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INTERACTION OF LEU-ENKEPHALIN WITH ISOLATED ENTEROCYTES FROM GUINEA PIG: BINDING TO SPECIFIC RECEPTORS AND STIMULATION OF cAMP ACCUMULATION

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SUMMARY: The specific binding of Leu-enkephalin and the stimulatory effect of the peptide on cAMP accumulation have been assessed in isolated enterocytes of guinea pig. The binding was reversible as well as time and temperature dependent. Two classes of binding sites could be defined: a class with a relatively high affinity (K_d=0.7 μM) that represented 1% of total binding capacity, and another class with low affinity (K_d=55.5 μM). The stimulation of cAMP accumulation was also shown to depend on time and temperature and was potentiated by a phosphodiesterase inhibitor. Half-maximal stimulation of cAMP accumulation was observed at 119 μM and maximal stimulation (27-fold basal level) at 300 μM Leu-enkephalin. Both steps of the interaction were not modified by Na^+ but exhibited a high specificity since modifications in the structure of Leu-enkephalin resulted in an important loss of binding affinity and stimulatory activity.

Enkephalins, originally isolated from the pituitary (1), have now been demonstrated in the gut, both in nerves and in endocrine cells (2-4). However, studies of opiate actions on intestinal functions have yielded conflicting results. One possibility is that the enkephalins act as neuromodulators influencing intestinal motility (5-7). Another possibility is that the enkephalins may exert a direct effect on the intestinal mucosa after local release by endocrine cells or nerve terminals (7-9).

A prerequisite for the demonstration of peptide action in target cells is the existence of a specific binding site and the induction of some biochemical response due to the coupling peptide-binding site. In this context, studies performed in intact or homogenized neuroblastoma and neuroblastoma x glioma cell clones (10,11) as well as in preparations derived directly from neural tissues (12,13) have demonstrated that enkephalins and other opiates inhibit both basal and stimulated cAMP levels. On the other hand, a stimulatory role of enkephalins upon cAMP has been characterized in non-neural preparations such as adipocyte plasma membranes (14) and islets of Langerhans (15,16).

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The purpose of the present investigation was to study both the existence of specific binding sites for Leu-enkephalin and the effect of the opiate peptide on cAMP in isolated guinea pig enterocytes. This characterization of the first steps of Leu-enkephalin action in isolated enterocytes is of a particular interest in regard to the possible role of this opiate peptide as a neuromediator modulating the physiology of intestinal epithelium.

MATERIALS AND METHODS

Chemicals. Enkephalins, enkephalin analogues and bacitracin were purchased from Sigma (St. Louis, MO); naloxone from Winthrop Laboratories (Surbiton upon Thames, England); 3-isobutyl-1-methylxanthine from Aldrich (Milwaukee, WI); and |^{3}H|Leu-enkephalin (40 Ci/mmol) from the Radiochemical Centre (Amersham,England).

Cell preparation. Enterocytes were isolated as in (17) from the jejuno-ileum of male guinea pigs (350-400 g) fed *ad libitum*. About 90% cells were viable as measured by exclusion of trypan blue dye. Protein was estimated by the method of Lowry *et al.* (18).

Binding studies. The binding assay was conducted under standard conditions at 25°C in 0.5 ml of a medium consisting of 150 mM Tris-HCl buffer (pH 7.5), 1.4% bovine serum albumin, 0.5 mg/ml bacitracin and 15 nM |^{3}H|Leu-enkephalin in the absence or presence of increasing concentrations of Leu-enkephalin (up to 300 µM). The reaction was initiated by the addition of enterocytes (1 mg cell protein/ml). After 60 min incubation, cell-bound peptide was separated by centrifugation (17) and the radioactivity determined. Nonspecific binding was obtained from the amount of radioactivity bound in the presence of 1mM Leu-enkephalin and represented about 35% of the binding observed in the absence of unlabeled peptide. This nonspecific component was subtracted from the total radioactivity bound in order to obtain the corresponding specific binding.

Measurement of cAMP accumulation. Accumulation of cAMP was studied in standard conditions in an incubation solution (0.5 ml final volume) consisting of 150 mM Tris-HCl buffer (pH 7.5), 1.4% bovine serum albumin, 0.5 mg/ml bacitracin, 0.5 mM 3-isobutyl-1-methylxanthine and increasing concentrations (up to 300 µM) of Leu-enkephalin. The reaction was initiated by the addition of enterocytes (1 mg cell protein/ml). After 60 min incubation at 25°C, the reaction was stopped by the addition of 2.5 ml methanol and cAMP was extracted and determined as in (19).

