Journal of Chromatography, 529 (1990) 43–54
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5282

High-performance liquid chromatographic system for the separation of dynorphin A (1–17) fragments and its application in enzymolysis studies with rat nerve terminal membranes

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(First received December 13th, 1989; revised manuscript received February 9th, 1990)

ABSTRACT

A high-performance liquid chromatographic method capable of separating a large number of C- and N-terminal degradation fragments of dynorphin A (1–17) (dyn 1–17) in 1 h has been developed. The system has been applied to study the metabolism profiles of various dyn 1–17-derived peptides following in vitro incubation with rat striatum and spinal cord nerve terminal membranes. In addition to the removal of the N-terminal amino acid Tyr, major sites of cleavage between the following amino acids could be established: Leu^5-Arg^6 in dyn 1–7 (formation of dyn 1–5); Arg^6-Arg^7 and Leu^5-Arg^6 in dyn 1–8 (formation of dyn 1–6 and dyn 1–5, respectively); Arg^7-Ile^8 in dyn 1–9 (formation of dyn 1–7) and Arg^9-Pro^{10} in dyn 1–10 (formation of dyn 1–9). Studies with inhibitors of the enzymes involved show that dyn 1–5 is formed directly from dyn 1–8 via an endopeptidase insensitive to the angiotensin-converting enzyme inhibitor MK 422 acting on the scissile Leu^5-Arg^6 bond in dyn 1–8. The method circumvents the use of [^{3}H]Tyr-labelled dynorphins, which have the inherent drawback that fragments lacking the N-terminal Tyr cannot be detected. Owing to the high resolution, also for the larger dynorphins dyn 1–14, dyn 1–15 and dyn 1–16, the chromatographic system should prove especially useful in the elucidation of the enzymolysis pattern of dyn 1–17. Furthermore, the method offers a way to evaluate simultaneously the selectivity of new enzyme inhibitors for several cleavage sites in the same assay.

INTRODUCTION

Endogenous opioid peptides play a role in a wide variety of physiological functions. Of these neuropeptides, the dynorphins, a series of peptides derived

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from the precursor prodynorphin (proenkephalin B) have attracted attention owing to their pain-regulating properties (for a review see ref. 1). Especially at the spinal level, acute analgesic effects of several dynorphins have been reported and increased biosynthetic activity of dynorphinergic neurons in the spinal cord has been demonstrated under chronic pain [2–4]. Post-translational processing of large precursor peptides and enzymic degradation of dynorphins occur via the action of numerous proteases, of which several have been purified [5–7]. However, it is unclear which enzymes are physiologically relevant, and considerable controversy exists as to the selectivity and the main pharmacological actions of the various fragments derived from dynorphin A. Therefore, there is great interest in the enzymic degradation of opioid peptides as a potential mechanism for regulating the concentration of these compounds in vivo, and also with regard to the design of mechanism-based inhibitors as pharmacological agents.

Although several chromatographic systems for the separation of products following in vitro degradation have been published [6,8,9], those studies deal only with a limited number of substrates, using enzyme preparations from different sources, and importantly, N-terminal degradation fragments were not analysed in most of those studies.

This paper describes a high-performance liquid chromatographic (HPLC) system capable of separating 36 synthetic peptides related to dynorphin A (1–17) in a 1-h chromatogram, and its application in the enzymolysis of various dynorphins with enzyme preparations from rat striatum and spinal cord nerve terminals. The method offers a way both to monitor the dynamics of the relations between precursor, intermediates and products, and also to evaluate simultaneously the selectivity of enzyme inhibitors for several cleavage sites in the same assay.

