Vol. 156, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1988 Pages 54-60

RECONSTITUTION OF PARTIALLY PURIFIED OPIOID RECEPTORS WITH A GTP-BINDING PROTEIN

Toshiyuki Fujioka, Fumiko Inoue, Sachiyo Sumita, and Masayuki Kuriyama

Faculty of Pharmaceutical Sciences, Mukogawa Women's University, Nishinomiya, 663 Japan

Received August 16, 1988

SUMMARY: Partially purified opioid receptors, obtained from rat brains using an affinity resin, AF-Amino Toyopearl with [D-Ala^2, Leu^5]enkephalin, were reconstituted with an inhibitory GTP-binding protein (G_i). In the reconstituted system, the displacement curve for the binding of a δ-agonist, [D-Ala^2, D-Leu^5]enkephalin, showed two states, high and low affinity binding ones, with different affinities for the agonist. The high affinity binding was eliminated by the addition of a guanine nucleotide analog to the system. These results directly showed that opioid receptors, at least the δ-type, could interact with G_i.
© 1988 Academic Press, Inc.

The GTP-binding proteins (G proteins) function as transducers in transmembrane signaling systems; G proteins link the activation of receptors to the regulation of effectors. There are several known G proteins, controlling several different intracellular systems that cause the changes of the activities of specific effectors (1-3). In some opioid receptor systems, transformation of the signals is transduced via G proteins. An inhibitory GTP-binding protein G_i links the activation of the receptors to the inhibition of adenylyl cyclase, thus lowering the concentration of cAMP (4-6). A G protein with unknown function G_o and,

Abbreviations: G proteins, guanine nucleotide-binding regulatory proteins; G_i, a G protein that inhibits adenylyl cyclase; G_o, a G protein of unknown function, originally isolated from bovine brain; $G_{PP}(NH)_p$, guanyl-5'-yl imidodiphosphate; Tes, N-tris-[hydroxymethyl]methyl-2-aminomethanesulfonic acid; DALE, [D-Ala^2, Leu^5]enkephalin; DADLE, [D-Ala^2, D-Leu^5]enkephalin; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

0006-291X/88 $1.50
Copyright © 1988 by Academic Press, Inc.
All rights of reproduction in any form reserved.

to a lesser extent, G_i may be involved in the functional coupling of the receptors to neuronal voltage-dependent calcium channels (7). To more directly clarify the interaction among the components of the transmembrane signaling pathways in the opioid receptor systems, the components need to be isolated and reconstituted.

Opioid receptors have recently been purified using affinity chromatography in several laboratories (8-12). We have reported the purification of opioid-binding materials using an affinity resin with an enkephalin derivative as the affinity ligand for the receptors (13). However, the functional reconstitution of the purified receptors with G proteins has not been reported. Therefore, we attempted to reconstitute partially purified opioid receptors with G_i and to examine the interaction between the receptors and G_i in a membrane state.

MATERIALS AND METHODS

All radiochemicals were purchased from New England Nuclear Co. Guanyl-5'-yl β,γ-imidodiphosphate (Gpp(NH)p) and phosphatidylinositol were obtained from Sigma. AF-Amino Toyopearl was a generous gift from TOSOH Co. Enkephalin derivatives were synthesized as previously reported (14). All other chemicals and chromatographic media were obtained from commercial sources and used as supplied.

An affinity resin, AF-Amino Toyopearl with DALE, was prepared as previously reported (14) except using AF-Amino Toyopearl instead of AH-Sepharose as an insoluble support.

The opioid-binding materials were purified as in our previous study (13) with minor modifications. Details of the preparation of the resin and the purification of the materials will be reported elsewhere (15).

