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Interaction of Enkephalin with Opiate Receptors in Intact Cultured Cells

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SUMMARY

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The interaction of enkephalins with opiate receptors in intact neuroblastoma cells (N4TG1) was studied by the use of a derivative of high specific activity (^{125}I-labeled) of the metabolically stable enkephalin analogue, (D-Ala^2, D-Leu^5)-enkephalin. The binding is specifically inhibited by low concentrations of the natural (Leu^5)- and (Met^5)-enkephalins and by β-endorphin, but not by β-lipotropin. The binding data clearly show a single class of homogeneous binding sites without evidence for cooperative interactions at any ligand concentration. The association and dissociation rate constants of binding to cells are 3×10^7 M^{-1}min^{-1} and 0.035 min^{-1}, respectively, at 24°. In cells, the binding of [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin is saturated when 30 fmoles of the peptide are bound per 10^6 cells. The K_d is 1 to 2 nM. No evidence for receptor or ligand-receptor complex internalization could be demonstrated on incubating the cells at 37°. The receptors in intact cells are very resistant to digestion by proteases and phospholipase A, in sharp contrast to the results observed in membrane preparations which show exquisite sensitivity to these enzymes. This resistance is apparently not due to the intracellular localization of a pool of receptors since cell homogenates have the same number of receptor sites as intact cells. In cells, the affinity of the receptor for enkephalin is very sensitive to the cationic composition of the medium. Removal of the divalent cations, Mg^{2+} and Ca^{2+}, in the presence of Na^+ reduces the affinity by about 3.5-fold. Narcotic agonists and antagonists are more potent in inhibiting [^{3}H]naloxone binding than [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin binding. The opposite is true for enkephalins, although etorphine and β-endorphin compete with both labeled ligands equally well. These results suggest that opiates and enkephalins bind differently to the same receptor or bind to different receptors with overlapping specificity.

INTRODUCTION

Tyr-Gly-Gly-Phe-Leu ([Leu^5]-enkephalin) and Tyr-Gly-Gly-Phe-Met ([Met^5]-enkephalin) are two recently isolated peptides exhibiting opiate-like properties (1, 2). (Met^5)-enkephalin represents the sequence 61-65 amino acids of β-lipotropin (3). Several fragments of β-lipotropin, which are longer than 61-65 and represent various portions of the C-terminal fragment of β-lipotropin, also have potent opiate agonist activity (4, 5). The 31 amino acid sequence residues 61-91, known as β-endorphin, has particularly potent analgesic activity (6). It is possible that β-lipotropin may act as a precursor for (Met^5)-enkephalin and other larger opiate-like peptides (7). Hypophysectomy does not change the brain (Leu^5)- and (Met^5)-enkephalin levels, indicating that brain (Leu^5)- and (Met^5)-enkephalins cannot be derived from pituitary β-lipotropin and β-endorphin (8-10).

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Both (Met^5)- and (Leu^5)-enkephalin are rapidly metabolized by serum, tissue homogenates, or tissue membrane preparations (11–15). Both (Met^5)- and (Leu^5)-enkephalin exhibit opiate receptor binding activity with affinity comparable to that of morphine. Hundreds of enkephalin derivatives and analogues have been synthesized, and their metabolic stability and opiate-like activities have been examined for opiate receptor-binding activity, inhibition of mouse vas deferens and guinea pig ileum contraction, analgesic and antidiarrheal activities (16–18). Analogues with a D-Ala substituted at position 2, and those with a D-Ala and D-Leu substituted at positions 2 and 5, have been found to be extremely stable and yield more potent analogues.

^{3}H-labeled (Leu^5)-enkephalins have been employed recently to study enkephalin-receptor interactions in brain membrane preparations (19–22). ^{125}I-labeled derivatives of high specific activity of the metabolically stable enkephalin analogues, (D-Ala^2, D-Leu^5)- and (D-Ala^2, L-Leu^5)-enkephalin, have been prepared and shown to bind to opiate receptors (22). Such derivatives show biological activity and stereospecific binding to opiate receptors in brain homogenates and some neuroblastoma cells. The availability of enkephalins of such high specific radioactivity has facilitated the study of enkephalin-receptor interactions. Neuroblastoma cells bearing large numbers of opiate receptors provide a convenient model for the study of opiate receptors. This report presents detailed characterization and some unusual properties of opiate receptors in intact cells.