EXPERIMENTAL

Reagents and chemicals

Dynorphin A (1–17) (dyn 1–17 = Tyr^{1}-Gly^{2}-Gly^{3}-Phe^{4}-Leu^{5}-Arg^{6}-Arg^{7}-Ile^{8}-Arg^{9}-Pro^{10}-Lys^{11}-Leu^{12}-Lys^{13}-Trp^{14}-Asp^{15}-Asn^{16}-Gln^{17}) and its fragments and enzyme inhibitors were obtained from the following sources: Bachem (Feinchemikalien, Bubendorf, Switzerland): dyn 1–2, 1–3, 1–4, 1–5, 1–6, 1–7, 1–8, 1–9, 1–10, 1–13, 1–17; 2–3, 2–4, 2–5, 2–13; 3–4, 3–5; 4–5, 4–6, 4–7, 4–8; 5–6; 6–7, 6–8; 7–8, 7–10; 8–9, 8–17; 10–11; 11–12, 11–17; 13–14; 14–15, 14–17 and bestatin; Peninsula Labs. (Belmont, CA, U.S.A.): dyn 1–11, 1–12; 2–17; 3–13; 6–17; 9–17; 13–17 and thiorphan (racemic). Bissendorf Biochemicals (Hannover, F.R.G.): dyn 1–15 and 1–16. Dyn 1–14; 2–6, 2–7, 2–8, 2–9, 2–10, 2–14; 3–14; 4–14; 9–12; 12–15 were synthesized by Dr. A. Jonczyk, and dyn 3–6 and 16–17 by Dr. G. Hölzemann (both from the Department of Medicinal Chemistry, E. Merck, Darmstadt, F.R.G.). MK 422 1-[N-[(*S*)-1-carboxy-3-phen-

ylpropyl]-2-alanyl]-2-proline, was kindly donated by Merck, Sharp and Dohme (Rahway, NJ, U.S.A.) and GEMSA (guanidinoethylmercaptosuccinic acid) was purchased from Calbiochem (Frankfurt, F.R.G.). All reagents and solvents were at least of analytical-grade purity. HPLC-grade water (E. Merck, 15333) or water, deionized and double quartzdistilled, and acetonitrile (LiChrosolv®, E. Merck, 30) were used for the chromatography.

Chromatography

Chromatography was carried out with a 5-μm C_8 column (Hibar®, LiChrospher® 100 CH-8/2, 250 mm×4.6 mm I.D., E. Merck) at 25°C and a flow-rate of 1.0 ml/min. The column was equilibrated with 0.1 *M* KH_2PO_4 buffer, pH 3.20 (phase A) and eluted with the following gradient of phase A and phase B (60% acetonitrile, v/v, in phase A): 0 to 34% B in 21 min, 34% B for 10 min and 34 to 100% B in the following 29 min. For higher resolution at retention times of 14–17 min, i.e. the separation of some Trp-containing short fragments, the following gradient was applied: 0 to 22% B in 12 min, 22% B for 10 min and 22 to 100% B in the following 38 min. Peptides were detected at 210 nm (Merck-Hitachi UV monitor 655A-22) with a sensitivity setting of 0.01 a.u.f.s., and absorbance was recorded (Merck-Hitachi data processor 655-61) at a detection limit of 0.01 nmol synthetic peptide.

Samples

For the preparation of striatal or spinal cord synaptosomal membranes, the procedure of Whittaker [10] was followed closely. Briefly, 1 g of striatum (ca. ten rats) or 3.5 g of spinal cord (ca. eight rats) from Wistar rats was homogenized by eight up-and-down pestle strokes in 10 or 35 ml, respectively, of 0.32 *M* sucrose with the pestle rotating at 3000 rpm. After centrifugation at 1100 *g* for 11 min, the supernatant was recentrifuged at 20 000 *g* for 30 min. The pellets were resuspended in 6.5 ml (striatum) or 11 ml (spinal cord) of 0.32 *M* sucrose and purified by density-gradient centrifugation as described previously [10]. About 7 ml per tube of the layer between 0.8 and 1.2 *M* sucrose were collected, mixed with 5 ml of distilled water and centrifuged at 20 000 *g* for 1 h. The resulting pellet was washed and resuspended in 0.5 ml of 50 m*M* Tris–HCl buffer (pH 7.6) containing 0.25% (v/v) Triton X-100, and frozen in 100-μl aliquots. These preparations contained ca. 5 mg of protein per ml. For incubations, 100 μl of synthetic dynorphin substrate (final concentration 50 or 125 μM), 10 or 100 μl of striatal or spinal cord enzyme, respectively, and 50 μl of inhibitor (final concentration 125 μM) were mixed and diluted to 800 μl in the above buffer. After incubation times varying between 5 and 180 min at 37°C, 100 μl were pipetted and mixed with 20 μl of 1% perchloric acid in an ice-bath. After centrifugation, 20 μl of the clear supernatant were injected into the HPLC column. Peaks were assigned by spiking with appropriate reference dynorphins. Further evidence for the identity of peaks results from the disap-

pearance or appearance of particular peaks following incubation with various selective enzyme inhibitors (see Results). Incubation with enzyme preparations alone resulted in only a few peaks in the chromatograms, which disappeared after longer incubation times.

