Naunyn-Schmiedeberg's Arch Pharmacol (1986) 333:381–386

Naunyn-Schmiedeberg's
Archives of
Pharmacology

Opiates induce long-term increases in prodynorphin-derived peptide levels in the guinea-pig myenteric plexus

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Summary. The subcutaneous administration of a single dose of an opiate agonist (levorphanol) or antagonist (naloxone) to guinea pigs results in an at least 3-fold elevation of dynorphin and alpha-neoendorphin-immunoreactivity in the longitudinal muscle myenteric plexus preparation. The effects are time- and dose-dependent, significant elevations first being observed 6 h after treatment and lasting for up to 24 h. Pretreatment levels of opioid peptides were observed after 8 days. Combined injection of the narcotic agonist and antagonist, at sufficiently high doses, resulted in an additive effect of the individual drugs. The respective stereoisomers dextrorphan and (+)-naloxone did not affect prodynorphin-derived peptide concentrations. An increase of endogenous opioids was also observed after administration of the non-opiate clonidine, a compound which, like opiates, alters the activity of the myenteric plexus. It is suggested that feedback mechanisms in the myenteric plexus are responsible for the elevation of endogenous opioid peptides following exposure to exogenous opiates.

Using a monoclonal antibody (3-E7), which recognizes virtually all endogenous opioid peptides, it was found that levels of higher molecular material were also increased upon opiate challenge. This suggests that a single dose of an exogenous opiate results in an increase in peptide synthesis.

Key words: Guinea-pig myenteric plexus – Prodynorphin – Dynorphin A – Alpha-neoendorphin – Feedback – Opiates

Introduction

Endogenous opioids are believed to have neurotransmitter functions in both the central and peripheral nervous systems (for reviews see Akil et al. 1984; Schulz 1985). Since neuronal activity is controlled by feedback mechanisms, Kosterlitz and Hughes (1975) postulated the occurence of adaptive processes after the application of exogenous opioids to neuronal systems. This concept has previously been tested by a number of investigators who reported either no change of brain enkephalin levels upon morphine treatment (Wesche et al. 1977; Childers et al. 1977; Fratta et al. 1977; Höllt et al. 1978), a decrease of β-endorphin concentrations following long-term morphine exposure (Przewlocki et al. 1979) or an increase of enkephalin levels during the state of morphine tolerance/dependence (Simantov and Snyder 1976). One investigation of the peripheral nervous system revealed no changes in enkephalin concentrations in the myenteric plexus of the guinea-pig ileum upon chronic morphine exposure; however, moderately increased levels of β-endorphin fragments were observed (Opmeer et al. 1980).

The data reported so far thus suggest that treatment of animals with exogenous opioids may affect the concentrations of some endogenous opioids but not of others. The primary aim of the present investigation was to study more closely the effect of acute actions of an opiate agonist and antagonist upon opioid peptides. We selected the guinea-pig myenteric plexus for these experiments, since it contains functional opioid receptors (Leslie et al. 1980; Paterson et al. 1983; Schulz et al. 1981) which are involved in the control of intestinal propulsion (Kromer et al. 1980, 1981; Clark and Smith 1981). The prodynorphin-derived peptides dynorphin A (DYN) and alpha-neoendorphin (ANE) have been indentified in the myenteric plexus of several species and are believed to be endogenous ligands of these receptors (Goldstein et al. 1979; Tachibana et al. 1982; Watson et al. 1981).

In the present experiments, DYN- and ANE-immunoreactivities (i.r.) were measured following their separation from biological material using the 3-E7 monoclonal antibody (Gramsch et al. 1983) which recognizes virtually all known opioid peptides. In an earlier study we showed the usefulness of this antibody in immunochromatographic studies for the identification of opioid peptides (Dandekar et al. 1985). In the present experiments we demonstrated that a single opiate treatment results in a considerable increase of DYN- and ANE-i.r. in the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum.