MATERIALS AND METHODS

Peptides were synthesized by Dr. Sam Wilkinson, Wellcome Research Laboratories, Beckenham, England (17, 18). All peptides were synthesized by the conventional solution method, using dicyclohexylcarbodiimide coupling, or by the repetitive anhydride method described by Tilak (23) and by Beyerman (24).

[^{125}I] (D-Ala^2, D-Leu^5)-enkephalin was prepared essentially as described previously (22). Briefly, enkephalin (1.5 μg) and carrier-free [^{125}I]Na (5 mCi) (Union Carbide) were added to 0.1 ml of 0.25 M sodium phosphate buffer, pH 7.5. Twenty microliters of chloramine T (0.5 mg/ml) were added, and after 20 sec, 20 μl of sodium metabisulfite (1 mg/ml) were added to stop the reaction. Monoiodinated enkephalin was purified by Bio-Gel P2 and DEAE-Sephadex chromatography (22). The specific activity of [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin is about 2 Ci per μmol. [^{3}H]naloxone (23.6 Ci/mmol) was purchased from New England Nuclear Corporation.

Neuroblastoma cells (N4TG1) obtained from Dr. Gilman, University of Virginia, Charlottesville, Virginia, were grown in monolayer with 10% fetal calf serum and DulBecco's modified minimum essential medium and scraped off and centrifuged at low speed. The cells were washed three times with KRP[1] buffer, pH 7.4, which contained sodium chloride (128 mM), calcium chloride (1.4 mM), magnesium chloride (1.4 mM), potassium chloride (5 mM) and sodium phosphate (5 mM). The cells were finally suspended in KRP at a cell concentration of about 5×10^6 cells per ml. Cell homogenates were prepared by homogenization with a Polytron PT-20 homogenizer in 50 mM Tris-HCl, pH 7.7, and the crude membrane preparations were then obtained by centrifugation at 40,000 $\times$ g for 30 min. Crude brain membranes were prepared from male Sprague-Dawley rat (150–200 g) as described previously (22).

Binding assays were essentially the same as described previously (10, 22). Briefly, 0.2 ml or 2 ml of cell suspension or membrane preparation were incubated with ^{125}I-labeled enkephalin or [^{3}H]naloxone and test substance at 24° for 1 hr. Two milliliters of ice-cold 50 mM Tris-HCl (pH 7.8) were added and the sample was filtered through Whatman filter paper GF/C under vacuum and quickly washed twice with 10 ml of cold buffer. The radioactivity on the filter was determined. Nonspecific binding is determined in the presence of a large excess (1 μM) of native enkephalin.

RESULTS

Kinetic studies. Previously we have demonstrated that monoiodinated (D-Ala^2,

[1] Abbreviations: KRP, Krebs-Ringer phosphate;

D-Leu^5)-enkephalin binds to opiate receptors in brain membrane preparations and neuroblastoma cells (N4TG1) and the neuroblastoma-glioma hybrid NG108-15 (22). The binding is specifically inhibited by low concentrations of levorphanol but not by dextrorphan. Natural (Met^5)- and (Leu^5)-enkephalins, β-endorphin, but not β-lipotropin, compete the binding of [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin to cultured cells and brain membranes. The binding is reduced by Na^+ and increased by $MnCl_2$ and $MgCl_2$ (22). These results confirm that [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin indeed binds to opiate receptors.

The time course of binding of [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin to cells was determined (Fig. 1). Binding in the presence of excess (D-Ala^2, D-Leu^5)-enkephalin (i.e., nonspecific binding) was complete within 1 min at 24° and was unchanged during a further 90 min period of incubation. In the absence of native enkephalin, binding required about 60 min to reach equilibrium (Fig. 1). The rate of specific binding was temperature dependent (Fig. 2). The initial

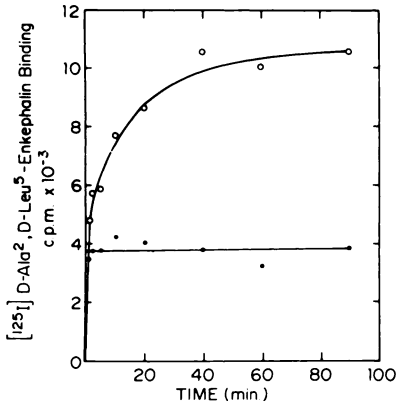

FIG. 1. *Rate of association of [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin to receptor sites.*