RESULTS

Specific binding of |^{3}H|Leu-enkephalin to enterocytes was a time and temperature dependent process, as observed at 37, 25 and 15°C (Fig.1, left). Reduction of the incubation temperature resulted in decreases of both the rate and the percentage of binding. At 25°C, an apparent steady-state was observed between 45-60 min. The decline observed in the extent of binding after long incubation periods at all temperatures tested is likely to be related, at least in part, to inactivation of |^{3}H|Leu-enkephalin (Fig.1, right). A 60 min incubation at 25°C was performed in subsequent studies.

Binding of |^{3}H|Leu-enkephalin to enterocytes was reversible (Fig.2): once formed, the complex tracer-cells could dissociate upon dilution and addition of an excess of unlabeled Leu-enkephalin. The dissociation process did not follow a first-order pattern, 50% being observed by 15 min. Interestingly, Na^+ was

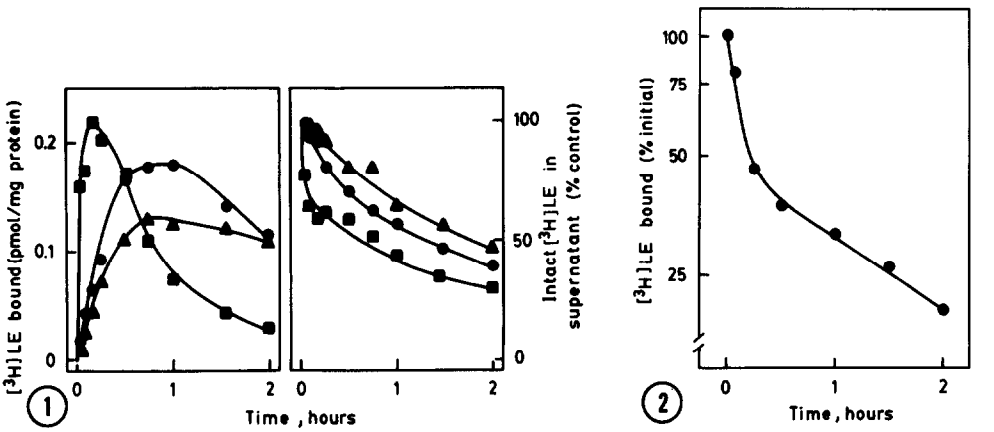

Fig.1: Time course of binding (left) and degradation (right) of |^{3}H|Leu-enkephalin (|^{3}H|LE) by guinea pig enterocytes as a function of temperature. Cells were incubated with 15 nM tracer at 15°C (▲), 25°C (●) or 37°C (○). Left: the specific binding of tracer was measured at the time intervals indicated. Right: at the same time periods, the integrity of the labeled peptide remaining free in the supernatant was estimated by its ability to bind to fresh enterocytes and expressed as the percentage of control incubated without cells. Each point is the mean of triplicates.

Fig.2: Time course of dissociation of |^{3}H|Leu-enkephalin (|^{3}H|LE) from guinea pig enterocytes. Cells were preincubated with 15 nM tracer for 60 min at 25°C; after centrifugation, the supernatant was replaced by 10 volumes of a similar medium containing 0.1 mM Leu-enkephalin. Results are expressed as the percentage of tracer specifically bound when new medium was added. Each point is the mean of triplicates.

devoid of any significant effect on the binding reaction when tested in the 25-200 mM range.

Increasing concentrations of Leu-enkephalin competitively inhibited tracer binding to enterocytes (Fig.3). Half-maximal inhibition was observed at 13.2 ±

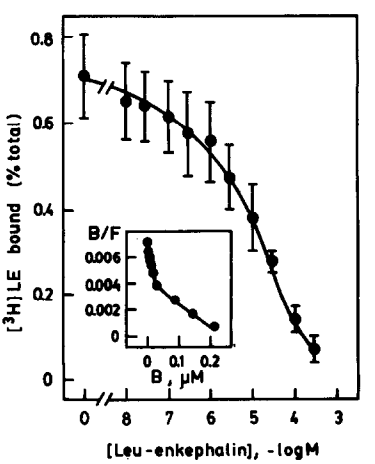

Fig.3: Competitive displacement of |^{3}H|Leu-enkephalin (|^{3}H|LE) by unlabeled Leu-enkephalin from guinea pig enterocytes. Cells (1 mg protein/ml) were incubated with 15 nM tracer at 25°C for 60 min in the absence or presence of native peptide. Results are the mean ± S.E.M. of seven triplicate experiments. The inset shows a Scatchard (20) analysis of the data.

Table I: Ability of enkephalin analogues (0.3 μM) to inhibit the binding of 15 nM |^{3}H|Leu-enkephalin and to stimulate cAMP accumulation in guinea pig enterocytes (1 mg protein/ml).

