regulatory proteins, G_i and G_o

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ABSTRACT **Reconstitution of purified μ opioid receptors with purified guanine nucleotide-binding regulatory proteins (G proteins) was investigated. μ opioid receptors were purified by 6-succinylmorphine AF-AminoTOYOPEARL 650M affinity chromatography and by PBE isoelectric chromatography. The purified μ opioid receptor (pI 5.6) migrated as a single M_r 58,000 polypeptide by NaDodSO$_4$/PAGE, a value identical to that obtained by affinity cross-linking purified μ receptors. When purified μ receptors were reconstituted with purified G_i, the G protein that mediates the inhibition of adenylate cyclase, the displacement of [^{3}H]naloxone (a μ opioid antagonist) binding by [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (a μ opioid agonist) was increased 215-fold; this increase was abolished by adding 100 μM (guanosine 5′-[γ-thio]triphosphate. Similar increases in agonist displacement of [^{3}H]naloxone binding (33-fold) and its abolition by guanosine 5′-[γ-thio]triphosphate were observed with G_o, the G protein of unknown function, but not with the v-Ki-ras protein p21. In reconstituted preparations with G_i or G_o, neither [D-Pen^2,D-Pen^5]enkephalin (a δ opioid agonist; where Pen is penicillamine) nor U-69,593 (a κ opioid agonist) showed displacement of the [^{3}H]naloxone binding. In addition, the μ agonist stimulated both [^{3}H]guanosine 5′-[β,γ-imido]triphosphate binding (in exchange for GDP) and the low-K_m GTPase in such reconstituted preparations, with G_i and G_o but not with the v-Ki-ras protein p21, in a naloxone-reversible manner. The stoichiometry was such that the stimulation of 1 mol of μ receptor led to the binding of [^{3}H]guanosine 5′-[β,γ-imido]triphosphate to 2.5 mol of G_i or to 1.37 mol of G_o. These results suggest that the purified μ opioid receptor is functionally coupled to G_i and G_o in the reconstituted phospholipid vesicles.**

Opioid receptors trigger various responses, including changes in levels of second messengers and ion-channel activities (1–5). Many receptor-mediated reactions are mediated through the activation of guanine nucleotide-binding regulatory proteins (G proteins) (6, 7). Opioid receptors in neuroblastoma–glioma hybrid cells and in the rat caudate nucleus may be functionally coupled to an inhibition of adenylate cyclase through G proteins that are pertussis toxin, islet-activating protein (IAP)-sensitive (8, 9). As there are also reports that IAP blocks the G-protein-mediated signal transduction by ADP-ribosylating α subunits of G_i and G_o, the G protein that inhibits the adenylate cyclase and the G protein of unknown function, respectively (10), G_i may be involved in the opioid receptor-mediated inhibition of adenylate cyclase (6).

On the other hand, little is known of the functional role of G_o, another IAP-sensitive G protein. Hescheler *et al.* (11) reported that the ligand-induced changes in Ca^{2+}-channel activities mediated through the opioid receptor in neuroblastoma–glioma hybrid cells were blocked by IAP treatment of cells and were recovered predominantly by reconstitution with G_o rather than G_i. Their report suggests that opioid receptors may be functionally coupled to G_o, as well as to G_i. We have purified (12) μ opioid receptors, which were identified as a M_r 58,000 protein, by affinity cross-linking. In the present study, we investigated the direct coupling between purified μ receptors and purified G_i or G_o.

MATERIALS AND METHODS

Chemicals. [D-Ala^2,MePhe^4,Gly-ol^5]Enkephalin (EK) was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland); morphine hydrochloride was from Takeda Chemical Industry (Osaka, Japan); [D-Pen^2,D-Pen^5]EK, where Pen is penicillamine in which the two Pens are linked, was from Peninsula Laboratories (San Carlos, CA); U-69,593 was from Amersham; naloxone hydrochloride was from Sankyo; and [^{3}H]naloxone hydrochloride (41.4 Ci/mmol; 1 Ci = 37 GBq), [γ-^{32}P]GTP, and [^{3}H]guanosine 5′-[β,γ-imido]-triphosphate (p[NH]ppG) were from New England Nuclear. AF-AminoTOYOPEARL 650M was purchased from TOSOH (Tokyo, Japan), digitonin with a high solubility in water was from Wako Pure Chemicals (Osaka, Japan), and other reagents were from Nakarai Chemical (Kyoto, Japan).

