Morphine-like Activity of Natural Human IgG Autoantibodies Is because of Binding to the First and Third Extracellular Loops of the \( \mu \)-Opioid Receptor

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We have previously demonstrated that randomly selected healthy individuals express anti-human \( \mu \)-opioid receptor antibodies which behave as agonist in vitro. In this study, we show that the activity of these antibodies was not affected by the deletion of the amino-terminal region of the receptor. Using agarose-bound peptide columns, we affinity-purified IgG specifically directed toward each extracellular loop. Whatever its specificity, each anti-human \( \mu \)-opioid receptor (hMOR) extracellular loop peptide IgG preparation was unable, when examined individually, to reduce adenylate cyclase activity. Activation of the hMOR was, however, achieved by the simultaneous binding of IgG to the first and third extracellular loops of the receptor. Our results suggest that the simultaneous binding of IgG antibodies to these two loops mimics morphine-induced receptor activation by triggering a coordinated shift of the third and sixth transmembrane helices.

The presence of low levels of circulating autoantibodies is a normal feature of the immune system. Numerous biological functions for these so-called natural autoantibodies have been proposed such as first line host-defense against pathogens, senescent cell removal, immunoregulatory activity (1). Alternatively, it has also been considered that disease-associated autoantibodies are derived from natural autoantibody repertoires. Autoantibodies directed against G protein-coupled receptors have been characterized in a number of diseases including Graves’ disease (2), Chagas’ disease (3), idiopathic dilated cardiomyopathy (4), malignant hypertension (5), and preeclampsia (6). In each of these pathological situations, the autoantibodies behave as agonists by interacting with extracellular domains of the corresponding receptors. We have previously characterized the presence of IgG autoantibodies directed against the human \( \mu \)-opioid receptor (hMOR) in serum of at least 42% healthy individuals. These autoantibodies also displayed an agonistic activity (7).

In this study, we have investigated the functional relevance of extracellular segments of the hMOR in the receptor activation induced by anti-hMOR IgG autoantibodies. Deletion of the amino-terminal domain of the receptor did not alter the ability of anti-hMOR IgG to inhibit adenylate cyclase activity. The role of the three extracellular loops of the receptor was examined by measuring, on intact recombinant hMOR/CHO cells, the agonistic activity of IgG affinity-purified against peptides corresponding to each extracellular loop. A G\(_i/G_{\alpha_i}\)-mediated inhibition of adenylate cyclase activity was observed with an equimolar mixture of IgG specifically directed against the first and the third extracellular loops. Our results show that efficient ligand/hMOR interactions involving the first and third extracellular loops of the hMOR can mimic the signal elicited by small alkaloid agonist as morphine. This alternative pathway for receptor activation opens new perspectives in the conception of analgesic drugs.

**EXPERIMENTAL PROCEDURES**

**Transfection**—The cDNA encoding the hMOR and the hMOR lacking the first 61 amino acids in the amino-terminal segment (hMOR\(_{1–61}\)) (8) were inserted into the pcDNA3.1 vector. CHO-K1 cells were transfected by the calcium phosphate procedure (9). Cell clones obtained by limiting dilutions were screened for their ability to bind \([3H]diprenorphine\) (Amersham Pharmacia Biotech, Little Chalfont, UK).

**Binding Assays**—Intact cells (3 x 10\(^5\)) were incubated with increasing amounts of \([3H]diprenorphine\) for 60 min at 25 °C. Nonspecific binding was determined in the presence of 1 μM unlabeled diprenorphine. For competition experiments, 1 nM \([3H]diprenorphine\) was added to the cells together with increasing amounts of morphine (Francoipia, Paris, France).

**Antibody Purification**—IgG preparation obtained from a large pool of plasma from normal donors was used as a source of human IgG (Sanoglobulin\(^\text{TM}\), Novartis, Basel, Switzerland). Lyophilized IgG preparation was reconstituted in H\(_2\)O and extensively dialyzed against PBS before use.

IgG directed against hMOR (anti-hMOR IgG) were purified on hMOR/CHO cells (7). Briefly, IgG were incubated with confluent hMOR/CHO cells for 1 h at 37 °C. Bound antibodies were eluted using 0.1 M sodium-citrate, pH 3.5, purified by chromatography on protein G-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) and then incubated with untransfected CHO cells.