Opiate	|^{3}H|Leu-enkephalin bound (% of maximum)	cAMP (maximum increase above basal)
None	100	0
Leu-enkephalin	9	100
Leu-enkephalinamide	33	13
des-Tyr,Leu-enkephalin	27	5
D-Ala^2,D-Leu^5-enkephalinamide	69	15
D-Ala^2,Leu-enkephalinamide	47	5
Met-enkephalin	22	5
Met-enkephalinamide	24	3
D-Ala^2,Met-enkephalin	50	2
D-Ala^2,Met-enkephalinamide	44	2
Naloxone	80	2

Binding data are expressed as the percentage of radioactivity specifically bound in the absence of unlabeled ligand. Maximum cAMP response was taken as 100%. Values are the means of triplicates.

2.7 μM. The Scatchard plot (20) of the binding data was curvilinear with an upward concavity (Fig.3, inset). The interpretation of the data in terms of two populations of Leu-enkephalin receptors results in the definition of a class with a relatively high affinity (K_d=0.7±0.2 μM) and low binding capacity (2.0 ± 0.1 pmol Leu-enkephalin/mg cell protein), and another class with a low affinity (K_d=55.5±17.2 μM) and high binding capacity (0.23±0.05 nmol Leu-enkephalin/mg cell protein).

The potency of various peptides structurally related to Leu-enkephalin in inhibiting the binding of |^{3}H|Leu-enkephalin to enterocytes is shown in Table I. Met-enkephalin, the other naturally occurring opiate pentapeptide, and the two corresponding enkephalinamides were able to inhibit tracer binding but with a lower potency than that of Leu-enkephalin. A similar loss of binding activity was observed by the removal of the N-terminal amino acid from Leu-enkephalin. However, the presence of D-amino acids in the enkephalin analogues evidenced drastic losses in binding affinity. Finally, naloxone behaved as a ligand with a extremely low affinity.

With respect to cAMP accumulation, Leu-enkephalin increased the level of the cyclic nucleotide in enterocytes in all conditions tested (Fig.4). In the presence of 0.5 mM 3-isobutyl-1-methylxanthine as a phosphodiesterase inhibitor, the stimulatory effect of Leu-enkephalin on cAMP accumulation depended on time and temperature. At 37°C, 10 μM Leu-enkephalin elicited a rapid increase of cAMP levels that became maximal at 15 min (4-fold increase above basal) and decreased slowly thereafter. Lowering the temperature to 25°C caused an increase in the extent of cAMP accumulation (6-fold increase above basal with 10 μM peptide) and an apparent steady-state could be observed from 60 to 120 min. A 60 min incubation at 25°C in the presence of 0.5 mM 3-isobutyl-1-methylxanthine represented the best conditions for subsequent experiments. In other context, Na^+ behaved

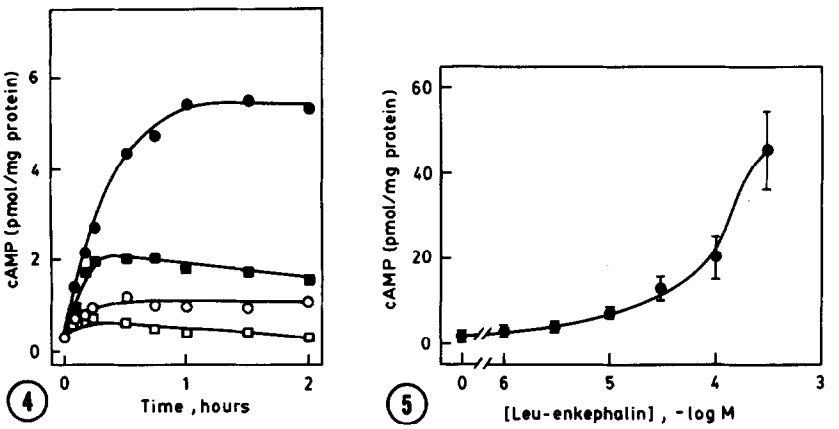

Fig.4: Accumulation of cAMP in guinea pig enterocytes as a function of time and temperature. Cells were incubated in the presence of 0.5 mM 3-isobutyl-1-methylxanthine with (●, ■) or without (○, □) 10 µM Leu-enkephalin at 25°C (●, ○) or 37°C (■, □), and the reaction was stopped at the indicated times. Results are the mean of three experiments performed in triplicate.

Fig.5: Effect of increasing concentrations of Leu-enkephalin on cAMP levels in guinea pig enterocytes. Values are the mean ± S.E.M. of eight separate experiments, each performed in triplicate.

as an activator of cAMP accumulation in enterocytes but did not potentiate the stimulation by Leu-enkephalin: at any Na^+ concentration between 50-200 mM, the effects of both Na^+ and Leu-enkephalin were purely additive (not shown).

Leu-enkephalin stimulated cAMP accumulation in enterocytes in a monophasic and dose-dependent way (Fig.5). The concentration of peptide giving half-maximal stimulation was 119±14 µM. The maximal stimulation of cAMP accumulation was observed at 0.3 mM Leu-enkephalin and represented a 27-fold increase of basal level.

