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Characterization of Opioid Receptors Mediating Stimulation of Adenylate Cyclase Activity in Rat Olfactory Bulb

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SUMMARY

We have investigated the pharmacological profile of the opioid stimulation of adenylate cyclase activity in rat olfactory bulb, in order to identify the opioid receptor subtype(s) involved in this response. The synthetic δ-selective agonists (D-Ala^2)deltorphin I, (2-D-penicillamine,5-D-penicillamine)-enkephalin, and (D-Ser-Leu^5-enkephalyl)-threonine were effective stimulators of the enzyme activity, with EC_{50} values of 6.7, 420, and 63 nM, respectively. A significant increase was also observed with the μ-selective agonists (*N*-methyl-Phe^3,D-Pro^4)-morphiceptin, dermorphin, and (D-Ala^2-*N*-methyl-Phe^4-Gly-ol^5)-enkephalin (DAGO). The latter two agonists displayed biphasic concentration-response curves, with high affinity components accounting for 75–80% of the maximal responses. The κ-selective agonists U-50,488 and U-69,593 were ineffective, whereas (D-Ala^2)dynorphin A-1–11, dynorphin A, dynorphin A-1–13, and dynorphin A-1–6 acted with a rank order of potency consistent with their affinity for δ receptors. The stimulatory responses of Leu-enkephalin, β-endorphin, dynorphin A, and δ-selective agonists were counteracted by naltrindole with pA_2 values of 9.39–8.93, whereas naloxone was less potent (pA_2 = 8.17–7.59). The κ-selective antagonist nor-binaltorphimine was the least potent. The inhibition by naltrindole and naloxone of DAGO stimulation showed biphasic curves, with 90% of the response being antagonized more potently by naloxone than by naltrindole. These results demonstrate that δ- and μ- but not κ-opioid receptor subtypes stimulate basal adenylate cyclase activity in rat olfactory bulb.

In the central nervous system, μ-, δ-, and κ-opioid receptor subtypes have been shown to be coupled to inhibition of adenylate cyclase activity, and many of the central opioid effects have been interpreted as being a consequence of decreased formation of cAMP (1). However, in certain tissues, stimulation of opioid receptors may increase, rather than decrease, basal adenylate cyclase activity. Thus, in mouse spinal cord-ganglion explants, the opioid receptor agonist levorphanol was found to stimulate basal cAMP formation (2). Moreover, we have recently reported that, in rat olfactory bulb, the naturally occurring opioid peptides β-endorphin, Leu-enkephalin, and dynorphin A cause stimulation of adenylate cyclase activity by a mechanism that is counteracted by naloxone, is GTP dependent, and is pertussis toxin sensitive (3). Studies on the distribution of opioid receptors by *in vitro* autoradiography have shown the presence of both δ and μ receptor subtypes in the rat olfactory bulb (4) and a high concentration of κ opiate binding sites in the external plexiform layer of the guinea pig olfactory bulb (5).

With the aim of identifying the receptor subtype(s) involved in the opioid stimulation of adenylate cyclase activity in rat olfactory bulb, in the present study we have investigated the effects of a number of agonists and antagonists with different selectivities for the distinct opioid receptor subtypes.

Materials and Methods

[α-^{32}P]ATP (30–40 Ci/mmol) and [2,8-^{3}H]cAMP (25 Ci/mmol) were purchased from DuPont de Nemours (Bad Homburg, FRG). Opioid peptides were obtained from either Peninsula Laboratories (Merseyside, UK) or Bachem (Bubendorf, Switzerland). Naltrindole, nor-binaltorphimine, U-50,488 {*trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide methanesulfonate salt}, U-69,593 {(5α, 7α, 8β)-(−)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4–5]dec-8-yl]benzeneacetaamide}, and (+)-*N*-allylnormetazocine hydrochloride were purchased from Research Biochemicals Inc. (Natick, MA). Naloxone hydrochloride was obtained from Salars (Como, Italy); dextrorphan hydrochloride and etorphine hydrochloride were from Hoffmann-La Roche (Nutley, NJ). The other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Adenylate cyclase assay. Male Sprague-Dawley rats (200–300 g) were sacrificed by decapitation, and olfactory bulbs were homogenized in 10 volumes (v/w) of ice-cold buffer containing 10 mM HEPES-NaOH, 1 mM EGTA, 1 mM $MgCl_2$, and 1 mM dithiothreitol (pH 7.4).

ABBREVIATIONS: HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DPDPE, (2-D-penicillamine,5-D-penicillamine)-enkephalin; DSLET, (D-Ser-Leu^5-enkephalyl)-threonine; DADLE, (D-Ala^2, D-Leu^5)-enkephalin; DAGO, (D-Ala^2-*N*-methyl-Phe-Gly-ol^5)-enkephalin; PL017, (*N*-methyl-Phe^3, D-Pro^4)-morphiceptin.