Materials and methods

Male guinea pigs (350–400 g) were housed at 22° C on a 12–12 h light-dark cycle. The animals were treated subcutaneously with the drugs under investigation or with saline. Food was withdrawn 15 h before sacrifice. After decapitation, the jejunum and ileum was dissected rapidly, and the longitudinal muscle with attached myenteric plexus was prepared. These procedures were carried out in the cold (4° C), and the strips were kept in Ringer solution (4° C). After the wet weight was taken, the strips were extracted in 0.1 N HCl (95° C, 0.1 g tissue in 20 ml HCl) for 15 min. Five minutes after starting the extraction the tissue was

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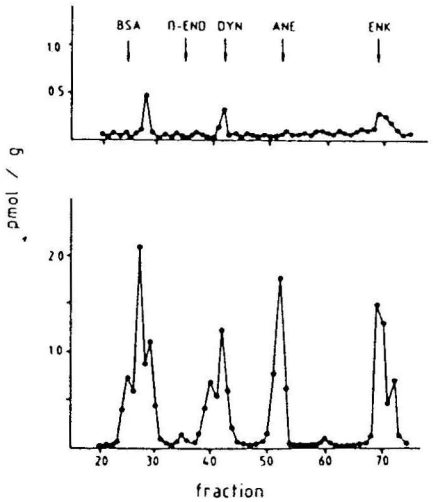

Fig. 1. Solid phase RIA ("opioid screening") of gel chromatographed extracts from longitudinal muscle/myenteric plexus strips of guinea pigs treated 6 h prior to sacrifice with either saline (*upper panel*) or 20 mg/kg morphine (*lower panel*). Strip material of two guinea pigs was combined for each run. Calibration of the Sephadex column was conducted with bovine serum albumin (BSA), β-endorphin (β-END), dynorphin A (DYN), alpha-neoendorphin (ANE) and methionin-enkephalin (ENK). *Ordinate*: pmol β-endorphin-immunoreactivity per g as indicated by the 3-E7 monoclonal antibody. *Abscissa*: fraction number

homogenized (Ultra Turrax, rod N 10, 10 s, at maximal speed). The samples were then centrifuged (10,000 × *g*, 15 min, 4°C) and the pH of the supernatant adjusted to 7.0 (NaOH). The recovery of opioid peptides after this extraction procedure was tested by incubating either ^{125}I-ANE or ^{125}I-DYN with 1 g striated muscle; 75–85% of the radioactive neuropeptides were recovered in the supernatant.

The separation of opioid peptides, including DYN and ANE, was achieved by immunoaffinity chromatography, employing the 3-E7 monoclonal antibody, as previously described (Dandekar et al. 1985). Briefly, the neutralized supernatant (20 ml) was run in the cold over an immunoaffinity column (3-E7 antibody linked to Sepharose). Thereafter, the affinity gel was washed with 0.1 M ammonium acetate (pH 7.0, 5 ml), and the opioid peptides eluted using 2 M acetic acid (2 ml). The eluted material was lyophilized, taken up in 1 M acetic acid and submitted to gel chromatography (Sephadex G 50, superfine, column 10 × 900 mm; 1 M acetic acid, containing 0.1% bovine serum albumin; flow rate 10 ml/h). One milliliter fractions were collected and lyophilized (Speed Vac Concentrator, Savant Instruments). β-Endorphin eluted in fractions 33–37, DYN in 39–44 and ANE in 50–54. Specific RIA's were employed to detect the individual peptides. Details of the RIA-technique and characteristics of the antisera have been published (Dandekar et al. 1985). The detection limit for DYN- and ANE-i.r. was 5 and 8 fmoles per tube, respectively. High pressure liquid chromatography (HPLC) was employed in a few cases in order to verify DYN- and ANE-i.r. (see Dandekar et al. 1985, for details).

Recovery of DYN and ANE, determined after samples have been submitted to the extraction procedure, immunoaffinity chromatography and gel chromatography, was approximately 50% detection of each peptide determined by RIA. All data presented in this paper refer to the non-corrected values determined by RIA.

In a pilot study the extracts of longitudinal muscle myenteric plexus strips were purified by 3-E7 immunoaffinity chromatography and subsequently submitted to gelchromatography. All fractions obtained were subjected to a solid phase 3-E7 RIA, using ^{125}I-β-endorphin as tracer (for details see Schulz and Gramsch 1985). The detection limit was 200 fmol/well.

