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ALTERED LEVELS OF β-ENDORPHIN FRAGMENTS AFTER CHRONIC MORPHINE TREATMENT OF GUINEA-PIG ILEUM IN VITRO AND IN VIVO

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Summary

The isolated myenteric plexus-longitudinal muscle of the guinea-pig ileum (GPI) was used as testsystem to study the influence of chronic morphine treatment on the levels of enkephalins, β-endorphin and some of its fragments. The peptides were assayed by means of a combination of high pressure liquid chromatography and radioimmunoassays. It was found that the levels of methionine- and leucine-enkephalin and β-endorphin were not altered by chronic morphine treatment of guinea-pigs in vivo nor in GPI exposed to morphine in vitro. However, the levels of some β-endorphin fragments i.c. γ-endorphin and des-tyrosine-γ-endorphin were elevated after morphine treatment in vitro and in vivo respectively. It is suggested that β-endorphin and its fragments are involved in homeostatic processes during development of opiate tolerance.

The presence of peptides with morphine-like properties in the brain (1,2,3) suggested that these "endorphins" may be involved in the development of tolerance to and physical dependence on opiates (4). Endorphins and related peptides appear to play a critical role in brain homeostasis and consequently in adaptive behavioral processes (5,6). According to Himmelsbach's theory (7), opiate tolerance is due to neural adaptation that develops in homeostatic compensation for the inhibitory action of morphine. This theory stimulated attempts to measure brain- and pituitary levels of β-endorphin (β-LPH_{61-91}) and enkephalins in animals chronically treated with morphine. However, the results obtained so far (8,9, 10,11) are not convincing probably due to the complexity of the brain and because dynamic changes in the metabolism of β-endorphin and enkephalins may not be reflected by the steady state levels.

In an attempt to circumvent these problems we have used the myenteric plexus-longitudinal muscle of guinea-pig ileum (GPI), a widely used model system for opiate action and for development

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of opiate tolerance (12,13). The levels of enkephalins, β-endorphin and of various β-endorphin fragments were assayed using a combination of high-pressure liquid chromatography (HPLC) and different specific radioimmunoassay systems (RIA). We found that chronic exposure to morphine led to altered levels of β-endorphin fragments It is suggested that β-endorphin and its fragments may be involved in opiate tolerance.

Methods

Induction of tolerance in vivo: Male guinea-pigs were injected with morphine twice daily at 8:30 a.m. and 5:30 p.m., using the following treatment schedule: (morphine dose in mg/kg) day 1: 20; day 2: 40; day 3: 80; day 4: 80; and day 5: 80 at 8:30 a.m. only. Control animals received injections with placebo (0.5 ml saline) according to the same schedule.

Induction of tolerance in vitro: Male guinea-pigs were killed by decapitation and the small intestine was removed and longitudinal muscle-myenteric plexus strips were prepared. These strips were incubated overnight (18-20 h, 20°C) in Krebs solution without (controls) or with 8 μM of morphine as described previously (14).

Extraction from longitudinal muscle-myenteric plexus tissue: Two hours after the last morphine or saline injection, the guinea-pigs were decapitated and muscle strips were prepared from the ileum (15) and wet weight was estimated. Immediately thereafter, strips were transferred to polypropylene tubes containing 5 ml 1 M acetic acid, which were preheated to 95°C in a boiling water bath. The muscle strips which were incubated overnight in order to induce tolerance to morphine in vitro were treated in the same way. After 10 min the tubes were chilled in ice. Homogenization of the tissue was achieved using a motor-operated teflon-glass homogenizer (twelve strokes) followed by sonification (Branson sonifier: 20 KHz for 30 sec). The homogenates were centrifuged (15 min, 30,000xg). Supernatants were lyophilized and the residues were dissolved in 0.1 M acetic acid (1.0 ml) and extracted using Vycor glass powder as described elsewhere (16). This Vycor glass extraction was performed twice. Recovery of this extraction procedure was between 70 and 80% for the β-endorphin-related peptides and about 95% for the enkephalins. No correction has been made for these recoveries. Small aliquots were taken for protein determination (17). The dry extracts were dissolved in 0.01 M ammonium acetate pH 4.15 (1 ml) and fractionated by HPLC after removal of insoluble residues by centrifugation.