The homogenate was diluted 6-fold with homogenization buffer and centrifuged at 27,000 × *g* for 20 min at 4°. The pellet was resuspended in 10 volumes of homogenization buffer, diluted to give a protein concentration of 1.0–1.4 mg/ml, and used immediately for adenylate cyclase assay. Unless otherwise specified, the enzyme activity was assayed in a 100-μl reaction mixture containing 50 mM HEPES-NaOH (pH 7.4), 2.3 mM $MgCl_2$, 1.3 mM dithiothreitol, 0.2 mM [α-^{32}P]ATP (45 cpm/pmol), 1 mM [^{3}H]cAMP (80 cpm/nmol), 0.3 mM EGTA, 1 mM 3-isobutyl-1-methylxanthine, 5 mM phosphocreatine, 50 units/ml creatine phosphokinase, 100 μM GTP, 50 μg of bovine serum albumin, 10 μg of bacitracin, and 10 kallikrein inhibitor units of aprotinin. The reaction was started by addition of the tissue preparation (40–60 μg of protein) and was carried out at 30° for 10 min. [^{32}P]cAMP was isolated according to the method of Salomon *et al.* (6). Assays were performed in duplicate.

Protein content was determined by the method of Bradford (7), using bovine serum albumin as the standard.

Statistical analysis. Results are reported as mean ± standard error. Data from concentration-response curves were analyzed by a least squares curve-fitting computer program (GraphPad; ISI Software, Philadelphia, PA). The antagonist pA_2 values were determined from the ratios (*DR*) between the EC_{50} (concentration producing half-maximal effect) values of the agonist estimated in the absence and in the presence of multiple concentrations of the antagonist, according to Schild analysis (8). The *x*-intercepts were calculated by linear regression analysis of the plots, where the logarithm of $DR - 1$ is plotted as a function of the antagonist concentration. When a single concentration of antagonist was tested, the antagonist inhibitory constant (K_i) was calculated from the equation:

$$EC_{50_b} = EC_{50_a}\,(1 + I/K_i) \qquad (1)$$

where EC_{50_a} and EC_{50_b} are the concentrations of the agonist producing half-maximal effect in the absence and in the presence of the antagonist, respectively. *I* is the concentration of the antagonist. In some experiments, the antagonists were also examined for their ability to reverse completely the opioid stimulation of the adenylate cyclase activity. In these cases, the effects of multiple concentrations of the antagonist on the response elicited by a fixed concentration of an agonist were determined. The data were analyzed as competition curves, by nonlinear regression analysis, for models of one or two noninteracting sites. The antagonist K_i value was calculated according to the equation:

$$K_i = IC_{50}/1 + (A/EC_{50}) \qquad (2)$$

where IC_{50} is the concentration of antagonist producing half-maximal inhibition, *A* is the concentration of the agonist, and EC_{50} is the concentration of the agonist producing half-maximal effect. The goodness of fit of the data to a one- versus two-site model was evaluated by the *F* test of the sum of squares of residuals from both fittings, considering a level of significance of $p < 0.05$. The K_i values were converted to logarithmic form (pK_i). Statistical significance of the difference between means was determined by Student's *t* test.

Results

Effects of δ receptor-preferring agonists. The δ-selective receptor agonists (D-Ala^2)deltorphin I (9) and DPDPE (10) and the δ-preferring agonist DSLET (11) stimulated basal adenylate cyclase activity of rat olfactory bulb by 36.2 ± 0.9%, 39.8 ± 0.7%, and 40.9 ± 1.2%, respectively (Fig. 1A). Their EC_{50} values were 6.7, 420, and 63 nM, respectively (Table 1). Two other opioid agonists with higher affinity for δ receptors, Met-enkephalin and DADLE, were also found to be effective stimulators of the enzyme activity (Fig. 1A; Table 1).

Effect of μ receptor-preferring agonists. The adenylate cyclase responses to the μ-selective agonists dermorphin (12), DAGO (13), and PL017 (14) are reported in Fig. 1B. Both dermorphin and DAGO elicited biphasic concentration-response curves with high and low affinity components. The high affinity component of the dermorphin curve displayed an EC_{50} value of 11.1 nM, reached a plateau at 300 nM, and accounted for 76 ± 3% of the whole stimulatory response, whereas the low affinity component (EC_{50} = 3 μM) saturated at 50–100 μM dermorphin. The major portion (79 ± 2%) of the DAGO stimulatory response displayed an EC_{50} value of 64 nM and appeared to saturate at 1 μM. Above this concentration, DAGO elicited a further increase of the enzyme activity, which reached a plateau at 130 μM (EC_{50} = 10 μM). On the other hand, PL017 produced a monophasic stimulatory response (slope value = 1.10), with an EC_{50} value of 350 nM. The μ-preferring agonist morphine was as effective as DAGO and dermorphin and showed an EC_{50} value of 220 nM.

Effect of κ receptor-selective agonists and dynorphins. The κ agonists U-50,488 and U-69,593 (15, 16) failed to affect the adenylate cyclase activity of rat olfactory bulb significantly, at any concentration tested (Fig. 1C). We also examined the effect of different dynorphin A peptides, which are known to have higher affinity for κ than δ and μ receptors (17, 18). (D-Ala^2)Dynorphin A-1–11, dynorphin A-1–13, and dynorphin A-1–6 increased the enzyme activity with EC_{50} values of 18, 60, and 240 nM, respectively (Fig. 1C; Table 1).