G_i was purified from bovine brain essentially using reported methods (16-18). The concentrations of Gi were determined by [^{35}S]guanosine-5'-[γ-thio]triphosphate binding activities (19). The purified opioid-binding materials and Gi were reconstituted essentially as reported in the literature (20, 21); ten- to twenty-fold excess of G_i over receptors was used in the reconstitution experiments. The brain extract (Folch Fraction I, 0.5 mg) and phosphatidylinositol (1.5 mg) was suspended in 2 ml of a buffer solution (Tes-Mg: 10 mM Tes-KOH (pH 7.5), 10 mM $MgCl_2$) containing 0.3% n-octyl-β-D-thioglucoside. The resulting suspension was sonicated in a bath-type sonicator in an atmosphere of nitrogen. This lipid mixture was added to the concentrated purified receptors (100 μl) and allowed to stand at room temperature (15 min). The resulting mixture was gel filtered, and void-volume fractions were collected. Next, Gi was added to the frac-

tions and the mixture was allowed to stand on ice (1 hr) followed by dilution with two volumes of Tes-Mg. The obtained suspension was used as a reconstituted system.

The binding of [^{3}H]DADLE (2 nM; 43.9 Ci/mmol; NEN) to the purified receptors or the reconstituted mixture was assayed by the membrane filter method, essentially as described previously (13). For the purified materials, the non-specific binding was measured in the presence of 10^{-5} M of cold ligands.

Protein was determined by staining with Amidoblack (22).

RESULTS

AF-Amino Toyopearl with DALE was used in the present study. AF-Amino Toyopearl has two outstanding characteristics; it contains more free amino groups than AH-Sepharose (the ratio of free amino groups of AF-amino Toyopearl to AH-Sepharose, 15:1) and it is more stable to pressure than AH-Sepharose. In the previous studies (14, 15), DALE or DADLE was used as an affinity ligand for opioid receptors. However, the content of DADLE (0.4 μmol/ml resin) was lower than that of DALE (1 μmol/ml resin), because of the solubility of the DADLE derivative in the solvent used in the coupling step to AH-Sepharose. Thus, DALE was used as an affinity ligand in this study. The content of DALE in this resin (10 μmol/ml resin) was ten times more than that in AH-Sepharose (15). The higher ligand content in the affinity resin enables more efficient purification of the receptors, especially in large-scale preparations.

Because the materials showed little or no GTPase activity originating from G protein (data not shown), the partially purified materials were reconstituted with purified G_i in liposome. The addition of G_i to the purified materials resulted in an increase in an apparently high affinity state for an agonist. Fig. 1 shows the displacement of [^{3}H]DADLE from isolated materials by DADLE in the absence or presence of G_i. In its presence, two states with different affinities were observed: a high affinity state with IC_{50} of 1 nM and a low affinity one with IC_{50} of 50

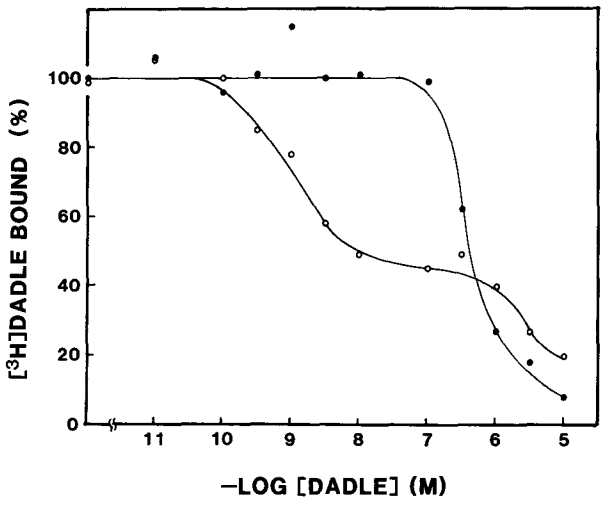

Fig. 1. Displacement of [^{3}H]DADLE binding by DADLE. The purified materials were reconstituted with (o—o) or without G_i (●—●) in liposome and the reconstituted systems were incubated with [^{3}H]DADLE (2 nM) and various concentrations of DADLE at 30°C, 1 hr. The fractions of protein-bound [^{3}H]DADLE were trapped by nitrocellulose membranes (BA-85, S & S). The points are the averages of triplicate determinations.

μM. The latter value was almost the same as in the absence of G_i.

The specific binding activity of the purified materials was ca. 150 pmol/mg protein for [^{3}H]DADLE, which was a little smaller than that of the previously purified materials (13) and much lower than expected for pure receptors (15). However, the activity increased to ca. 2,000-3,000 pmol/mg protein if the materials were reconstituted with G_i in liposome. The same phenomenon of an increase in the agonist-binding activity has been observed in the reconstituted system of muscarinic cholinergic receptors and G proteins (23).