Fractionation by HPLC: To measure the levels of enkephalins (methionine- and leucine-enkephalin) and of β-endorphin and its main metabolites (α-endorphin, γ-endorphin and their des-tyrosine fragments) (18), the samples were first subjected to an HPLC procedure which allows the separation of these very similar peptides (18). The extracts were fractionated on a reversed-phase μBondapak C18 column (0.39 x 30 cm; Waters Ass.). Because the levels of endogenous endorphins were too low to detect directly by UV absorbance (210nm) the retention times of selected synthetic reference peptides (α-MSH and $ACTH_{4-10}$) were used as a guide to collect fractions corresponding to the respective endorphin peptides as described elsewhere (20). These fractions were dried at 60°C using an evaporator (Büchler, vortex). The pooled fractions were lyophilized, the resi-

dues dissolved in phosphate buffered saline and subjected to radioimmunoassay.

Analysis by radioimmunoassay (RIA): The amount of peptide in the fractions was measured using RIA systems specific for various endorphins (methionine-enkephalin, α-, β- and γ-endorphin). These assay systems together with the preparation of antigens and antisera and cross-reactivity data have been described in detail previously (19). Since the des-tyrosine peptide fragments cross-react completely in the RIA systems, developed for the parent peptides (19), it is possible to measure these peptides in HPLC fractions corresponding to the retention times of synthetic reference β-endorphin fragments. The minimal detectable peptide dose using the HPLC-RIA combination is 20 pg and 200 pg for methionine- and leucine-enkephalin respectively, 20 pg for β-endorphin, α-endorphin and des-tyrosine-α-endorphin and 5 pg for γ- and des-tyrosine-γ-endorphin (19). Synthetic peptides were a generous gift of Dr. H. M. Greven, Scientific Development Group, Organon, Oss, The Netherlands.

Results and Discussion

Treatment of guinea-pigs with twice daily injections of morphine led to development of tolerance and physical dependence in the ileum strips as reported previously (21). This could be inferred from the reduced sensitivity to opiates and the withdrawal signs upon naloxone challenge of the isolated ileum preparation (22,23,24,25). Incubation of GPI from opiate naive guinea-pigs *in vitro* (18-20 h, 20°C) in Krebs solution containing morphine (8 μM) resulted in tolerance to morphine as measured by reduced sensitivity to a test dose of morphine (400 nM). This tolerance lasted for at least one hour (14).

The muscle strip from placebo treated animals (*in vivo* controls) contained considerable amounts of methionine-enkephalin and leucine-enkephalin (95 ± 10 and 68 ± 9 ng/g wet weight respectively). Comparable amounts of these peptides were estimated in extracts of *in vitro* incubated strips (*in vitro* controls). These levels were in the same range as in other reports involving various assay systems (26,27,28). The high resolving power of the HPLC technique allows separation of closely resembling peptides (e.g.: des-tyrosine-α-endorphin from α-endorphin, des-tyrosine-γ-endorphin from γ-endorphin and α-endorphin from γ-endorphin). Since des-tyrosine-α-endorphin and des-tyrosine-γ-endorphin cross-react completely in the RIA systems developed for respectively α-endorphin and γ-endorphin, the des-tyrosine fragments can be measured in biological tissue by employing the combination of HPLC fractionation and RIA (19). Using these methods we found detectable levels of β-endorphin (6.7 pg/mg protein) and its fragments in the ileum strips. The concentrations of these peptides hardly differed between the *in vivo*- and *in vitro* controls (Figures 1 and 2). These data indicate that enkephalins and β-endorphin and its fragments are present in the guinea-pig ileum. These peptides may be formed in the ileum or taken up from the general circulation. Evidence for the biosynthesis of enkephalins by the myenteric plexus *in vitro* has been presented (29), but information about β-endorphin and its fragments in this respect is lacking. Although uptake of these peptides from the circulation cannot be excluded, it is not likely that such a mechanism is accounted for the