Membrane Preparation and Solubilization of μ Opioid Receptors. The whole brain minus the cerebella from male Sprague–Dawley rats [250–300 g (body weight)] was homogenized first in 10 vol of 0.32 M sucrose with a Polytron (Kinematica, Lucerne, Switzerland) at a minimal setting for 5 s and second with a Potter–Elvehjem homogenizer and then was centrifuged at 1000 × *g* for 10 min. The supernatant (S_1) was centrifuged for 100,000 × *g* for 60 min, and the pellet (≈1 g of protein) was washed with 20 mM Tris·HCl (pH 7.5; buffer A), resuspended in about 50 ml of 0.32 M sucrose, and stored at −80°C. For solubilization, the suspension (≈1 g of protein) was diluted in 200 ml of ice-cold 0.32 M sucrose containing several enzyme inhibitors (0.002% soybean trypsin inhibitor, 1 μM leupeptin, 0.2 μM phenylmethylsulfonyl fluoride, and 0.01% bacitracin), sonicated for 10 min, and incubated with 0.1 mM dithiothreitol, 1% digitonin, and 0.1% sodium cholate at 0°C for 45 min. After centrifugation of the solubilized mixture at 100,000 × *g* for 1 hr, the supernatant was

Abbreviations: G protein, guanine nucleotide-binding regulatory protein; G_i, G protein that mediates the inhibition of adenylate cyclase; G_o, a G protein of unknown function; U-69,593, (5α,7α,8β)-(+)-*N*-methyl-*N*-(7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl)benzeneacetamide; p[NH]ppG, guanosine 5′-[β,γ-imido]triphosphate; CHAPS, 3 - [(3 - cholamidopropyl) dimethyl ammonio] propanesulfonate; Pen, penicillamine; GTP[γS], guanosine 5′-[γ-thio]triphosphate; IAP, islet-activating protein; EK, enkephalin.

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concentrated and diluted with buffer A to adjust the digitonin concentration to 0.1–0.3% in 100 ml, by using an ultrafiltration kit with a LaboCassette (Millipore) and UF membrane (type PTGC; 10,000 NMWL; Millipore).

Affinity Chromatography. 6-Succinylmorphine (12 g), prepared by the method of Simon *et al.* (13), was coupled to 200 ml of settled, washed AF-AminoTOYOPEARL 650M, by using 20 g of 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide, according to a conventional method (14). The solubilized and concentrated material was subjected to 6-succinylmorphine AF-AminoTOYOPEARL 650M column (2 × 30 cm) chromatography. Material was eluted at a flow rate of 1 ml/min with 20 mM Tris·HCl (pH 7.5) containing 0.5 mM 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS) and 0.1 mM dithiothreitol (buffer B). After a 250-ml wash with buffer B, the μ opioid receptors were eluted with 15 ml of buffer B containing 1 mM morphine and then with 100 ml of buffer B without morphine, and the eluate was concentrated to 100–200 μl by using a LaboCassette and UF membrane and a Centricon 30 (Amicon).

Isoelectric Chromatography. Affinity-purified μ opioid receptors were immediately applied to a PBE 94 (Pharmacia) column (0.5 × 10 cm) and isoelectric chromatography was performed with Polybuffer 74. Eluates (2-ml fractions) were collected and used for reconstitution experiments and for $NaDodSO_4$/PAGE with 10% gels.

Reconstitution of Affinity-Purified μ Opioid Receptors with G Proteins. G_i and G_o were purified (>95%) from the cholate extract of rat and porcine brain membranes, respectively, as reported (15). Another GTP-binding protein, the v-Ki-ras protein p21 was purified, as described (16). The μ opioid receptor from the PBE column (1 pmol) and purified G proteins, such as G_i (10 pmol), G_o (10 pmol), or p21 (10 or 100 pmol), were mixed with 50 μg of phosphatidylcholine in 150 μl of buffer A containing 100 mM NaCl, 0.1 mM EDTA, and 5 mM CHAPS and applied to a Sephadex G-50 (0.4 × 60 cm). Reconstituted vesicles were eluted with buffer A containing 100 mM NaCl and 0.1 mM EDTA in the void volume (1.2–2.4 ml).