Three peptides NYLMGTVFGITLLCK, KRYQGIDICTLFTSHP- WWENLVK, and KALVTIPETTFQT corresponding to the hMOR extracellular loops 1, 2, and 3 (hMOR EL1, hMOR EL2, hMOR EL3), respectively, (10), were immobilized on AminoLink\(^\text{TM}\) coupling gel (Pierce). Anti-hMOR EL peptide IgG were affinity-purified according to the instructions of the manufacturer.

Anti-laminin IgG autoantibodies were purified from the original IgG pool by affinity chromatography on a column of Sepharose-bound laminin as described elsewhere (11). Antibody activity was assessed by ELISA. Plates were coated with mouse laminin (5 μg/ml) (E-Y Laboratories, San Mateo, CA) or hMOR EL peptides (10 μg/ml) overnight at 4 °C in PBS. Uncoated sites were saturated with PBS containing 1% gelatin (PBS gel) for 90 min at 37 °C. Plates were washed before incubation for 60 min at 37 °C with IgG diluted in PBS-gel.

1 The abbreviations used are: hMOR, human \( \mu \)-opioid receptor; hMOR\(_{1–61}\), human \( \mu \)-opioid receptor deleted from its first 61 amino acids; CHO, Chinese hamster ovary cells; hMOR/CHO, hMOR-expressing CHO-K1 cell; hMOR\(_{1–61}/\text{CHO}\), hMOR\(_{1–61}\)-expressing CHO-K1 cell; EL, extracellular loop; PTX, pertussis toxin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

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Expression and binding properties of wild-type hMOR and NH2-terminal domain truncated hMORΔ1–61 on transfected CHO cells

Saturation isotherm binding of [3H]diprenorphine was performed for each receptor with 300,000 intact recombinant CHO cells. Cells were incubated with eleven concentrations of [3H]diprenorphine ranging from 0.05 to 14 nM, and the specifically bound radioactivity as function of the ligand concentrations was determined. \( K_d \) values and maximal binding (\( B_{max} \)) were estimated from Scatchard transformation of the data (one-site mode). \( K_d \) values for morphine were obtained by displacement of [3H]diprenorphine (1 nM) binding using increasing amounts of morphine ranging from 2 nM to 20 M. 

Experiment results are expressed as means ± S.E. of three independent experiments performed in triplicate.

|                | \( B_{max} \) (sites/cell) | \( K_d \) (nM) | \( K_i \) (nM) |
|----------------|-----------------------------|---------------|---------------|
| Wild-type hMOR | 57,000                      | 0.08 ± 0.01   | 83 ± 9        |
| hMORΔ1–61      | 70,000                      | 0.30 ± 0.03*  | 292 ± 11*     |

RESULTS

The NH2-terminal Region of the hMOR Does Not Contribute to the Receptor Activation Induced by Anti-hMOR Antibodies

The lack of the NH2-terminal extracellular domain in the hMOR did not prevent the biological effect of morphine as assessed by specific inhibition of adenylate cyclase activity (Fig. 1). In agreement with previous reports (8, 13, 14), when compared with the wild-type hMOR the 3.5-fold decrease in the affinity of morphine for the NH2-terminal-truncated hMOR (\( p < 0.05 \); Table I) was associated with a 6-fold increase in the concentration responsible for a 50% inhibition of adenylate cyclase activity (\( EC_{50} = 0.5 ± 0.3 \times 10^{-7} \) M and 2.9 ± 1.3 \( \times 10^{-7} \) M, respectively, for hMOR/CHO and hMORΔ1–61/CHO) (\( p < 0.05 \)).

Anti-hMOR IgG were affinity-purified from a normal human IgG pool on intact hMOR/CHO cells (7). Affinity-purified anti-laminin IgG, or affinity-purified anti-hMOR IgG. Background corresponded to cell staining with labeled goat anti-human IgG F(ab’)2-specific antibodies and phycoerythrin-labeled streptavidin alone. The figure shows results of one representative experiment.
IgG-induced μ-Opioid Receptor Activation

hMOR/CHO and in hMORΔ1–61/CHO cell clones, indicating that the activity of these antibodies was not dependent on the NH₂-terminal extracellular region of the receptor (Fig. 3). Because a weak residual binding of anti-hMOR IgG on control CHO cells was observed (Fig. 2), we have checked out that CHO-reactive IgG had no effect on the inhibition of the adenylate cyclase (not shown) (7).

