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Dynorphin A(1–17) biotransformation in striatum of freely moving rats using microdialysis and matrix-assisted laser desorption/ionization mass spectrometry

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Abstract

The biotransformation of the opioid peptide dynorphin A(1–17) was investigated in striatum of freely moving Fischer rats, by direct infusion of this peptide, followed by recovery of the resulting biotransformation products via microdialysis and identification using matrix-assisted laser desorption/ionization mass spectrometry. The observed peptides are consistent with enzymatic cleavage at the Arg^7-Ile^8 position of dynorphin A(1–17), followed by terminal degradation of the resulting dynorphin A(1–7) and dynorphin A(8–17) peptides. Unexpectedly, novel post-translational modifications were found on C-terminal fragments of dynorphin A(1–17). Using tandem mass spectrometry, a covalent modification of mass 172 Da, the nature of which is not understood, was found on the tryptophan residue of C-terminal fragments (Trp^{14}). Additional modifications, of mass 42 and 113 Da, were also found on the N-terminus (Ile^8 or Pro^{10}) of these same C-terminal fragments. The role of these modifications of C-terminal fragments has not yet been characterized.

Keywords: Fischer rat, opioid peptide, post-translational modification, tryptophan modification.

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Dynorphin A(1–17) is a peptide that arises from the processing of preprodynorphin and that can be further processed to dynorphin A(1–8) (Seizinger *et al*. 1984). Both dynorphin A(1–17) and dynorphin A(1–8) are present in the brain and pituitary, with the relative amounts of each varying depending upon the particular region, as determined using polyclonal antisera with minimal cross-reactivity against other dynorphin fragments (Weber *et al*. 1982; Cone *et al*. 1983). Both are endogenous agonists of opioid receptors, showing selectivity for the κ opioid receptor (Chavkin *et al*. 1982; Corbett *et al*. 1982). Dynorphin, and the other endogenous opioids, play an intriguing, as yet not fully understood, role in addiction to psychoactive drugs (Kreek *et al*. 2002). Dynorphin is expressed by neurons of the striatum, where κ receptor agonists have been shown to block dopamine release in rats, counteracting the effects of drugs of abuse such as cocaine and morphine (Di Chiara and Imperato 1988; Werling *et al*. 1988). Moreover, dynorphin A(1–13), which has activity similar to that of dynorphin A(1–17), has been shown in humans to lower tuberoinfundibular dopaminergic tone (Kreek *et al*. 1999). The role of dynorphin in the striatum is particularly intriguing in light of the increases in peptide levels and preprodynorphin gene expression following the administration of psychostimulants and opiates (Smiley *et al*. 1990). In humans, polymorphisms in the promoter region of the preprodynorphin gene have been associated with cocaine dependence and abuse (Chen *et al*. 2002).

The present study investigates the biotransformation of exogenously applied dynorphin A(1–17) in the rat striatum *in vivo*. Using a microdialysis probe containing a central infusion port, dynorphin was applied directly to the striatum of freely moving Fischer rats. Microdialysis was used to recover the biotransformation products, which in turn were identified using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. This combination of microdialysis with MALDI mass spectrometry is a unique methodology in the investigation of *in vivo* peptide

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Abbreviations used: aCSF, artificial cerebrospinal fluid; Dyn A, dynorphin A; MALDI, matrix-assisted laser desorption/ionization.

biotransformation; similar methodologies have been used in the investigation of the biotransformation of substance P and peptide E using microdialysis coupled with HPLC–electrospray ionization mass spectrometry (Andren and Caprioli 1995; Zhang *et al*. 1999). The fragments resulting from the biotransformation of dynorphin A(1–17) are of interest because of the possibility of activity at both opioid and non-opioid binding sites (Shukla and LeMaire 1994; Mansour *et al*. 1995).

Materials and methods

Materials

Unless otherwise stated, all chemicals were obtained from Sigma (St Louis, MO, USA). Dynorphin A 1–1 7 was synthesized by the Rockefeller University Protein/DNA Technology Center using 9-Fluorenylmethoxycarbonyl chloride (Fmoc) solid-phase synthesis, with purity checked by HPLC and MALDI time-of-flight spectrometry. Artificial cerebrospinal fluid (aCSF) was obtained from CMA Microdialysis AB (North Chelmsford, MA, USA). E-2078 was obtained from Eisai (Tsukuba, Japan). Model IBR guide cannulae for microdialysis with accompanying probes were obtained from Bioanalytical Systems (West Lafayette, IN, USA). POROS 50 R2 beads were obtained from PerSeptive Biosystems (Framingham, MA, USA). Eppendorf 10-μl gel loading tips were purchased from Brinkmann Instruments (Westbury, NY, USA).