Four milliliters of cell suspensions (4×10^6 cells/ml) were incubated with [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin (0.5 nM) in the presence (●) and absence (○) of 1 μM native (D-Ala^2, D-Leu^5)-enkephalin at 24°. The reactions were terminated at various times by transferring 200 μl of the cell suspension to 2 ml of cold buffer and immediately filtering through GF/C filters under vacuum and washing twice with 10 ml of ice-cold buffer. The half-time of association under such conditions is 8 min. The calculated rate constant of the bimolecular reaction is 3×10^7 M^{-1}min^{-1}.

binding rate seems slower at 24° than at 37° but the steady state of binding was higher at 24° than at 37°. Presumably, at 37° degradation of the peptide occurred during long periods of incubation. It was also possible that receptor transition was achieved at 37°. Those problems are currently under investigation. The binding at 4° was slower and did not reach equilibrium during 90 min of observation.

The rate of association of [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin (0.5 nM) to cells at 24°, using a receptor site concentration of 0.12 nM, is very rapid, showing a half-time (T½) to reach equilibrium of 8 min. The calculated rate constant (k_1), according the second-order equation (25)

$$k_1 = \frac{2.303}{T½\,(H - R)} \cdot log \frac{R(H - RH)}{H(R - RH)}$$

is 3×10^7 M^{-1}min^{-1}, where R, H, and RH are the concentrations of the receptor, labeled ligand, and bound labeled ligand, respectively.

In order to determine the rate of dissociation of the specifically bound [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin, binding was first allowed to take place for 60 min at 24°. The cells were then pelleted and washed twice with KRP. Dissociation was then initiated by the addition of KRP containing 5 μM (D-Ala^2, D-Leu^5)-enkephalin. The decrease in the amount of bound [^{125}I]-enkephalin was followed over the next 2 hr. The dissociation of cell-bound enkephalin was temperature dependent (Fig. 3). At 0° about 20% dissociation was found over a 2 hr period; at 37° over 95% of the bound enkephalin was dissociated within 1 hr. Similar results were observed when the cells were first labeled with [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin at 37° for 15 min. The half-time of dissociation at 24° was 18 min. The binding was reversible with a dissociation rate constant (k_{-1}) of 0.035 min^{-1}. The dissociation constant, $K_d = \frac{k_{-1}}{k_1}$, calculated from the rate constants, was 1.3 nM. This value is very close to the independently derived equilibrium constant. The rate of dissociation measured by adding a large excess of (D-Ala^2, D-Leu^5)-enkephalin is not significantly different from that ob-

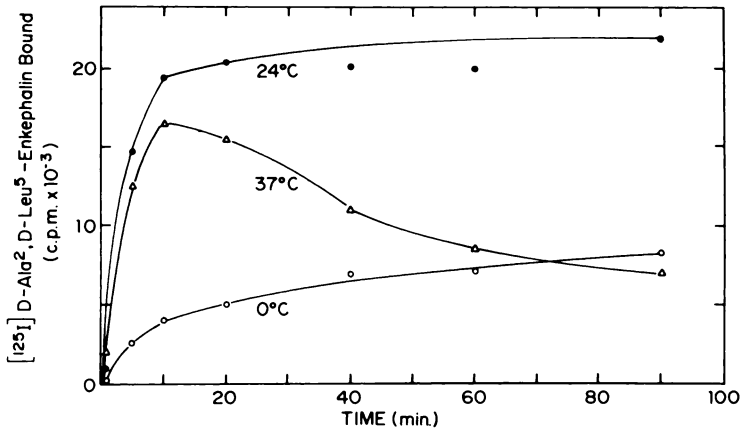

FIG. 2. *Effect of temperature on the rate of association of enkephalin binding.*

Four milliliters of cell suspensions (3.5 × 10^6 cells/ml) were incubated with 1 nM [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin at 0° (○), 24° (●), and 37° (△). The nonspecific binding was determined in the presence of 1 μM native (D-Ala^2, D-Leu^5)-enkephalin. At various times, 200 μl of the incubation were transferred to 2 ml ice-cold buffer and filtered and washed through GF/C filters.