The data shown here represent mean ± SEM. When no standard errors are given, the values are the mean of only two determinations. Each guinea pig provided a single extract in which DYN and ANE levels were determined; individual values shown are the means of duplicate determinations. Student's *t*-test was employed to calculate significance of differences between mean values.

The following drugs were used: Dynorphin A, alpha-neoendorphin, met-enkephalin, β-endorphin (all from Bachem, Bubendorf, Switzerland), bovine serum albumin (Sigma, St. Louis, MO, USA), morphine-HCl (Merck, Darmstadt, FRG), levorphanol and dextrorphan (Roche, Grenzach-Wyhl, FRG), ethylketazocine methanylsulfonate (Sterling-Winthrop, Rensselaer, NY, USA), fentanyldihydrogentartrate (Janssen, Düsseldorf, FRG), (−)naloxone-HCl (Endo, Garden City, NY, USA), clonidine-HCl (Boehringer, Mannheim, FRG), (+)-naloxone-HCl (a gift of Dr. A. Jacobson).

Results

3-E7 "opioid-screening"

The 3-E7 monoclonal antibody recognizes all endogenous opioid peptides. Therefore, it was here utilized to screen for these neuropeptides in preparations of the longitudinal muscle/myenteric plexus. Figure 1 demonstrates the results of screening in extracts of strips from naive (upper panel) and morphine treated (lower panel) guinea pigs. When ^{125}I-β-endorphin was used as the tracer, a considerable increase in the i.r. profile was observed 6 h after morphine treatment (20 mg/kg). Since pilot studies also suggested an elevation of i.r. DYN and ANE, the relevant fractions were subjected to the appropriate RIA's so as to determine the amount of these peptides. There was indeed a significant increase in the levels of i.r. DYN and ANE in morphine exposed preparations.

Effect of narcotic agonists

Figure 2 displays the temporal profile of the effect of a single dose of the μ-ligand levorphanol (1 mg/kg) on DYN- and ANE-i.r. Neither peptide-concentration was affected by levorphanol 1 h after injection. In contrast, 6 h following levorphanol challenge, i.r. of both DYN and ANE were significantly increased (3-fold for DYN, 6-fold for ANE), and similar increases were seen 24 h later. Dextrorphan (inactive isomer of levorphanol) treatment did not alter peptide levels.

Figure 3 shows that the effect of levorphanol upon DYN- and ANE-i.r. in strips prepared 6 h after treatment is dose-dependent. Controls were treated with dextrorphan. While no effect was observed at 0.01 mg/kg levorphanol on

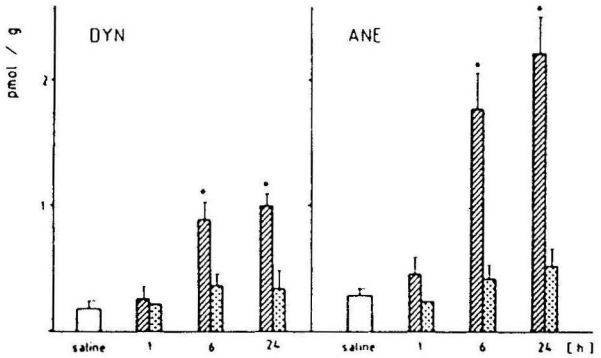

Fig. 2. The effect of 1 mg/kg levorphanol (▨) and dextrorphan (▩), respectively, upon DYN- and ANE-i.r. in strips taken at different times after treatment of guinea pigs. Saline treated animals were killed after 6 h. Columns indicating SEM consist of data from at least three guinea pigs * $P < 0.01$