Animals

Fischer rats were used in all studies (weight 175–250 g, aged 13–14 weeks; Charles River Laboratories, Wilmington, MA, USA). Animals were individually caged, given free access to food and water, and kept in temperature- (25°C) and light-controlled rooms. All animal care and experimental procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. The experimental protocol was approved by the Institutional Animal Care and Use Committee of The Rockefeller University.

Microdialysis

A guide cannula (pin style, with an additional central cannula for infusion directly into the tissue) was stereotaxically implanted into the striatum (bregma coordinates: anterior (A) + 0.5 mm, lateral (L) ± 3.0 mm, ventral (V) − 3.5 mm) of each rat following anesthesia with pentobarbital. The rats were allowed to recover for 72–96 h. Probes were implanted the evening before the experiments, and dialysis begun in the morning with freely moving animals. Immediately before infusion, baseline microdialysate was obtained by perfusion of aCSF at 1 μl/min, in 20-min fractions (total volume 20 μl). Dynorphin A(1–17) was infused via the central cannula at a rate of 0.1 μl/min for 20 min (total volume 2 μl, 500 μM solution, total amount 1.0 nmol). In some instances, 1.0 nmol of the protease-resistant Dyn A(1–8) analog E-2078 was infused, separately or with Dyn A(1–17). Dialysate was collected in 20-min fractions (total volume 20 μl) during the infusion and up to 5 h after the Dyn A(1–17) infusion. In a control experiment to test the stability of Dyn A(1–17), 1 nmol Dyn A(1–17) was infused into 50 μl aCSF in a 1.5-mL eppendorf tube and maintained at 37°C, followed by collection of 20-min fractions for up to 1 h after infusion. Following collection, dialysate fractions were immediately placed on dry ice.

Sample preparation

The dialysate fractions were prepared for mass spectral analysis as follows. Samples were first concentrated and desalted in a procedure similar to that described previously (Krutchinsky *et al*. 2001). To microdialysate tubes containing 20-μl fractions was added 20 μl of 10 nM thymopoietin fragment peptide (molecular weight 1611 Da) as an external standard in aqueous 7% formic acid and 0.1% trifluoroacetic acid. Next was added 2 μl of a 25% slurry of POROS 50 R2 beads in 50% methanol, to bind peptides present in the microdialysate fractions. The mixture was vortexed and placed in Eppendorf 10-μl gel loading tips that were pulled and cut to act as a frit. A 1-mL syringe was used to pack the beads. These beads with bound peptides were then washed with 50 μl 0.1% trifluroacetic acid. Bound peptides were eluted directly on to the Voyager DE-STR sample plate (Applied Biosystems, Foster City, CA, USA) for MALDI time-of-flight mass spectrometric analysis using 1 μl 2 : 1 acetonitrile : water (0.1% trifluoroacetic acid), saturated with α-cyano-4-hydroxycinnamic acid as the MALDI matrix. Alternatively, when partial sequencing for peptide identification was necessary, the concentrated peptides were eluted directly on to a MALDI ion trap mass spectrometer target disk (treated as described previously; Krutchinsky *et al*. 2001) using 1 μl 60% methanol/5% acetic acid, saturated with 2,5-dihydroxybenzoic acid matrix.

Mass spectrometry

For MALDI time-of-flight analysis, we used a Voyager-DE STR Mass Spectrometer (Applied Biosystems), both in linear and reflectron modes, with delayed extraction. This instrument is configured with a nitrogen laser (337 nm) that delivers 3-Hz pulses. Each spectrum represents a sum of 100 laser shots, and has been smoothed, calibrated and analyzed using the program M-over-Z (Genomic Solutions, Lansing, MI, USA), with peptide identification using the program PAWS (Genomic Solutions).

For tandem mass spectrometry experiments, we employed a MALDI ion trap mass spectrometer (Krutchinsky *et al*. 2001) that incorporated a commercial ion trap mass analyzer (Thermo Finnigan LCQ Classic; San Jose, CA, USA). The configuration and settings of the instrument for tandem mass spectrometry of peptides have been described previously (Krutchinsky *et al*. 2001; Krutchinsky and Chait 2002).

Results

An example of the time course of the fragments resulting from infusion of dynorphin A(1–17) (total 1.0 nmol, 20-min infusion, 0.1 μl/min) is shown in Fig. 1. A summary of fragments observed in all nine of the rats for which recovery of dynorphin fragments was successful is shown in Table 1. No dynorphin was found in the baseline fractions, which indicates that the basal extracellular level of dynorphin was below the detection limits of this method. In the example shown in Fig. 1, no fragments are observed in the first 0–20-min fraction during infusion; this fraction probably contains

