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DIFFERENTIAL EFFECTS OF GTP AND CATIONS ON BINDING OF LABELED DIMERIC AND MONOMERIC ENKEPHALINS TO NEUROBLASTOMA-GLIOMA CELL DELTA OPIATE RECEPTORS

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SUMMARY. Binding of radio-labeled enkephalin monomers [D-Ala^2,Met^5]Enkephalin Amide (DAMEA) and [D-Ala^2,D-Leu^5]Enkephalin (DADLE) and a dimer of [D-Ala^2,Leu] Enkephalin Amide (DPE_2) to neuroblastoma-glioma (NG108-15) cells was examined in the presence and absence of GTP and/or cations. We found that: (1) binding occurs to a single class of homogeneous and non-interacting membane sites; (2) the affinity of the enkephalin dimer is reduced 50% in the presence of Mn^{2+} and 65% in the presence of both Mn^{2+} and GTP; (3) GTP alone either increases or does not change affinity of DPE_2; (4) Na^+ and GTP significantly decrease the affinities of monomers, but not that of the dimer; and (5) a higher concentration (0.1 mM) of GTP increases the binding of DPE_2 but significantly decreases binding of monomers. Conclusion: Changes in binding of a dimeric enkephalin by Na^+, Mn^{2+} and GTP are significantly and qualitatively different than those occurring for monomers.

INTRODUCTION. Two opiate receptor subtypes, enkephalin (δ) and morphine (μ) selective sites, were identified in guinea pig and rat brain membranes by the use of radiolabeled opiates and enkephalin analogs (1,2). In addition to the coexistence of both subtypes in the brain from some animals, either subtype may also exist predominantly alone. Thus, the toad brain appears to contain mainly the μ subtype (3) and the mouse neuroblastoma-rat glioma NG108-15 cell line may contain only the δ subtype (4,5). Because of the presumed homogeneity of its δ receptors, this cell line was chosen for the present study of enkephalin-receptor interaction.

An enkephalin dimer of increased biological potency was described by Coy et al (6). We have studied the properties of two new series of enkephalin dimers (pentapeptide and the des-Leu^5 tetrapeptide) as a function of length of the connecting methylene bridge (7-9). The dimeric pentapeptide with a two-carbon connecting bridge, designated DPE_2 = [H-Tyr-D-Ala-Gly-PheLeu-NH]$_2(CH_2)_2$,

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has the highest affinity for the δ enkephalin receptor yet reported (7,8). This compound is a very potent agonist in bioassays and displays no detectable antagonist properties. Hazum et al (10) have described dimers of oxymorphine and of enkephalyl-lysine. It is postulated that the increased receptor binding and/or biological potency and selectivity of the dimers may be due to the ability of the dimers to attach bivalently to two receptors simultaneously (7-10). In the present report, we employ the dimeric pentapeptide enkephalin DPE_2 labeled with tritium to high specific activity, to a) verify that monomers and dimers compete for a single class of δ enkephalin receptors on NG108-15 cells; b) directly demonstrate the high affinity of the dimer relative to monomers previously observed (8) in indirect displacement studies with the unlabeled dimer; c) show significant qualitative differences in the binding of monomers and dimers to the δ-receptor when perturbed by systematic changes in Na^+, Mn^{++}, and GTP alone or in several combinations.

Studies of the influence of nucleotides and ions on opiate receptor binding was prompted by the demonstration that GTP and certain monovalent and divalent cations were essential for inhibition of adenylate cyclase activity by opiate agonists (11). Nucleotides and ions selectively affect the binding of agonists, whereas binding of antagonists is largely unaffected. Na^+ and guanine nucleotides decrease opiate agonist binding to membrane from rat brain and neuroblastoma-glioma cells (12-15). In contrast, divalent cations such as Mn^{2+} and Mg^{2+}, increase opiate binding. Because the dimer DPE_2 has been observed to be equipotent with the known δ-specific agonist DADLE in inhibiting cyclic AMP formation in PGE_1-stimulated NG108-15 cells (16), it was of interest to compare the effects of GTP and ions on binding of monomers and dimers.

