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Electrospray ionization mass spectrometric and liquid chromatographic–mass spectrometric studies on the metabolism of synthetic dynorphin A peptides in brain tissue in vitro and in vivo

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Abstract

Metabolic stability of synthetic dynorphins [N-terminal fragments of dynorphin A (Dyn A)] were evaluated in vitro and in vivo. These peptides were applied at concentrations 100–1000 times higher than those of the endogenous dynorphins. Degradation kinetics of these peptides were studied in rat brain homogenate by using microbore gradient RP-LC assay, and limited information on their metabolism was obtained by electrospray ionization mass spectrometry (ESI-MS) of the isolated metabolites. In vivo cerebral microdialysis, in which the peptides were introduced via the probe placed in striatum region of the brain of the experimental animals, was used to circumvent contamination arising from autoproteolysis of brain during incubation of the samples in vitro. Metabolites of Dyn A (1-13) and Dyn A (1-11) were identified from electrospray ionization mass spectra of the microdialysates without chromatographic separation; the identification of peptides in the mixtures were supported by medium resolution ESI Fourier-transform ion cyclotron resonance MS. LC–MS was used to fully characterize the complex peptide mixture obtained after the striatal perfusion of Dyn A (1-12). © 1998 Elsevier Science B.V.

Keywords: Mass spectrometry; Peptides; Dynorphins; Neuropeptides

1. Introduction

The dynorphin (Dyn) family of neuropeptides [1] is considered the endogenous ligands for the κ-opioid receptor [2]. Dyn-like peptides have effects on many physiological and behavioral parameters such as pain perception, pituitary hormone release, blood pressure, body temperature, drinking and feeding [3]. The endogenous Dyn A (1-17), whose structure is Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro–Lys–Leu–Lys–Trp–Asp–Asn–Gln according to the three-letter abbreviation of the amino acid residues, and its synthetic fragments have also shown to attenuate opiate withdrawal and antinociceptive tolerance in experimental animals [4]. In particular, Dyn A (1-13) [5] has been effective in various animal models [6–9], and has been evaluated for the management of opioid withdrawal in heroin addicts [9]. Therefore, these peptides have been considered potential neuropharmaceuticals [10].

Neurochemical and pharmacological effects of various dynorphins have been evaluated by directly

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delivering the peptides into the brain of experimental animals [11,12]. Administration of these peptides causes increase in the extracellular levels of glutamate and aspartate [11], and they produce body temperature changes and analgesia in rats [12]. Because of the rapid breakdown of dynorphins, it may be critical to examine their metabolism in brain tissue to correlate with the observed neurochemical and pharmacological effects [13]. The involvement of various peptidases in the metabolism of dynorphins in the central nervous system (CNS) has been shown [14–16]. Metabolism can alter the pharmacological profile of these peptides, and their action may be mediated through conversion to metabolites [17]. However, most neurochemical and CNS pharmacological studies on dynorphins have not included experiments probing their eventual metabolism under the specific experimental conditions.

Incubation in brain homogenate [18], intact pieces of brain [4] or appropriate tissue fraction [19] can be used to evaluate the possible fate of neuropeptides in the CNS in vitro, because both soluble and membrane-bound peptidase will probably cleave neuropeptides. Dyn A (1-13) is rapidly degraded by membrane-bound exopeptidases [20] in rat membrane preparations. Tissue homogenates, however, contain many cytosolic peptidases to which the peptides may not be exposed during in vivo pharmacological evaluation, and autoproteolysis of the tissue may represent a serious challenge to most techniques used for the subsequent analysis of peptide metabolites [19]. The in vitro approaches has recently been complemented by in vivo techniques such as ventriculocisternal perfusion [14] and microdialysis to circumvent this problem.

Cerebral microdialysis combined with mass spectrometry (MS) is especially attractive for studying CNS metabolism of peptides [21], because it enables continuous monitoring of biochemical events in brain extracellular tissue space in vivo, and provides positive identification of the metabolites through the high molecular specificity of the spectrometric method. In this approach, the peptide dissolved in the perfusion medium is continuously delivered into the brain through the semipermeable membrane of a microdialysis probe, and the extracellular metabolites that enter the probe from the tissue are simultaneously collected in the dialysates. Microbore LC was used off-line to separate the obtained peptide mixture for subsequent electrospray ionization mass spectrometry (ESI-MS), or a reversed-phase (C_{18}) packed fused-silica capillary was applied for on-line desalting of the microdialysates prior analysis. However, the peptides were eluted from the column with no or minimal chromatographic separation, and identification of the metabolites were done from a single (averaged) mass spectrum. Synthetic dynorphins are highly cationic (due to multiple Arg and Lys residues), and doubly- and triply-charged ions are commonly obtained during ESI [22]. Complex peptide mixtures obtained from studies on the brain-metabolism of these peptides may, therefore, produce complicated mass spectra and chromatographic separation prior to ionization and mass analysis may be beneficial.