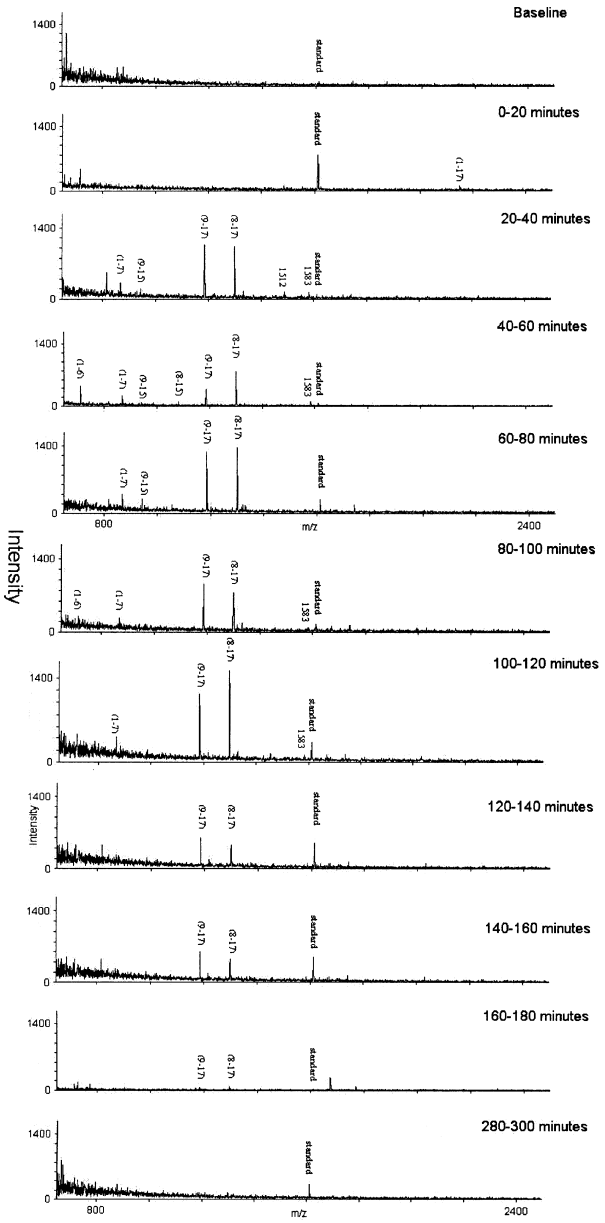

Fig. 1 Time course of dynorphin biotransformation in a typical rat striatum following *in vivo* administration, collection of 20-min fractions (total volume 20 μl), and MALDI mass spectrometric analysis using a Voyager DE-STR in reflectron mode. All spectra are normalized to an ion intensity of 1500, and set to display a *m/z* range of 650–2500. The 0–20 fraction was collected during the 20-min Dyn A(1–17) infusion. The appearance of the Dyn A(8–17), Dyn A(9–17), Dyn A(1–7) and Dyn A(1–6) fragments occurs early after administration. The smaller N-terminal fragments Dyn A(1–7) and Dyn A(1–6) are cleared quickly relative to the C-terminal fragments Dyn A(8–17) and Dyn A(9–17). The modified fragments of *m/z* 1583 and 1512 (see Fig. 3) both appear in this particular example, with the 1583 fragment exhibiting higher levels and longer duration following infusion than the 1512 fragment.

largely baseline dialysate fluid (the volume of the tubing from the outflow of the dialysis probe is as large as 15 μl). Initially, Dyn A(1–7) and Dyn A(8–17) fragments are observed, consistent with cleavage at the Arg^7-Ile^8 bond (Fig. 2). Comparable levels of Dyn A(1–6) and Dyn A(9–17) fragments are also observed in the first time point. However, the complements of these peptides, Dyn A(7–17) and Dyn A(1–8) respectively, were typically not observed. The initial assignment of peptide fragment identity was based on the observed *m/z* and the possible fragments generated from hydrolysis of Dyn A(1–17). Generally speaking, the mass accuracy for peptide identification, in reflectron-mode time-of-flight mass spectrometry, was better than 100 ppm. Owing to low levels of peptide, in some cases the mass accuracy was lower; the lowest mass accuracy which we accepted for peptide identification (Table 1) was 300 ppm. To confirm these initial assignments, tandem mass spectrometry was performed on fragments obtained in parallel experiments (data not shown). The biotransformation of Dyn A(1–17) is rapid, as none of the full-length peptide is recovered after 20 min of infusion. In control experiments, in which Dyn A(1–17) was infused directly into aCSF at 37°C, no fragments were observed, indicating that the peptide itself is stable under these conditions (data not shown). All fragments have apparently been cleared to levels below the sensitivity of the method within 5 h following infusion.