MATERIALS AND METHODS. [^{3}H]-[D-Ala^2,Met^5]Enkephalin Amide (^{3}H-DAMEA; 32.8 Ci/ mmol) and [D-Ala^2,D-Leu^5]Enkephalin (^{3}H-DADLE; 31.2 Ci/mmol) were obtained from New England Nuclear, Boston, MA. Dimeric Pentapeptide Enkephalin, DPE_2, synthesized by Dr. Y. Shimohigashi (7,8) was obtained by crosslinking of [D-Ala^2, Leu^5]Enkephalin at the carboxy-terminal leucine with α,β-diaminoethane. Radiolabeling was performed by New England Nuclear by the exchange method after bromination (37.8 Ci/mmol; 97% radionuclidic purity). DADLE was obtained from Peninsula Laboratories, San Carlos, CA.; DAMEA, the nucleotides, guanosine triphosphate (GTP) and guanyl-5'-yl-imidodiphosphate (Gpp(NH)p) and all other chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

Mouse neuroblastoma-rat glioma hybrid cell line NG108-15 of passage 16 was generously donated by Dr. M. Nirenberg. The cells were grown in flasks

in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 5% fetal bovine serum, 0.1 mM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine in a humidified atmosphere of 5% CO_2 at 37° and passaged weekly. The cells were grown to confluency and harvested immediately before use by shaking the cells loose from the flasks in growth medium. Following centrifugation at 200 g for 5 min at 4°, the cells from one T-150 flask were washed once with 20 ml of ice-cold DMEM containing bovine albumin (2 mg/ml). The washed cells were then resuspended at room temperature at $2x10^6$ cells/ml in a solution of 0.25 M sucrose in 50 mM Tris.HCl (pH 7.4) containing bovine serum albumin (2 mg/ml) and bacitracin (100 μg/ml). For brevity, this solution is referred to as A, and the same solution without albumin and bacitracin as B. Large batches of cells were grown in similar way for membrane preparations. At harvest, cells were pelleted and frozen immediately at -70°. Cell membranes were prepared as described by Chang and Cuatrecasas (17). Binding to membrane preparations was performed essentially as described by Miller et al.(18), using a filtration method. Binding to intact cells was performed by a modification of the same filtration method. Binding was initiated by adding $2x10^6$ cells suspended in A to tubes containing solution A with and without the indicated amounts of unlabeled enkephalins. The final concentration of labeled enkephalin was: 0.2 nM of either [^{3}H]-DADLE or [^{3}H]-DAMEA and 0.05 nM of [^{3}H]-DPE_2. Total volume of the reaction mixture was 2 ml. Each tube was vortexed at the beginning of the 1 h incubation period used (25°), and again prior to filtration over Whatman GF/B filters under vacuum, followed by two rapid 5 ml washes with B. Cells of passages 16-30 were assayed in duplicate or triplicate.

RESULTS. Specificity of Binding. It was first necessary to demonstrate that binding of DPE_2, DADLE, and DAMEA involves the same site, presumably the δ-receptor, the only class of opiate binding sites identified in NG108-15 cells. DAMEA, DADLE, and DPE_2 are able to completely displace each other (Fig. 1, left). Mathematical modelling of the binding data using program "LIGAND" (19) confirmed that a model involving only a single class of sites was sufficient. Affinity: Equilibrium binding studies using ^{3}H-DPE_2, ^{3}H-DADLE, or ^{3}H-DAMEA

Table 1. Comparison of binding affinities of enkephalins to suspended NG108-15 cells and their membrane preparations

Enkephalin		Affinity constant[a] (K) (10^8 L/M)	
		cells[b]	membranes[c]
Monomers			
[D-Ala^2, Met^5]enkephalin amide	(DAMEA)	6 ± 2	8 ± 1
[D-Ala^2, D-Leu^5]enkephalin	(DADLE)	7 ± .5	10 ± 1
Dimer			
[H-Tyr-D-Ala-Gly-Phe-Leu-NH]$_2$(CH_2)$_2$	(DPE_2)	42 ± 3	42 ± 3

[a] Affinity constants were computed using the "LIGAND" program (19).
[b] Cells of passages 16 through 30.
[c] Cells of passages 20 and 22.