[^{3}H]Naloxone Binding Assay. Preparations containing 25 fmol of purified μ opioid receptors were incubated at 30°C for 60 min with or without G proteins (250 fmol) in 300 μl of buffer A containing 100 mM NaCl, 5 mM $MgCl_2$, and 5 nM [^{3}H]naloxone in the presence of competing ligands. Incubation was terminated by adding 2 ml of ice-cold buffer A, and the reaction mixture was rapidly passed through a nitrocellulose membrane (BA85; Schleicher & Schuell). The filter was washed three times with ice-cold buffer A and the radioactivity retained on the filter was measured in a Beckman LS-7500 scintillation counter. Specific [^{3}H]naloxone binding was defined as the difference between [^{3}H]naloxone bound in the absence and presence of 100 μM unlabeled naloxone. The amount (mol) of purified μ opioid receptor was determined relative to the specific [^{3}H]naloxone binding at 20 nM [^{3}H]naloxone (approximately maximal binding).

p[NH]ppG Binding Assay. Reconstituted vesicles containing 25 fmol of purified opioid receptor and 250 fmol of purified G protein (except for 250 fmol per assay of p21) were incubated at 30°C as indicated in 100 μl of buffer A containing 100 nM [^{3}H]p[NH]ppG, 100 mM NaCl, 1 mM $MgCl_2$, and 0.1 mM EDTA. The incubation was terminated by adding of 2 ml of ice-cold buffer A containing 5 mM $MgCl_2$ and then rapidly passed through a BA85 filter. The filter was washed eight times with 2 ml of buffer A containing 5 mM $MgCl_2$ and put into a vial containing Bray's solution to measure radioactivity.

GTPase Assay. The GTPase activity was assayed by a modification of the method of Ueda *et al.* (17). Reconstituted vesicles containing purified opioid receptors (12.5 fmol per assay) and purified G proteins (125 fmol per assay, except for p21 at 1.25 pmol per assay) were incubated with a solution of 0.1 μM [γ-^{32}P]GTP (≈70,000 cpm), 0.5 mM adenosine 5′-[β,γ-imido]triphosphate, 1 mM ATP, 5 mM $MgCl_2$, 100 mM NaCl, 0.1 mM EDTA in 100 μl of buffer A. The incubation was carried out at 30°C for 20 min. The low-K_m GTPase activity was calculated from the difference between the cpm of $^{32}P_i$ released in the absence and presence of 50 μM unlabeled GTP. The high-K_m GTPase activity was measured in the presence of 50 μM unlabeled GTP. The amount of P_i released from 0.1 μM GTP increased linearly as incubation time was increased. In reconstituted preparations, the P_i released at 50 μM GTP was <10% of that released at 0.1 μM GTP.

RESULTS

Solubilization. We have solubilized (12) the μ opioid receptor by using 0.5% Triton X-100, after sonication with a 10% yield. When 0.5% Triton X-100 was replaced by 1% CHAPS or 1% digitonin plus 0.1% sodium cholate, the yield was improved to 30%. As CHAPS markedly inhibited the [^{3}H]naloxone binding at concentrations >0.05%, the solubilized materials with 1% CHAPS had to be diluted to concentrations <0.05%, when used for affinity chromatography. On the other hand, digitonin did not inhibit the binding at 0.1–0.5%, and sodium cholate at 0.1% was added to aid in the solubilization (18).

Isoelectric Chromatography and $NaDodSO_4$/PAGE. When the affinity-purified materials were further separated by PBE 94 isoelectric chromatography. [^{3}H]naloxone binding activity was observed only in the fraction with a pI value of 5.6 ± 0.1 (mean ± SEM, n = 4) (Fig. 1*A*). This fraction contained a single M_r 58,000 protein by $NaDodSO_4$/PAGE under reducing conditions (50 mM dithiothreitol) with silver staining (Fig. 1*B*), as described (12). The molecular weight of the μ binding protein, estimated under nonreducing conditions, was the same as that under reducing conditions (data not shown).