Table 1 shows a compilation of data from all the rats studied. Dynorphin fragments were recovered from nine of a total of 15 rats from which fractions were collected for at least 3 h. The levels of dynorphin fragments from these rats showed a high degree of variability, compared with the internal standard (thymopoietin fragment II, 1611 Da; total 200 fmol added). Microdialysate samples from the remaining six rats were analyzed but showed no recovered dynorphin fragments. Similar variability was found in the recovery of the proteolysis-resistant Dyn A(1–8) analog E-2078 (data not shown). Microdialysis, in general, has a higher success rate; for instance, investigations of extracellular dopamine levels, with analysis by HPLC with electrochemical detection, typically 95–100% of animals yield detectable levels (Zhang *et al*. 2001). Possible reasons for the low success rate for dynorphin recovery in this study are discussed below.

Generally, Dyn A(8–17) and (1–7) are both observed, consistent with cleavage at the Arg^7-Ile^8 position (Table 1). Although Dyn A(1–6) is typically observed, the complement peptide Dyn A(7–17) was observed in only a single rat. Likewise, Dyn A(9–17) is typically observed; however, the complement of this peptide, Dyn A(1–8), was observed in only a single rat. The structure of the peptides observed following infusion of dynorphin A(1–17) are shown in Fig. 2, with the exception of those peptides that have been modified (see Fig. 3). In addition to the predominant peptides [Dyn A(8–17), Dyn A(9–17), Dyn A(1–7), and Dyn A(1–6)],

Table 1 Summary of time course of biotranformation fragments recovered via microdialysis during and following a 20-min infusion of dynorphin

	Time (min)								
Fragment	0–20	20–40	40–60	60–80	80–100	100–120	120–140	140–160	160–180
Dyn A(1–6)	2	3	4	3	2	2	0	0	0
Dyn A(1–7)	5	5	5	4	4	2	2	2	2
Dyn A(1–8)	1	1	1	1	1	1	1	1	0
Dyn A(7–17)	1	1	1	1	1	1	1	1	1
Dyn A(8–17)	6	8	8	8	8	7	7	7	5
Dyn A(9–17)	6	7	7	7	7	6	6	6	6
Dyn A(8–15)	1	3	4	3	3	3	3	3	2
Dyn A(9–15)	2	3	4	5	4	4	4	4	3
Dyn A(1–17)	4	3	3	3	3	2	2	2	2
1242	5	4	3	2	0	0	0	0	0
1313	1	3	1	0	0	0	0	0	0
1512	1	2	2	1	1	0	0	0	0
1583	1	5	3	3	2	1	1	0	0

Number of animals for which the recovery of each dynorphin fragment was successful in each 20-min sample following infusion. Fraction 0–20 is the fraction recovered during the 20-min dynorphin infusion. Dynorphin fragments were recovered successfully from nine of 15 animals from which fragments were collected for at least 3 h. Fragments are listed by sequences, and were identified within 300 ppm of the predicted exact mass (for reflectron spectra using Voyager DE-STR) or average mass (for linear spectra using Voyager DE-STR, or for spectra obtained using the in-house MALDI–ion trap mass spectrometer) based on the calculations of Dyn A(1–17) mass (using PAWS software). Fragments listed that were found in more than one animal are shown in Fig. 2. The modified fragments (Fig. 3) listed correspond to *m/z* 1242, 1313, 1512 and 1583.

Dyn A(8–15) and Dyn A(9–15) are also observed. The most likely predominant pathway for the formation of the fragments is indicated by bold arrows in Fig. 2. We cannot rule out some contribution from alternative pathways of biotransformation, indicated by the dotted arrows. Note, Dyn A(1–5) (leucine enkephalin) was not observed, and is not therefore included in Table 1. We have verified using a leucine enkephalin standard that this peptide can be concentrated and detected using our sample preparation methodology and mass spectrometry instrumentation (data not shown).

Using tandem mass spectrometry, it was shown that masses that do not correspond to the sequence of unmodified Dyn A were novel, previously unreported, modified Dyn A biotransformation fragments (Fig. 3). Peptides of *m/z* 1242 (Fig. 3a) and 1313 (Fig. 3b) were identified as modified versions of a Dyn(10–17) fragment. The differences in mass were accounted for by two different N-terminal modifications, of nominal mass 42 and 113 Da respectively. In the case of the 1242 *m/z* fragments, the 42-Da modification is localized to the first amino acid, Pro^{10}, at the N-terminus of the peptide, based on the observation of the y_7 fragment. For the 1313 *m/z* fragment, the 113-Da modification is localized to the first two N-terminal amino acids, Pro^{10} or Lys^{11}. In addition, there is a modification of mass 172 to Trp^{14}. Only peptides containing the Trp modification along with one of the two N-terminal modifcations were observed; peptide fragments containing only the 172-Da modification of Trp^{14} or only the 42- or 113-Da modification of Pro^{10} were not observed.

A second pair of peptides, of *m/z* 1512 (Fig. 3c) and 1583 (Fig. 3d), are modified versions of the (8–17) fragment. Again, consistent with the observation of the shorter pair of modified fragments, the differences in mass are accounted for by two different N-terminal modifications, of nominal mass 42 and 113 Da respectively. In this case, these modifications are localized to the first amino acid at the N-terminus of the peptides, Ile^8, based on the observation of the y_9 fragment in both cases. Although there are differences in the time course of appearance and disappearance of each of these modified peptides, they each exhibit a decrease and absence from later fractions which is different from that of the unmodified N-terminal fragments mentioned above (Table 1).