RESULTS

Fig. 1A shows the chromatographic separation of various dyn 1–17 fragments. Table I lists the retention times of 55 fragments and some amino acids. Thus, most C-terminally fragmented dynorphins can be clearly separated, although overlapping occurs with several smaller fragments, especially at retention times ca. 16 min and in the range 24–29 min. Although higher resolution can be obtained with longer run-times, peak-broadening can become a problem under these conditions. Therefore, in degradation studies we routinely analyse the sample with different gradients with higher resolution for separation and spiking of certain peaks. One example of a separation with such a special gradient is given in Fig. 1B, which shows the separation of dyn 14–17, 13–17, 14–15 and 13–14 and Trp. The first three fragments have virtually identical retention times with the standard gradient. Although the relative retention times differ only ca. 0.3 min, the absolute retention times can differ by 1–2 min within several months, owing to slight changes in the column.

The time-courses of the enzymolysis of dyn 1–8 and dyn 1–9 by striatal membranes are shown in Fig. 2A and B, respectively. Under the conditions used

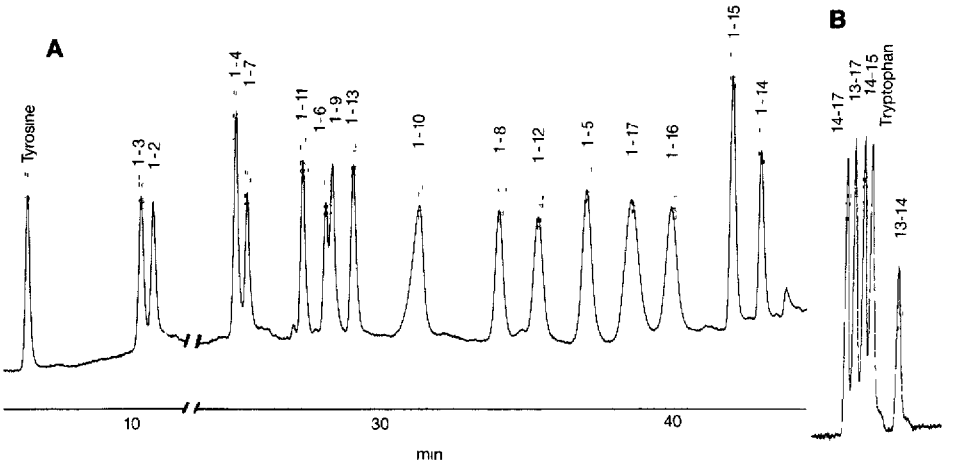

Fig. 1. (A) Separation of various C-terminal fragments of dyn 1–17 with the standard gradient described in Experimental: ca. 0.05 nmol of the pure standards was applied to the column. (B) Separation of dyn 14–17, dyn 13–17, dyn 14–15, Trp and dyn 13–14 with the special gradient described in Experimental: ca. 0.1 nmol of the pure standards was applied to the column.

TABLE I

RETENTION TIMES OF VARIOUS DYNORPHIN 1-17 (DYNORPHIN A) FRAGMENTS

Compound	Retention time (min)	Compound	Retention time (min)	Compound	Retention time (min)
Gly	2.33	16-17	17.10	4-5	26.70
Arg	2.45	4-7	17.10	2-10	26.86
Dyn 2-3	2.45	2-4	17.34	1-11	26.92
6-7	2.68	9-12	18.00	1-6	27.58
10-11	2.78	13-14	18.31	1-9	27.84
Pro	2.85	4-6	19.41	2-8	28.54
Leu	6.85	2-7	21.42	1-13	28.64
Tyr	6.87	11-17	22.38	2-5	29.50
8-9	7.31	6-17	23.09	3-5	29.63
5-6	7.45	2-6	23.95	4-14	30.02
11-12	9.32	3-6	24.03	2-17	30.05
1-3	10.50	2-9	24.20	1-10	30.68
1-2	10.90	9-17	24.25	1-8	33.40
7-8	11.10	3-4	24.62	1-12	34.24
Phe	12.13	8-17	24.71	3-14	35.72
6-8	12.19	4-8	24.72	1-5	36.19
7-10	15.74	1-4	24.74	1-17	36.55
14-15	16.26	12-15	25.28	2-14	36.88
13-17	16.28	1-7	25.29	1-16	38.50
14-17	16.35	3-13	25.39	1-15	41.69
Trp	17.08	2-13	25.75	1-14	42.87