In this study, we evaluate in vitro and in vivo approaches to investigate the metabolic fate of selected synthetic dynorphins in the brain under conditions commonly used in neurochemical and CNS pharmacological studies involving these peptides [11,12]. Our primary method for identifying peptides is ESI-MS. We also demonstrate the benefits of on-line liquid chromatography (LC)–ESI-MS that simplifies the task of identifying peptide metabolites present in complex mixtures produced in these experiments.

2. Experimental

2.1. Chemicals

Dyn A (1-13), (1-10), (1-6) and Leu-enkephalin were obtained from Sigma (St. Louis, MO, USA), Dyn A (1-12) and (1-11) were purchased from Peninsula Labs. (Blemont, CA, USA), and Dyn A (2-13) was acquired from Bachem Bioscience (King of Prussia, PA, USA). Each peptide was of >98% purity based on gradient reversed-phase (RP) LC. Water was purified by ion-exchange and filtering through active-carbon packed cartridges. Acetonitrile was of HPLC grade, other chemicals were of analytical reagent grade and were obtained from Fisher Scientific (Pittsburgh, PA, USA).

2.2. In vitro metabolism studies

For in vitro metabolism studies, about 100 nmol of peptide was dissolved in 1 ml rat brain homogenate (20%, w/w, in pH 7.4 phosphate buffer), and the mixtures was incubated in a temperature-controlled shaking water bath at 37°C. Samples (100 μl) were removed periodically for analysis and added to 100 μl aqueous 5% (w/v) $ZnSO_4$ and 5% AcOH (v/v) solution for protein precipitation. After centrifugation at 12 500 *g* for 10 min, the supernatant was removed and analyzed by gradient microbore RP-LC, and the metabolites were collected for ESI-MS.

2.3. In vivo cerebral microdialysis in rats

In vivo cerebral microdialysis experiments were done after modifying a protocol described elsewhere [21]. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Florida, and were consistent with the National Institutes of Health guidelines detailed in the Guide for the Care and Use of Laboratory Animals and the Federal Animal Welfare Act. Briefly, a CMA/12 microdialysis probe (CMA, Acton, MA, USA) was used both to introduce the peptide into the brain and to collect metabolic products. Male Sprague-Dawley rats (300–350 g body mass) were used throughout the experiments. The implantation of a guide cannula was done after the animal is completely anesthetized (sodium pentobarbital, 100 mg/kg, intraperitoneally). The top of the head was shaved and a midline cut was made through the skin. Fascia over the skull was scraped away, and the cut was protracted. With a hand-held dental drill, a hole was drilled through the skull, and a guide cannula was inserted into the striatum (anterior–posterior +2.4 mm, medial-lateral +3.0 mm to bregma and sagittal suture, −6.5 mm dorsal–ventral depth) [23]. The insertion of the microdialysis probe guide was done by using a stereotaxic frame equipped with a micromanipulator. Two additional holes were also drilled posterior to the cannula for stainless steel anchor screws. The guide cannula was held permanently in place with cranioplastic cement filling the protracted area. The actual process of insertion of the probe into the brain did not start until we had allowed adequate time (3–5 days) for healing after the implantation of the guide cannula.

After inserting the microdialysis probe into the guide, the animal was placed into an animal containment unit (BAS, West Lafayette, IN, USA). Unlike in the reference protocol [21], the animals were conscious and unrestrained. An artificial cerebrospinal fluid (CSF, 146 m*M* Na^+, 2.7 m*M* K^+, 155 m*M* Cl^-, 1.2 m*M* Ca^{2+} and 1.0 m*M* Mg^{2+}) was perfused through the probe via a 1-ml syringe pump (BAS) at 0.8 μl/min for 2 h. Then a liquid switch was used to start perfusion with the peptide solution (100 to 1000 pmol/μl in artificial CSF, 0.8 μl/min flow-rate; both optimized during a series of experiments varying Dyn A 1-13 concentration and perfusion rate) via a second syringe pump without disrupting the flow to the probe. The concentration of dynorphins in the perfused solution were selected based on protocols related to neurochemical and pharmacological studies [11,12] which also employed microdialysis for the delivery of these peptides into the brain. Upon perfusion of the peptide solution, no apparent changes in the animals' behaviour were observed. After equilibration for 30 min, the sample was collected for 60 min into a HoneyComb (BAS) refrigerated fraction collector. The amount (flux, *J*) of peptide entering the brain during perfusion was determined by microbore HPLC analysis for the loss of the compound from the probe as follows:

$$J = (c_{in} - c_{out})V$$

where, c_{in} is the peptide concentration entering the probe (pmol/μl), c_{out} is the peptide concentration in the microdialysate (pmol/μl), and *V* is the perfusion rate (μl/min). The percentage of the perfused peptide that ended up in the brain (*R*, %) was calculated as [21]:

$$R, \% = (c_{in} - c_{out})/c_{in} \cdot 100$$

Control experiments were also done to confirm the chemical stability of compounds during microdialysis. The peptide solution was perfused through probes placed into a small volume (50 μl, glass autosampler insert) of artificial CSF at 37°C under dialysis conditions identical to those of the in vivo experiments.

2.4. Sample preparation

For ESI-MS and LC–ESI-MS, the supernatant or microdialysate was transferred into a pre-wetted (2 ml methanol, then 2 ml aqueous 3% acetic acid), 1-ml Supelclean LC-18 cartridge (Supelco, Bellefonte, PA, USA), washed with 2 ml 3% (v/v) aqueous acetic acid, then eluted with 300 μl methanol–water–acetic acid (69:28:3, v/v/v) solution to obtain salt-free samples.

2.5. Chromatography

Microbore RP-LC analyses were done on a system consisting of a ThermoSeparation–SpectraPhysics (Fremont, CA, USA) SpectraSERIES P200 binary gradient solvent delivery system, a Rheodyne (Cotati, CA, USA) Model 7125 injector valve equipped with a 5-μl sample loop, a Spectroflow 757 variable-wavelength UV–Vis detector (Kratos Analytical, Manchester, UK) operated at 216 nm, and a Hewlett-Packard Model HP 3395 computing integrator (Palo Alto, CA, USA). A 30 cm×1.0 mm I.D. Supelcosil LC-18 (d_p=5 μm) reversed-phase column (Supelco) was used at a flow-rate of 50 μl/min that was maintained by using a dynamic split (the flow was split before the injection valve, and a 25 cm× 4.6 mm I.D. balance column was operated in parallel with the microbore column). The mobile phase was mixed from 0.1% (v/v) trifluoroacetic acid (TFA) in water and 0.08% (v/v) TFA in acetonitrile, and gradient elution was done from 5% to 95% of organic modifier that changed in a linear profile at a rate of 0.5%/min or 1%/min. HPLC calibration curves for determining concentrations were obtained by adding known amount of peptide into aliquots of brain homogenate that had been transferred into ice-cold aqueous 5% (w/v) $ZnSO_4$ and 5% (v/v) acetic acid solution and analyzing the supernatant after centrifugation. Concentration–time profiles were analyzed by exponential fitting, assuming a pseudo first-order degradation. Half-lives ($t_{1/2}$) were calculated from the rate constants (k) as $0.693/k$.

For LC–ESI-MS, a packed capillary HPLC system was applied. Two Model 2150 pumps was controlled by a Model 2152 controller (both from Pharmacia, Bromma, Sweden). The 0.5 ml/min flow-rate from the pumps was reduced to ca. 6.3 μl/min by an Acurate AC-70 (LC Packings, San Francisco, CA, USA) mixer-splitter device before entering the injector. A Model 7125 injector with a 10-μl loop (Rheodyne) was used. A 15 cm×0.3 mm I.D. packed C_{18} (d_p=5 μm) capillary column (LC Packings) was used. The mobile phase was proportioned by controlling the operation of the two pumps; one that delivered water–acetonitrile (95:5, v/v) containing 0.1% (v/v) TFA (A), and another that supplied water–acetonitrile (5:95, v/v) containing 0.05% (v/v) TFA (B). Analytes were eluted by a linear gradient from 0% B to 100% B in 45 min.

2.6. Mass spectrometry

ESI-MS without chromatographic separation was done by using a Vestec 200 ES instrument (PerSeptive Biosystems, Framingham, MA, USA). Ionization was achieved by applying a potential of 2.7 kV to a flat tipped (120 μm I.D.) stainless steel needle. The sample solution was delivered to the needle by a syringe pump at 5 μl/min flow-rate. A flat stainless steel plate with a 0.4-mm orifice functioned as the counterelectrode (nozzle) in the probe insertion tube. The source block was heated to 250°C to reach 55–60°C in the spray chamber. The collimator was held at 10 V, and the repeller potential was maintained at 16 V. A Vector/Two data system (Teknivent, St. Louis, MO, USA) installed on an IBM-compatible (486, 33 MHz) personal computer was used to control the mass spectrometer and acquire mass spectra between m/z 200 to 2000. Mass spectra were acquired with a scan speed of 3 ms/Da. The mass scale was calibrated by using multiply-charged positive ions from electrosprayed horse-heart myoglobin (M_r 16 951; Sigma) dissolved in aqueous methanolic solution containing acetic acid (3%).