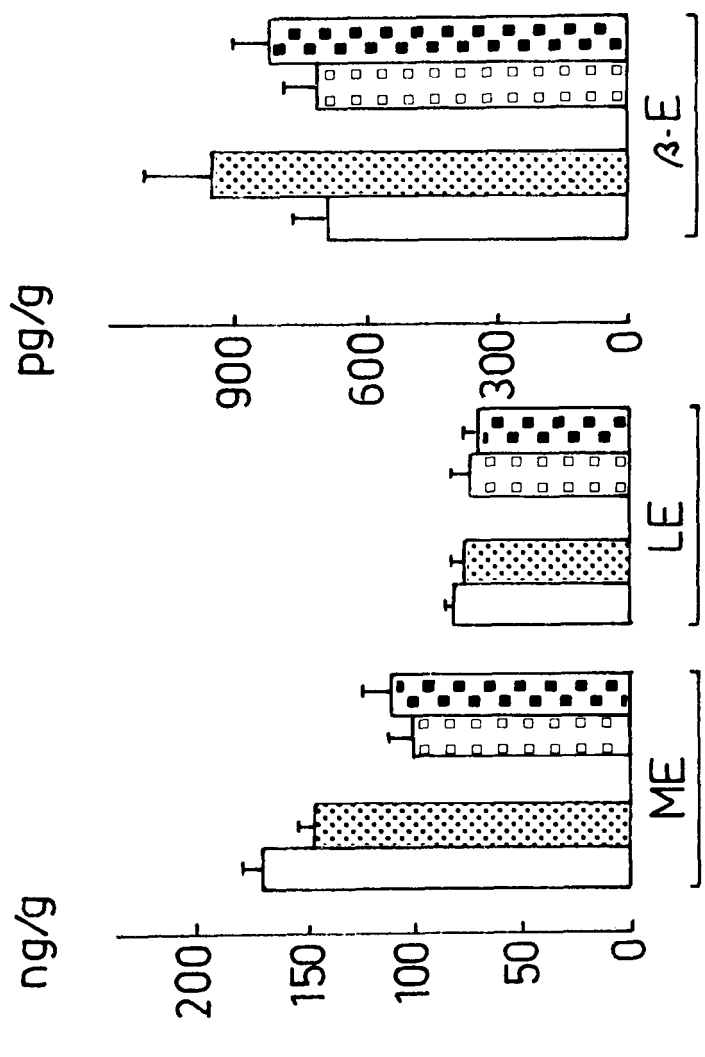

FIG. 1

Effect of tolerance to morphine induced in vivo and in vitro on the levels of methionine-enkephalin (ME); leucine-enkephalin (LE) in ng/g wet weight and β-endorphin (β-E) in pg/g in extracts of smooth muscle strips of guinea-pig ileum. □ : in vitro control strips; ▩ : in vitro tolerant strips; ▣ : in vivo control strips; ■ : in vivo tolerant strips. Values are means of 5 determinations + SEM.