(50 μM substrate), degradation of dyn 1–8 is rapid ($t_{1/2} \approx 4$ min), whereas dyn 1–9 is slightly more stable ($t_{1/2} \approx 7$ min). For both substrates, less than 10% of the original dynorphin was recovered after 15 min of incubation (results not shown). In accordance, the Tyr peak has reached almost its maximum value at 15 min, and formation of the respective des-Tyr fragments is noted due to aminopeptidase activity, which seems to be prevalent with these two substrates. Smaller steady-state concentrations of dyn 1–6 and 1–7 accumulate from dyn 1–8 and 1–9, respectively, by the action of a dipeptidylcarboxypeptidase, i.e. angiotensin-converting enzyme (ACE) or an ACE-like enzyme, which cleaves dipeptides from the C-terminus of peptides. Also, the corresponding des-Tyr fragments of dyn 1–6 and 1–7 can be observed. These fragments are likely to be formed predominantly from dyn 2–8 and 2–9, respectively, by ACE, although concurrent formation from the Tyr-containing precursors and subsequent action by an aminopeptidase is not excluded. Neither dyn 1–7/dyn 2–7 nor dyn 1–8/dyn 2–8 could be detected in the degradation of dyn 1–8 and 1–9, respectively; the absence of these two pairs indicates low activities of peptidylcarboxypeptidase(s). Interestingly, in the degradation of both substrates, dyn 1–5 and 2–5 are formed, and the latter seems to be

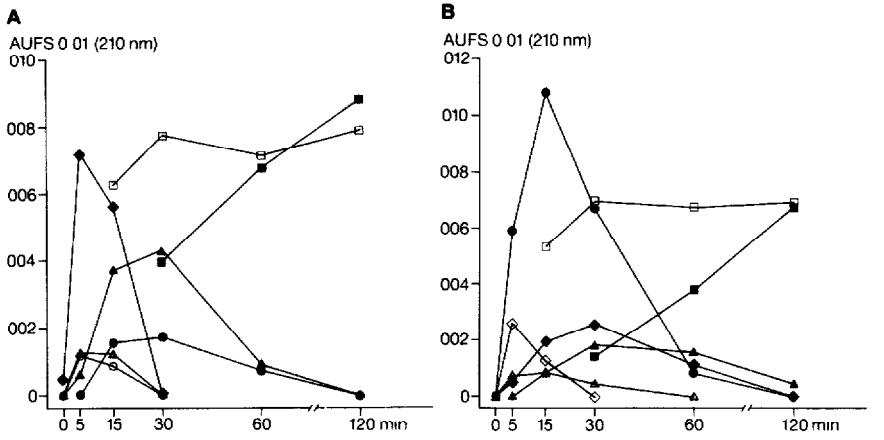

Fig. 2. (A) Enzymic degradation of dyn 1-8 (50 μM) with rat striatal nerve terminal membranes in vitro. The value at 0 min was obtained by mixing the components followed by immediate deproteinization of the 100-μl aliquot of the incubation mixture; for dyn 1-8 a value of 0.019 A.U. was obtained at 0 min, which corresponds to 0.83 nmol dyn 1-8. (□) Tyr; (■) Phe; (△) dyn 1-5; (▲) dyn 2-5; (○) dyn 1-6; (●) dyn 2-6; (♦) dyn 2-8. For clarity, the curves for dyn 1-8 itself and for low levels of dyn 1-4 and dyn 2-4 have been omitted. (B) A similar experiment with dyn 1-9. At 0 min, a value of 0.029 A.U. was obtained for dyn 1-9. For clarity, the curves of dyn 1-9 and of two small, unidentified peaks have been omitted. (◇) dyn 1-7; (♦) dyn 2-7; (●) dyn 2-9; other symbols in (A).

remarkably stable. With dyn 1-8, small amounts of dyn 1-4 and 2-4 are also detected; however, for clarity of presentation, these curves were omitted from the diagram. Also with dyn 1-9, small peaks at retention times of 24 and 24.5 min appeared, but were not identified.