LC–ESI-MS and MS–MS analyses were performed with a Quattro (Micromass, Altrincham, UK) instrument under the control of the manufacturer's Lab-Base data system. The source temperature was 65°C, and the cone voltage was held at 38 V. The mass spectrometer was scanned from m/z 200 to 1600 in 10 s. The mass scale was calibrated by using multiply-charged positive ions from horse-heart myoglobin. Tandem mass spectra were obtained by collision-induced dissociation in the radiofrequency (RF) only (second) quadrupole analyzer pressurized

with argon (to 0.12 Pa) and using 55 to 65 eV collision energy.

Electrospray Fourier-transform ion-cyclotron resonance mass spectrometry (ESI-FTICR-MS) experiments were done on a Finnigan FT/MS (Madison, WI, USA) Newstar system operating at 3.0 T with the standard dual-trap configuration and fitted with the manufacturer's Ultrasource I ESI accessory. Experimental control, data processing and spectral interpretation were accomplished by the Odyssey (Finnigan FT/MS) software running on a Sun Microsystems (Mountain View, CA, USA) workstation. External calibration for accurate mass measurement at medium resolving power (~15 000) was done by using monosodiated molecular ions of poly(methylmethacrylate) (average molecular mass 1830, Polymer Labs., Shropshire, UK) obtained by ESI (the calibrant was sprayed with methanol–tetrahydrofuran solution containing 0.1 m*M* NaI). The syringe pump delivering the sample was operated at 2 μl/min flow-rate.

3. Results

Metabolism of synthetic dynorphins have been studied in 10% brain homogenate in vitro. Kinetic data on their degradation in brain homogenate are given in Table 1. Identification of the major metabolites by collecting fractions after gradient microbore RP-LC only revealed certain major, relatively long-lived metabolites, as shown in Fig. 1. The smaller or rapidly appearing then disappearing metabolites could not be isolated in amounts necessary for positive identification by their ESI mass spectra. The tissue also gave numerous peaks that interfered with the HPLC analysis, and this interference increased with the time of incubation. Even after on-line LC–ESI-MS analysis, identification of many metabolites were possible only via systematic screening for their presence (i.e., displaying reconstructed ion chromatograms of the expected metabolites), and several samples had to be analyzed for complete metabolic profiling.

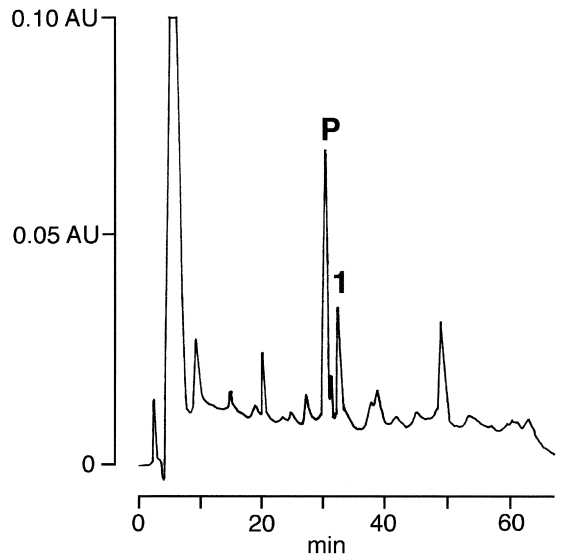

Fig. 1. Microbore HPLC analysis of the degradation products from incubation of Dyn A (1-13) (Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro–Lys–Leu, YGGFLRRIRPKLK) in rat brain homogenate (20%, w/w, pH 7.4, 37°C). HPLC conditions: 30 cm×1.0 mm I.D. Supelcosil LC-18 column, 50 μl/min flow-rate, injection volume 5 μl, gradient elution from 5 to 95% acetonitrile (0.1 to 0.08% TFA) at 1%/min, UV detection at 216 nm. P: Parent peptide (Dyn A 1-13), 61 pmol/μl homogenate; 1: identified as Dyn A (1-12) based on ESI mass spectrum (*m*/*z* 740.4 and 492.9; M_r 1477.3), 28 pmol/μl homogenate.