Discussion

The combination of mass spectrometry with microdialysis confers several advantages for the investigation of the biotransformation or degradation of peptides. Several previous investigations of peptide biotransformation have used antibodies against the peptide of interest in radioimmunoassay detection (e.g. Maidment *et al.* 1991). To increase the confidence of radioimmunoassay detection of peptides, HPLC is typically used; co-elution of immunoactivity with the peptide of interest is highly suggestive of identity. For the detection of a particular peptide, and changes in the

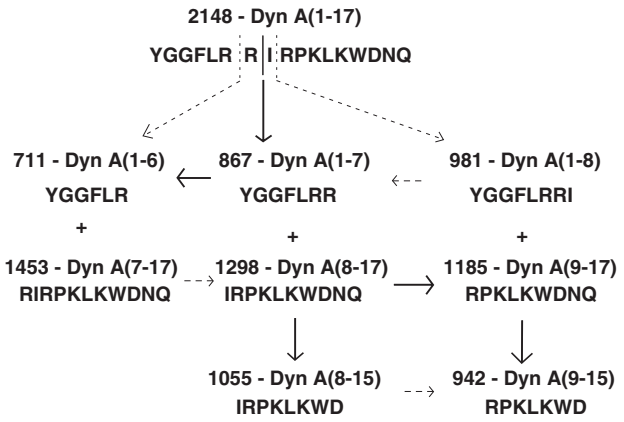

Fig. 2 Fragments of dynorphin observed, along with possible pathways for their production (primary pathways, consistent with data summarized in Table 1, are represented by bold solid arrows; minor pathways are represented by dotted arrows). The primary peptides are shown as Dyn A(1–7) and Dyn A(8–17). At least two processing steps are required for production of Dyn A(8–15) and Dyn A(9–15), namely cleavage at the Arg^7-Ile^8 or Ile^8-Arg^9 positions respectively, and cleavage at the Asp^{15}-Asn^{16} position. The production of Dyn A(1–6) might result directly from Dyn A(1–17), but the complement peptide Dyn A(7–17) is not generally observed, and this is therefore not likely to be a major processing pathway for dynorphin biotransformation. Likewise for Dyn A(9–17), the complement peptide Dyn A(1–8) is not generally observed. Hence the production of both Dyn A(1–6) and Dyn A(9–17) from Dyn A(1–17) is shown to occur through intermediaries, namely the initial fragments Dyn A(1–7) and Dyn A(8–17) respectively, rather than directly from Dyn A(1–17) (dotted arrows). The enzymes responsible for each step are not known. One letter abbreviations for amino acids are used: Y, tyrosine; G, glycine; F, phenylalanine; L, leucine; R, arginine; I, isoleucine; P, proline, K, lysine, W, tryptophan, D, aspartate, N, asparagine, Q, glutamine.

concentration thereof, radioimmunoassay coupled with HPLC provides suitable sensitivity and selectivity (Maidment *et al*. 1991). However, for the study of fragments resulting from peptide biotransformation, radioimmunoassay combined with HPLC requires the synthesis of a large number of potential degradation products. For instance, a previous study involving the administration of Dyn A(1–17) to the periaqueductal gray showed significant formation of an unidentified immunoactive product, based on HPLC elution time (Young *et al*. 1987). Moreover, the epitope selectivity, which may or may not be fully defined, of any antibody used for radioimmunoassay may preclude recognition of many possible biotransformation products.

By contrast, mass spectrometry is not limited with regard to selectivity, as the nature of this analytical technique allows the detection of peptide fragments and differentiation simply according to their mass (although there are potential limitations with regard to differential ionization efficiencies of different peptides; Kratzer *et al*. 1998). A further advantage of the use of MALDI for peptide ionization is that prior separation of the peptide biotransformation products is not necessarily required, resulting in increased throughput compared with methods that required separation by HPLC. The combination of a MALDI ion source with an ion-trap mass spectrometer allows tandem mass spectrometry, further increasing both the selectivity and sensitivity of peptide detection (Krutchinsky *et al*. 2001; Krutchinsky and Chait 2002).

The present work is based on previous developments using MALDI mass spectrometry to investigate Dyn A(1–17) biotransformation in blood from non-human primates and humans (Chou *et al*. 1996; Yu *et al*. 1996). These previous studies indicated that the predominant point of cleavage was at the Tyr^1-Gly^2 site, suggesting the activity of an aminopeptidase of unknown specificity. Further N-terminal degradation of the resulting Dyn A(2–17) was also found. Moreover, there also appeared to be an enzyme cleaving dynorphin at the Arg^6-Arg^7 site, possibly the dynorphin-converting enzyme reported in CSF (Silberring *et al*. 1992). The differences in the major cleavage fragments of dynorphin in this previous study from those found here may result from the use of different systems (human and rhesus monkey blood vs. extracellular fluid in the striatum of the freely moving rat). Also use of different concentrations of Dyn A(1–17) in the studies may lead to predominant cleavage by different enzymes.