Effect of other opioid receptor agonists. The nonselective opioid receptor agonist etorphine increased the enzyme activity by 40.5 ± 1.1%, with an EC_{50} value of 2.2 nM (Fig. 1D). Dextrorphan and the σ receptor agonist (+)*N*-allylnormetazocine were without effect at concentrations up to 10 μM.

Table 1 summarizes the properties of all the opioid agonists tested. When compared with the effect of etorphine, most of the compounds behaved as full agonists. (D-Ala^2)Deltorphin I, dermorphin, DAGO, and morphine displayed efficacies that corresponded to 75–85% of that of etorphine, whereas PL017 was the least effective.

Antagonism by selective opioid receptor blockers. The addition of increasing concentrations of the δ-selective antagonist naltrindole (19) progressively shifted to the right the concentration-response curve of Leu-enkephalin, β-endorphin, and dynorphin A (Fig. 2). The analysis of the Schild plots yielded pA_2 values of 9.20, 8.82, and 8.56, respectively (Table 2), and slope values not significantly different from unity. Naltrindole antagonized the stimulatory effect of (D-Ala^2)deltorphin I with a pA_2 value of 8.93 and a slope of 1.10 ± 0.05 (Table 2).

The κ-selective antagonist nor-binaltorphimine (20), tested at the concentrations of 100 nM, 1.0 μM, and 5.0 μM, counteracted the stimulatory effect of Leu-enkephalin with a pA_2 value of 7.25 (Fig. 3). At 100 nM, this antagonist produced a parallel shift in the dose-response curves of β-endorphin and dynorphin A and increased their EC_{50} values by 4.6- and 3.5-fold, respectively (results not shown). The corresponding pK_i values were 7.57 and 7.39, respectively (Table 2).

Naloxone, an antagonist with higher affinity for μ-opioid receptors (21), antagonized the stimulatory effects of PL017, (D-Ala^2)deltorphin I, and DSLET with pA_2 values of 8.64, 7.59, and 7.59, respectively (data not shown). The slope values of the Schild plots were 0.85 ± 0.07, 1.01 ± 0.04, and 1.06 ± 0.03. The