Guanine Nucleotide-Sensitive μ Agonist Binding in Reconstituted Preparations of Purified μ Receptors and Purified G Proteins. Scatchard analysis of [^{3}H]naloxone binding in vesicles without G proteins gave a K_d of 11.7 nM and an IC_{50} for the displacement of 5 nM [^{3}H]naloxone of 10 nM (details not shown). In contrast, the IC_{50} of [D-Ala^2,MePhe^4,Gly-ol^5]EK (a μ opioid agonist; for review, see ref. 19) was 58 μM, as shown in Fig. 2. When G_i at 250 fmol per assay was reconstituted, the agonist displacement was markedly increased with an IC_{50} of 0.27 μM. Such an increase in μ agonist binding was abolished by the addition of 100 μM guanosine 5′-[γ-thio]triphosphate (GTP[γS]), a stable GTP analogue. A similar GTP[γS]-sensitive increase in μ agonist binding was observed in reconstitution with G_o at 250 fmol per assay (Fig. 2*B*). The IC_{50} of [D-Ala^2,MePhe^4,Gly-ol^5]EK in G_o-reconstituted vesicles was 1.4 μM. However, [D-Pen^2,D-Pen^5]EK (a δ opioid agonist; for review, see ref. 19) and U-69,593 (a κ opioid agonist, ref. 20) showed no significant displacement of [^{3}H]naloxone binding in vesicles reconstituted with G_i or G_o. On the other hand, reconstitution with G_i (or G_o) showed no significant change in the antagonist binding with [^{3}H]naloxone (data not shown).

μ Agonist Stimulation of [^{3}H]p[NH]ppG Binding in Exchange for GDP Bound to G_i or G_o in Reconstituted Preparations. When 100 nM [^{3}H]p[NH]ppG was added to preparations reconstituted with G_i at 250 fmol per assay, the binding of [^{3}H]p[NH]ppG in exchange for GDP increased with time (Fig. 3*A*). The addition of 100 μM [D-Ala^2,MePhe^4,Gly-ol^5]EK stimulated the [^{3}H]p[NH]ppG binding within 4 min. The apparent initial velocity of [^{3}H]p[NH]ppG binding within 1 min was increased from 42.4 to 70.3 fmol/min by 100 μM [D-Ala^2,MePhe^4,Gly-ol^5]EK. However, there was a slight increase in the binding at 4–10 min. The maximal stimulation

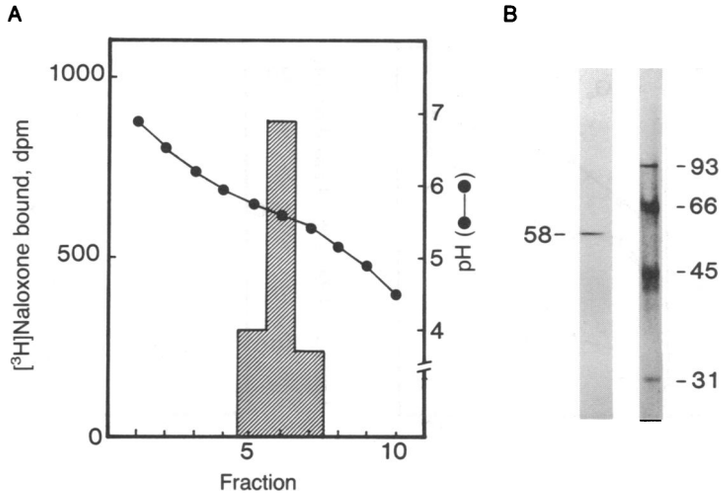

FIG. 1. Isoelectric chromatography of affinity-purified μ opioid receptors. (*A*) [^{3}H]Naloxone binding (hatched bars) in eluates of isoelectric chromatography (PBE column). The pH of eluates (●) represents the isoelectric point of the eluted proteins. The pI value of fractions with [^{3}H]naloxone binding activity was 5.6 ± 0.1 (n = 4). (*B*) NaDodSO$_4$/PAGE and silver staining of active fractions in the PBE chromatography. NaDodSO$_4$/PAGE (10% gel) was performed as described (12). A kit for molecular weight marker proteins was purchased from Bio-Rad. Molecular weights are presented $\times 10^{-3}$.

of the binding by [D-Ala^2,MePhe^4,Gly-ol^5]EK was calculated to be 62.5 fmol of [^{3}H]p[NH]ppG bound per assay in the G_i reconstituted preparations, as determined from double-reciprocal plots (1/binding vs. 1/time). The [D-Ala^2, MePhe^4,Gly-ol^5]EK stimulation of [^{3}H]p[NH]ppG binding was antagonized by 100 μM naloxone.