The identities of the cleavage fragments of dynorphin resulting from biotransformation (Fig. 2) are important for understanding the pharmacological activity of this peptide. For instance, whereas Dyn A(1–17) and Dyn A(1–7) show preferential binding for the κ opioid receptor compared with the μ and δ receptors, the Dyn A(1–6) fragment is essentially equipotent at the three cloned opioid receptors (Mansour *et al*. 1995). Generally, Dyn A(1–7) and Dyn (8–17) are both observed, consistent with cleavage at the Arg^7-Ile^8 position. Although Dyn A(1–6) is typically observed, the complement peptide Dyn A(7–17) was observed in only a single rat, indicating that the Arg^6-Arg^7 position is not a predominant cleavage site for dynorphin in the dorsolateral rat striatum *in vivo* (Table 1; Fig. 2). Likewise, Dyn A(9–17) is typically observed; however, the complement of this peptide, Dyn A(1–8), was observed in only a single rat, indicating that the Ile^8-Arg^9 position is probably not a predominant cleavage site in the extracellular fluid of the rat striatum. In addition to the predominant peptides [Dyn A(8–17), Dyn A(9–17), Dyn A(1–7), and Dyn A(1–6)], Dyn A(8–15) and Dyn A(9–15) are also observed. These probably represent biotransformation products of the intermediates Dyn A(8–17) and Dyn A(9–17). No Dyn A(8–16) or Dyn A(9–16) is observed; if they arise they are probably short-lived intermediates. It is possible that an aspartate-specific protease, possibly a caspase (Troy and Salvesen 2002), is involved, cleaving directly at Asp^{15}-Asn^{16}, without the need for successive carboxypeptidase activity, to form the products Dyn A(8–15) and Dyn A(9–15). No Dyn A(1–15) is observed; the enzymatic activity cleaving the Arg^7-Ile^8 position is

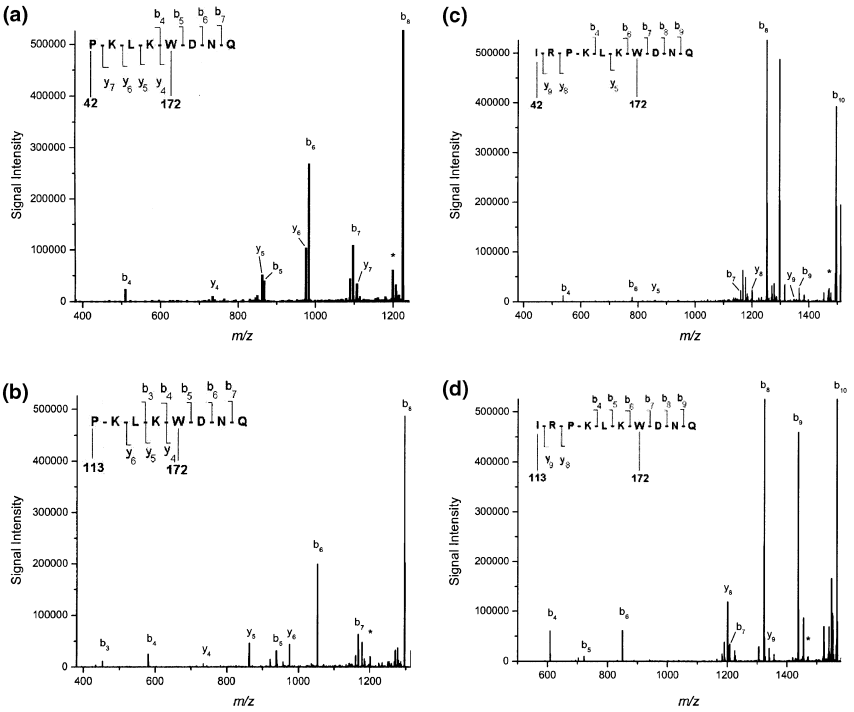

Fig. 3 Tandem mass spectrometry of observed fragments found to represent modified C-terminal dynorphin A(1–17) peptides, obtained with a 20-Hz laser pulse (337 nm), 30% activation energy, 300 ms activation time. The N-terminal series is denoted by 'b', and the C-terminal series is denoted by 'y', according to the conventions of Biemann and Martin (1987). In each case, the 'b' series is consistent with modification of the tryptophan residue. The 'y' series is generally incomplete but is consistent with N-terminal modification by 42 Da (a and c) or 113 Da (b and d). *Denotes the peak resulting from simple loss of this N-terminal modification. (a) Tandem mass spectrometry of peptide of mass 1242.4, representing an average of 12 mass spectra. (b) Tandem mass spectrometry of peptide of mass 1313.2, representing an average of seven mass spectra. (c) Tandem mass spectrometry of peptide of mass 1512.3, representing an average of four mass spectra. (d) Tandem mass spectrometry of peptide of mass1583.4, representing an average of 11 mass spectra.

evidently much more rapid that that at the C-terminus. Alternatively, it is possible that the full-length peptide Dyn A(1–17) does not fulfill the structural requirements for the C-terminal enzymatic activity.