Activation of the hMOR Is Triggered by Antibodies Directed against the First and Third Extracellular Loops of the hMOR—We next investigated the contribution of the three other extracellular regions of the hMOR in the anti-hMOR IgG-mediated opioid signal. For this purpose, peptides corresponding to the three extracellular loops of the hMOR (EL1, EL2, and EL3) were synthesized. The amino acid sequence of each peptide was defined relative to the putative positioning of transmembrane domains of the receptor (10). Three preparations of IgG directed toward EL1, EL2, and EL3 peptides, respectively, were prepared from the human IgG pool by affinity chromatography on peptide-bound agarose columns. Enrichment of the antibody activity against each peptide was estimated in ELISA. Affinity purification of IgG against each hMOR EL peptide resulted in enhancement of specific antibody activity as compared with unpurified IgG (Fig. 4). The ability of each anti-hMOR EL peptide affinity-purified IgG to bind to the hMOR was then estimated by cytofluorometry analysis using hMOR/CHO, hMORΔ1–61/CHO, and control CHO-K1 cells. The anti-hMOR EL peptide activity of IgG was associated with recognition of the hMOR in native configuration (i.e., expressed on cell surface) (Fig. 5). We then examined each anti-hMOR EL peptide IgG preparation, individually or in combination, for their inhibitory effect on forskolin-stimulated adenylate cyclase activity in intact hMOR/CHO cells. 150,000 hMOR/CHO cells were incubated with 10 μM forskolin alone (control) or together with increasing amounts of either individual or combined anti-hMOR EL peptide IgG fractions (A). □, anti-hMOR EL1 IgG; ◇, anti-hMOR EL2 IgG; ▲, anti-hMOR EL3 IgG; ◆, anti-hMOR EL1 + anti-hMOR EL2 IgG; ●, anti-hMOR EL1 + anti-hMOR EL3 IgG; ▼, anti-hMOR EL2 + anti-hMOR EL3 IgG. Panel B depicts the reversion by naloxone (○) and by PTX (▲) of the effect of anti-hMOR EL1 and anti-hMOR EL3 IgG mixture (●). Each experimental point represents mean ± S.E. of three independent experiments performed in triplicate.
cific activation of the hMOR associated with a G/Go-dependent transduction signal.

DISCUSSION

In this study, we showed that the morphine-like activity of antibodies present in normal IgG pools (7) is elicited by their simultaneous binding to the first and third extracellular loops of the hMOR. These findings confirm and extend our previous observations on the pivotal role of EL1 and EL3 in the formation of the μ-opioid binding site; by studying chimeric μ-opioid/angiotensin receptor, we had indeed suggested that these two loops may constrain the relative positioning of the connected transmembrane α-helices that contain receptor/ligand contact points (8).

Previous reports have already documented that extracellular domains of G protein-coupled receptors such as thyrotropin (15, 16), α1-adrenergic (5), angiotensin AT1 (6), and β2-adrenergic (4) receptors could be instrumental in transducing external signals to intracellular compartments. The latter study also illustrated that activation of β2-adrenergic receptor could be achieved either by catecholamines interacting with transmembrane α-helices buried within the membrane bilayer (17) or by autoantibodies recognizing the second extracellular loop of the receptor (4).

Point mutagenesis experiments have shown that key residues of the μ-opioid binding site may be distributed over the third, sixth, and seventh transmembrane domains (18). It seems, however, unlikely that the agonistic behavior of IgG is because of interaction with such residues. Recent reports demonstrated that activation of rhodopsin required rearrangement of the relative positions of the third and sixth transmembrane helices (19, 20). Similar conformational changes are expected to be involved in the activation of other G protein-coupled receptors including μ-opioid receptor (21, 22). Accordingly, modeling of opioid receptors three-dimensional structure has suggested that interaction of morphine with Asp23 within the third transmembrane helix might induce such a repositioning of the third and sixth transmembrane helices (23). It is interesting to note that G protein-coupled receptor activation such as rhodopsin and tachykinin NK-1 receptor is prevented by reducing flexibility of either the third and sixth or the fifth and the sixth transmembrane helices using zinc binding to engineered metal ion binding sites (24, 25). Based on these studies, it could be speculated that similar movements of the third and sixth transmembrane segments are triggered by the binding of IgG to the adjacent first and third extracellular loops. The requirement for hMOR activation of a coordinated shift of the third and sixth transmembrane helices would explain the ineffectiveness of each anti-hMOR EL peptide IgG when individually tested.