Similar μ agonist stimulation of [^{3}H]p[NH]ppG binding and its blockade by 100 μM naloxone were also observed in preparations reconstituted with G_o at 250 fmol per assay. [D-Ala^2,MePhe^4,Gly-ol^5]EK (100 μM) stimulated the apparent initial velocity of [^{3}H]p[NH]ppG binding from 61.3 to 85.9 fmol/min (Fig. 3*B*). The maximal stimulation by [D-Ala^2, MePhe^4,Gly-ol^5]EK was 34.3 fmol of [^{3}H]p[NH]ppG bound per assay.

μ Agonist Stimulation of Low-K_m GTPase in Reconstituted Preparations. In purified μ opioid receptor preparations not reconstituted with G proteins, no significant basal low-K_m GTPase activity was detected. The low-K_m GTPase activity in vesicles reconstituted with G_i at 125 fmol per assay was 680 fmol of P_i released per 20 min (control activity). As shown in Fig. 4*A*, [D-Ala^2,MePhe^4,Gly-ol^5]EK at 10–1000 μM stimulated the low-K_m GTPase in G_i-reconstituted vesicles to 105–182% of the control value, and this effect was antagonized by 100 μM naloxone, whereas the μ agonist showed no effect on low-K_m GTPase activity in vesicles without G proteins. The [D-Ala^2,MePhe^4,Gly-ol^5]EK (100 μM) stimulation of P_i released was 93.5 fmol per 20 min in the G_i-reconstituted preparations.

On the other hand, the control level of low-K_m GTPase activity was 969 fmol of P_i released per 20 min in vesicles reconstituted with G_o at 125 fmol per assay. The μ agonist stimulation of low-K_m GTPase (102–195% of the control value) and its blockade by naloxone were also observed in the G_o-reconstituted preparations (Fig. 4*B*). The [D-Ala^2, MePhe^4,Gly-ol^5]EK (100 μM) stimulation of P_i released was 103.0 fmol per 20 min.

Reconstitution of Purified μ Opioid Receptors with v-Ki-ras p21. To examine selective coupling of the purified μ receptor with G proteins, reconstitution with purified v-Ki-ras p21 was carried out. As shown in Fig. 5*A*, 100 μM [D-Ala^2,MePhe^4,

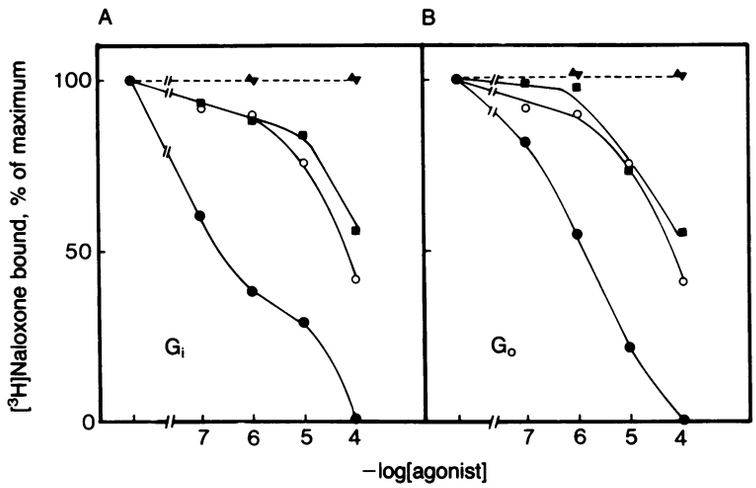

FIG. 2. Displacement of [^{3}H]naloxone binding by opioid agonists in vesicles reconstituted with G_i or G_o. Results are presented as the percentage of specific [^{3}H]naloxone (5 nM) binding in vesicles (25 fmol of μ receptor per assay) reconstituted with G_i (*A*) or G_o (*B*) at 250 fmol per assay, determined by the difference in [^{3}H]naloxone bound in the presence and absence of 100 μM naloxone. ○, [D-Ala^2,MePhe^4,Gly-ol^5]EK without G proteins; ●, [D-Ala^2,MePhe^4,Gly-ol^5]EK with G proteins; ■, [D-Ala^2,MePhe^4,Gly-ol^5]EK with G proteins and 100 μM GTP[γS]; ▲, [D-Pen^2,D-Pen^5]EK with G proteins; ▼, U-69,593 with G proteins.

