Identification of Stabilized Dynorphin Derivatives for Suppressing Tolerance in Morphine-Dependent Rats

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Purpose. Modulatory actions on morphine-induced effects, such as tolerance and withdrawal, have been noted for dynorphin A(1-13) [Dyn A(1-13)] and similar peptides. These are currently of limited therapeutic potential due to extensive metabolism by human metabolic enzymes resulting in a half-life of less than 1 min in human plasma. The purpose of this study was to identify stabilized dynorphin A (Dyn A) derivatives, to determine their metabolic routes in human plasma, and to assess whether the pharmacodynamic activity is retained.

Methods. The stability of peptides in human plasma was tested using *in vitro* metabolism studies with and without enzyme inhibitors. Identification of the generated metabolites was performed by mass spectrometry after high performance liquid chromatography (HPLC) separation. The *in vivo* activity of a stabilized dynorphin was tested by tail-flick assay in morphine-tolerant rats.

Results. Though amidation of the Dyn A(1-13) was able to stop the majority of C-terminal degradation, metabolism of Dyn A(1-10) amide continued by captopril sensitive enzymes, suggesting that Dyn A(1-13) amide is a better candidate for additional stabilization. Two Dyn A(1-13) amide derivatives further stabilized at the N-terminal end, [D-Tyr^1]-Dyn A(1-13) amide and [N-Met-Tyr^1]-Dyn A(1-13) amide, showed half-lives in plasma of 70 and 130 min, respectively. The most stable derivative [N-Met-Tyr^1]-Dyn A(1-13) amide was tested successfully for retention of the pharmacological activity in modulating antinociceptive activity.

Conclusions. [N-Met-Tyr^1]-Dyn A(1-13) amide showed significant stability and antinociceptive activity in the tail-flick test, thus pointing to the clinical potential of this derivative in the management of pain as well as its potential activity in suppressing opiate tolerance and withdrawal.

KEY WORDS: dynorphin A(1-13); dynorphin A(1-10) metabolism; enzyme inhibition study; suppression of morphine tolerance.

INTRODUCTION

Quite often, small linear peptides show markedly short half-lives in biological fluids. To make these peptides pharmacologically useful, it is necessary to increase their biological half-life by increasing their resistance to proteolysis. *In vitro* metabolism in plasma, which is considered the major site for peptide metabolism, has been a useful tool for predicting the *in vivo* metabolism of peptides (1).

Dynorphin (Dyn) A(1-13), a trideca-endogenous opioid peptide, and other fragments such as Dyn A(1-10), modulate opioid induced effects, in particular, tolerance and withdrawal in opiate-dependent human and animal models (2–6). It has been reported that despite the lack of analgesic activity on its own, Dyn A(1-13) antagonizes morphine-induced analgesia in naive mice and potentiates it in tolerant animals when given i.c.v. (intracerebroventricularly) (7). These effects are not reversed by naloxone, suggesting a nonopioid mechanism of action (6). Hooke *et al.* (6) observed an inhibition of withdrawal symptoms by intravenously (i.v.) administered Dyn A(1-13) in morphine-dependent mice for up to 1 h, whereas studies conducted in morphine-tolerant rats showed a shorter effect for i.v.-administered dynorphin (20–40 min) (8). The therapeutic potential of Dyn A(1-13) was also studied in the treatment of opiate addicts. Wen *et al.* observed a significant decrease in withdrawal symptoms of heroin addicts after intravenous administration of 60 μg/kg Dyn A(1-13) (5,9). Uneklabh *et al.* found reduction in craving after administration of 60 and 180 μg/kg of Dyn A(1-13) (10). Specker *et al.* have also found Dyn A(1-13) well tolerated even in 1000 μg/kg in reducing withdrawal symptoms (11). One metabolic fragment that has also shown to possess a similar pharmacological profile as Dyn A(1-13) is Dyn A(1-10). Upon these findings, Dyn A(1-10) and Dyn A(1-13) may have a role in treating addict patients. However, both peptides have a markedly short half-life in human plasma (<1 min) because of the extensive metabolism by carboxypeptidases and aminopeptidases, which limits its therapeutic potential (12). One clinical study that has determined the pharmacokinetic profile of Dyn A(1-13) *in vivo* has shown an initial half-life of 0.56 min. (13), which agrees well with an apparent half-life of less than 1 min for Dyn A(1-13), as previously determined by *in vitro* metabolism studies conducted in our lab (12). While the pharmacological profile seems promising, pharmacokinetic stability is also important for being a potential drug candidate.