During microdialysis, 10 to 15% of the perfused

Table 1
Stability of selected synthetic dynorphins in rat brain homogenate at pH 7.4 and 37°C (determined by microbore RP-LC assay, conditions as in Fig. 1 Sections 2.2 and 2.5)

Peptide	Rate constant (min^{-1})	Half-life (min±S.E., $n=3$)
Dyn A (1-13)	0.24	2.9±0.7
Dyn A (1-12)	0.13	5.6±0.5
Dyn A (1-11)	0.47	1.5±0.5
Dyn A (1-10)	0.48	1.5±0.5
Dyn A (2-13)	0.23	3.0±0.8
Dyn A (1-5)[a]	1.39	0.5±0.2

[a] Leu-enkephalin (YGGFL), natural brain peptide [1].

peptide was delivered to the tissue (based on calculating *R*, %). The maximum peptide flux entering the brain in our experiments was, therefore, 120 pmol/min. ESI mass spectra obtained from the microdialysates collected under these conditions revealed the presence of numerous brain metabolites, as shown in Fig. 2 (these products were not present in the corresponding control samples obtained by perfusion of the probes with artificial CSF containing no peptide). Several major metabolites of Dyn A (1-13) and Dyn A (1-11) could be identified based on their molecular ions ($[M+H]^+$, $[M+2H]^{2+}$ and/or $[M+3H]^{3+}$) from the mass spectra of the peptide mixtures obtained. The assignment of these molecular ions to the particular peptides were supported by ESI-FTICR-MS; this technique afforded resolution high enough to resolve the isotope peaks and, thus, allowed the unequivocal verification of the number of positive charges of the ion. Fig. 3 shows selected ions in the ESI-FTICR mass spectrum of the sample obtained after perfusion of the probe with Dyn A (1-13). The 0.5 u difference between the isotope peaks clearly indicates that they were doubly-charged ions. The high mass accuracy of FTICR-MS (<10 ppm with external calibration) also allowed us to compute molecular formulae for the compounds present in the samples after perfusion of the peptide solution into rat brain by microdialysis. The measured monoisotopic mass of the perfused peptide was 1605.009 u with two protons attached; this represented a +6.1 ppm error based on the theoretical value of 1604.998 u. The tentative major metabolite gave a measured monoisotopic mass of 1476.915 u. The molecular formula calculation for ^{12}C (0-75 atoms), ^{1}H (0-128 atoms), ^{16}O (0-15 atoms) and ^{14}N (0-24 atoms) at an error of 10 ppm produced 12 possibilities. On the other hand, subjecting the mass difference (128.094 u) between the measured mass of the infused peptide (1605.009 u) and that of its in vivo metabolite (1476.915 u) to molecular formula calculation (also at 10 ppm error allowed) gave a single composition: $C_6H_{12}N_2O$, which corresponds to the mass of a Lys residue (−7.5 ppm error). The only plausible metabolite obtained from Dyn A (1-13) was, obviously, Dyn A (1-12) due to cleavage by a carboxypeptidase. Although similar calculation for the mass difference (369.272 u) between the measured mass of the infused peptide (1605.009 u) and that of its other in vivo metabolite (1235.737 u) shown in Fig. 3 gave 19 possible compositions, only one possibility ($C_{18}H_{35}N_5O_3$, the removal of a Leu and two Lys residues, −5.3 ppm error) remained when the list was compared to the mass differences obtained after considering amino acid residues (three or four) removed from the parent peptide. From the sequence of Dyn A (1-13), the only plausible metabolic product was Dyn A (1-10). Comparisons of the isotopic patterns for the formulae of the metabolites (shown in insets of Fig. 3) with the