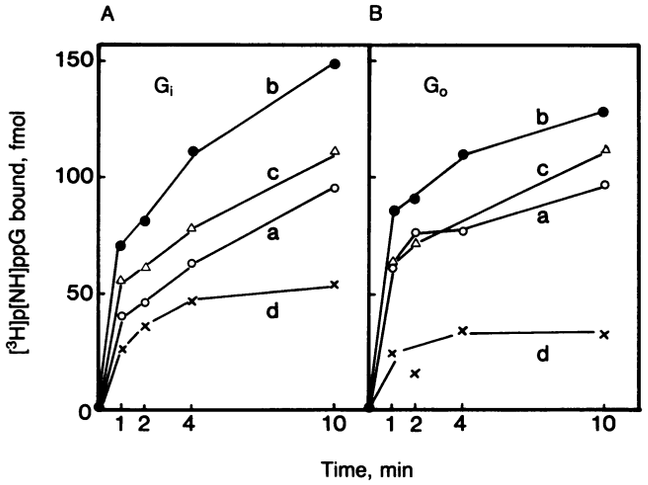

FIG. 3. [D-Ala^2,MePhe^4,Gly-ol^5]EK stimulation of [^{3}H]p[NH]ppG binding in vesicles reconstituted with G_i or G_o. Results are presented as the time course of 100 nM [^{3}H]p[NH]ppG binding (in fmol per assay) in exchange for GDP after addition of 100 μM [D-Ala^2,MePhe^4,Gly-ol^5]EK in vesicles (25 fmol of μ receptor per assay) reconstituted with G_i (*A*) and G_o (*B*) at 250 pmol per assay. Curves: a, control (without drugs); b, [D-Ala^2,MePhe^4,Gly-ol^5]EK; c, [D-Ala^2,MePhe^4,Gly-ol^5]EK plus 100 μM naloxone; d, difference curve—curve b − curve a ([D-Ala^2,MePhe^4,Gly-ol^5]EK stimulation).

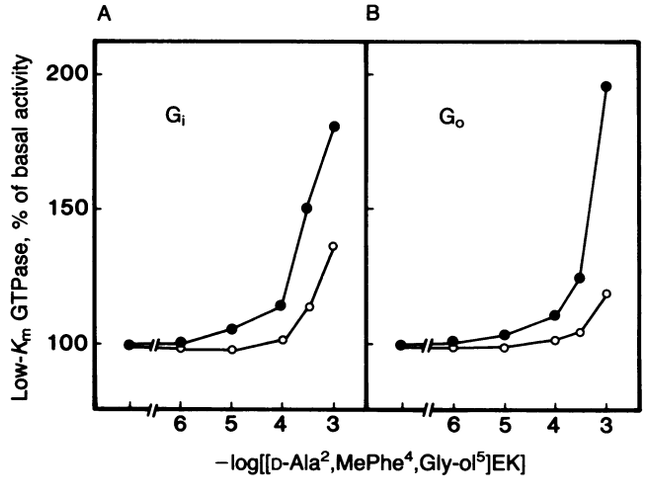

FIG. 4. [D-Ala^2,MePhe^4,Gly-ol^5]EK stimulation of low-K_m GTPase in vesicles reconstituted with G_i or G_o. Results are presented as the concentration dependence of [D-Ala^2,MePhe^4,Gly-ol^5]EK stimulation (as the percentage of control) or low-K_m GTPase (P_i release) in vesicles (12.5 fmol of μ receptor per assay) reconstituted with G_i (*A*) and G_o (*B*) at 125 fmol per assay. ●, [D-Ala^2,MePhe^4,Gly-ol^5]EK alone; ○, [D-Ala^2,MePhe^4,Gly-ol^5]EK plus 100 μM naloxone.

Gly-ol^5]EK led to no significant change in the [^{3}H]p[NH]ppG binding in preparations with 25 fmol of μ receptor and 250 fmol of p21 per assay. The μ agonist (1 mM) did not change the low-K_m GTPase activity in preparations with 12.5 fmol of μ receptor and 1.25 pmol of p21 per assay (Fig. 5*B*). In addition, there was no increase in the displacement of [^{3}H]naloxone binding by 100 μM [D-Ala^2,MePhe^4,Gly-ol^5]-EK in preparations with 25 fmol of μ receptor and p21 at 250 fmol or 2.5 pmol per assay (data not shown).