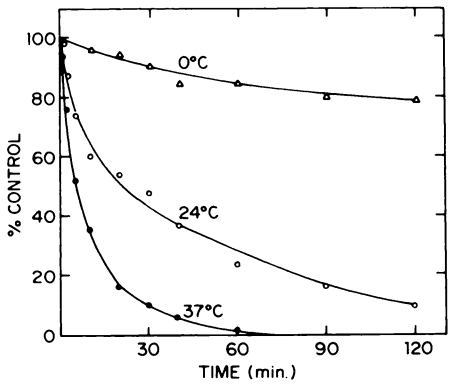

FIG. 3. *Rate of dissociation of the enkephalin-receptor complex and the effect of temperature*

Ten milliliters of cell suspensions (1.2 × 10^6 cells/ml) were labeled with [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin (1.6 nM) at 24° for 1 hr. The labeled cells were then washed two times with ice-cold buffer to remove unbound enkephalin and then divided equally into three parts. The dissociation of enkephalin-receptor complexes was then determined at 0° (△), 24° (○) and 37° (●) in the presence of 5 μM (D-Ala^2, D-Leu^5)-enkephalin. The dissociation was terminated by transferring and washing 200 μl of cells as described for Fig. 1. The rate constant of dissociation at 24° is calculated to be 0.035 min^{-1}.

tained by a large dilution. This result indicates a lack of negative cooperativity of enkephalin-opiate receptor interactions.

Equilibrium binding and number of binding sites. Figure 4 shows the saturation curves of binding of [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin to neuroblastoma cells and membrane preparations. The binding was readily saturable and followed a simple Langmuir absorption isotherm. Maximal binding was reached at 5 nM concentration and was the same for both cell and membrane preparations. The number of binding sites for enkephalin was about 18,000 sites per cell, as calculated from Scatchard's analysis (Fig. 4 insert). However, the affinity (0.8 nM) of enkephalin in cell membranes was higher than that (2 nM) in intact cells. The former value was very close to the affinity estimated from brain membrane preparations. The reason for the differences in affinity between cells and membranes was not clear, although it may be due to differences in the ionic composition of the medium. As will be shown below, the affinity of the opiate receptors of intact cells was dependent upon the cationic composition of medium.

It is notable that the Scatchard's plots (Fig. 4 insert) are linear, suggesting a single or homogeneous binding site without cooperative interactions, in both intact cells and membrane preparations. The Hill coefficient (n) is close to 1 (Fig. 5), further supporting this contention.

A second binding site could not be detected using ligand concentrations as high as 20 nM. Recently, Blume *et al.* (21) also

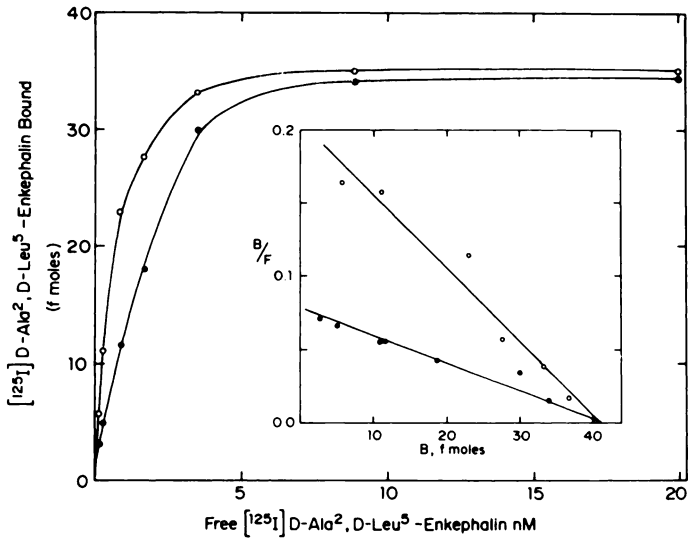

FIG. 4. *Saturation curves and Scatchard plots of [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin binding to intact cells (●) and cell membranes (○).*

The cell concentration was 5 × 10^6 cells/ml. An equal number of cells was homogenized in cold 5 mM Tris-HCl buffer. The membrane was pelleted by centrifugation at 40,000 cpm for 30 min and the pellet was resuspended in 50 mM Tris buffer. The cells and membranes were then incubated with various concentrations of [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin for 60 min at 24°. Specific binding was determined as described in Fig. 1.

