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Characterization of β-Endorphin-Immunoreactivity in Limbic Brain Structures of Rats Self-Administering Heroin or Cocaine

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Abstract—The effects of intravenous self-administration of 30 μg infusions of either heroin or cocaine, or saline on the concentrations of β-endorphin-immunoreactivity (βE-IR) in the anterior part of the rat brain limbic system were studied. Self-administration of heroin and cocaine for 5 daily sessions resulted in a marked reduction of the concentrations of βE-IR in the nucleus accumbens, rostral striatum, septum and hippocampus at the time of the scheduled next session on day 6. In pooled extracts of these regions from rats receiving saline, combined application of high-pressure liquid chromatography (HPLC) fractionation and specific radioimmunoassays revealed the presence of a number of βE-related peptides co-chromatographing with synthetic non-acetylated and acetylated α, β- and γ-type endorphins. Similar profiles were found after HPLC fractionation of extracts of these regions from rats self-administering heroin and cocaine. Rats self-administering heroin or cocaine, however, showed decreased amounts of all detected forms of β-endorphin as compared to saline rats. These findings indicate that both self-administration of an opiate that induces psychic as well as physical dependence and of a non-opiate stimulant inducing psychic but not physical dependence, results in a significant decrease of βE and related peptides in limbic brain regions of the rat. All forms of βE detected after HPLC were equally affected, suggesting an overall effect of the drugs on peptide turnover. These results suggest that βE and related peptides may be involved in the neurochemical mechanisms underlying psychic dependence to drugs.

Introduction

There is indirect evidence suggesting that β-endorphin (βE) may be involved in the biochemical mechanisms underlying drug addiction. Human heroin and alcohol addicts display altered βE-IR levels in cerebrospinal fluid and in plasma (1-6). Rats will press a lever in order to obtain intraventricular injections of βE, suggesting that βE has rewarding and addictive properties (7). The involvement of endogenous opioids in the process

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of drug self-administration, a reliable animal model for drug dependence (8), is suggested by the fact that pretreatment of rats with the opiate receptor antagonists naloxone or naltrexone affects self-administration of both opiate drugs e.g. heroin (9-10) and non-opiate compounds e.g. alcohol and cocaine (11-13). Recently, we have found effects of heroin and cocaine self-administration on the concentrations of βE-IR in a number of rat brain regions (Sweep et al., submitted). Particularly, the βE-IR levels in tissues constituting the anterior part of the rat brain limbic system were decreased. Since the limbic system is thought to be involved in self-administration of both opiate and non-opiate psychoactive compounds (14, 15), these findings point to a role of βE-like peptides in mechanisms underlying drug dependence.

Pro-opiomelanocortin (POMC) is the common precursor molecule for corticotropin (ACTH), α-melanocyte stimulating hormone (α-MSH) and the opioid βE. βE is liberated from this precursor by proteolytic cleavage, and further post-translational processing of this peptide results in the formation of a variety of N^{α}-acetylated and C-terminally shortened endorphins (16-19). A variety of βE-related peptides has been found in the rat brain (20-24). The enzymatic processing of βE and related peptides is paralleled by marked changes in the biological activities of the molecules. The acetylated endorphins are devoid of opiate activity (24, 25). C-terminal shortening of βE results in the formation of α- and γ-type endorphins, peptides with different central nervous system activities, as evidenced by their effects in a variety of behavioral paradigms (26, 27). In the present study we investigated the nature of the βE-IR in the anterior part of the rat brain limbic system of rats self-administering heroin or cocaine.