DISCUSSION

We have noted (12) that opioid receptors from rat brain were purified with a major M_r 58,000 protein and a minor M_r ≈40,000 protein by use of a 6-succinylmorphine-Affi-Gel 102 column and that the former is a μ subtype, determined by the affinity cross-linking experiment with the μ agonist [^{3}H][D-Ala^2,-MePhe^4,Gly-ol^5]EK (12). In the present study, M_r 58,000 protein was purified by 6-succinyl-morphine-AF-AminoTOYOPEARL 650M affinity chromatography and then by PBE isoelectric chromatography. From the starting membranes (1 g of protein), ≈0.5 pmol of purified μ receptor was obtained.

[D-Ala^2,MePhe^4,Gly-ol^5]EK showed a very weak displacement of [^{3}H]naloxone binding (μ agonist binding) in preparations reconstituted without G proteins. However, in preparations reconstituted with G_i or G_o, there was a significant increase (215-fold for G_i and 33-fold for G_o) in the μ agonist binding, but not in δ agonist ([D-Phe^2,D-Phe^5]EK or κ agonist (U-69,593) binding. Taking into account the reports that the opioid δ receptor inhibits adenylate cyclase in NG 108-15 cells, possibly by way of G_i (1, 6) and that the opioid κ receptor is also coupled to G_i in guinea pig brain membranes (21), it appears that the purified receptor is a μ receptor subtype, without δ or κ subtypes. In addition, the increase in μ agonist binding was abolished by GTP[γS], thereby indicating that the high-affinity receptor–G_i (or–G_o) complex was formed in a GTP-dependent manner in reconstituted vesicles.

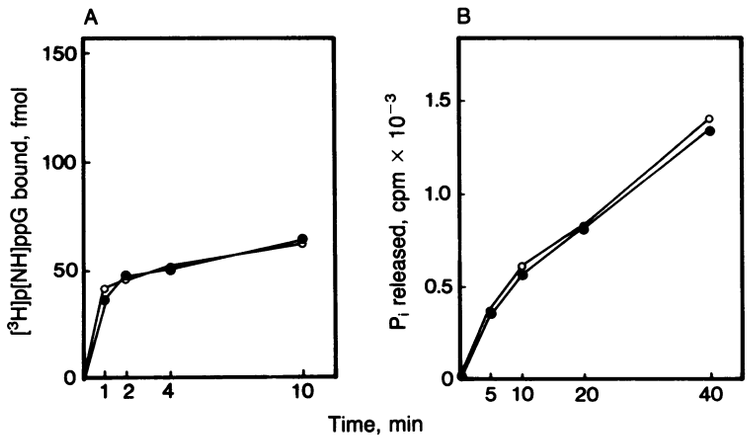

FIG. 5. Effects of [D-Ala^2,MePhe^4,Gly-ol^5]EK on [^{3}H]p[NH]ppG binding and low-K_m GTPase assays in vesicles reconstituted with p21 protein. (*A*) [^{3}H]p[NH]ppG binding assay. Results are presented as the time course of [^{3}H]p[NH]ppG binding (in fmol per assay) in vesicles (25 fmol of μ receptor per assay) reconstituted with p21 at 250 fmol per assay. ○, Control (without drugs); ●, 100 μM [D-Ala^2,MePhe^4,Gly-ol^5]EK. (*B*) Low-K_m GTPase assay. Results are presented as the time course of P_i released (in cpm per assay) in vesicles (12.5 fmol of μ receptor per assay) reconstituted with p21 at 1.25 pmol per assay. High-K_m GTPase activity was not detected. Control low-K_m GTPase activity was 490 fmol per 20 min. ○, Control (without drugs); ●, 1 mM [D-Ala^2,MePhe^4,Gly-ol^5]EK.

The "functional" coupling between the purified μ receptor and purified G proteins was also demonstrated. [D-Ala^2, MePhe^4,Gly-ol^5]EK at 100 μM stimulated [^{3}H]p[NH]ppG binding within 4 min, in preparations reconstituted with G_i and G_o, but the marked stimulation between 4 and 10 min of incubation was absent in both cases. This finding indicates that the signal transduction from the μ receptor to G_i (or G_o) may be completed within 4 min in the presence of [^{3}H]p-[NH]ppG. As 100 μM [D-Ala^2,MePhe^4,Gly-ol^5]EK seems to saturate the purified μ receptors in G_i (or G_o) reconstituted preparations (Fig. 2), the [D-Ala^2,MePhe^4,Gly-ol^5]EK-stimulated amount of [^{3}H]p[NH]ppG bound is equal to the amount of G protein coupled to 25 fmol of μ receptor. Accordingly, the stoichiometry revealed that 1 mol of μ receptor is coupled to 2.5 mol of G_i or 1.37 mol of G_o and the G_i/G_o molar ratio was $\approx$2:1. Although it is expected that the coupling between receptors and G proteins depends on their densities in phospholipid vesicles, it is at least likely that the μ opioid receptor is coupled to several molecules of G_i or G_o.