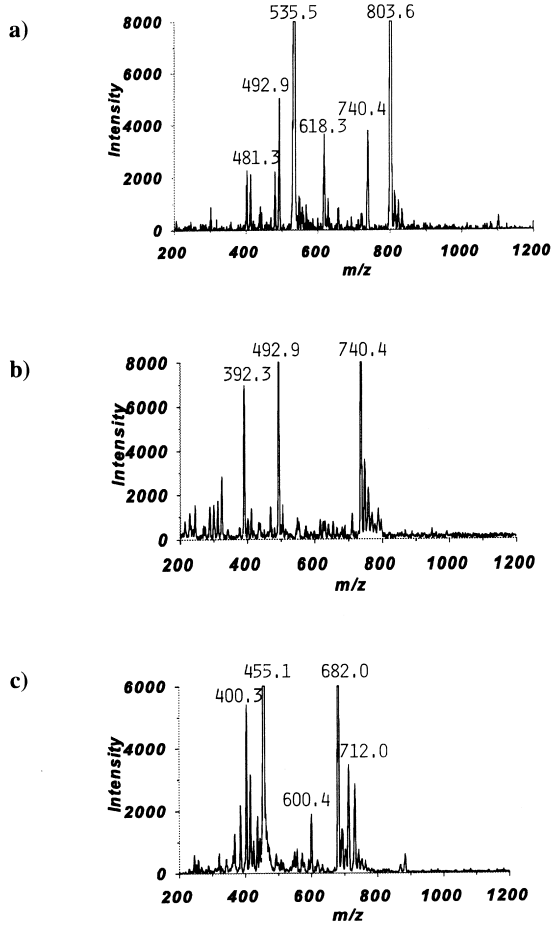

Fig. 2. ESI-MS analysis of the microdialysates collected by perfusion of the probes with (a) Dyn A (1-13), (b) Dyn A (1-12) and (c) Dyn A (1-11). Perfusion conditions: 1 nmol/μl peptide in artificial CSF, 0.8 μl/min flow-rate, sample collected 30 min after starting peptide; duration: 60 min. The dialysates were desalted on a reversed-phase cartridge (1-μl Supelclean LC-18) before analysis.

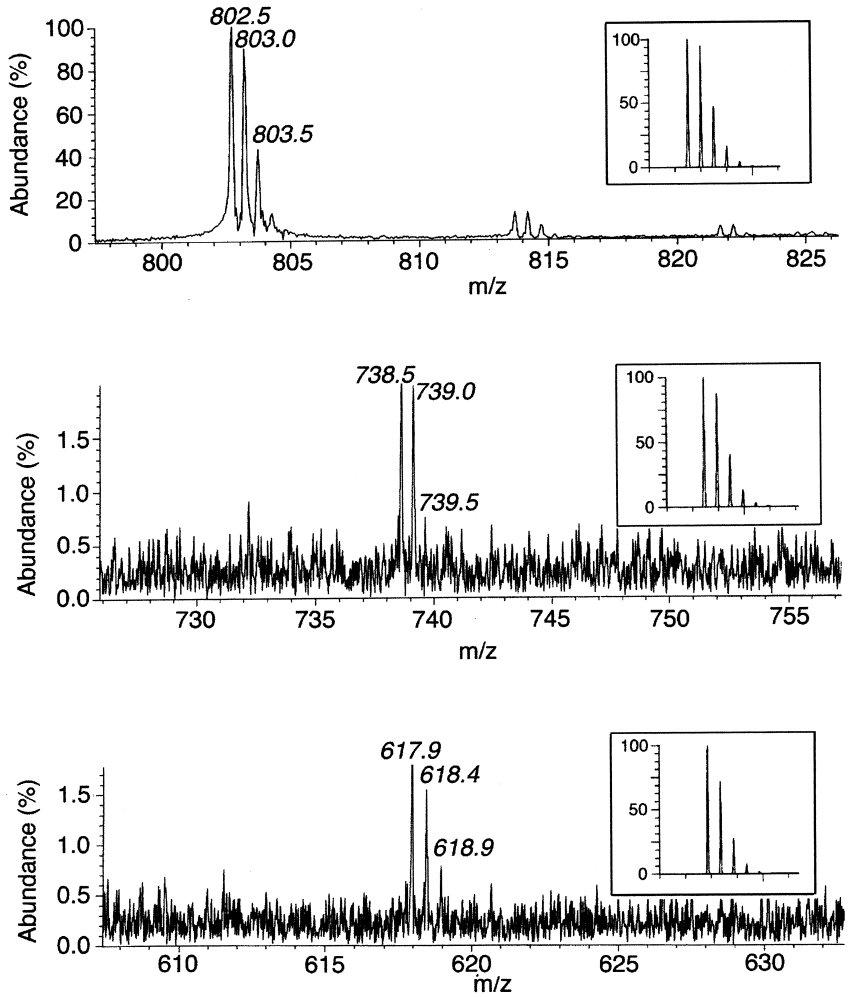

Fig. 3. Medium resolving power (FTICR) ESI mass spectra of ions selected from the spectrum shown in Fig. 2a [sample obtained after the perfusion of the probe with Dyn A (1-13) solution]. Insets: predicted isotopic patern for Dyn A (1-13), Dyn A (1-12) and Dyn A (1-10), respectively.

experimental patterns [24] also supported our assignment. Prominent ESI ions identified in the microdialysates of Dyn A (1-13), Dyn A (1-12), and Dyn A (1-11) are given in Table 2. Shallow-gradient (0.5%/min increase in the organic modifier of the mobile phase) microbore RP-LC analyses were also used to confirm the presence of the major metabolites for Dyn A (1-13) and Dyn A (1-11) in the microdialysates based on coelution with the corresponding synthetic peptides.