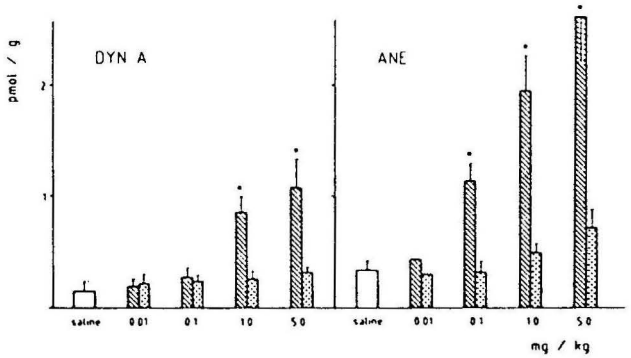

Fig. 3. Dose-dependent effect of levorphanol and dextrorphan, respectively, upon DYN- and ANE-i.r. in strips obtained from guinea pigs 6 h after treatment. For further explanation see Fig. 2

either of the peptides, 0.1 mg/kg caused an increase in ANE-i.r. only, and both DYN- and ANE-i.r. were elevated by 1.0 mg/kg levorphanol. Treatment with 5 mg/kg of the narcotic did not further elevate peptide levels. Again, dextrorphan failed to affect peptide concentrations.

Further experiments were conducted with the μ-receptor agonist fentanyl and the k-ligand ethylketazocine. After 6 h of each of these drugs (1 mg/kg) there was an increase in i.r. ANE and DYN which was similar to that seen in response to the same dose of levorphanol (two animals per drug).

Extracts were prepared from the strips of three guinea pigs and submitted to gelchromatography. Eluted material which corresponded to synthetic DYN and ANE was subjected to HPLC. The retention times of those materials on HPLC were identical to these for authentic DYN and ANE, respectively.

Effect of narcotic antagonist

(−)-Naloxone was tested on DYN- and ANE-i.r.: controls were treated with the inactive (+)-isomer. Increasing doses were applied and the strips were prepared 6 h later. Figure 4 demonstrates a dose-dependent effect of the antagonist in elevating DYN- and ANE-i.r. (+)-Naloxone did not affect tissue concentrations of ANE- and DYN-i.r. In addition, there was a time-dependent effect of a fixed dose of naloxone (1 mg/kg) upon DYN- and ANE-i.r. (Fig. 5).

Effect of naloxone/levorphanol on DYN- and ANE-i.r.

Table 1 demonstrates the effects of naloxone and levorphanol given either separately or simultaneously. 0.1 mg/kg naloxone failed to affect the peptide levels, whereas the same dose of levorphanol elevated the concentration of ANE-i.r. Employing naloxone/levorphanol at a 10-fold higher dose (1 mg/kg), there was an elevation of both DYN- and ANE-i.r.; this increase was greater than that found in response to each drug given on its own.

Recovery of peptide levels to the pre-treatment state

A time course study of DYN- and ANE-i.r. responses to the single opiate exposure was made. Figure 6 shows that maximal peptide levels were determined one day after 1 mg/kg levorphanol, and that there was a decline in i.r. to almost normal levels by 8th day after opiate exposure.

Effect of clonidine on DYN- and ANE-i.r.

Table 2 demonstrates that clonidine (1 mg/kg) elevated both DYN- and ANE-i.r. 6 h after its administration. However, at this high dose the blood pressure is extremely low and the animals are unable to move. Lower doses also effectively cause a lowering of the blood pressure, but failed to affect peptide concentrations.

Discussion

The findings reported here suggest that a single treatment of guinea-pigs with an opiate significantly alters the levels of endogenous opioids in the myenteric plexus longitudinal muscle preparation. The fact that not only activation but also blockade of opioid receptors caused an increase of prodynorphin derived peptides in the myenteric plexus throws light upon the function of these neuropeptides in the intestine. From the isolated gut ("Trendelenburg preparation") it is known that DYN is released during peristalsis and that naloxone interferes with intestinal propulsion (Kromer et al. 1981; van Nueten et al. 1976). In postulating a neurotransmitter function of the opioid peptides in the myenteric plexus, feedback mechanisms are likely to operate following the administration of exogenous opiates, resulting in changes in the levels of endogenous opioids. These mechanisms would operate in response to both agonists and antagonists. While neurotransmitter receptor blockade might be expected to result in an elevation of neurotransmitter levels, an increase of transmitter levels following administration of receptor agonists is less plausible. However, it should be noted that another peripheral organ, the pituitary, has been shown to respond to morphine challenge with increased DYN-concentrations; in that preparation, however, β-endorphin levels were found to be reduced (Herz and Höllt 1982). In addition, Kachur et al. (1985) reported an elevation of endogenous opioid-like activity in cerebrospinal fluid upon acute opiate challenge.