On the other hand, the μ agonist stimulated low-K_m GTPase in G_i (or G_o) reconstituted preparations. However, there was no significant change in the μ agonist stimulations between the two preparations. In the GTPase assay, the GTP-bound form of G protein, as a result of GDP–GTP exchange, may be converted to the GDP-bound form by the GTPase activity of G protein *per se* and recoupled to the receptor. Accordingly, the signal will be transduced to the G protein coupled repeatedly within a 20-min incubation. Indeed, the [D-Ala^2,MePhe^4,Gly-ol^5]EK (100 μM) stimulation of P_i release ($\approx$100 fmol/12.5 fmol of the μ receptor in G_i or G_o preparations) was apparently higher than that of [^{3}H]p-[NH]ppG binding (62.5 and 34.3 fmol/25 fmol of μ receptor for the G_i and G_o, respectively). Taking into account the data that the intrinsic "turn-over rate" factors in the G_o preparation, such as the rate of association of GTP (initial velocity was 61.3 fmol of [^{3}H]p[NH]ppG bound per min) and basal GTPase activity (969 fmol of P_i released per 20 min) were both $\approx$1.5 times higher than those in the G_i (42.4 fmol of [^{3}H]p[NH]ppG bound per min and 680 fmol of P_i released per 20 min, respectively), it is likely that the receptor stimulation of low-K_m GTPase mediated by a ligand is more effective in the G preparation than that in the G_i, resulting in a similar [D-Ala^2,MePhe^4,Gly-ol^5]EK stimulation of low-K_m GTPase, despite the stoichiometry of these couplings. Furthermore, the [D-Ala^2,MePhe^4,Gly-ol^5]EK stimulation of low-K_m GTP-ase was not saturated even at 100 μM. The [D-Ala^2,MePhe^4, Gly-ol^5]EK (100 μM) displacement of [^{3}H]naloxone binding may not have been complete in preparations reconstituted with G_i (or G_o) in a GTP (or GTP[γS])-bound form (Fig. 2).

To examine the specificity of coupling of the μ receptor with G proteins, we performed reconstitution experiments with v-Ki-ras p21. p21 is a GTP-binding protein purified from *Escherichia coli* and expressed from a plasmid encoding 189 amino acids of the Kirsten murine sarcoma virus oncogene (22). [D-Ala^2,MePhe^4,Gly-ol^5]EK showed no stimulation of [^{3}H]p[NH]ppG binding or low-K_m GTPase in preparations reconstituted with p21. As increases in [D-Ala^2,MePhe^4,Gly-ol^5]EK displacement of [^{3}H]naloxone binding were never apparent, the high-affinity receptor–p21 protein complex in such phospholipid vesicles may not be formed. On the other hand, there are reports that several G proteins other than G_i and G_o have been identified or predicted by screening cDNA libraries by using probes derived from purified G proteins (7, 15, 23) and perhaps reconstitution experiments with these proteins would be informative.

There are reports that the membrane α_2-adrenergic receptor (24) and purified muscarinic receptor (25) were reconstituted with G_i and G_o. There is also a question of whether or not such couplings to G_i and G_o are due to the homology in their primary structures (26). The abolition of μ agonist stimulation of low-K_m GTPase in membranes by pretreatment with *N*-ethylmaleimide was recovered by reconstitution with G_i or G_o (19), and such a recovery was additive when both G proteins were present in amounts required for each maximal recovery (unpublished data). In addition, we have found that both the κ opioid receptor (21) and kyotorphin receptor (27) are selectively reconstituted with G_i but not with G_o in such *N*-ethylmaleimide- or IAP-treated membrane preparations. Thus, it is likely that the μ receptor is functionally coupled to G_i and G_o *in vivo*. However, it remains to be determined whether the μ receptors that couple to G_i are the same as those that couple to G_o. If these μ receptors are different, then the ratio of μ receptors could be predicted from the number of receptors coupled to G_i vs. those coupled to G_o, which was 2:1 in the present study. The determination of primary structure(s) of μ receptor(s) with cDNA cloning techniques should aid in the elucidation of this problem.

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