In contrast to dyn 1-8 and 1-9, attack at the C-terminal end of dyn 1-7 by ACE-like enzymes is the pre-eminent reaction, as evidenced by the almost exclusive formation of dyn 1-5 from dyn 1-7 during the first 5 min of incubation (Fig. 3A). At this time-point, only ca 5% of the dyn 1-7 could be recovered ($t_{1/2} \approx 2.5$ min) and, consonant with the primary formation of dyn 1-5, Tyr formation was rather low. Therefore, aminopeptidase activity is clearly less pronounced with dyn 1-7 than with the longer substrates, a fact also indicated by the low levels of dyn 2-7 found and the protracted formation of dyn 2-5 from dyn 1-5 (note the shift in maximum concentration in the curves). Apart from small amounts of dyn 1-4 (results not shown), neither dyn 1-6 nor dyn 2-6 could be detected, indicating insufficient accumulation in the dynamic state. Therefore, either none is formed, or all is degraded. The latter seems to be the case, as evidenced by the degradation pattern of dyn 1-7 in the presence of the ACE-inhibitor MK 422 (125 μM) (Fig. 3B). As expected, dyn 1-5 and 2-5 are no longer observed, but rather the (low) activities of aminopeptidase and peptidylcarboxypeptidase become apparent, resulting in a slow build-up of dyn 2-7 and, less so, dyn 1-6. Unfortunately, the broad peak of MK 422 interferes

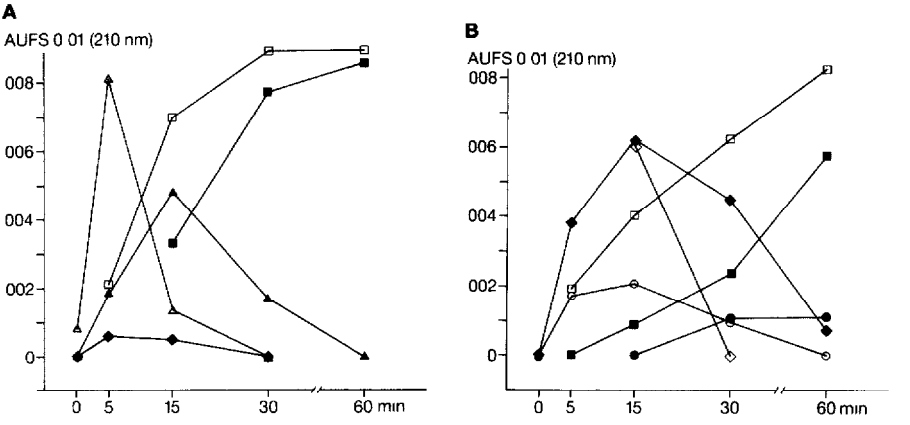

Fig. 3. (A) Enzymic degradation of dyn 1-7 (125 μM) with rat striatal nerve terminal membranes in vitro. Experimental details are given in Fig. 2. At 0 min, an A.U. value of 0.031 was obtained for dyn 1-7. For clarity, the curves for dyn 1-7 and for small amounts of dyn 1-4 have been omitted. (□) Tyr; (■) Phe; (△) dyn 1-5; (▲) dyn 2-5; (♦) dyn 2-7. (B) The same experiment in the presence of MK 422 (125 μM). At 0 min an A.U. value of 0.029 was obtained for dyn 1-7. (◇) dyn 1-7; (○) dyn 1-6; (●) dyn 2-4; other symbols as (A). Owing to the large MK 422 peak close to dyn 2-6, the latter could not be quantified.

with the quantification of dyn 2–6. Small amounts of dyn 2–4 are present at long incubation times, probably formed by consecutive removal of single amino acids from dyn 2–6.

To clarify the question of whether or not dyn 1–6 and dyn 2–6 are intermediates in the formation of dyn 1–5 and dyn 2–5 in the breakdown of dyn 1–8, experiments with appropriate inhibitors were carried out. In the presence of 125 μM MK 422 (Fig. 4A), degradation of dyn 1–8 (125 μM) was considerably retarded and, as expected, formation of dyn 1–6 and dyn 1–4/dyn 2–4 were both reduced to ca. 20 and 40% of control values, respectively. However, accumulation of dyn 1–5 was practically unchanged. Similarly, production of Phe, which is an index of the degradation of dyn 1–5/dyn 2–5 (via the "enkephalinase pathway" and further degradation of the resulting fragment Phe-Leu) was also unchanged. Thus, the synthesis rate of the dyn 1–5 and 2–5 must be uninfluenced by MK 422. Quite different results were obtained with the metalloendopeptidase inhibitor thiorphan, which inhibits enkephalinase, an enzyme cleaving the Gly–Phe bond (positions 3 and 4) of enkephalins in brain tissue. The K_i value of thiorphan for this enzyme is 3.5 nM [11]. As shown in Fig. 4B, 125 μM thiorphan increases the production of dyn 1–5 and 2–5 after 60 min of incubation with dyn 1–8 (125 μM). In line with this finding, Phe formation is reduced dramatically. Because thiorphan also inhibits to a lesser extent ACE (K_i=140 nM [11]), the static concentrations of dyn 1–6/dyn 2–6 and dyn 1–4/dyn 2–4 are reduced in a similar way as with MK 422, corroborating the results with MK 422. In contrast to MK 422 and thiorphan,