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REFERENCES

1. Avrameas, S., and Ternynck, T. (1995) Res. Immunol. 146, 235–248
2. Strakosch, C. R., Wenzel, B. E., Row, V. V., and Volpe, R. (1982) N. Engl. J. Med. 307, 1499–1507
3. Borda, E., Pascual, J., Cosio, P., De La Vega, M., Arana, R., and Sterin-Borda, L. (1984) Clin. Exp. Immunol. 57, 679–686
4. Magnusson, Y., Wallukat, G., Waagstein, F., Hjalmarson, A., and Hoebeke, J. (1994) Circulation 89, 2760–2767
5. Fu, M. L. X., Herlitz, H., Wallukat, G., Hilme, E., Hedner, T., Hoebeke, J., and Hjalmarson, A. (1994) Lancet 344, 1660–1663
6. Wallukat, G., Homuth, V., Fischer, T., Lindschau, C., Horstkamp, B., Jupner, A., Baur, E., Nissen, E., Vetter, K., Neichel, D., Dudenhausen, J. W., Haller, H., and Luft, F. C. (1999) J. Clin. Invest. 103, 945–952
7. Macé, G., Blanpied, C., Emorine, L. J., Druet, F., and Dietrich, G. (1999) Eur. J. Immunol. 29, 997–1003
8. Dietrich, G., Galbelet, G., Capeyrou, R., Boutour, J.-L., Pontet, F., and Emorine, L. J. (1998) J. Neurochem. 70, 2106–2111
9. Okayama, H., and Berg, P. (1992) Bio/Technology 24, 270–279
10. Kieffer, B. L. (1995) Cell. Mol. Neurobiol. 15, 615–635
11. Druet, E., Pradaude, F., Druet, F., and Dietrich, G. (1998) Eur. J. Immunol. 29, 183–192
12. Alvarez, R., and Daniels, D. V. (1990) Anal. Biochem. 187, 98–103
13. Wang, J. B., Imai, Y., Eppler, C. M., Gregor, P., Spivak, C. E., and Uhl, G. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10230–10234
14. Wang, W. W., Shahrestanifar, M., Jin, J., and Howells, R. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12436–12440
15. Nagayama, Y., Wadsworth, H. L., Russo, D., Chazenbalk, G. D., and Roop, B. (1991) J. Clin. Invest. 88, 336–340
16. Morris, J. C., Gibson, J. L., Haas, E. J., Bergert, E. R., Dallas, J. S., and Frabhakar, B. S. (1994) Autoimmunity 17, 287–299
17. Tota, M. R., and Strader, C. D. (1995) J. Biol. Chem. 270, 16891–16897
18. Mansour, A., Taylor, L. F., Rose, J. L., Thompson, R. C., Hovestorf, M. T., Mosberg, H. I., Watson, S. J., and Akil, H. (1997) J. Neurochem. 68, 344–353
19. Farrens, D. L., Altenbach, C., Yang, K., Hubbel, W. L., and Khornana, H. G. (1996) Nature 374, 768–770
20. Dunham, T. D., and Farrens, D. L. (1993) J. Biol. Chem. 278, 1683–1689
21. Geber, U., and Kublika, B. K. (1988) J. Biol. Chem. 263, 17979–17982
22. Bockaert, J., and Pin, J. P. (1999) EMBO J. 18, 1723–1729
23. Pogozheva, I. D., Lomize, A. L., and Mosberg, H. I. (1998) Biophys. J. 75, 612–634
24. Sheikh, S. P., Zvyaga, T. A., Lichtarge, O., Sakmar, T. P., and Bourne, H. R. (1996) Nature 383, 347–350
25. Eiling, C. E., Nielsen, S. M., and Schwartz, T. W. (1995) Nature 374, 74–77