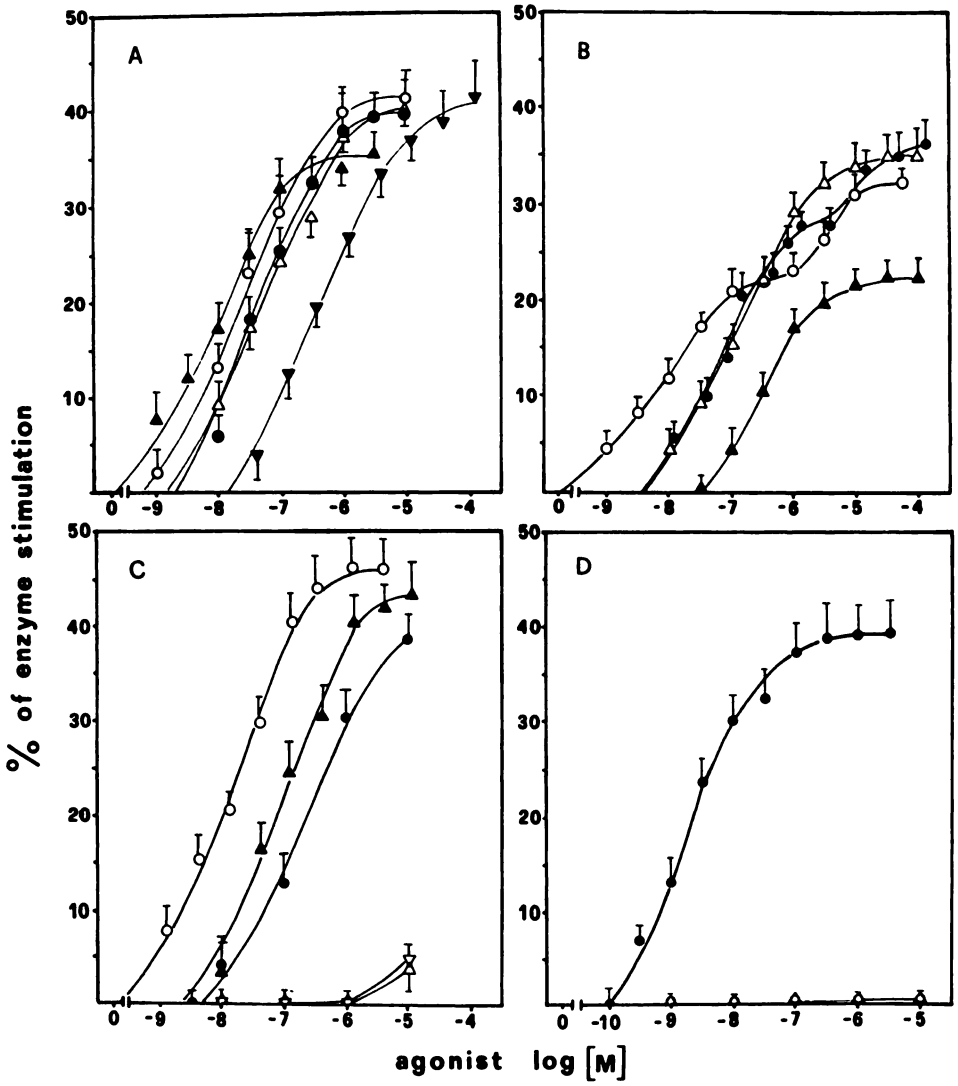

Fig. 1. Concentration-dependent stimulation of adenylate cyclase activity of rat olfactory bulb by different opioid receptor agonists. A, (D-Ala²)Deltorphin I (▲), Met-enkephalin (○), DSLET (△), DADLE (●), and DPDPE (▼); B, dermorphin (○), DAGO (●), morphine (△), and PL017 (▲); C, (D-Ala²)dynorphin A-1–11 (○), dynorphin A-1–13 (▲), dynorphin A-1–6 (●), U-50,488 (▽), and U-69,593 (△); D, ethorphine (●), dextrorphan (△), and (+)-*N*-allylnormetazocine (○). The stimulation of the enzyme activity is expressed as the mean ± standard error of percentage stimulation above basal enzyme activity (122.1 ± 2.4 pmol of cAMP/min/mg of protein). The number of experiments for each agonist is reported in Table 1.

potencies of naloxone in antagonizing the effects of Leu-enkephalin, β-endorphin, and dynorphin A were previously determined under the same experimental conditions (3) and are reported in Table 2.

Naltrindole reversed the effects of Leu-enkephalin (10 μM), DPDPE (120 μM), and PL017 (30 μM), with monophasic inhibition curves (Fig. 4A). The Hill coefficients were 0.87 ± 0.07, 1.01 ± 0.02, and 1.10 ± 0.05, respectively, and did not differ significantly from unity. The pK_i of naltrindole in reversing the Leu-enkephalin stimulation (9.21 ± 0.01) was similar to the pA_2 determined by Schild analysis. The pK_i values of the antagonist versus DPDPE and PL017 were 9.39 and 7.90, respectively (Table 2). On the other hand, the inhibition curves of naltrindole in antagonizing the effect of DAGO (10 μM) and dermorphin (10 μM) were biphasic and better fitted ($p < 0.01$) to a two-site competition model. For DAGO, a small fraction of the response (10.1%) was antagonized with high affinity by naltrindole (IC_{50} = 3.20 ± 0.10 nM), whereas the remaining major portion was counteracted with low affinity (IC_{50} = 2.50 ± 0.15 μM). The latter value corresponded to a pK_i of 7.79, calculated according to eq. 2 of Materials and Methods and using the EC_{50} value of the high affinity component of the DAGO concentration-response curve (64 nM). Similarly, 90.8% of the dermorphin response was antagonized by naltrindole with a IC_{50} of 6.92 ± 0.25 μM and the remaining portion with an IC_{50} of 3.3 ± 0.5 nM. The calculated pK_i value of the major lower affinity component was 8.12, using the EC_{50} value of dermorphin of 11.1 nM.

Naloxone inhibition of the stimulation elicited by (D-Ala²)deltorphin I (3 μM) yielded a monophasic curve (Hill coefficient = 0.88 ± 0.06; not significantly different from unity) (Fig. 4B) and a pK_i value of 7.67. On the other hand, the naloxone antagonism of the simulation produced by 10 μM DAGO was better fitted to a two-sites model ($p < 0.01$), with 10% of the response being antagonized with an IC_{50} of 5.2 ± 1.2 nM and the remaining, greater, portion with an IC_{50} of 400 ± 25 nM. The latter value corresponded to a pK_i of 8.72.

Effect of the combined addition of δ- and μ-selective agonists. The combined addition of (D-Ala²)deltorphin I and PL017, each at a maximally effective concentration, elicited an increase of the enzyme activity that was significantly lower ($p < 0.05$) than that expected if the effects of the individual agents were additive (Fig. 5).

TABLE 1

Properties of opioid agonists in stimulating adenylate cyclase activity of rat olfactory bulb

Agonist	n[a]	EC_{50}	Slope	Efficacy[b]
		nM		%
Ethorphine	3	2.2 ± 0.1	0.83 ± 0.07	100
(D-Ala²)Deltorphin I	3	6.7 ± 0.3	0.83 ± 0.05	82
Dermorphin	4	11.1 ± 0.4 (H)[c]	0.54 ± 0.05	86
		3,000 ± 400 (L)[d]		
β-Endorphin	10	21.0 ± 0.9	0.95 ± 0.05	100
(D-Ala²)Dynorphin A-1–11	3	18.3 ± 1.2	0.80 ± 0.10	120
Met-Enkephalin	3	25.1 ± 2.3	0.87 ± 0.08	100
Leu-Enkephalin	10	56.0 ± 1.1	1.10 ± 0.06	100
Dynorphin A-1–13	3	60.0 ± 1.9	0.81 ± 0.09	100
DSLET	3	63.0 ± 0.8	0.75 ± 0.08	100
DAGO	6	64.1 ± 3.2 (H)[c]	0.46 ± 0.07	90
		10,000 ± 1,000 (L)[d]		
DADLE	4	68.2 ± 2.1	1.10 ± 0.06	100
Dynorphin A	6	127.5 ± 15.3	0.70 ± 0.08	100
Morphine	3	220.1 ± 18.7	0.70 ± 0.10	88
Dynorphin A-1–6	3	240.9 ± 13.9	0.78 ± 0.11	100
PL017	3	350.2 ± 11.8	1.10 ± 0.04	60
DPDPE	4	420.7 ± 25.1	0.80 ± 0.09	100
U-50,488	3	NA[e]		
U-69,593	3	NA		