The opiate effect of DYN- and ANE-i.r. is characterized by a delay in the onset of the effect (several hours) and by the long duration of the effect. Although the exact underlying

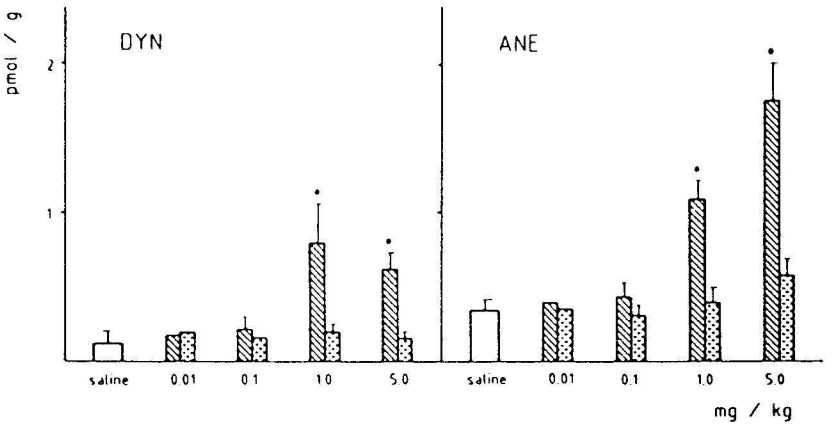

Fig. 4. Dose-dependent effect of (−)-naloxone (▨) and (+)-naloxone (▩) upon DYN- and ANE-i.r. in strips of animals sacrificed 6 h after treatment. For further explanations see Fig. 2

Table 1. Effect of combined application (s.c.) of naloxone and levorphanol on DYN and ANE i.r. (fmol/g)

	Dynorphin A	alpha-Neo-endorphin
Saline	180; 205	330; 395
Naloxone		
0.1 mg/kg	203; 219	410; 385
1.0 mg/kg	630; 840	1200; 910
Levorphanol		
0.1 mg/kg	230; 281	856; 1300
1.0 mg/kg	885; 990	2100; 2430
Naloxone (0.1 mg/kg)		
+ Levorphanol (0.1 mg/kg)	260; 310	803; 920
Naloxone (1.0 mg/kg)		
+ Levorphanol (1.0 mg/kg)	1370; 1560	2800; 2318

6 h after drug injection; data were obtained from 14 guinea pigs; each guinea pig provided a single extract which served to determine a single value for DYN and ANE

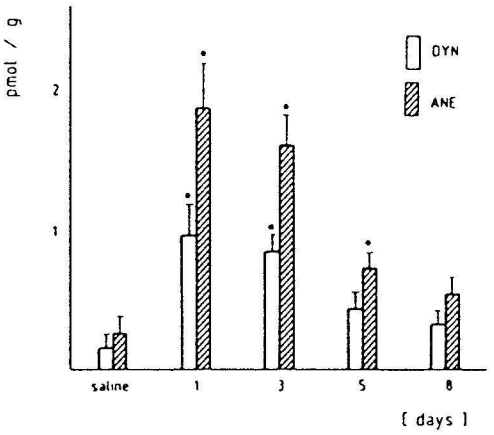

Fig. 6. Recovery of DYN- and ANE-i.r. concentrations in strips of guinea pigs treated with 1 mg/kg levorphanol. Saline injected animals were sacrificed on the 3rd and 8th days after treatment. For further explanations see Fig. 2

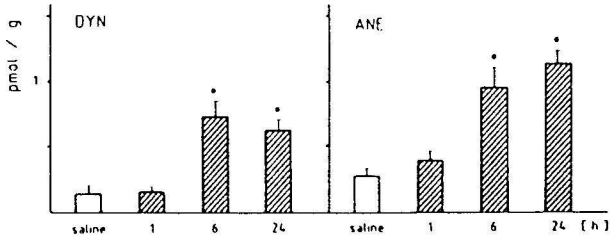

Fig. 5. Time-dependent effect of (−)-naloxone (1 mg/kg) upon DYN- and ANE-i.r. in strips of guinea pigs. For further explanation see Fig. 2