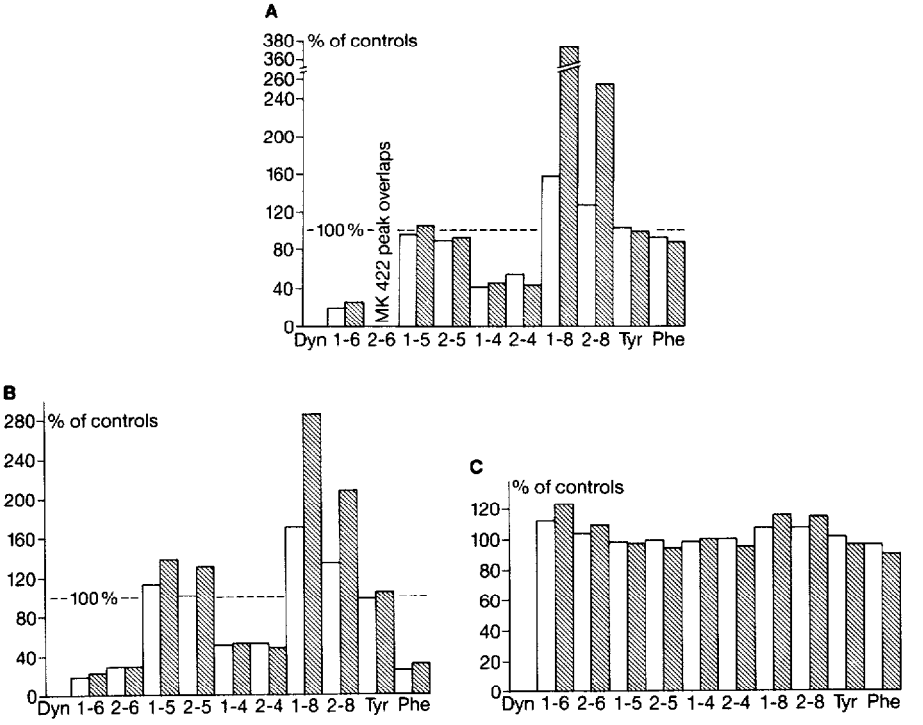

Fig. 4. (A) Effect of MK 422 (125 μM) on the degradation pattern of dyn 1-8 (≈ 125 μM) with rat spinal cord nerve terminal membranes in vitro. The curves were similar to those obtained with striatal membranes (Fig. 2A), although enzyme activity is lower in spinal cord than in striatal membranes and thus significant amounts of dyn 1-8 could still be measured at longer incubation times. Experimental details as in Fig. 2. Two separate experiments were carried out. The A.U. value of dyn 1-8 at 0 min for the control experiment was 0.068, and for the experiment with MK 422 the value was 0.065. The A.U. values of the various products of the control experiment were set at 100%. Open bars: 30 min incubation; hatched bars: 60 min incubation. (B) Same experiments except that thiorphan (125 μM) was used instead of MK 422. The A.U. value of dyn 1-8 at 0 min for the control experiment was 0.062, and for the experiment with thiorphan the value was 0.065. Other details as for (A). (C) Same experiments except that GEMSA (125 μM) was used instead of MK 422. The A.U. value of dyn 1-8 at 0 min for the control experiment was 0.055, and for the experiment with GEMSA the value was 0.051. Other details as for (A).

GEMSA, an inhibitor of "enkephalin convertase" (see below) had only minor effects on the degradation profile of dyn 1–8 (Fig. 4C).