The metabolic fate of Dyn A 1-12 was evaluated by using an RP-LC–ESI-MS analysis, because direct analysis of its microdialysate (i.e., no chromatographic separation) yielded an extremely complex mass spectrum (Fig. 2b) due to the large number of primary and secondary metabolites formed in vivo. No complete separation of the metabolites was achieved by chromatography, but the number of peptides whose elution overlapped during the acquisition of the successive scans usually did not exceed three. Thus, deconvolution of the mass spectra to obtain relative molecular masses of the cleavage products was straightforward. We also confirmed the assignment of the molecular masses to metabolites of

Table 2
Major ESI ions of synthetic dynorphins and their major metabolites

Peptide	Sequence	ESI ions
Dyn A (1-13)	YGGFLRRIRPKLK	803.6 (2+), 535.5 (3+), 401.8 (4+)
Dyn A (1-12)	YGGFLRRIRPKL	740.4 (2+), 492.9 (3+)
Dyn A (2-13)	GGFLRRIRPKLK	722.9 (2+), 481.3 (3+)
Dyn A (1-10)	YGGFLRRIRP	618.3 (2+), 412.5 (3+)
Dyn A (1-11)	YGGFLRRIRPK	682.0 (2+), 455.1(3+)
Dyn A (2-11)	GGFLRRIRPK	600.4 (2+), 400.3 (3+)
Dyn A (1-6)	YGGFLR	712.0 (1+)
Dyn A (7-12)	RIRPKL	392.3 (2+)

the parent peptide in selected cases by obtaining MS–MS product ion spectra such as for m/z 470.4. Based on the presence of several sequence ions (e.g., b_6^{2+} at m/z 403.9, y''^{2+}_4 at m/z 254.4, a_1^{1+} at m/z 128.8, etc.) expected based on the proposed amino acid sequence (Arg–Arg–Ile–Arg–Pro–Lys–Leu), this metabolite could be positively identified.

After compiling a list of the metabolites identified, ion (mass) chromatograms were reconstructed for further confirmation. Fig. 4 shows the reconstructed ion chromatograms (RICs) for the metabolites obtained from the parent peptide (Dyn A 1-12) through the cleavage of a single peptide bond (primary products). Although the RIC peak areas may indicate the relative rates of metabolite formation by the individual peptidases involved, one must be aware of the differing dialysis efficiencies as the function of peptide size [18], and that ionization efficiencies under electrospray conditions also vary among peptides. On the other hand, mass-specific detection after RP-LC was essential, because complete RP-LC separation of the metabolites was not achieved even by using a shallow gradient (+0.5%/min in acetonitrile).

4. Discussion

In our experiments, the peptides were applied at concentrations much (at least 100–1000 times) higher than those of the endogenous dynorphins. The in vitro approach was satisfactory only for studying the degradation kinetics of synthetic dynorphins. Using brain homogenate, self-proteolysis of the brain tissue during incubation was a serious source of contamination, which made the HPLC chromatograms and ESI mass spectra difficult to evaluate for the presence of several key (especially short-lived) peptide metabolites. The value of this approach is that biological stability of dynorphins can be compared (Table 1), although homogenates may also contain many cytosolic peptidases to which these peptides may not be exposed during neurochemical and CNS pharmacological studies.

In vivo cerebral microdialysis circumvents the problems associated with the in vitro approach. During these experiments, sample collection is done during the simultaneous delivery of the peptide into the tissue. The formation and removal (by further metabolism, as well as by diffusion into the probe and into the tissue surrounding the probe) of metabolites from the dynorphin entering the brain probably reaches a steady-state. Accordingly, even short-lived products (e.g., Leu-enkephalin, whose in vitro half-life is only 30–35 s, as shown in Table 1) may reach adequate concentration in the dialysates for detection and identification. For Leu-enkephalin formed as a minor metabolite of Dyn A (1-12) as shown in Fig. 4 (m/z 556.7, chromatogram f), 0.5 pmol could be detected at 5:1 signal-to-noise (S/N) ratio upon acquiring full-scan ESI mass spectra under the LC–MS conditions described, which may permit a significant reduction in the concentration of the microdialyzed peptide in the perfusion medium. Decreasing the amount of peptide entering from the probe into the brain may be desirable for the study of the metabolism of endogenous peptides, where the effect of the exogenously administered compound should not influence physiological processes significantly [21]. Should increased sensitivity be re-