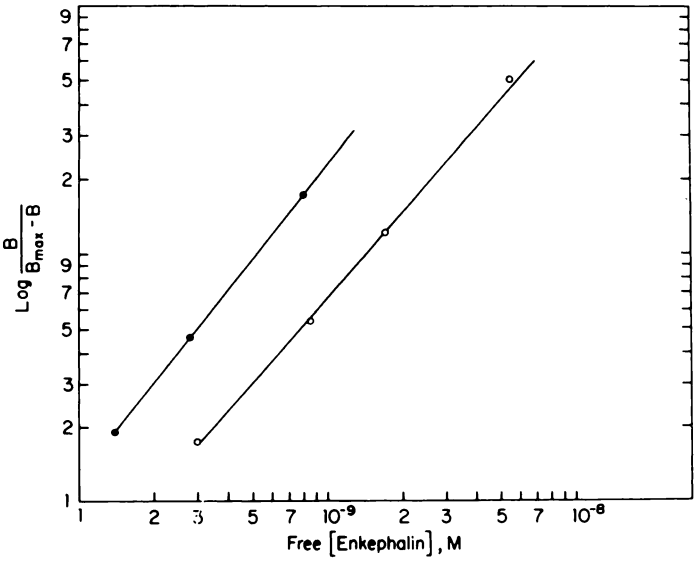

FIG. 5. *Hill plots of the enkephalin-receptor interaction.*

The data is from Fig. 4. Note that the Hill coefficient, *n*, is close to 1 for both cells and membranes. Intact cells (○), cell membrane (●).

reported that the neuroblastoma-glioma hybrid cell line NG108-15 binds [^{3}H] (Leu^5)-enkephalin, and Scatchard's analysis and Hill's plots of the specific binding demonstrated the existence of only one class of noncooperative sites. These results are different from those described previously in brain membranes using ^{3}H-labeled nalox-

TABLE 1

Comparison of the potency of narcotic agonists, antagonists, endorphin, and enkephalin in competing with the binding of [^{3}H]naloxone (1.5 nM) and [^{125}I](D-Ala^2, D-Leu)-enkephalin (1 nM) to brain membrane and neuroblastoma cells at 24°

The potencies are expressed as the concentration which causes 50% inhibition of binding (IC_{50}).

Drug	IC_{50} (M)		
	[^{3}H]Naloxone binding	[^{125}I] (D-Ala^2, Leu^5)-enkephalin binding	[^{125}I] (D-Ala^2, D-Leu^5)-enkephalin binding
	Rat brain	Rat brain	N4TG1 cells
Naloxone	1×10^{-9}	4×10^{-9}	9×10^{-9}
Nalorphine	3×10^{-9}	7×10^{-9}	4×10^{-8}
Levorphanol	1.5×10^{-9}	3.6×10^{-9}	1.4×10^{-8}
Etorphine	8×10^{-10}	5×10^{-10}	8×10^{-10}
Morphine	3×10^{-9}	4×10^{-8}	7×10^{-8}
Levallorphan	2×10^{-9}	9×10^{-9}	6×10^{-9}
Pentazocine	9×10^{-9}	2×10^{-8}	1×10^{-7}
Phenazocine	4×10^{-9}	7×10^{-9}	8×10^{-9}
Phentanyl	1×10^{-9}	5×10^{-8}	4×10^{-7}
Pethidine	1×10^{-6}	2×10^{-5}	9×10^{-5}
Dextrorphan	3×10^{-5}	$>10^{-6}$	7×10^{-5}
Methadone	1×10^{-8}	8×10^{-8}	9×10^{-8}
Etonitazene	8×10^{-9}	5×10^{-7}	2×10^{-6}
Codeine	7×10^{-5}	8.5×10^{-6}	5×10^{-5}
(Met^5)-enk[a]	2.0×10^{-8}	6×10^{-9}	9.8×10^{-10}
(Leu^5)-enk	1.1×10^{-8}	9.6×10^{-9}	2.2×10^{-9}
(D-Ala^2, D-Leu^5)-enk	2.6×10^{-9}	1×10^{-9}	7×10^{-10}
(D-Ala^2, L-Leu^5)-enk	3.2×10^{-9}	9×10^{-10}	1×10^{-9}
β-endorphin	8×10^{-10}	2×10^{-9}	8×10^{-10}
β-lipotropin	$>10^{-6}$	$>10^{-6}$	$>10^{-6}$

[a] Enkephalin.

one, dihydromorphine and (Met^5)- and (Leu^5)-enkephalins (26, 27). Thus, it may be that the second site seen in brain membranes is not in fact related to the physiological opiate receptor. Alternatively, neuroblastoma cells may lack some additional opiate receptor function that exists in the central nervous system or myenteric plexus.