The possibility of pharmacological activity of the C-terminal fragments, Dyn A(8–17) and Dyn A(9–17), their truncation fragments Dyn A(8–15) and Dyn A(9–15) respectively, as well as of the modified fragments (Fig. 2), is difficult to assess. Non-opioid activity, such as antagonism of melanocortin receptors and modulation of NMDA receptor activity, has been shown for Dyn A(1–17), as well as for fragments that lack the N-terminal Tyr^1 residue [Dyn A(2–17)] (Quillan and Sadee 1997; Zhang *et al*. 1997; Tang *et al*. 1999). The C-terminal fragments may show binding to non-opioid sites similar to that of the full-length peptide (Dumont and Lemaire 1993). It is of note that no Dyn A(2–17) was observed in the studies here, given our previous findings of this product as a major biotransformation product in blood (Yu *et al*. 1996) and speculation that Dyn A(2–17) may play a role as a modulator of NMDA receptor activity (Tang *et al*. 1999).

Previous investigations of dynorphin biotransformation in the striatum using electrospray ionization mass spectrometry yielded results that differ from the present findings. Homogenates from both the striatum and substantia nigra of rats showed relatively low levels of Dyn A(1–17) biotransformation, with the only detected product being Dyn A(1–5) (leucine enkephalin) (Sandin *et al*. 1997). The differences in results probably arise from the fact that the previous *in vitro* study used homogenized tissue; homogenization may lead to the release of prohormone convertase enzymes, predominantly localized intracellularly, which can process the paired basic residues Arg^6-Arg^7, as is found in extracts from the pituitary (Kilpatrick *et al*. 1983).

A similar study to that performed here, which investigated the biotransformation of Dyn A(1–13), used conventional microdialysis probes to deliver Dyn A(1–11), Dyn A(1–12) and Dyn A(1–13) separately to the striatum of Sprague–Dawley rats, and electrospray ionization mass spectrometry for identification of biotransformation products (Prokai *et al*. 1998). A dominant cleavage of the Arg^7-Ile^8 bond was not observed; rather, the majority of the biotransformation products appeared to result from the action of aminopeptidases and carboxypeptidases on the full-length peptide (Prokai *et al*. 1998). The discrepancy with the results reported here probably arises from the use of different peptides. We have shown that,

Table 2 Summary of results found for the biotransformation of dynorphin

Primary dynorphin fragments observed	Cleavage sites	Enzymes	Reference
Dyn A(1–9)	Arg^9-Pro^{10}	Proprotein convertase 2 (EC 3.4.21), Carboxypeptidase E (EC 3.4.17.10)	Day *et al.* (1998)
Dyn A(1–8)	Ile^8-Arg^9		
Dyn A(1–6), Dyn A(7–17) – predominant	Arg^6-Arg^7	Dynorphin A converting enzyme (EC 3.4.22)	Silberring *et al.* (1992)
Dyn A(1–5), Dyn A(6–17) – trace	Leu^5-Arg^6		
Dyn A(1–7), Dyn A(8–17) – trace	Arg^7-Ile^8		
Dyn A(1–8)	Ile^8-Arg^9	Dynorphin A(1–17) processing enzyme (EC 3.4.24)	Berman *et al.* (1999)
Dyn A(2–17)	Tyr^1-Gly^2	Aminopeptidase (?)	Chou *et al.* (1996); Yu *et al.* (1996)
Dyn A(1–6), Dyn A(7–17)	Arg^6-Arg^7	Dynorphin converting enzyme (?)	
Dyn A(1–7), Dyn A(8–17)	Arg^7-Ile^8	Unidentified enzyme	Present study
Dyn A(1–6)	Arg^6-Arg^7	Carboxypeptidase (?)	
Dyn A(9–17)	Ile^8-Arg^9	Aminopeptidase (?)	

Results of previous investigations into the biotranformation of Dyn A(1–17), listing the fragments, cleavage sites and enzymes responsible. Question mark indicates when the enzymes responsible for cleavage have not been specifically defined.

in human blood, the full-length Dyn A(1–17) is more resistant to proteolytic degradation than Dyn A(1–13) (Chou *et al.* 1996).