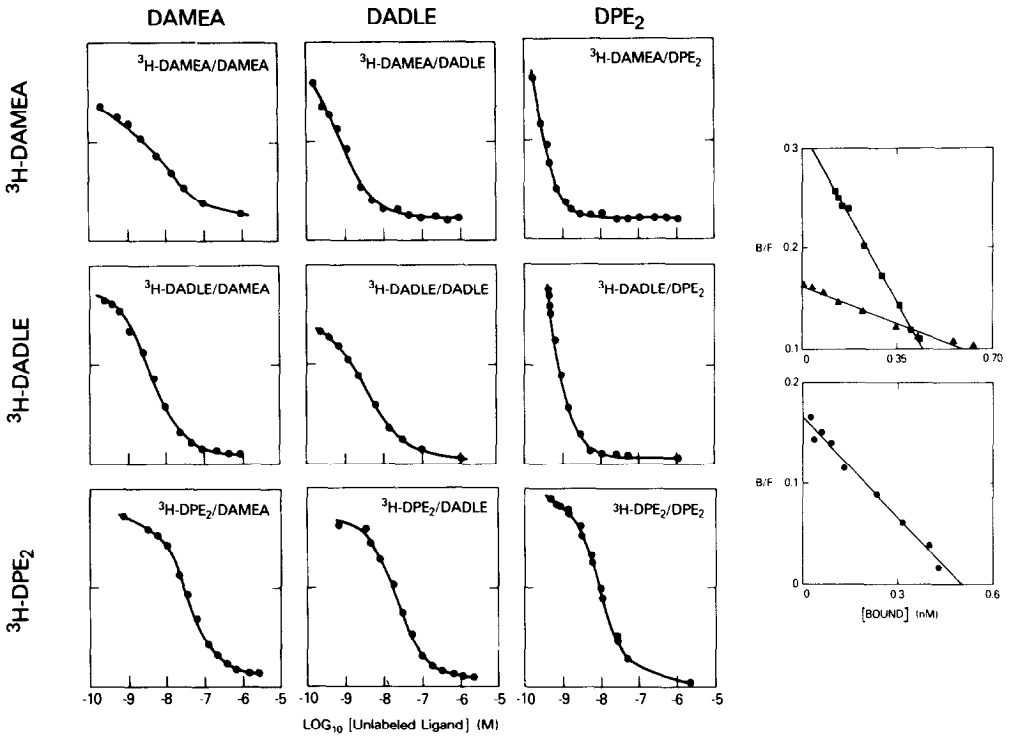

Fig. 1. Left: Competitive inhibition of binding of enkephalin monomers. (^{3}H-DAMEA and ^{3}H-DADLE) and dimer (^{3}H-DPE$_2$) to intact NG108-15 cells by unlabeled DAMEA, DADLE, and DPE$_2$ (group of 9 panels). Right: Scatchard plots of the binding data: DPE$_2$ (■) and DADLE (▲)(upper panel); DAMEA (lower).

were performed with both intact cells and membrane preparations. The Scatchard plots of the binding data to cells were linear, consistent with binding of ligands to a homogeneous class of non-interacting sites (Fig. 1, right). The affinity constants (K) for binding of monomers and the dimer to both intact cells and membrane preparations are shown in Table 1.

There was excellent agreement between the results for cells and membrane preparations.

Effect of GTP and cations on binding. When binding to membrane preparations was studied in the presence of different concentrations of Na^+, Mn^{2+}, and/or GTP (Table 2), the following was observed: 1) Binding of ^{3}H-DAMEA decreased in the presence of either 100 mM Na^+ (23%) or 0.1 mM of GTP (40%), and decreased further in the presence of both (62%), each added at lower concentrations. This effect could be partially reversed by replacing Na^{2+} with Mn^{2+}.