[a] n, number of determinations.
[b] Based on maximal observed response, compared with ethorphine (considered as 100).
[c] High affinity site.
[d] Low affinity site.
[e] NA, not active up to 10 μM.

Discussion

Besides being sensitive to the naturally occurring opioid peptides, the adenylate cyclase activity of rat olfactory bulb can be stimulated by a number of synthetic subtype-selective peptide agonists, thus providing useful information on the pharmacological nature of the opioid receptors involved in this response. The δ agonist (D-Ala²)deltorphin I is the most potent among the subtype-selective peptides, with an EC_{50} value that is in the low nanomolar range and only 3-fold higher than that of etorphine. Because the agonist has been shown to bind with high affinity to δ receptors and to activate μ receptors only at micromolar concentrations (9), the high potency of (D-Ala²)deltorphin I in stimulating adenylate cyclase activity in the olfactory bulb constitutes important evidence for the involvement of δ receptors in this response. Another selective δ agonist, DPDPE, is also able to stimulate the enzyme activity, with a potency 62-fold lower than that of (D-Ala²)deltorphin I. This difference is consistent with the results previously obtained in radioligand binding and functional studies, where DPDPE has been shown to be 60-fold and 40-fold less potent, respectively, than (D-Ala²)deltorphin I at δ receptors (9, 22).

The possible involvement of μ receptors has been investigated by testing the response to dermorphin, DAGO, and PL017, three agonists that possess higher affinity for μ than for δ receptors. Both dermorphin and DAGO elicit biphasic concentration-response curves that display high and low affinity components, with the high affinity component accounting for the major portion of the maximal response. The ratio between the apparent EC_{50} values for the low and the high affinity components is 272 and 156 for the dermorphin and DAGO response, respectively. These values agree with the