Several enzymes have been implicated previously in the processing of dynorphin A(1–17) (Table 2). Proprotein convertase 2, in conjunction with carboxypeptidase E, results in the production of Dyn A(1–8), following cleavage at the Arg^9-Pro^{10} position and C-terminal processing of the terminal Arg^9 residue of Dyn A(1–9) (Day *et al.* 1998). This processing sequence is typical of prohormone processing, and the enzymes involved are thought to be localized primarily in secretory granules, intracellularly, although there is the possibility of some relevant extracellular localization (Seidah *et al.* 1998). On the other hand, dynorphin A converting enzyme, isolated from the spinal cord, cleaves dynorphin at the Arg^6-Arg^7 bond, resulting in Dyn A(1–6) and Dyn A(7–17), with only trace amounts of Dyn A(1–7) and Dyn A(8–17) (Silberring *et al.* 1992). A third enzyme, dynorphin A-17 processing enzyme, cleaves Dyn A(1–17) predominantly at the Ile^8-Arg^9 position, resulting directly in Dyn A(1–8), and shows relatively high activity in the striatum (Berman *et al.* 1999). This predominantly membrane-bound metallopeptidase exhibits similarities with other metallopeptidases involved in the extracellular degradation of neuropeptides, but the biological significance of this activity in Dyn A(1–17) biotransformation remains to be determined. However, the results described here contrast with those that would be expected based on these previous studies, indicating the predominant involvement of a different enzyme.

There was considerable inter-animal variability with regard to the extent of recovery of dynorphin fragments. The use of E-2078, a degradation-resistant Dyn A(1–8) analog, showed similar variability in recovery (data not shown); therefore the variability in recovery of Dyn A(1–17) biotransformation products is probably not primarily the result of inter-animal variability in biotransformation. Although there is inter-animal variability in the quantity of peptides recovered, the qualitative profile of peptides resulting from dynorphin biotransformation was maintained (Table 1). It is likely that the variability in the recovery via the microdialysis probes reflects variability in the response of the injured brain tissue, such as fibrin coating of the probe (Grabb *et al.* 1998), consistent with the identification here of fibrinopeptide A (*m/z* 1739), a marker of fibrinolysis, in some baseline fractions (data not shown), or the initiation of an inflammatory immune response due to the introduction of the microdialysis probe (Clapp-Lilly *et al.* 1999). In a previous study that investigated changes in microdialysis recovery, probes were inserted 3 or 24 h before the determination; significant decreases in the recovery of certain compounds were found (Grabb *et al.* 1998).

The nature of the modification, of mass 172 Da, of the tryptophan residue has not yet been characterized. There have been some reports of peptide tryptophan residues being modified as a result of oxidation (Deterting *et al.* 1998). In fact, peptides bearing tryptophan and/or tyrosine residues have been proposed to play a role as endogenous antioxidants (Moosmann and Behl 2002). This possibility may be supported by the fact that the modified dynorphin fragments appear early after infusion and are absent from later microdialysate fractions. If oxidants are present in the extracellular fluid surrounding the dialysis probe (possibly owing to an inflammatory reaction to the probe), reaction with dynorphin fragments immediately following infusion may deplete the oxidants. Later microdialysate fractions would therefore be expected to contain lower amounts of modified peptides. Whether or not endogenous dynorphin plays a role as an antioxidant in the striatum is not known, but it is provocative considering that some models of Parkinson's disease propose a central role for oxidized dopamine (Drukarch and van Muiswinkel 2000). The role of dynorphin in Parkinson's disease has not been well defined.

The additional modifications of the N-termini of the C-terminal fragments of dynorphin (Fig. 3) have not been reported previously. The modification of mass 42 Da (Figs 3a and c) may represent acetylation of the N-terminal amine. We did not observe a metastable loss of the acetyl group, 42 Da, in MALDI reflectron-mode time-of-flight mass spectrometry, which has been interpreted as indicative of acetylation by some investigators (Floyd *et al*. 1999). However, the loss of 42 Da, using MALDI ion trap mass spectrometry, is indicative of the lability of this modification, consistent with acetylation. The nature of the modification of mass 113 Da (Figs 3b and d) is not known. The fragmentation resulting from tandem mass spectrometry of the fragment of *m/z* 1313 (Fig. 3b) did not allow definitive specification of the localization of the 113 modification to the N-terminal residue (Pro^{10}). In light of the localization of the modification of mass 113 Da on the modified peptide of *m/z* 1584 (Fig. 3d) to the first N-terminal amino acid, it is likely that the modification of mass 113 Da occurs on the Pro^{10} residue. The mass of 113 Da is the same as that of an isoleucine or leucine residue, but the fragmentation that results from tandem mass spectrometry does not suggest that this modification results from N-terminal addition of an isoleucine or leucine residue. Again, the role of these modifications in dynorphin biotransformation remains to be elucidated.

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