Materials and Methods

Animals and surgery

Male albino Wistar rats (Cpb:WU, bred from own stock) weighing 200-280 g were used. The animals were anesthetized with Hypnorm^{R} and a silicone rubber tubing was implanted into a jugular vein (28). During the post-operative recovery period of 5 days, the animals were housed individually under a reversed day/night light regimen (12 h dark; 12 h light, lights on at 7.00 p.m.). Three days before the start of the self-administration, animals were partially food deprived in order to obtain a weight reduction of about 20%. Weight reduction was introduced in order to facilitate acquisition of self-administration (29). Standard diet and water were only available in the home cages. Five to seven days after the operation, the self-administration procedure was started. The animals were placed in sound-attenuated two-lever operant chambers during 6 h of the dark period of the illumination cycle (10.00 a.m.-4.00 p.m.) and the cannulae were connected to infusion pumps (28). Pressing the left lever resulted in the delivery of an intravenous injection of 0.25 ml of either 0.125 mg ml^{-1} cocaine hydrochloride or heroin hydrochloride, or saline (pH 7.35). Infusion fluid was only available for 60 injections. The animals were allowed to self-administer drugs during 5 consecutive daily sessions. At day 6, about 18 h after the last session, at the time of the scheduled next session, the animals were taken one by one to an adjacent sound-attenuated room, and decapitated. The brain was quickly removed from the skull and dissected into anatomically defined regions (30). Tissue samples were stored at −80 °C until further processing.

Extraction of βE-IR from tissue

Tissues were heated for 10 min in 1 M acetic acid (1:10, w/v) in a boiling water bath, cooled on ice and homogenized by ultrasound. After centrifugation the supernatants were lyophilized and the residues dissolved in 1 ml phosphate buffered saline (pH 7.4) containing 0.25% (w/v) bovine serum albumin and 0.2% (w/v) sodium-azide (PBSA). βE and related peptides were extracted and concentrated according to the method of Ratcliffe and Edwards (31) with slight modifications. In short, heat activated (24 h at 700 °C) Vycor^{R}-glass powder was added to tissue extracts (35 mg ml^{-1}) and rotated end over end for 4 h at 4 °C. After centrifugation, the supernatants were aspirated and the pellets washed consecutively with 1 ml bidistilled water and 1 ml 1 M HCl. The peptides were eluted from the glass beads by

rotation for 30 min at 4°C with a mixture of acetone/bidistilled water/acetic acid (700 μl, 60:40:1, v/v). The acetone fraction was collected by centrifugation and 600 μl aliquots were dried under a mild nitrogen stream at 50°C. The recovery of the extraction procedure was determined by simultaneous extraction of [^{125}I]βE and amounted to 70-80%. The data were corrected for recovery.

HPLC fractionation of βE-IR

Extracts of nucleus accumbens, rostral striatum, septum and hippocampus were pooled for each experimental group and subjected to HPLC, to achieve a separation of βE and related peptides. The contribution of the βE-IR from each tissue to the βE-IR of the pools was: 21%, 41%, 13% and 25% for nucleus accumbens, rostral striatum, septum and hippocampus respectively, for the saline group, 13%, 36%, 23% and 28% for the heroin group and 18%, 45%, 16% and 21% for the cocaine group. Chromatography was performed using a μBondapack C18 reversed-phase column (Waters Associates, Milford, USA) and an ammoniumacetate/methanol gradient. Extracts (10.4 ml), adjusted to pH 4.15 with HCl, were applied to the column via one of the gradient pumps at a flow of 1 ml min^{-1}. Elution was carried out with 0.01 M ammoniumacetate (pH 4.15) and acidified methanol (0.15% v/v, acetic acid) using a linear gradient running from 0% to 100% methanol in 100 min. The flow rate was 2 ml min^{-1}. Fractions of 30 s were collected in tubes containing 100 μl 0.1% BSA and subsequently evaporated in vacuo at 55°C. Alternating with sample runs, blanc HPLC runs were performed where only ammoniumacetate was injected. No carry over of βE-IR from previous runs was detected in these runs. The retention times of endorphins were determined in separate runs using synthetic peptides. No corrections were made for recovery.

Radioimmunoassays

Dried HPLC fractions were dissolved in PBSA containing 0.1% triton X-100 (PBSAT; RIA buffer). Radioimmunoassays (RIA's) were performed as described elsewhere (32). The antisera used, were raised in rabbits against human β-endorphin-(1-31) ($β_h$E-(1-31); antiserum B4), camel β-endorphin-(1-31) ($β_c$E-(1-31); antiserum X5), γendorphin (γE (βE-(1-17)); antiserum L10), α-endorphin (αE (βE-(1-16)); antiserum A2) and $N^α$-acetyl-γ-endorphin (AcγE; antiserum NAG7).