Table 2. Effect of clonidine on DYN- and ANE-i.r. (fmol/g)*

	Dynorphin A	alpha-Neoendorphin
Saline	181; 153	290; 310
Clonidine		
0.01 mg/kg	190; 220	210; 330
0.1 mg/kg	170; 198	243; 310
1.0 mg/kg	430; 480	930; 1080

* Strips prepared 6 h after injection; data were obtained from 8 guinea pigs; for further explanations see Table 1

biochemical mechanisms are not known at present, several speculations may be made. The changes observed may result from: Increased synthesis of the prodynorphin precursor, an altered processing of the percursor molecule, or the attenuated release of the peptide. The notion of an increased rate of protein synthesis is favoured for the following reasons: firstly, use of the 3-E7 monoclonal antibody revealed an overall elevation of high and low molecular weight opioid peptid-i.r.; secondly, there was a delay of several hours before increased peptide levels were observed. Furthermore, the long lasting effect may be indicative of changes in protein- and corresponding mRNA-levels (Thoenen and Barde 1980; Greenberg et al. 1985).

The increase of endogenous opioid peptides seems to be mediated via opioid receptors. This view is supported by the dose- and time-dependence of the effects as well as by the inefficacy of the inactive stereoisomers dextrorphan and (+)-naloxone. However, the commonly applied approach of blocking opioid effects with a specific antagonist did not

work in the classical way in the present experiments. The antagonist, like the agonist, elevated tissue DYN- and ANE-i.r. concentrations. It is surprising that the combined administration of levorphanol and naloxone produced increases in levels of DYN- and ANE-i.r. above those observed when the drugs were injected separately. Since naloxone and levorphanol interfere primarily with μ-receptors, it may be that the rather high naloxone dose applied here (1 mg/kg), resulted in the blockade of κ-receptors too (Paterson et al. 1983). In this case, opioidergic transmission would be more effectively blocked, possibly resulting in an intensification of the feedback regulation.

Our data also show that opioid peptide levels in the myenteric plexus may be increased by non-opiate compounds. Clonidine, an adrenergic agonist, affected the levels of DYN and ANE in a way similar to that found for the opiates. Since clonidine inhibits electrically evoked twitches of the isolated guinea-pig ileum, it seems to exert similar functions to those described for opiates in this preparation. The data are thus in line with the notion that drugs which interfere with peristalsis may affect opioid peptide levels in the myenteric plexus. However, the data reported here conflict with those by Opmeer et al. (1980). These investigators observed no change in enkephalin and β-endorphin concentrations (but a moderate increase of β-endorphin fragments) in the longitudinal muscle myenteric plexus preparation of guinea pigs challenged with morphine. Although we did not measure enkephalin levels, "opioid screening" with the 3-E7 antibody suggested a significant increase in the concentrations of enkephalin upon morphine challenge. These discrepant results may be explained by differences in preparation methods and in the absolute levels reported by Opmeer et al. (300 pmol/g). If the difference results from immunoreactive material not related to the enkephalins, changes in the concentrations of these peptides would be masked. Lastly, we were not able to detect β-endorphin i.r. in this preparation; this raises the question as to whether this neuropeptide is synthesized in the guinea-pig myenteric plexus as suggested by Jessen et al. (1980).

The experiments described here do not prove that the increased peptide concentrations found in the myenteric plexus following a single opiate challenge are indeed generated in this tissue. Alternative explanations may be considered. However, the enteric plexus contains multiple opioid receptors and high concentrations of endogenous opioids (including DYN and ANE). It is, thus, highly probable that the increase of neuropeptide levels results from an activation of peptide synthesis within the myenteric plexus itself.

Acknowledgement. We are grateful to Dr. A. Jacobson (Bethesda, USA) for providing (+)-naloxone. We like to thank Dr. O. F. X. Almeida for stylistic revision of the text. Supported by Deutsche Forschungsgemeinschaft, Bonn (SFB 220).

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Received March 17, 1986/Accepted May 2, 1986