In opioid receptor systems, guanine nucleotides decreased the affinities of agonists for the receptor binding site (24-27). We therefore tested the effect of guanine nucleotides in our system; a nonhydrolyzable analog of GTP, $G_{pp}(NH)_p$, decreased the opioid-binding activity of the reconstituted purified materials

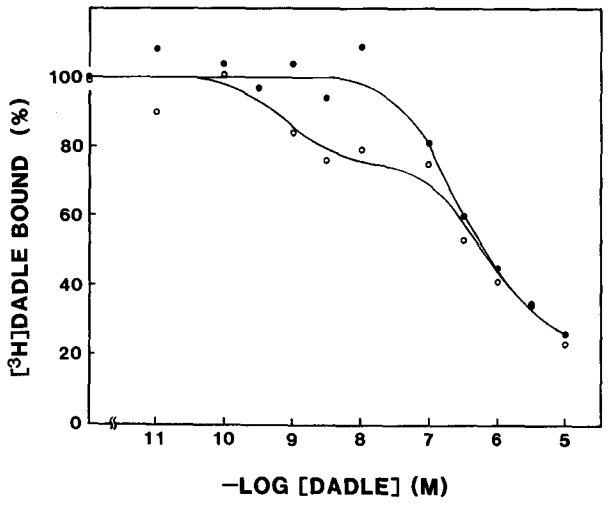

Fig. 2. Effect of $G_{pp}(NH)_p$ on agonist binding to reconstituted receptors. [^{3}H]DADLE binding to the reconstituted system with G_i was assayed in the presence (●—●) and absence (○—○) of 0.1 mM $G_{pp}(NH)_p$. The conditions of the assay were the same as cited in Fig. 1.

with G_i. Fig. 2 shows the effect of $G_{pp}(NH)_p$ in the reconstituted system; addition of $G_{pp}(NH)_p$ to the reconstituted system with G_i eliminated the high affinity state for the agonist. No effect of $G_{pp}(NH)_p$ was observed on the reconstituted system without G_i (data not shown).

DISCUSSION

In the previous study (13), one major polypeptide with specific opioid-binding activity was purified using the affinity resin. However, the opioid-binding activity was the only measure for the materials which might be opioid receptors. To find whether or not the materials were primarily composed of opioid receptors, the purified materials had to be functionally reconstituted with the proposed compornent involved in the state of the cell membrane.

In opioid receptor systems, extracellular signals received with receptors are transformed into intracellular responses produced by effectors, such as adenylyl cyclase (4-6) or ion chan-

nels (7), via the transducers, G_i and/or G_o. Therefore, the purified materials were reconstituted with G_i in liposome; two binding states, a high and low affinity one, for the δ-agonist of opioid receptors were observed in the reconstituted system with G_i, but only a low affinity state was observed without G_i. Because the coupling efficiencies of the receptors and G_i differed in each reconstitution experiment, the percentage of the high affinity state differed (compare Fig. 1, o—o, and Fig. 2, o—o). The low affinity state showed very large IC_{50} value (50 μM), but physiological importance for this state is not clear. From the preliminarily experiments, the reconstituted system with G_i showed no high affinity binding activity to a μ-agonist Tyr-D-Ala-Gly-(N-Me)Phe-Gly-ol (DAGO) and a κ-agonist ethylketocyclazocine. These results indicate that the purified materials contain mainly δ-type opioid receptors.

Guanine nucleotides cause dissociation of the subunits of G_i, simultaneously decoupling of G_i and receptors, resulting in a change in the affinity of the agonists for the receptors from high to low (26-29). In the reconstituted system with G_i, the addition of $G_{pp}(NH)_p$ changed the high affinity state for the agonist to a low one. These results also suggested that the δ-type of opioid receptors could functionally couple with G_i in the membrane state as in other transmembrane signaling pathways transduced with G_i (1-3). We stand at the first step in the elucidation of transmembrane signaling pathways in opioid-signaling systems.