Table 2. Effects of Na^+, Mn^{2+}, and GTP on binding of enkephalin monomers and dimer to membrane preparations of NG108-15 cells. Binding is expressed as percentage of tracer binding in Tris.HCl buffer (control).

	Monomers[a]		Dimer[a]
Additions (mM)	^{3}H-DAMEA	^{3}H-DADLE	^{3}H-DPE$_2$
Na+ (50)	80 ± 1[b]	114 ± 18	80 ± 7
Na^+ (100)	77 ± 1[b]	120 ± 5[b]	79 ± 1[b]
Mn^{2+} (3)	84 ± 18	128 ± 3	57 ± 1
Mn^{2+} (6)	109 ± 1[b]	130 ± 39	54 ± 1[b]
GTP (0.02)	86 ± 6	119 ± 21	106 ± 8
GTP (0.10)	60 ± 3[b]	79 ± 3[b]	115 ± 2[b]
Na^+ (50) + GTP (0.02)	38 ± 10	63 ± 5	71 ± 5
Mn^{2+} (3) + GTP (0.02)	71 ± 16	132 ± 24	55 ± 2

[a] DAMEA = [D-Ala^2, Met^5]Enkephalin Amide; DADLE = [D-Ala^2,D-Leu^5]Enkephalin; and DPE$_2$ = [H-Tyr-D-Ala-Gly-Phe-Leu-NH]$_2$(CH$_2$)$_2$.
[b] Mean ± S.D. within assay, otherwise between assays.

2) Similarly, ^{3}H-DADLE binding showed the largest decrease in the presence of both Na^+ and GTP (37%). Addition of either 6 mM Mn^{2+} or 100 mM Na^+ resulted in 30 and 20% increased binding, respectively; 3) ^{3}H-DPE$_2$ binding remained elevated in the presence of either 0.02 or 0.1 mM GTP, in contrast to monomers; 4) In the presence of 50 or 100 mM Na^+, binding of DPE$_2$ decreased, similar to that of DAMEA; (5) Binding of DPE$_2$ was maximally decreased (46%) in the presence of Mn^{2+}, and GTP had no effect in the presence of Mn^{2+}. The results shown in Table 2 were obtained using a single "tracer" concentration of each of the three labeled ligands. These data alone do not permit one to conclude whether the ions and GTP produce their effects by changes in affinity, binding capacity, or both. Accordingly, we constructed complete Scatchard plots, for all 3 labeled ligands in triplicate at 12 dose levels under 6 sets of conditions (Table 3). Results were analyzed by the "LIGAND" computer program (19). The effects of the ions and GTP were found to be entirely due to changes in affinity (Table 3) and the Scatchard plots remained linear in every instance. GTP dramatically increases the affinity for DPE$_2$ but not for DAMEA and moderately for DADLE. In the presence of sodium, GTP reduces the affinity of the monomers but increases the affinity of DPE$_2$; Mn^{2+} reduces the affinity for DPE$_2$ but not

Table 3. Affinity constants of enkephalin monomers and dimer to membrane preparations of NG108-15 cells in the absence and presence of Na^+, Mn^{2+}, and GTP.

	Affinity constant (K) (10^8 L/M)		
	Monomers[a]		Dimer[a]
Additions (mM)	^{3}H-DAMEA	^{3}H-DADLE	^{3}H-DPE_2
Tris.HCl Buffer (Control)	8 ± 1[b]	10 ± 1[b]	42 ± 3[b]
Na^+ (50)	8 ± .1	11 ± .5	28 ± 1
Mn^{2+} (3)	8 ± .3	9 ± 1	20 ± 4
GTP (0.02)	8 ± .4	13 ± .3	64 ± 7
Na^+ (50) + GTP (0.02)	4 ± .4	8 ± .5	35 ± 5
Mn^{2+} (3) + GTP (0.02)	7 ± .4	11 ± .5	15 ± 2

[a] DAMEA = [D-Ala^2,Met^5]Enkephalin Amide; DADLE = [D-Ala^2,D-Leu^5]Enkephalin; DPE_2 = [H-Tyr-D-Ala-Gly-Phe-Leu-NH]$_2$(CH_2)$_2$
[b] Mean ± S.D. within assays for DAMEA and DADLE, but between assays for DPE_2.

significantly for the monomers; the presence of Mn^{2+} completely blocks the effect of GTP for the dimer, while GTP and Mn^{2+} have minimal effects on the affinities of the monomers. The binding capacities were affected minimally (and not statistically significantly) by any of the additions of cations and/or GTP.