Inhibition of [^{125}I]enkephalin binding by opiate agonists, antagonists, and endogenous morphine-like peptides. Lord *et al.* (26) and Simantov and Snyder (27) have reported that when the potency of a series of drugs in competing [^{3}H]naloxone binding is compared to their potency in displacing [^{3}H]enkephalin binding in brain membranes, the potency of the drugs in the two series varies considerably, although certain fundamental features such as stereospecificity are still maintained. Most narcotics are less effective in competing enkephalin binding than narcotic binding (Table 1) in rat brain membrane. In contrast, enkephalins are in general more effective in competing enkephalin binding than naloxone binding. In cells, the potencies of narcotics and enkephalins in inhibiting the binding of [^{125}I](D-Ala^2, D-Leu^5)-enkephalin are similar to brain membrane (Table 1). However, the potency of naloxone and enkephalin in inhibiting the binding of [^{3}H]naloxone is not different significantly from that of [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin in cells.[2] This further suggests that neuroblastoma cells may lack some opiate receptor site that exists in the rat brain membrane. There are some notable exceptions. Thus, β-endorphin and etorphine are equally effective against both types of labeled ligands. Such effects were also observed in the present studies (Table 1). Beta-endorphin and etorphine compete with the binding of [^{125}I]

[2] Chang, K.-J., & Cuatrecasas, P., manuscript submitted.

(D-Ala^2, D-Leu^5)-enkephalin from N4TG1 cells as well as they compete with the binding of [^{3}H]naloxone and [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin in brain membrane preparation (Table 1).

Cationic effect on [^{125}I]enkephalin binding. In Tris-HCl buffer, Na^+ decreases the binding of narcotic agonists and enkephalins and increases the binding of antagonists in brain membranes (28) and cells (22). Since KRP was used in this study, the effect of cations was examined. Table 2 shows that removal of Mg^{2+} from the medium did not affect the binding significantly. Removal Mg^{2+} plus the addition of 1 mM EDTA increased the binding by 50%. Removal of Ca^{2+} alone significantly reduced the binding, and this reduction was potentiated by 1 mM EDTA. Removal of both Mg^{2+} and Ca^{2+} reduced the binding to 36% of the control, and the subsequent addition of 1 mM EDTA reduced the binding even further. The effect of removing cations is to change the affinity of enkephalin binding while the maximal binding is not affected (Fig. 6). The effect of removal of cations is reversible. A normal binding is found after the cells are washed and resuspended in normal medium.

Effect of enzymatic digestions. Binding of [^{3}H]naloxone and [^{3}H]dihydromorphine to brain membranes is very sensitive to proteolytic digestion by trypsin and chymotrypsin, and to treatment with phospholipase A, but far less sensitive to other phospholipases or to neuraminidase (29). The binding of [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin to brain membrane preparations is also sensitive to trypsin, chymotrypsin, and phospholipase A (Figs. 7, 8). However, in intact cells the binding is maintained in spite of the action of these enzymes. However, once the cells are subjected to homogenization the binding becomes very sensitive to enzymatic digestion.

DISCUSSION

Due to their complexity and heterogeneous cell origin, brain membranes are not ideal systems for elucidating the receptor nature or mechanism of action of opiates. Cell culture systems are simpler working models, as demonstrated by Klee and Nirenberg (30) and Brandt *et al.* (31), who used neuroblastoma-glioma hybrid cell lines to study the action of opiates at the molecular level. These cells have a high density of opiate receptors (22).

The high specific activity of [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin has allowed detailed studies of opiate receptors in intact cells under physiological conditions (KRP buffer). The affinity for enkephalin in intact cells in KRP medium is very similar to that of brain membrane preparations in Tris-HCl buffer. Pert and Snyder (28) have

TABLE 2

Effect of removing cations on the binding of [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin to neuroblastoma (N4TG1) cells

N4TG1 cells (3×10^6 cells/ml), suspended in media lacking various cations, or containing EDTA (1 mM), were incubated with [^{125}I] (D-Ala,^{2} D-Leu^5)-enkephalin at 24° for 60 min. Some of cells were returned to normal medium after exposure to the medium lacking cation. Specific binding was then determined as described in the text.