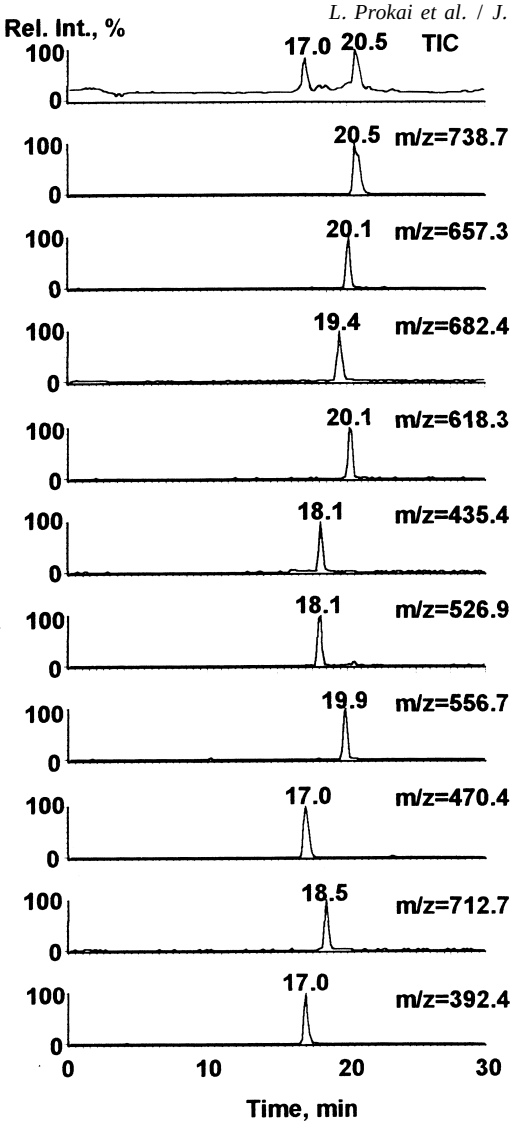

Fig. 4. Total ion current (TIC, a) and reconstructed ion chromatograms (b–g) from the gradient reversed-phase HPLC–ESI-MS analysis of the microdialysates collected from rat striatum after perfusion of the probe with 100 pmol/μl Dyn A 1-12 (Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro–Lys–Leu, YGGFLRRIRPKL) at 0.8 μl/min. HPLC conditions: 15 cm×0.3 mm I.D. C_{18} reversed-phase column, 6.3 μl/min flow-rate, gradient elution from 5 to 95% acetonitrile (0.1 to 0.05% TFA) at 2%/min. Reconstructed ion chromatograms: (b) parent peptide: Dyn A 1-12 (*m*/*z* 738.7, doubly-charged); metabolites: (c) Dyn A 1-10 (*m*/*z* 618.3, doubly-charged); (d) Dyn A 1-7 (*m*/*z* 435.4, doubly-charged); (e) Dyn A 5-12 (*m*/*z* 526.9, doubly-charged); (f) Dyn A 1-5 (*m*/*z* 556.7, singly-charged); (g) Dyn A 6-12 (*m*/*z* 470.4, doubly-charged); (h) Dyn A 1-6 (*m*/*z* 712.7, singly-charged); (i) Dyn A 7-12 (*m*/*z* 392.4, doubly-charged); (j) Dyn A 2-12 (*m*/*z* 657.3, doubly-charged); (k) Dyn A 1-11 (*m*/*z* 682.3, doubly-charged).

quired from the mass spectrometric method, micro- or nano-electrospray ionization [25], as well as matrix-assisted laser desorption/ionization may be considered [26]. Adequate sensitivity is provided by routine ESI-MS for studies such as ours where metabolism of synthetic peptides with intended pharmacological effects on the CNS is to be studied, and the peptide enters the tissue by microdialysis in a quantity commonly administered during neurochemical and pharmacological experiments [11,12].

ESI-MS (after desalting) have revealed the major extracellular metabolites directly for certain synthetic dynorphins such as Dyn A (1-13) and Dyn A (1-11). A follow-up ESI-FTICR-MS analysis, as well as microbore RP-LC study (coelution with reference peptides) were used to confirm the assignment of ESI ions to the in vivo metabolic products of the parent peptide delivered to the brain by microdialysis. The extracellular metabolism of Dyn A (1-13) was mainly due to carboxypeptidase (to the 1-12 fragment) [20], while Dyn A (1-11) was degraded by aminopeptidase (to the 2-11 fragment) and, in part, by dynorphin-converting enzyme [15] (to the 1-6 fragment).

Metabolic profiling by LC–ESI-MS was necessary to fully characterize complex mixtures, such as the one obtained during the study on striatal metabolism of Dyn A (1-12), where nine primary metabolites have been identified. The rich spectrum of the extracellular metabolism of this peptide by exopeptidases (amino- and carboxypeptidase) and various endopeptidase in the brain may be connected to its relatively higher stability in the tissue, compared to related synthetic dynorphins (Table 1), facilitating the attack by a variety of peptidases. In conclusion, in vivo microdialysis combined with ESI-MS and LC–ESI-MS has proven to be a powerful technique for the elucidation of extracellular metabolism involving highly cationic synthetic peptides, such as dynorphins, in the brain.

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