The stimulatory effect of Leu-enkephalin on cAMP accumulation appeared to be highly specific (Table I). In fact, removal of the N-terminal amino acid or substitution of one or more residues led to a drastic loss in the corresponding ability to stimulate cAMP accumulation, essentially rendering the peptides inactive at a concentration as high as 0.3 mM. A similar lack of effect was shown by naloxone. Furthermore, 0.3 mM naloxone did not antagonize the action of Leu-enkephalin.

DISCUSSION

Present data show that Leu-enkephalin possesses specific receptors and behaves as an efficient stimulator of cAMP accumulation in guinea pig enterocytes.

Kinetic and stoichiometric experiments showed that the binding reaction exhibited properties such as dependence on time and temperature, reversibility, saturability and specificity. These are common features in other enkephalin binding systems such as brain membranes (21,22), clonal cell lines of neurotumor origin (23) and peripheral tissue preparations (24,25). As suggested by disso-

ciation experiments and Scatchard (20) analysis of stoichiometric data, two classes of Leu-enkephalin receptors with different affinities can be defined in enterocytes. These binding sites bear most similarity to what has been described as δ-opiate receptors (25); however, there appear to be some differences as well, such as the reported lack of effect of Na^+ on ligand binding. In fact, Na^+ reduces opiate binding (26) and is required for the expression of an opioid-directed inhibition of adenylate cyclase (10) in neural preparations. However, present results correspond to a peripheric tissue and demonstrate a stimulatory role of Leu-enkephalin on cAMP accumulation in enterocytes. Furthermore, it should also be noted the extremely low affinity of naloxone for Leu-enkephalin receptors and the high degree of specificity showed by these binding sites. Interestingly, a previous study on the effect of opioid peptides and narcotic drugs on transepithelial potential difference and short circuit current in guinea pig ileal mucosa suggested the presence of a modified δ-receptor (9).

With respect to cAMP accumulation, the time and temperature dependence of the stimulation and its potentiation by a phosphodiesterase inhibitor are features that have been also described in other systems such as vasoactive intestinal peptide with enterocytes (27,28). These features have been considered in these systems to be characteristic of an involvement of cAMP in the first step of peptide action in cells. Opiate peptides have been shown to inhibit cAMP levels in cells and membranes of neural origin by a Na^+-dependent mechanism (10,13). However, present results demonstrate an opposite effect of Leu-enkephalin in enterocytes. It agrees with a similar action of various opiate peptides in non-neural systems such as adipocyte membranes (14) and pancreatic islets (15,16). Then, it appears that, at least in a number of cases, occupancy of opiate peptide receptors in peripheral tissues leads to cAMP stimulation.

Half-maximal stimulation of cAMP accumulation in enterocytes was observed at 119 μM Leu-enkephalin, a value that is near of the K_d (55.5 μM) of the corresponding low affinity receptors. These results suggest that the action of the opiate peptide on cAMP results mainly from the interaction of Leu-enkephalin with the low affinity receptors. On the other hand, Leu-enkephalin showed a remarkably high efficiency in stimulating cAMP (27-fold increase above basal level at a maximally effective dose of peptide). This feature is similar to that offered by vasoactive intestinal peptide in rat (27) and guinea pig (28) enterocytes. However, the opiate peptide possessed a relatively low potency as compared with that of vasoactive intestinal peptide (27,28) but it was proximal to the potency of prostaglandin E_1 in the same cell system (28). On the contrary, Leu-enkephalin acted with a high potency (in the nM range) when inhibiting cAMP in neural preparations (10-13).

Interestingly, the effect of Leu-enkephalin on cAMP accumulation in guinea pig enterocytes possessed a high degree of specificity. In fact, substitution,

removal or modification of some of the amino acid residues in the Leu-enkephalin molecule resulted in an almost complete loss of the ability to stimulate cAMP accumulation. This agrees with the low affinity showed by the same analogs at the receptor level. In other context, the practical absence of effect of naloxone on both basal and Leu-enkephalin stimulated cAMP levels does not agree with previous reports on adenylate cyclase inhibition in preparations of neural origin (10,13). However, it is in accordance with previous data (16) on that naloxone did not reverse the stimulatory action of Leu-enkephalin on both cAMP content and insulin secretion in islets of Langerhans.

Present results together to the known presence of a great density of enkephalinergic nerve fibers and endocrine cells in the small intestine (5,8) strongly suggest that Leu-enkephalin can exert a direct action at the mucosal level. The peptide could be liberated at high local concentrations at the vicinity of the intestinal epithelial cells and, after binding to specific receptors and stimulation of cAMP levels, it would initiate a cascade of events leading to the corresponding final responses.

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