ACKNOWLEDGMENT. We thank Professor M. Ui of University of Tokyo, Dr. T. Katada of the Industrial College of Tokyo and Dr. T. Higashijima of University of Texas Health Science Center at Dallas for their useful suggestions and discussions. This work was supported in part by a Grant-in-Aid for Special Project Research from the Ministry of Education, Science, and Culture, Japan (1987, 1988) and a research grant from the Uehara Memorial Foundation (1986).

REFERENCES

1. Stryer, L. and Bourne, J. (1986) Ann. Rev. Cell Biol. 2, 391-419.
2. Gilman, A. G. (1987) Ann. Rev. Biochem. 56, 615-649.
3. Neer, E. J. and Clapham, D. E. (1988) Nature 333, 129-134.
4. Sharma, S. K., Nirenberg, M., and Klee, W. A. (1975) Proc. Natl. Acad. Sci. USA 72, 590-594.
5. Law, P. Y., Wu, J., Koehler, J. E., and Loh, H. H. (1981) J. Neurochem. 36, 1834-1846.
6. Cooper, D. M. F., Londos, C., Gill, D. L., and Rodbell, M. (1982) J. Neurochem. 38, 1164-1167.
7. Hescheler, J., Rosenthal, W., Trautwein, W., and Schultz, G. (1987) Nature 325, 445-447.
8. Bidlack, J. M., Abood, L. G., Osei-Gyimah, P., and Archer, S. (1981) Proc. Natl. Acad. Sci. USA 78,636-639.
9. Maneckjee, R., Zukin, R. S., Archer, S., Michael, J., and Osei-Gyimah, P. (1985) Proc. Natl. Acad. Sci. USA 82, 594-598.
10. Cho, T. M., Ge, B.-L., and Loh, H. H. (1985) Life Sci. 36, 1075-1085.
11. Gioannini, T. L., Howard, A. D., Hiller, J. M., and Simon, E. J. (1985) J. Biol. Chem. 260, 15117-15121.
12. Cho, T. M., Hasegawa, J., Ge, B.-L., and Loh, H. H. (1986) Proc. Natl. Acad. Sci. USA 83, 4138-4142.
13. Fujioka, T., Inoue, F., and Kuriyama, M. (1985) Biochem. Biophys. Res. Commun. 131, 640-646.
14. Fujioka, T., Inoue, F., and Kuriyama, M. (1985) Chem. Pharm. Bull. (Tokyo) 33, 4633-4636.
15. Fujioka, T., Inoue, F., and Kuriyama, M., submitted.
16. Sternweis, P. C., Northrup, J. K., Smigel, M. D., and Gilman A. G. (1981) J. Biol. Chem. 256, 11517-11526.
17. Bokoch, G. M., Katada, T., Northrup, J. K., Hewlett, E. L., and Gilman, A. G. (1984) J. Biol. Chem. 259, 3560-3567.
18. Sternweis, P. C., and Robinshaw, J. D. (1984) J. Biol. Chem. 259, 13806-13813.
19. Ferguson, K. H., Higashijima, T., Smigel, M. D., and Gilman A. G. (1986) J. Biol. Chem. 261, 7393-7399.
20. Florio, V. A., and Sternweis, P. C. (1985) J. Biol. Chem. 260, 3477-3483.
21. Haga, K., Haga, T., and Ichiyama, A. (1986) J. Biol. Chem. 261, 10133-10140.
22. Schaffner, W. and Weissmann, C. (1973) Anal. Biochem. 56, 502-514.
23. Florio, V. A. and Sternweis, P. C. (1985) J. Biol. Chem. 260, 3477-3483.
24. Childers, S. R. and Snyder, S. H. (1978) Life Sci. 23, 759-762.
25. Childers, S. R. and Snyder, S. H. (1982) J. Neurochem. 34, 583-593.
26. Chang, K.-J., Blanchard, S. G., and Cuatrecasas, P. (1983) 80, 940-944.
27. Law, P.-Y., Hom, D. S., and Loh, H. H. (1985) J. Biol. Chem. 260, 3561-3569.
28. Blume, A. J. (1978) Life Sci. 22, 1843-1852.
29. Blume, A. J., Mullikin-Kilpatrick, D., and Kilian, P. L. (1984) in Methodol. Surv. Biochem. Anal. (Reid, E., Cook, G. M. W., and Morre, D. J., eds.) Vol. 13, pp.151-161, Plenum, New York.