Dermorphin Gene Sequence Peptide with High Affinity and Selectivity for δ-Opioid Receptors*

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Communication

The peptides DM, DGAP, and [D-Met]DGAP were synthesized at Farmitalia; DM was prepared by the solution method according to de Castiglione et al. (15), while DGAP and [D-Met]DGAP were synthesized by conventional solid phase methods. DAGO, DADLE, DSLET, (16), and DPDPE (17) were purchased from Peninsula Laboratories, Inc. (Belmont, CA) and Bachem (Torrance, CA).

The preparation of rat whole brain (minus cerebellum) synaptosomes was achieved by a slight modification (18) of that described by Chang and Cuatrecasas (19); the P2 fraction was initially preincubated at 22°C for 60 min in a dissociation solution containing 50 mM HEPES, pH 7.5, 50 μg of soybean trypsin inhibitor/ml, 100 mM NaCl, and 0.1 mM GDP in order to remove endogenously bound opioid peptides (20). The membrane fraction was then washed extensively in cold buffer (50 mM HEPES, pH 7.5, on ice) and stored at -70°C in buffer containing trypsin inhibitor (50 μg/ml) and 20% glycerol (v/v) (18).

Binding studies were performed by incubating an aliquot of synaptosomes (800 μg of protein) in an assay containing a final concentration of 50 mM HEPES, pH 7.5, 20 μg of trypsin inhibitor, 8% glycerol (v/v), 1 mg of bovine serum albumin, 16 μg of bacitracin, 1 μM bestatin, and 0.2 nM [3H]DAGO (α-receptor agonist) or 0.2 nM [3H]DADLE (δ-receptor agonist) plus 2.6 μM [NMMePhe²,D-Pro³] morpheaepin to suppress binding to μ-receptor sites (20) in a final volume of 100 μl. After 90 min at 22°C the membranes were collected on glass fiber filters (GF/C), presoaked in buffer containing 0.1% bovine serum albumin, and washed thrice in the same ice-cold buffer solution. Specific binding is the difference between total binding and non-specific binding (21). The IC₅₀ values (nM), representing the midpoint of the competition binding curves, are expressed as the mean ± S.E. for the indicated number (n) of assays performed in duplicate.

RESULTS AND DISCUSSION

The competitive binding curves for μ-receptors indicated that only DM and DAGO were effective in displacing the

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‡ The abbreviations used are: DM, dermorphin; DGAP, dermorphin gene-associated peptide; DAGO, [D-Ala²,NMe-Phe⁴,Gly-ol]enkephalin; DADLE, [D-Ala²,D-Leu⁴]enkaphalin; DSLET, [D-Ser⁴,Leu⁵,Thr⁶]enkephalin; DPDPE, [D-Pen²⁰]enkephalin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SR, δ-selectivity ratio; Pen, penicillamine.
labeled DAGO (Fig. 1) with IC₅₀ values of 1.04 and 1.51 nM, respectively (Table I). The apparent high affinities for synapticosomal µ-receptors for DM and DAGO are consistent with in vitro bioassay data involving relaxation of electrically stimulated guinea pig ileum preparations (16, 23–27), whose activity is regulated primarily by µ-type receptors (19, 24). In the µ-receptor assay, DADLE, DSLET, [D-Met]DGAP, DPDPE, and DGAP exhibited progressively weaker binding; DGAP was essentially inactive (Fig. 1). The IC₅₀ values for DAGO, DM, DADLE, and DSLET were comparable to published values (18–22), while that for DPDPE was about half the value reported by Mosberg et al. (17). Although DGAP poorly interacted with µ-receptors, [D-Met]DGAP had a high but measurable IC₅₀ value similar to that of DPDPE (Table I). It is known that δ-specific ligands, such as DADLE, DSLET, and DPDPE, have low affinities for µ-receptors (16, 17, 20).

Binding assays conducted with [³H]DADLE to label δ-receptors indicated that [D-Met]DGAP had δ-type receptor affinities similar to those of DADLE and DSLET (Fig. 2). Although δ-affinity of DPDPE was 4.5-fold less than those of the other δ-agonists (Table I), our IC₅₀ value was one-fourth of that published (17). The apparent δ-receptor affinities for DAGO and DM were more than 2 orders of magnitude less than that for DADLE (Table I). DGAP also exhibited low affinity for the δ-receptor with an IC₅₀ 930 times higher than that of DADLE, suggestive that DGAP has an intrinsic affinity for this receptor type. In contrast, [D-Met]DGAP exhibits dramatically high affinity for δ-receptors (Table I).

The double reciprocal plot of peptide affinities versus receptor type selectivity (SR) (21) provides a very convenient means for presenting relationships between each of the peptide ligands (Fig. 3). That figure shows that the SR of the µ-specific ligands DAGO and DM is typically <0.01, while the δ-specific ligands have SR values greater than 10. The respective SR values for DADLE and DSLET are approximately 20 and 40, twice the values reported by others (21). The estimated