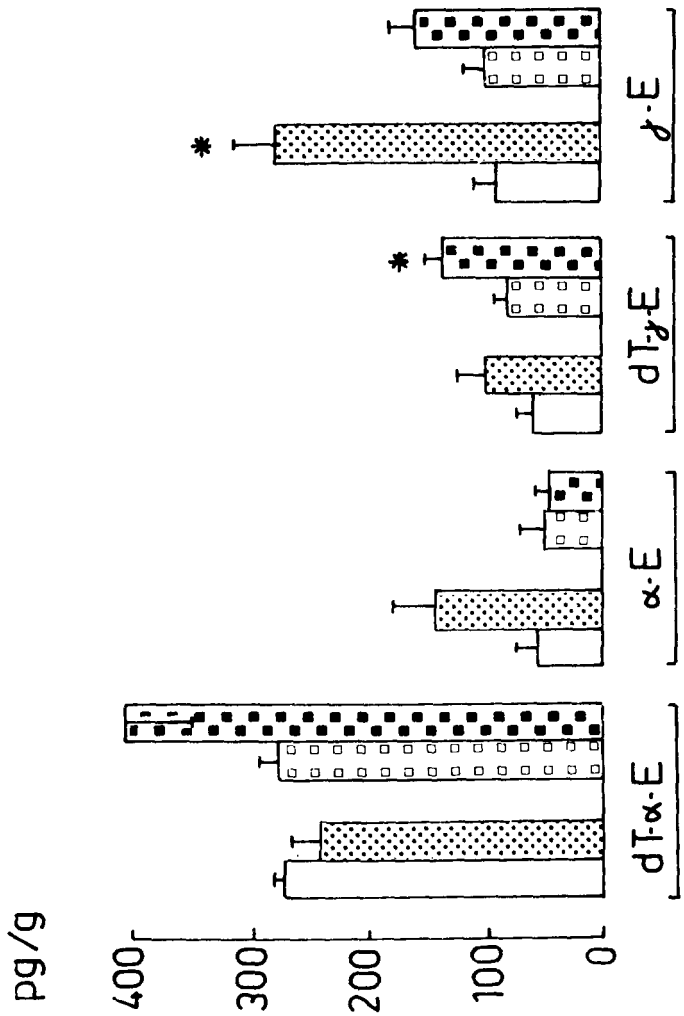

FIG. 2

Effect of tolerance to morphine induced in vivo and in vitro on the levels of some fragments of β-endorphin in pg/g wet weight in extracts of smooth muscle strips of guinea-pig ileum. dT-α-E = des-tyr-α-endorphin; α-E = α-endorphin; dT-γ-E = des-tyr-γ-endorphin; γ-E = γ-endorphin. For the meaning of the shaded bars see figure 1. Values are means of 5 determinations ± SEM (*: significantly different from respective controls; $P < 0.01$, Student's t-test).

measured amounts in the guinea-pig ileum. In vitro experiments indicated a very low, if any, conversion of β-endorphin to smaller peptide fragments by endo- or exo-peptidases in human plasma (18). Thus if β-endorphin present in the ileun is originated from pituitary β-LPH, the fragmentation of β-endorphin may take place locally in the ileum. Accordingly, this fragmentation can be affected by morphine treatment in vitro (see below). However, more experiments are needed before definite conclusions can be drawn about the biosynthesis and fragmentation of β-endorphin in the ileum. The similarity of the endorphin and enkephalin levels from in vivo and in vitro controls suggest that post-mortum degradation is not likely. Accordingly, Childers and Snyder (30) reported that post-mortum enkephalin degradation in brain tissue was not observed for at least six hours.

Morphine pretreatment in vivo or in vitro affected neither the level of the enkephalins nor that of β-endorphin. In contrast, the levels of some β-endorphin fragments appeared to be changed after morphine treatment (fig. 2). Thus, the amount of α-endorphin tended to be higher and that of γ-endorphin was significantly higher after in vitro morphine treatment. The amount of des-tyrosine-α-endorphin was elevated and that of des-tyrosine-γ-endorphin was significantly higher in muscle strips from morphine-treated animals as compared to those of in vivo controls (fig. 2). The present data indicate that both in vivo and in vitro morphine treatment led to increased levels of β-endorphin fragments. As the steady state level of β-endorphin was not affected, its turnover could have been stimulated by chronic morphine treatment or its fragmentation altered in some specific way. Evidence for a function of β-endorphin and/or its fragments in the guinea-pig ileum is not available as yet. However, the ileum strip preparation is one of the model systems used to study acute and chronic morphine action in the central nervous system. The potency of several opioids to inhibit electrically induced contractions of the ileum strip correlates well with their analgesic activity (31) and tolerance develops after chronic exposure of the ileum to morphine in vivo (22,23) as well as in vitro (13,14). In our experiments with ileum, as in those with brain tissue (9,11), steady state levels of enkephalins and β-endorphin were not affected by chronically applied morphine. However, the amounts of β-endorphin fragments seem to be increased by morphine treatment. Thus, α- and γ-endorphin accumulated during development of in vitro tolerance, while their des-tyrosine fragments accumulate during development of in vivo tolerance. This differential accumulation is not understood as yet. It might be that the incubation temperature (20°C) of the in vitro morphine-treated strips interferes with various enzyme systems, leading to a decreased tyrosine loss.

Fragments of β-endorphin e.g. α- and γ-endorphin and their des-tyrosine fragments have been demonstrated in rat brain and pituitary (19,20) and in human cerebrospinal fluid (32). Evidence has been presented that these peptides can be formed preferentially from β-endorphin by enzymes associated with an enriched synaptosomal plasma membrane fraction from brain tissue (18). Since these fragments may play an essential role in brain homeostatic and adaptive processes (5,6), it is tempting to speculate that they are also involved in neural adaptation during the development of tolerance to morphine in both the central nervous system and the ileum. However, fragmentation of β-endorphin has not been demon-

strated in ileum tissue, moreover, the mode of action of β-endorphin and its fragments with respect to morphine tolerance is not yet clear.

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