Unlike dyn 1–7, 1–8 and 1–9, the enzymolysis pattern of dyn 1–10 suggests that a peptidylcarboxypeptidase plays a prominent role in the C-terminal degradation of this substrate because considerable amounts of dyn 2–9 accumulate at longer incubation times (Fig. 5A). In spite of high aminopeptidase activity (dyn 2–10 formation), even small amounts of dyn 1–9 can be found. In the presence of 125 μM bestatin, an aminopeptidase inhibitor, only insignificant

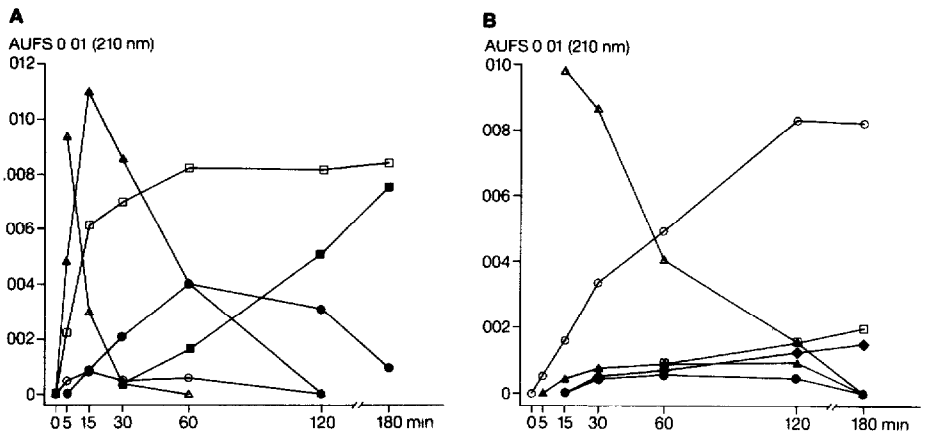

Fig. 5. (A) Enzymatic degradation of dyn 1-10 (50 μM) with rat striatal nerve terminal membranes in vitro. Details are given in Fig. 2. At 0 min, an A.U. value of 0.015 was obtained for dyn 1-10. (□) Tyr; (■) Phe; (△) dyn 1-10; (▲) dyn 2-10; (○) dyn 1-9; (●) dyn 2-9. (B) Same experiment in the presence of bestatin (125 μM). At 0 min, an A.U. value of 0.013 was obtained for dyn 1-10. (□) Tyr; (△) dyn 1-10; (○) dyn 1-9; (●) dyn 1-8; (▲) dyn 1-7; (♦) dyn 1-5.

amounts of dyn 1–8 are formed (Fig. 5B); however, accumulation of dyn 1–9 is increased substantially. Thus, under our conditions, ACE-like activities play virtually no role in the C-terminal degradation of dyn 1–10. Interestingly, more than 20% of dyn 1–10 is recovered following 15 min of incubation ($t_{1/2} \approx 9$ min), in contrast to some of the shorter dynorphins studied. This finding agrees with literature data [12] that show that removal of Tyr by aminopeptidase is hindered progressively by elongation of the dynorphin sequence.

DISCUSSION

The present chromatographic system allows the separation of a large number of synthetic dynorphin A fragments, including Tyr, Phe and Trp, which in contrast to other amino acids are detected with high sensitivity and represent important, stable landmarks in the elucidation of enzymolysis profiles. Time-course studies seem helpful in establishing precursor/product relationships as shown in the study with dyn 1–7 (Fig. 3A) because of the multiplicity of products. With dyn 1–8, three major or primary cleavage sites at the Tyr^1–Gly^2, Leu^5–Arg^6 and Arg^6–Arg^7 bonds were demonstrated, in agreement with studies on guinea-pig whole brain membranes [8]. In contrast, Benuck et al. [12], in studies with rat whole brain nerve terminal membranes, mention only the cleavage of the Tyr^1–Gly^2 and Arg^6–Arg^7 bonds as major pathways. One possible explanation could be the long retention time of dyn 1–5; which might have escaped detection in the study of Benuck et al. [12] because they used a different type of HPLC column. Our results strengthen the view that dyn 1–5 is

a major product of dyn 1–8 enzymolysis, and provide evidence that formation occurs directly from dyn 1–8 via the action of an endopeptidase different from the enkephalin convertase [13], which converts hexa- and heptapeptides to dyn 1–5 (Leu^5-enkephalin) and Met^5-enkephalin. Thus, GEMSA, a potent inhibitor of enkephalin convertase [14], or almost complete inhibition of dyn 1–6 formation by MK 422 does not influence dyn 1–5 synthesis or degradation. In addition, the possibility of dyn 1–7 being an intermediate in the formation of dyn 1–5 is excluded by the failure to detect adequate amounts of dyn 1–7 in the enzymic degradation pattern of dyn 1–8 (Fig. 2A), also in the presence of the ACE inhibitor MK 422 (Fig. 4A). Therefore consecutive removal of single amino acids from dyn 1–8 to form dyn 1–5 can at best be only a minor route.