Condition	Binding of ^{125}I-labeled enkephalin		
	cpm[a]	% of control	After return to normal medium (% of control)
Control KRP[b]	2545		100
$-Mg^{2+}$	2610	105	84
$-Mg^{2+}$ + 1 mM EDTA	3840	150	101
$-Ca^{2+}$	2080	81	93
$-Ca^{2+}$ + 1 mM EDTA	1636	64	102
$-Mg^{2+}$ and Ca^{2+}	920	36	93
$-Mg^{2+}$ and Ca^{2+} + 1 mM EDTA	660	27	101

[a] Counts per min.

[b] Krebs-Ringer phosphate buffer.

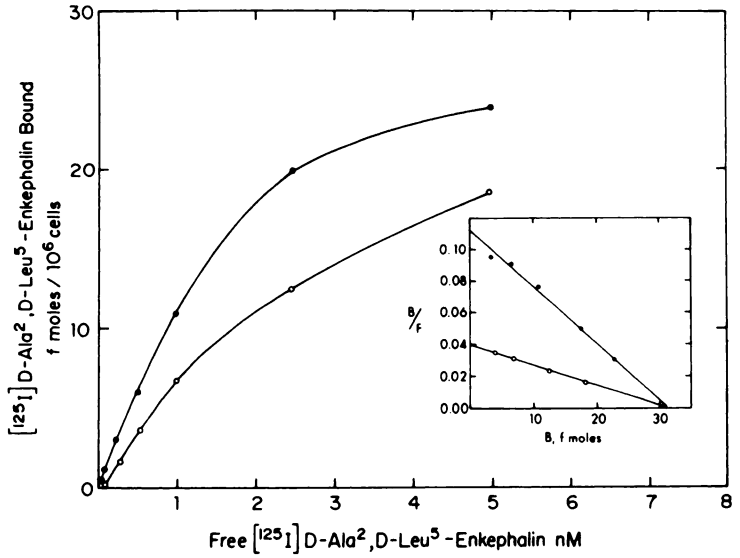

FIG. 6. *Effect of the removing divalent cations on the affinity of enkephalin to cells.*

The binding of enkephalin was determined in normal Kreb-Ringer phosphate buffer (●) and in the KRP medium lacking Ca^{2+} and Mg^{2+} (○). The cell concentration was 4×10^6 cells/ml. Note the difference in affinity as shown in the insert Scatchard plots. The maximum binding is not significantly different.

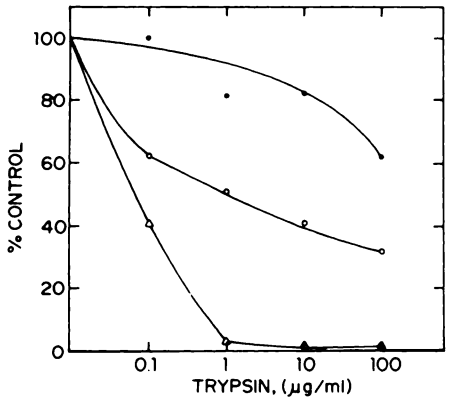

FIG. 7. *Differential effect of trypsin treatment on intact cells* (●), *cell membranes (N4TG1)* (○), *and brain membrane preparations* (△).

The intact cells (N4TG1), cell membranes, and brain membranes were incubated with trypsin at 24° for 30 min. The reaction was terminated by adding soybean trypsin inhibitor (5 mg/ml). Binding activities were determined by incubating with [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin for 60 min at 24° in the presence and absence of native (D-Ala^2, D-Leu^5)-enkephalin.

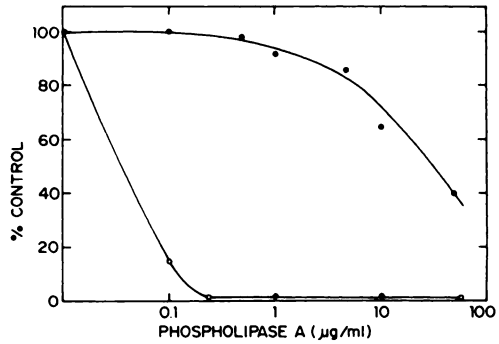

FIG. 8. *Differential effect of phospholipase A on intact cells* (●) *and brain membrane preparations* (○).