βE-IR was measured using antiserum B4 directed against the (9-16) sequence of the $β_h$E-(1-31) molecule or antiserum X5 directed against the C-terminal part of $β_c$E-(1-31). Synthetic $β_c$E-(1-31) was used as standard and iodinated $β_c$E-(1-31) as tracer. With the B4 antiserum, the following cross-reactivities (expressed as % on mass basis) were obtained: human β-LPH ($β_h$-LPH), 39%; camel $N^α$-acetyl-β-endorphin-(1-31) (Ac$β_c$E-(1-31)), 100%; camel $N^α$-acetyl-β-endorphin-(1-27) (Ac$β_c$E-(1-27)), 177%; camel β-endorphin-(1-27) ($β_c$E-(1-27)), 120%; γE, 391%; AcγE, 429%; αE, 170%; α-MSH, 0.7%. Cross-reactivity with ACTH and [Met]enkephalin (Met-Enk, βE-(1-5)) was <0.2%. Using the X5 antiserum, the following cross-reactivities were determined: $β_h$-LPH, 85%; Ac$β_c$E-(1-31), 85%; $β_c$E-(1-27), 2.0%; and Ac$β_c$E-(1-27), 0.6%. Cross-reactivity with αE and γE was <0.1%.

γE-IR was assayed with antiserum L10. Synthetic γE was used as standard and iodinated γE as tracer. The cross-reactivities with des-Tyr^1-γ-endorphin (DTγE, βE-(2-17)), des-enkephalin-γ-endorphin (DEγE, βE-(6-17)) were 103.0% and 94.4% respectively. L10 had full cross-reactivity with AcγE (113.3%) and 1.2% cross-reactivity with $β_h$-LPH, 0.3% with $β_c$E-(1-31), and 1.7% with $β_c$E-(1-27), while cross-reactivity with αE was <0.1%.

αE-IR was assayed with antiserum A2 directed against the C-terminus of the αE molecule. Synthetic αE was used as standard and iodinated αE as tracer. The following cross-reactivities were determined: des-Tyr^1-α-endorphin (DTαE, (βE-(2-16)), 140%; des-enkephalin-α-endorphin (DEαE, (βE-(6-16)), 100%; $α_h$-LPH, 0.6%; $β_c$E-(1-31), 1.2%; Ac$β_c$E-(1-31), 1.8%; $β_c$E-(1-27), 0.6% and γE, 6.9%.

$N^α$-acetylated-endorphins were assayed with antiserum NAG7, that specifically recognized the acetylated N-terminus of endorphins. AcγE was used as standard and iodinated AcγE as tracer. The following cross-reactivities were determined:

Acβ_cE-(1-31), 100%; Acβ_cE-(1-27), 35%; AcαE, 92%; β_h-LPH, 0.01%; β_cE-(1-31), 0.04%; γE, 0.3%; αE, 1.8%; Met-Enk, 0.2% and 0.04% with ACTH. All samples were assayed in duplicate.

Chemicals

Synthetic β_cE-(1-31), β_cE-(1-27) and Acβ_cE-(1-27) were obtained from Bachem Feinchemikalien AG, Switzerland. Synthetic Acβ_cE-(1-31) from Peninsula Lab. Inc. CA, and β_h-LPH from Dr F. Facchinetti, University of Modena, Italy. All other peptides used were synthesized and kindly provided by Organon International B.V., Oss, The Netherlands. All chemicals used were of analytical grade. The antiserum NAG7 was kindly donated by Drs S. Watson and H. Akil, Ann Arbor, USA.

Results

During 5 consecutive daily sessions of 6h rats were allowed to self-administer intravenous 30 μg infusions of either heroin or cocaine, or saline. The number of self-injections by rats receiving heroin or cocaine was considerably higher than that by rats receiving saline (mean number of infusions during the self-administration sessions on day 4 and 5; saline 37.4 ± 9.6 (n = 24)); heroin 66.6 ± 9.1 (n = 13) ($p < 0.05$ vs. saline); cocaine 68.7 ± 9.1 (n = 24) ($p < 0.05$ vs. saline).