DISCUSSION. These studies are the first to show the mutual cross-displace ability of DPE_2 and its monomers DAMEA, and DADLE, demonstrating that the new dimer binds to the same homogeneous class of receptor sites as the monomers. Binding of monomers and a dimer of enkephalin to NG108-15 membranes are differentially affected by cations and GTP. The effects demonstrated by the dimer, DPE_2 *viz* decreased affinity in the presence of Mn^{2+} and increased or no change in affinity in the presence of GTP, are contrary to the effects previously described for monomeric agonists (12-15). The effect of guanine nucleotides on DPE_2 binding is similar to the enhanced binding of antagonists in the presence of nucleotides (e.g., [^{3}H](-)3-quinuclidinyl benzylate binding to the muscarinic receptor of rat heart) (20).

The failure of GTP to reduce binding for an agonist (DPE_2) may be unique. IF DPE_2, being bivalent, were to interact simultaneously with two closely spaced receptors, the bivalent attachment could account for its high affinity.

The bivalency of DPE_2 might also account for the unusual modulation by GTP and Mn^{2+}, possibly associated with loss of flexibility of the ligand-receptor complex.

DAMEA, because of its C-terminal carboxy group modification, is a μ/δ non-selective ligand in contrast to the δ-specific DADLE (21). Only the combined presence of Na^+ and GTP appeared to reduce the binding affinity of DAMEA to an appreciable degree. This observation agrees with report of Blume et al. (22), who showed that the combination of Na^+ and Gpp(NH)p was more effective than Gpp(NH)p alone in reducing the potency of enkephalin analogs in a binding system employing and [^{3}H]-naltrexone and NG108-15 cell membranes. The binding of DAMEA varies to a greater extent than that of DADLE in the presence of cations and/or GTP.

Differences in binding as result of ion concentration changes may reflect changes in the conformational or stability of the receptor, the ligand, and/or the guanyl-nucleotide binding proteins. Pert et al, classifying the opiate receptors in part on their GTP-reactivity and adenylate cyclase coupling (23, 24), claim to have converted the "Type 2" subtype (GTP-resistant and enzyme-uncoupled) to the "Type 1" subtype (GTP sensitive and enzyme-coupled) by addition of Na^+ and GTP. Such additions resulted in reduction of binding of opiate agonists to Type 1 receptors. We have observed that DPE_2 and DADLE are about equally potent in inhibiting cAMP production in NG108-15 cells stimulated with PGE_1 (16, and submitted for publication). Both DPE_2 and DADLE showed about 20% reduction in affinity with addition of Na^+ and GTP (Table 3). Presumably, conditions favoring the "Type 1" conformation would be present in order for DADLE and DPE_2 to inhibit cAMP production (16). Whereas Na^+ and GTP promote enzyme-coupling of the receptor, Mn^{2+} has been found to uncouple the alpha adrenergic receptor-mediated inhibition of adenylate cyclase in human platelets (25), presumeably due to perturbation of interaction of an inhibitory regulatory unit with the enzyme rather than with the receptor. In the present system, perturbation might directly involve the receptor, in view of the changes in affinity observed with Mn^{2+}.

In conclusion, we note that our new dimeric pentapeptide enkephalin, DPE_2, displays extraordinarily high affinity for the delta enkephalin receptor, and displays unique properties in terms of perturbation by GTP, Mn^{2+} and Na^{+}. DPE_2 provides special properties for characterization of opiate receptors. Further studies to demonstrate that the dimer can crosslink two receptors are in progress.

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