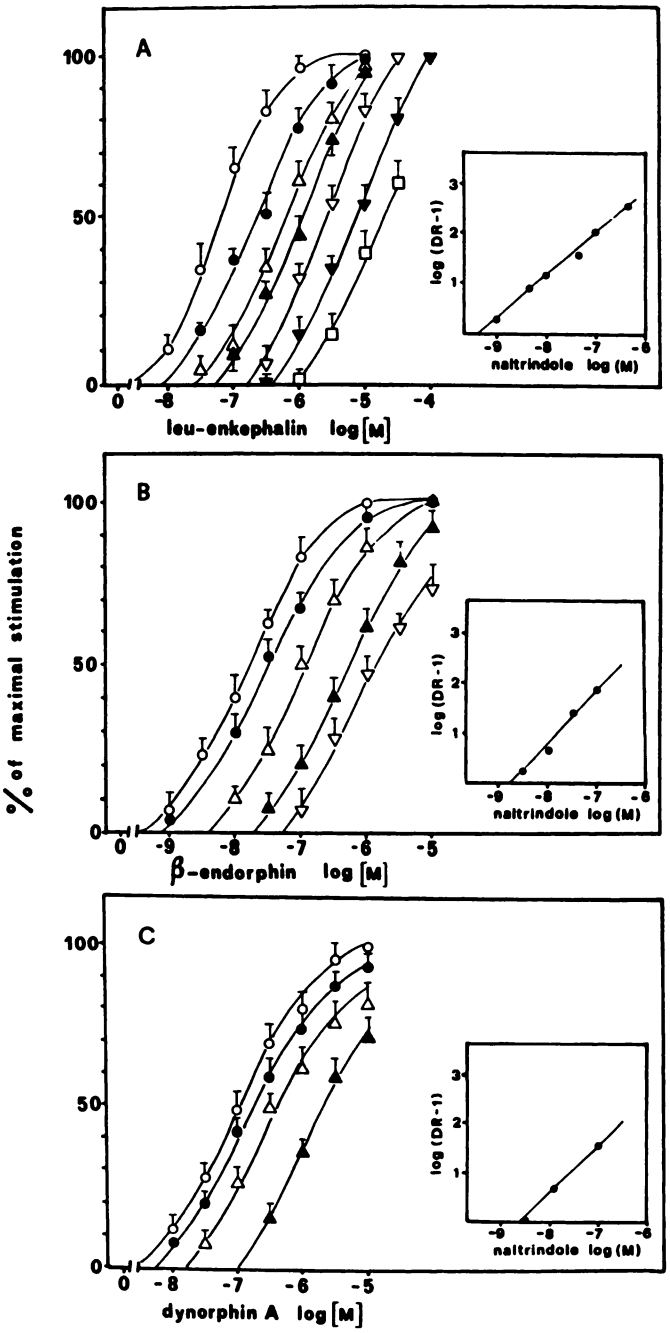

Fig. 2. Antagonism by naltrindole of adenylate cyclase stimulation elicited by Leu-enkephalin (A), β-endorphin (B), and dynorphin A (C). The enzyme activity was assayed with the indicated concentrations of the opioid receptor agonists, in the absence and in the presence of increasing concentrations of naltrindole. A, Vehicle (○) and naltrindole at 1 nM (●), 5 nM (△), 10 nM (▲), 50 nM (▽), 100 nM (▼), and 500 nM (□); B, vehicle (○) and naltrindole at 3 nM (●), 10 nM (△), 30 nM (▲), and 100 nM (▽); C, vehicle (○) and naltrindole at 3 nM (●), 15 nM (△), and 100 nM (▲). Data are the mean ± standard error of three experiments for each agonist. Enzyme activities (expressed as pmol of cAMP/min/mg of protein) (mean ± standard error) were basal, 118.1 ± 3.4; Leu-enkephalin (10 μM), 168.1 ± 4.7; β-endorphin (10 μM), 167.3 ± 5.1; and dynorphin A (10 μM), 169.3 ± 4.4. *Insets*, Schild plots of the naltrindole antagonism. Slope values of the lines were A, 0.87 ± 0.04; B, 1.02 ± 0.03; and C, 0.914 ± 0.06.

TABLE 2

Potencies of naltrindole, naloxone, and nor-binaltorphimine on the stimulation of adenylate cyclase activity by different opioid agonists

Agonist	Naltrindole	Naloxone	Nor-binaltorphimine
Leu-Enkephalin	9.20 ± 0.03[a]	7.70 ± 0.08[b]	7.25 ± 0.02[a]
β-Endorphin	8.82 ± 0.01[a]	8.00 ± 0.07[b]	7.57 ± 0.04[c]
Dynorphin A	8.56 ± 0.04[a]	8.10 ± 0.10[b]	7.39 ± 0.05[c]
(D-Ala²)Deltorphin I	8.93 ± 0.01[a]	7.59 ± 0.02[a]	
DSLET	8.88 ± 0.02[a]	7.59 ± 0.03[a]	
DPDPE	9.39 ± 0.04[d]	7.79 ± 0.06[d]	
PL017	7.90 ± 0.06[d]	8.64 ± 0.02[a]	
DAGO	7.79 ± 0.05[d] (89.9%)[e]	8.67 ± 0.03[d] (87.4%)[e]	
Dermorphin	8.12 ± 0.04[d] (90.8%)[e]		

[a] pA_2 value.
[b] pA_2 value taken from Ref. 3.
[c] pK_i value calculated according to eq. 1 described in Materials and Methods.
[d] pK_i value calculated according to eq. 2 described in Materials and Methods.
[e] Percentage of the response antagonized with the indicated potency.

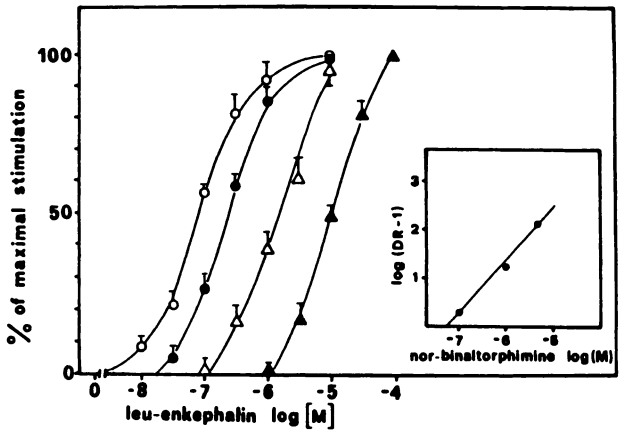

Fig. 3. Antagonism of Leu-enkephalin stimulation of adenylate cyclase activity in rat olfactory bulb by nor-binaltorphimine. The enzyme activity was assayed with the indicated concentrations of Leu-enkephalin, in the absence (○) and in the presence of 0.1 μM (●), 1.0 μM (△), and 5.0 μM (▲) nor-binaltorphimine. Data are the mean ± standard error of three experiments. Enzyme activities (expressed as pmol of cAMP/min/mg of protein) (mean ± standard error) were basal, 110.7 ± 6.7; and Leu-enkephalin (10 μM), 155.4 ± 5.7. *Inset*, Schild plot of nor-binaltorphimine antagonism. The slope value of the line was 1.07 ± 0.06.

selectivity ratios for μ versus δ sites for the agonists, as observed in radioligand binding studies (237 and 149 for dermorphin and DAGO, respectively) (23). The EC_{50} value of the DAGO high affinity component (64 nM) is close to the reported potency of this agonist in inhibiting adenylate cyclase activity via μ receptors in rat striatum (20 nM) (24) and in rabbit cerebellum (about 30 nM) (25). Thus, it is likely that the major, high affinity, component of the dermorphin and DAGO responses is mediated by activation of μ receptors, whereas the minor, low affinity, component is due to stimulation of δ receptors. Moreover, PL017 gives rise to a monophasic concentration-response curve, with a maximal effect corresponding approximately to the plateau of the high affinity components of dermorphin and DAGO responses. PL017 has been shown to possess high selectivity for binding to μ receptors and virtually no affinity for δ receptors (14). Therefore, the stimulatory effect of PL017 on adenylate cyclase activity may be exclusively mediated by occupancy of μ receptors. The 5-fold difference in the potencies of PL017 and DAGO compared favorably with the 3-fold difference in the affinities of these two agonists for μ sites of rat brain membranes (26). Taken together, the data obtained with the μ-preferring agonists strongly indicate that μ receptors can stimulate adenylate cyclase activity in rat olfactory bulb.