The concentrations of βE-IR in the nucleus accumbens, rostral striatum, septum and hippocampus of control rats (receiving saline) as determined with the B4-antiserum amounted to 1.51 ± 0.18 ng/tissue, 4.40 ± 0.92 ng/tissue, 4.43 ± 0.65 ng/tissue and 4.05 ± 0.48 ng/tissue respectively. Rats receiving heroin or cocaine showed significantly decreased concentrations of βE-IR in the nucleus accumbens (−47.3% in the heroin group and −36.6% in cocaine group, $F(2, 45) = 8.69$, $p < 0.01$), rostral striatum (−52.9% and −38.2%, $F(2, 45) = 5.41$, $p < 0.01$), septum (−49.4% and −24.1%, $F(2, 44) = 4.82$, $p < 0.05$) and hippocampus (−29.4% and −23.6%, $F(2, 44) = 3.49$, $p < 0.05$).

To characterize the molecular forms of βE and related peptides in these tissues, extracts of nucleus accumbens, rostral striatum, septum and hippocampus were pooled, subjected to HPLC and the resulting fractions were analyzed with specific radioimmunoassays. In tissue extracts from rats receiving saline 7 peaks were resolved that reacted with the antiserum recognizing the midportion of the βE molecule (antiserum B4; Peaks I-VII, Fig 1A). The peaks eluted with retention times of synthetic αE (peak I), AcαE (II), γE (III), AcγE (IV), β_cE-(1-31) (V). Peak VI eluted with the retention time of synthetic Acβ_cE-(1-31) and peak VII with the retention time of synthetic N^α-acetyl-β-endorphin-(1-26) (AcβE-(1-26)) and Acβ_cE-(1-27), peptides that were not resolved in the present HPLC system. In the HPLC fractions 85% of the βE-IR (antiserum B4) of the initial tissue extract was recovered. Peaks I and II showed complete cross-reaction with αE (A2) antiserum (Fig 1B), indeed suggesting that they contained α-type endorphins. Peaks III and IV completely cross-reacted with γE (L10) antiserum (Fig 1C), while peaks V and VI cross-reacted with the antiserum directed against the C-terminus of the βE-(1-31) molecule (X5; Fig 1D). Peaks II, IV, VI and VII showed complete cross-reaction with the antiserum recognizing AcE's (NAG7; Fig 1E) suggesting that these peaks contained the acetylated forms of αE, γE, βE-(1-31) and βE-(1-27) and/or βE-(1-26) respectively. Thus based on reactivity in the different RIA-systems for αE's, γE's, βE's and N^α-acetylated endorphins, and their retention times as compared to synthetic peptides, the endorphins constituting the βE-IR in the tissue extracts of the brain regions from animals receiving saline were characterized as αE (peak I), AcαE (II), γE (III), AcγE (IV), βE-(1-31) (V), AcβE-(1-31) (VI) and AcβE-(1-27) and/or AcβE-(1-26) (VII).

In Figure 2 the HPLC profiles of equal volumes of pooled extracts of nucleus accumbens, rostral striatum, septum and hippocampus of rats that had self-administered heroin or cocaine and of the controls receiving saline, are presented. All peaks of βE-IR detected in the saline group (Fig 2A) were also present in the other groups. All the peaks, however, appeared to be lower in extracts of rats that had self-administered heroin (Fig 2B), or cocaine (Fig 2C). The decrease was more pronounced in case of heroin than in case of cocaine. Under both conditions, all peaks seemed to be equally affected, and quantitatively the