The conditions were the same as in Fig. 7.

shown that the binding activities of brain homogenate to agonists and antagonists are differentially affected by Na^+ and Mg^{2+} ions. Sodium increases the binding of antagonists and decreases that of agonists while the opposite is true for divalent ions, such as Mn^{2+} and Mg^{2+}. Similarly, in intact cells the binding of enkephalin is altered by changes in the cationic composition of the medium. Removal of divalent cations in the presence of Na^+ decreases the binding of enkephalin. This effect is more profound in the presence of EDTA, indicating that tightly bound divalent ions may be required to retain the high affinity state of the binding sites. This reduction of the binding by removing divalent cations in the presence of Na^+ may be due to the antagonistic effect

between monovalent and divalent cations (32).

The ability of monoiodinated (D-Ala^2, D-Leu^5)-enkephalin but failure of diiodinated (D-Ala^2, D-Leu^5)-enkephalin to maintain affinity for opiate receptors (22) suggests that monoiodo atom is directed out of the receptor site without interfering with the interaction of phenolic ring with the binding site. The diiodo-substitution seems to prevent the interaction.

The fact that virtually all (>95%) cell-bound [^{125}I] (D-Ala^2, D-Leu^5)-enkephalin can be dissociated from cells (Fig. 3), even after incubation at 37° for as long as 60 min, suggests that the cell-bound enkephalin and opiate receptors are not internalized upon interaction.

It was surprising to find that the receptor sites of intact cells were extremely resistant to proteolytic digestion and to phospholipase A treatment. In crude brain membrane preparations, the binding is extremely sensitive to digestion by trypsin, chymotrypsin, and phospholipase A (29). Furthermore, membranes obtained from the same cells demonstrated greatly increased susceptibility to digestion (Figs. 7, 8). This apparent inaccessibility to enzymatic treatment of the intact cell could theoretically be explained by an intracellular location of binding sites. This theory cannot be accepted since cell homogenates bind the same amount of enkephalin as intact cells, although there are differences in the affinity (Figs. 4, 5). The receptor may be an integral plasma membrane component and the enzyme digestible portion is located on the internal side of the membrane. It is also conceivable that homogenization disrupts the membrane structure sufficiently to expose the receptor to enzyme treatment. It is also possible that the enzymatic treatments affect primarily some entity in the membrane which functions to "anchor" the receptor in the membrane and thus helps to link it to other membrane structures. Thus, it may be that treatment with proteases could solubilize (or release into the medium) the receptors.

Some interesting differences have been found with respect to the relative ability of various drugs to compete with the binding of radioactive ligands to the opiate receptor. When a series of drugs is examined for competition of [^{3}H]naloxone binding and this is compared to the potency in competing for ^{3}H- or ^{125}I-labeled enkephalin binding, in either cells or brain membranes, it is quite clear that the potencies of drugs in the two series vary a great deal, as does the order of potency, although certain features such as stereospecificity are still seen. In general, most narcotic drugs are less effective in competing with radioactive enkephalin than with naloxone (22, 26, 27, 33). There are some exceptions, however, since etorphine, for example, displaces both types of ligand with the same effectiveness. In contrast, enkephalins are in general more effective in competing with labeled enkephalin than with naloxone. Again, there are exceptions since β-endorphin is equally effective against both types of labeled ligand. Although a clear pattern is not yet apparent, naloxone and the enkephalins are probably binding to slightly different or "overlapping" regions of the receptor. A theoretical model of the opiate receptor has been proposed in which at least three specific ligand binding sites are present (34). It may be, therefore, that etorphine and β-endorphin can cover the regions that are accessible to both naloxone and the enkephalins with equal effectiveness. However, other explanations are also possible. For example, Lord *et al.* (26) prefer to account for anomalous binding data by invoking heterogeneity of opiate receptor sites in the brain membrane. However, the experiments in N4TG1 cells clearly demonstrate the presence of only a single class of enkephalin binding sites. Those possibilities are currently being investigated by us[3] and it seems that at least two opiate receptors exist in rat brain membrane preparation.

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