Table I

| Peptides  | Sequences               | IC₅₀ µ  | IC₅₀ δ  | SR  |
|-----------|-------------------------|---------|---------|-----|
| DAGO      | Tyr-D-Ala-Gly-NMe-Phe-Gly-ol | 1.51 ± 0.18 (8) | 376.3 ± 81.8 (4) | 0.0040 |
| DM        | Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH₂ | 1.04 ± 0.22 (4) | 190.0 ± 18.8 (4) | 0.0055 |
| DADLE     | Tyr-D-Ala-Gly-Phe-d-Leu-OH | 14.1 ± 2.30 (4) | 0.72 ± 0.10 (7) | 20  |
| DSLET     | Tyr-D-Ser-Gly-Phe-Leu-Thr-OH | 32.5 ± 2.35 (3) | 0.78 ± 0.07 (3) | 42  |
| DPDPE     | Tyr-D-Pen-Gly-Phe-d-Pen-OH | 1,228 ± 141 (4) | 4.08 ± 0.21 (3) | 301 |
| DGAP      | Tyr-Met-Phe-His-Leu-Met-Asp-NH₂ | 28,000 ± 4,221 (6) | 670.0 ± 142 (5) | 42  |
| [D-Met]DGAP | Tyr-D-Met-Phe-His-Leu-Met-Asp-NH₂ | 1,076 ± 113 (7) | 0.80 ± 0.11 (7) | 1,345 |

Fig. 1. Competitive µ-receptor binding curves. The assay for µ-receptors using rat brain synapticosomal membranes is detailed in the text. Each point in the peptide competition curves represents the average ± S.E. (denoted by the error bars) for the number of repetitions given in Table I. The symbols define the following peptides: ▲, dermorphin; ○, DAGO; ●, DADLE; □, DSLET; △, [D-Met]DGAP; ■, DPDPE and ▼, DGAP.

Fig. 2. Competitive binding assays for δ-receptors. The conditions for the δ-receptor assay are given in the text and in Ref. 18. The expressions of the binding data and definitions of symbols are identical to those listed in the legend to Fig. 1.
to influence interaction with a positively charged group in the assisted opioid receptor selection.

The C-terminal carboxyl function of the peptide is presumed to interact with a C-terminal carboxyl function of the receptor by [D-M~*~]DGAP exceeds that for DADLE by approximately 0.000036 nM~4, and on the ordinate, the IC50 values for δ-receptors (nM). Nonselective ligands (SR = 1) would fall within the stippled region of the graph, as reported (18, 21). The diagonal lines indicate 10-fold differences in the SR values >1 characterize δ-selective ligands, while values >1 indicate δ-selective ligands. The dashed lines connecting related structural sequence analogues emphasize the marked differences in opioid receptor selectivities: the DM prohormone-derived peptides (DM, DGAP, and [D-M~*~]DGAP) on the one hand, and the enkephalin-derived peptides (DAGO, DADLE, DSLET, and DPDPE) on the other.

SR of 42 for DGAP reflects a preference for δ-receptors primarily based upon its very minimal interaction with μ-receptors (Table I); on the other hand, [D-M~*~]DGAP exhibited a SR of over 1300. This SR value is 4.5-fold greater than that of DPDPE (SR = 301), which is considered to be one of the most effective synthetic δ-agonists; the SR for DPDPE (Table I) exceeds that value obtained under different assay conditions (17).

Our findings indicate that even though the selectivity for the δ-receptor by [D-M~*~]DGAP exceeds that for DADLE by over 1.5 orders of magnitude, the apparent receptor affinities for these peptides are identical (Fig. 2 and Table I). By analogy, the presence of the β-isomer of Ala~2~ in DM is absolutely essential for bioactivity (1, 2, 23, 25-28) and binding to μ-receptors (18, 25, 27); it also affects peptide conformation and stabilization in solution (29-31). The absence of Gly~1~ and Tyr~4~ residues of DM in [D-M~*~]DGAP (Table I) may prohibit this peptide from assuming the intramolecular H-bonded structure characteristic of DM (32) and DM N-terminal tetrapeptide (31). In the case of the enkephalin analogues, arguments have been presented that a more open molecular structure (33, 34) confers higher affinity to δ-receptors (16, 17, 20, 24; DAGO is the exception, being a μ-agonist (23, 24). These data tend to support the membrane-assisted opioid receptor selection model of Schwyzer (35) in that a C-terminal carboxyl function of the peptide is presumed to influence interaction with a positively charged group in the δ-type receptor site. The conformational properties imposed on DGAP by the inclusion of a δ-Met~2~ residue may permit the peptide to bind more tightly or selectively, or both, at a specific site in the δ-receptor.

At the present time, neither DGAP nor [D-M~*~]DGAP has been isolated from extracts of frog skins. Although it would be of considerable interest to know whether the occurrence of δ-amino acids in DM or DGAP reflects an unanticipated direct incorporation into the prohormone, or the more probable post-translational modification of the configuration around the α-carbon of L-Met~2~ (3), our results indicate that a single frog skin gene codes for two distinct heptapeptides which, after appropriate processing, possess unique structural information to allow them to exhibit both high affinity and selectivity for μ- and δ-type opioid receptors in rat brain. In addition to the primary sequence, secondary structural properties of the DM precursor may influence the processing which leads to an inversion of the configuration about the α-carbon of Ala~3~ and Met~2~ adjacent to the N-terminal tyrosine residues of the excised heptapeptides. In this regard, the amino acid sequence of the DM prohormone (3) resembles that of a de novo-designed α-helical protein (36) that could suggest a model in which the C-terminal cleavage sites for DM and DGAP lie within a loop between α-helices. Similarly, the two β-turns in pro-opiomelanocortin enable site-specific cleavage while the α-helical content confers a degree of rigidity that prevents proteolysis (11). It is conceivable then that the composite structural features of the DM prohormone may dictate the sites for enzymic excision and for enantiomer inversion.

In conclusion, the recognition of differential receptor type selectivities of two opioid peptides, DM and [D-M~*~]DGAP, coded by a single gene, is unprecedented. These peptides may prove invaluable in opioid research in the facilitation of the design of new high affinity peptide agonists and antagonists (28, 31, 37, 38), which may establish clinically relevant paradigms (39-43).

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