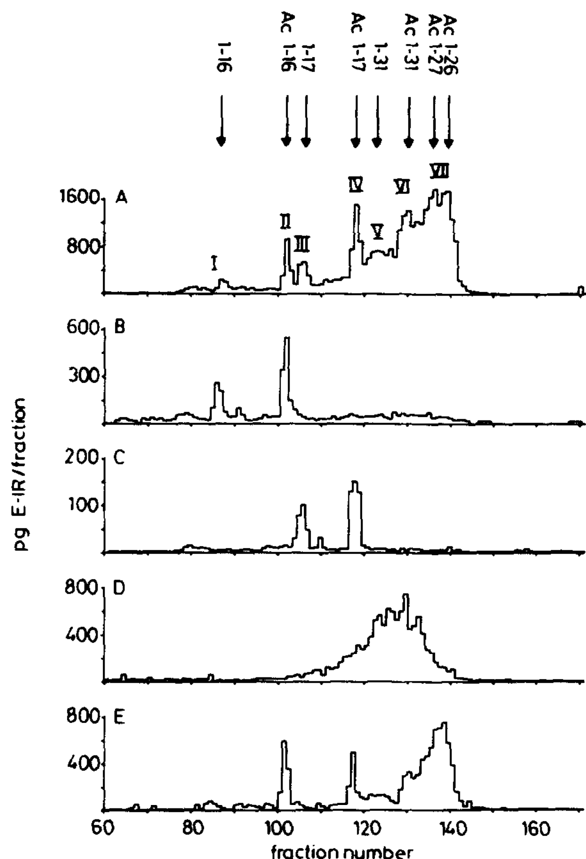

Fig 1 HPLC fractionation of βE-IR from pooled extracts of nucleus accumbens, rostral striatum, septum and hippocampus from saline rats. Fractions were analyzed by radioimmunoassays using antisera recognizing the midportion of βE (antiserum B4; panel A), the C-terminus of αE (A2; B), γE (L10; C) or β_cE-(1-31) (X5; D), and the N-terminus of acetylated endorphins (NAG7; E). Peaks are indicated with Roman numerals (Panel A; peak I-VII). The elution positions of synthetic reference β-endorphin sequences are indicated at the top. Each value represents the mean of duplicate determinations. Data are expressed as pg/fraction. No corrections were made for recovery of HPLC procedure, nor for cross-reactivity of peaks in the RIA's.

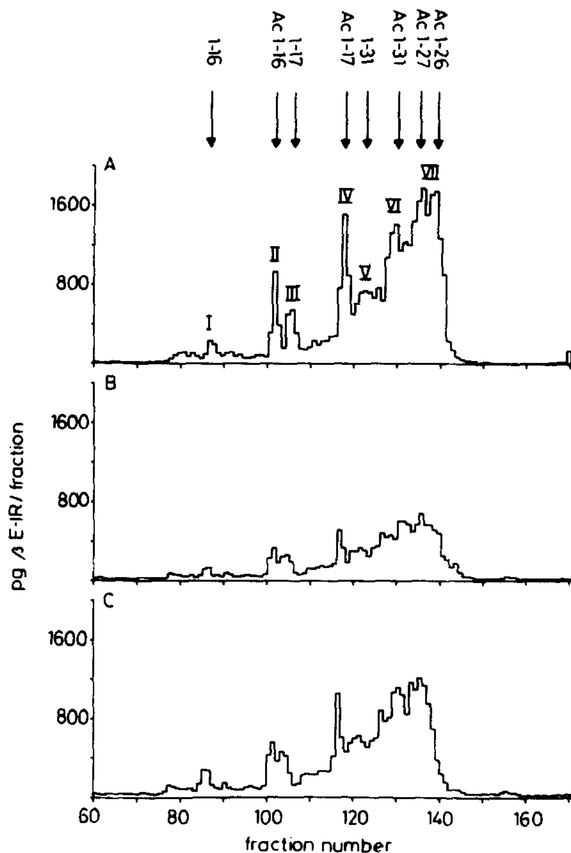

Fig 2 HPLC fractionation of βE-IR from pooled extracts of nucleus accumbens, rostral striatum, septum and hippocampus from rats that had self-administered saline (panel A), heroin (B) or cocaine (C). Fractions were assayed by radioimmunoassay using an antiserum (B4) directed against the midsequence of the β-endorphin molecule. Assays were performed in duplicate. Peaks are indicated with Roman numerals. At the top elution positions of synthetic reference β-endorphin sequences are shown. Data are expressed in pg/fraction. No corrections were made for recovery of chromatography.

decrease found after HPLC was comparable to that observed by direct assay of total βE-IR in the tissue extracts.