In contrast to the enkephalin convertase mentioned above, less is known about the putative enzyme(s) cleaving the Leu^5–Arg^6 bond of dyn 1–8. However, Gillan et al. [8] found that the dipeptide Leu–Arg potently inhibits the formation of dyn 1–5 from dyn 1–8 in the presence of bestatin and the ACE inhibitor captopril. Studies are in progress to identify dyn 6–8 as the second product of this reaction, but dyn 6–8 still has to be separated from Phe in our system (see Table I). In accordance with our results, Dixon and Traynor [15] showed that conversion of dyn 1–8 into dyn 1–5 by rat spinal cord slices in vitro is increased substantially if a mixture of bestatin, thiorphan and captopril is added to the incubation medium. Similarly, Miller et al. [16] reported conversion of dyn 1–9 into dyn 1–5 by slices of mouse vas deferens in the presence of the above inhibitors. Candidate enzymes for the scission of the Leu^5–Arg^6 bond in dyn 1–8 are a metalloendopeptidase (EC 3.4.24.15) and a cysteinyl-endopeptidase described recently [17,18]. These two enzymes prefer oligopeptides as substrates and produce enkephalins from several bioactive precursors, including dyn 1–5 from dyn 1–8.

With regard to dyn 1–7, the above enzyme, which is insensitive to MK 422, does not seem to be involved in its degradation because formation of dyn 1–5 from dyn 1–7 was inhibited completely by MK 422 (Fig. 3B). Removal of the dipeptide Arg^6–Arg^7 by ACE-like enzymes and/or the enkephalin convertase [13] (more appropriately named carboxypeptidase H [19]), which is a carboxypeptidase B-like enzyme, seems to be the major pathway in dyn 1–7 degradation. Nevertheless, because some dyn 1–6 formation was observed, concomitant sequential removal of single Arg residues is possible. Similarly, in the degradation of dyn 1–9, the precursor–product relationships (Fig. 2B) suggest that ACE and/or carboxypeptidase H is the major activity leading to dyn 1–5/dyn 2–5, with dyn 1–7/dyn 2–7 being intermediates. On the other hand, additional formation of dyn 1–5 by direct cleavage of dyn 1–9 by the metalloendopeptidase postulated in the direct cleavage of dyn 1–8 cannot be excluded; this is supported by the results obtained by Miller et al. [16] with mouse vas deferens.

The striking difference in the C-terminal degradation pattern of dyn 1–10

compared with those of dyn 1–7, 1–8 and 1–9 may be explained partly by the C-terminal, non-basic amino acid of dyn 1–10, i.e. Pro, which does not seem prone to attack by carboxypeptidase B-like enzymes, which preferentially remove basic C-terminal amino acids. Instead, the reaction might be catalysed by carboxypeptidase A. With purified carboxypeptidase A from rat brain, considerable formation of dyn 1–9 from dyn 1–10 has been reported [6]. On the other hand, one would expect formation of dyn 1–7 from dyn 1–8, whose C-terminal Ile seems highly qualified for interaction with the hydrophobic catalytic site of carboxypeptidase A. However, the present results do not favour this pathway for dyn 1–8 and show the difficulty of making predictions on the basis of theoretical consideration involving pure enzymes. Possibly, reaction rates of the other enzymes discussed in the enzymolysis of dyn 1–8 are too high to allow significant accumulation of dyn 1–7, especially in view of the rapid enzymic degradation of dyn 1–7.

In conclusion, the analytical method described represents a useful approach to the elucidation of enzymolysis profiles of dynorphins. It circumvents the use of tritiated dynorphins [8,15,16], which have the drawback that fragments lacking Tyr cannot be detected, because only [^{3}H]Tyr-labelled dynorphins are available presently. However, the detection of these fragments becomes critically important in studies with longer dynorphins, e.g. dyn 1–17 [20]. The use of subcellular fractions complicates the interpretation of chromatograms owing to the multiplicity of enzymes present. However, the patterns obtained with subcellular fractions or tissue slices reflect more closely the physiological situation than those obtained with purified enzymes. This approach may ultimately answer the question of which enzymes are relevant in the formation of the dynorphinergic transmitters or modulators, i.e. the biologically active species, and which are responsible for the termination of their pharmacological actions.

ACKNOWLEDGEMENTS

The skilful technical assistance of Marianne Ross and Karl-Heinz Heider is gratefully acknowledged. Silvia Krichbaum is thanked for typing of the manuscript and expert preparation of the figures.

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