The finding that U-50,488 and U-69,593, two highly selective agonists of κ-opioid receptors (15, 16), are ineffective in stimulating adenylate cyclase activity of rat olfactory bulb argues against the possible involvement of κ receptors. Also, the results obtained with the various dynorphin peptides are not consistent with the occurrence of a κ response. In fact, dynorphin A, dynorphin A-1–13, and dynorphin A 1–6, which possess much higher affinity for κ receptors than does Leu-enkephalin (18), are less potent than Leu-enkephalin in stimulating the enzyme activity. Moreover, (D-Ala²) dynorphin A-1–11, a stable dynorphin analogue that has been found to be 10-fold less potent than dynorphin 1–13 in inhibiting muscle contraction in guinea pig ileum (a κ-μ response) (27), is 3-fold more potent than dynorphin A 1–13 in increasing cAMP formation in the olfactory bulb. It is noteworthy that studies on the structure-activity relationship have demonstrated that the D-alanine substitution in position 2 lowers the affinity of the long dynorphin sequences for κ receptors and increases that for δ and μ receptors (27, 28). Moreover, radioligand binding data (18) have shown that dynorphin A and dynorphin A-1–13, compared with Leu-enkephalin, possess higher affinity for μ and lower and equal affinity, respectively, for δ sites, whereas dynorphin A-1–6 shows equal affinity for μ but lower affinity for δ sites. Therefore, the rank order of potency of dynorphin peptides in stimulating adenylate cyclase activity of the olfactory bulb better correlates with their relative affinities for the δ receptor, suggesting that their action is predominantly mediated via interaction with this receptor subtype.

The antagonist profile of the opioid stimulation of adenylate cyclase activity of the olfactory bulb further characterizes the pharmacological nature of the receptor subtypes involved. The stimulatory effects of Leu-enkephalin, β-endorphin, and dynorphin A are most potently blocked by the δ-selective antagonist naltrindole, which displays pA_2 values that are in the low nanomolar range and close to its affinity for δ receptors (19, 29). In agreement with the lack of activity of the κ-selective synthetic agonists, the κ antagonist nor-binaltorphimine is the least effective in antagonizing the responses to Leu-enkephalin, β-endorphin, and dynorphin A, displaying a potency equal to

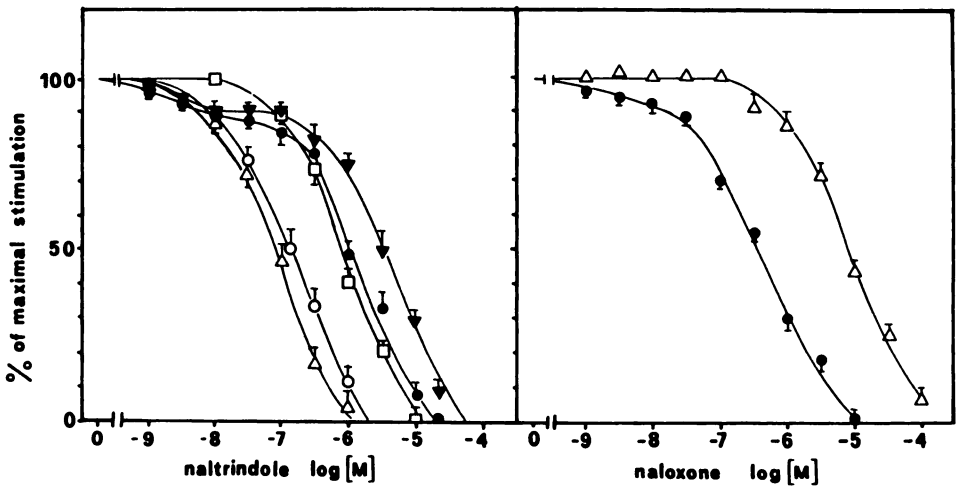

Fig. 4. Concentration-dependent inhibition by naltrindole and naloxone of the stimulation of adenylate cyclase activity by different opioid receptor agonists. A, The enzyme activity was assayed at the indicated concentrations of naltrindole, in the presence of 10 μM Leu-enkephalin (○), 120 μM DPDPE (△), 10 μM DAGO (●), 10 μM dermorphin (▼), or 30 μM PL017 (□). B, The enzyme activity was assayed at the indicated concentrations of naloxone, in the presence of 10 μM DAGO (●) or 3 μM (D-Ala²)deltorphin I (△). For each agonist, data are expressed as percentage of the enzyme stimulation elicited in the absence of antagonists and represent the mean ± standard error of three experiments. Enzyme activities (expressed as pmol of cAMP/min/mg of protein) (mean ± standard error) were basal, 118.2 + 4.0; Leu-enkephalin, 164.5 ± 3.9; DPDPE, 167.1 ± 5.3; DAGO, 156.1 ± 2.5; dermorphin, 157.3 ± 3.1; PL017, 146.8 ± 3.1; and (D-Ala²)deltorphin I, 160.2 ± 3.1. Within the range of concentrations tested, naltrindole or naloxone only failed to affect basal enzyme activity.