Discussion

The present findings demonstrate that self-administration of heroin and cocaine results in decreased levels of βE-IR in the nucleus accumbens, rostral striatum, septum and hippocampus. These anterior parts of the limbic system have been implicated in self-administration of both opiate and non-opiate psycho-stimulants (14, 15). Previously, we showed that βE-IR levels in other brain areas are not affected by heroin or cocaine self-administration (Sweep et al., submitted). It is generally assumed that, unlike heroin, the non-opiate cocaine does not induce physical dependence, as evidenced by the absence of withdrawal symptoms after drug discontinuation. Since the present effects of cocaine were closely similar to that of heroin, the alterations in βE-IR levels in

the brain tissues, are probably not related to physical dependence. Both drugs induce psychic dependence, a common denominator of dependence on various drugs, which may be assessed by the self-administration procedure (8). Thus, the similar effects of heroin and cocaine self-administration on the βE-IR levels in the anterior brain limbic system, suggest a general role for βE-like peptides in the biochemical mechanisms underlying psychic dependence on addictive drugs.

In the brain, βE and related peptides are synthesized as components of POMC in neurons originating in the nucleus arcuatus of the hypothalamus (33, 34). From this region βE containing axons project to many regions throughout the brain (35, 36). In the past few years a large number of β-endorphin related peptides have been identified in rat brain (21, 23, 37, 38). In the present study we employed an antiserum recognizing the aminoacid sequence (9-16) of the β-endorphin molecule, that detects βE-(1-31) as well as its N^{α}-acetylated and C-terminally shortened forms (e.g. βE-(1-27), βE-(1-26), γE, αE). Thus the βE-IR measured in extracts of brain tissues with this antiserum likely results from binding of a heterogenous pool of β-endorphin derived peptides to this antiserum. Indeed, HPLC analysis of the βE-IR in pooled extracts of the nucleus accumbens, rostral striatum, septum and hippocampus revealed a number of endorphins, with the chromatographic properties of αE, AcαE, γE, AcγE, βE-(1-31), AcβE-(1-31) and AcβE-(1-27) and/or AcβE-(1-26) as is shown in Figure 1A. Using specific antisera for α-, γ-, β- type-endorphins and AcE's we could confirm the identity of these peptides (Fig. 1B-1E). Clearly, the acetylated forms of the endorphins constitute the major portion of the IR in these brain regions, which is in line with previous reports (22-24, 37).

Self-administration of both the opiate heroin and the non-opiate cocaine resulted in decrease tissue concentrations of all β-endorphin-related peptides detected after HPLC. These effects fully accounted for the differences in total βE-IR in tissue extracts of animals receiving heroin or cocaine. No selective effects of either treatment on the occurrence of certain peptides were found. This suggests, that self-administration of heroin or cocaine had not induced selective effects on certain steps of the enzymatic processing of βE or the release of certain peptides in these tissues, but that the effects may represent overall changes in turnover of βE and related peptides.

Processing of βE and releated peptides results in marked changes in the biological activities of the peptides. N^{α}-acetylation results in complete loss of opiate activity (24, 25), while C-terminal truncation has also profound effects on the biological activities of endorphins. Removal of the C-terminal (28-31) sequence from βE-(1-31) results in the formation of βE-(1-27), which may be an endogenous antagonist for βE-induced analgesia (39). It has been postulated that α- and γ-type endorphins play a role as functional antagonists in maintaining homeostasis in the central nervous system. The activities of α-type endorphins resemble those of psycho-stimulants, whereas γ-type endorphins have neuroleptic-like properties (27). Presently, one can only speculate about the functional relevance of these different forms of endorphins in the brain for the self-administration properties of heroin and cocaine.

In conclusion, self-administration of both the opiate heroin and the non-opiate cocaine, resulted in markedly decreased levels of β-endorphin and its opiate-active and opiate-inactive fragments in regions of the limbic forebrain that are considered to be involved in the process of self-administration of psychoactive drugs. These data suggest that these peptides may play an important role in the neurochemical mechanisms underlying drug dependence.

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