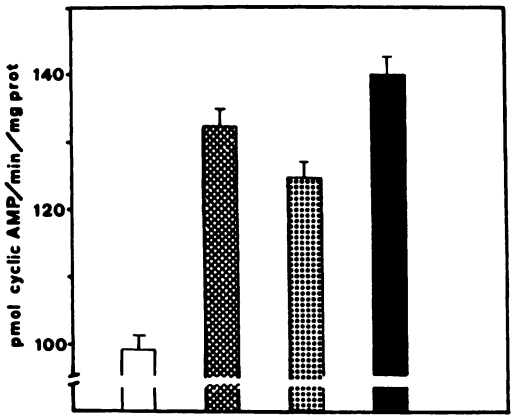

Fig. 5. Effect of combined addition of (D-Ala²)deltorphin I and PL017 on adenylate cyclase activity of rat olfactory bulb. Enzyme activity was assayed in the absence (□) and in the presence of 3 μM (D-Ala²)deltorphin I (■), 100 μM PL017 (□), or (D-Ala²)deltorphin I plus PL017 (■). Data are the mean ± standard error of three experiments.

its affinity for δ and μ sites (20). Naltrindole is 20–40-fold more potent than naloxone in blocking the effects of (D-Ala²)deltorphin I, DPDPE, and DSLET, whereas naloxone is more potent than naltrindole in antagonizing the stimulation of PL017, thus demonstrating the receptor selectivity of the responses elicited by these agonists. Moreover, naltrindole is 35-, 6.6-, and 2.9-fold more potent than naloxone in antagonizing the stimulatory effect of Leu-enkephalin, β-endorphin, and dynorphin A, respectively. Thus, Leu-enkephalin, like δ-selective agonists, shows high and low sensitivity to naltrindole and naloxone, respectively, indicating that its stimulatory effect is mostly, if not exclusively, mediated by activation of δ receptors. On the other hand, the higher naloxone sensitivity displayed by β-endorphin and dynorphin A may be explained by assuming that their action, although predominantly mediated by δ receptors, also involves interaction with a significant portion of μ receptors.

Naltrindole displays monophasic curves in antagonizing the stimulatory effect of Leu-enkephalin, DPDPE, and PL017, indicating that each of these agonists activates a homogeneous receptor population. This finding agrees with the observation that the concentration-response curves of Leu-enkephalin, DPDPE, and PL017 show slopes values close to unity. On the other hand, the biphasic profile of the curves produced by naltrindole and naloxone in counteracting the stimulations elicited by sufficiently high concentrations of DAGO and dermorphin further supports the idea that these agonists may activate two pharmacologically distinct opioid receptor subtypes. About 90% of the enzyme stimulation elicited by either 10 μM DAGO or 10 μM dermorphin, a fraction mostly represented by the high affinity component of their concentration-response curves, is antagonized by naltrindole with potencies (pK_i = 7.79–8.12) lower than its affinity for δ receptors. A similar percentage of the DAGO response is blocked by naloxone, with a potency (pK_i = 8.67) close to its affinity for μ receptors (21). Thus, the major portion of the stimulatory responses elicited by DAGO and dermorphin appears to be due to the activation of μ receptors. About 10–15% of the responses elicited by 10 μM DAGO and 10 μM dermorphin is antagonized by low concentrations of either naltrindole or naloxone. This portion likely corresponds to the low affinity component of the agonist concentration-response curves and may be mediated by activation of δ receptors. A more detailed characterization of the pharmacological nature of this minor component was hampered by the smallness of the response and the need to use high agonist concentrations.

The maximal stimulation of adenylate cyclase produced by the μ-selective agonist PL017 is significantly lower than that produced by the δ-selective agonists DPDPE and (D-Ala²)deltorphin I. This finding correlates with the observation

that δ sites are more concentrated than μ sites in the rat olfactory bulb, although both binding sites appear to be localized in the same anatomical structures within the bulb (4). The combined addition of PL017 and (D-Ala²)deltorphin I causes a less than additive effect, suggesting that a large portion of δ and μ receptors control a common pool of adenylate cyclase. On the basis of both functional and radioligand binding studies, it has been proposed that rat brain, in addition to the distinct opioid receptor subtypes, contains a δ/μ opioid receptor complex, where δ and μ sites interact allosterically (30, 31). In rat striatum, evidence has been provided that physically associated μ and δ receptors mediate opioid inhibition of dopamine D1 receptor stimulation of adenylate cyclase activity (32). Further studies are required to assess whether, in the olfactory bulb, δ and μ receptors control adenylate cyclase activity individually or a δ/μ receptor complex is involved.

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