Numerous examples have been cited in literature for the enhancement of peptide stability by chemical modification. Some common modifications include: N-methylation of the amino-terminus (14), replacement of L-Tyr^1 with D-Tyr^1 (15), amidation (15), substitution of amide bonds with the peptide bond surrogate ψ[CH_2-NH] (16,17), and cyclization (18). Ideally, the chemical modification introduced into the peptide should not compromise the activity. The N-terminally intact metabolic fragments of Dyn A(1-13) retain opioid receptor affinity (19), whereas N-terminally truncated dynorphin derivatives such as Dyn A(2-13), Dyn A(2-17) modulate somatic signs of withdrawal as well as the developing tolerance via nonopioid pathways, possibly the NMDA-receptor and/or melanocortin receptor (19,20). The ability of this nonopioid peptide to modulate the expression of morphine tolerance can be used in clinical practice for treating opioid addicts (19). Another step toward stabilizing peptides is amidation, which has also shown to be a modification effective in improving the stability of several peptides (15). Amidation of the C-terminus is expected to enhance the stability of Dyn A(1-13) and Dyn A(1-10) as it protects against carboxypeptidase attack. The amide of Dyn A(1-10) has been reported to be more potent and selective than Dyn A(1-13) in modulating opioid and nonopioid actions (21). Further stabilization of these peptides could be attempted by either N-methylation of the amino-terminus (14) or by replacing L-Tyr^1 with D-Tyr^1 (15).

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It is hypothesized that rational chemical modification of Dyn A(1-10) and Dyn A(1-13) will result in more stable derivatives that can exert enhanced *in vivo* activity. Therefore, the goals of this work were to identify stable Dyn A(1-10) and Dyn A(1-13) derivatives and to determine their metabolic routes in human plasma using *in vitro* metabolism studies. Finally, [N-Met-Tyr^1]-Dyn A(1-13) amide, the most stable derivative, was tested for the activity in modulating tolerance in a rat model.

MATERIALS AND METHODS

Materials

Dyn peptides were obtained from the American Peptide Company (Sunnyvale, CA, USA) except for [D-Tyr^1]-Dyn A(1-13) amide, which was custom synthesized by the Protein Chemistry Core Facility, Interdisciplinary Center for Biotechnology Research, University of Florida (Gainesville, FL). Peptide purity was higher than 70%. The following chemicals were obtained from the indicated sources: (2-guanidinoethylmercapto) succinic acid (GEMSA) from Fluka (Buchs, Switzerland), L-leucinethiol and pentanesulfonic acid sodium salt from Sigma (St. Louis, MO, USA), and acetonitrile and methanol from Fisher Scientific (Pittsburgh, PA, USA) as HPLC grade.

Drugs Used in Animal Studies

Morphine sulfate (Baxter Healthcare Corporation, Deerfield, IL, USA) was dissolved in sterile pyrogen-free isotonic saline, at a concentration of 10 mg/kg. N-Met-Tyr^1-Dynorphin amide was purchased from GenoMechanix, LLC (Gainesville, FL, USA) and dissolved in saline at a concentration of 5 μmol/kg.

Methods

In Vitro Metabolism

The Health Center Institutional Review Board of University of Florida approved the study protocol. Blood was collected from the antecubital vein of a caucasian, healthy, male volunteer (age 25) and transferred into heparinized (200 μl of 100 IU heparin/15 ml blood) polypropylene centrifuge tubes. It was then immediately centrifuged at 300 × *g* for 20 min and the supernatant (plasma) was either used directly for metabolism studies or stored at −70°C.

Nine hundred microliters of thawed human plasma (previously frozen at −70°C) was spiked with 100 μl dynorphine derivatives to achieve a final concentration of 100 μg/ml, then incubated at 37°C. Aliquots of 100 μl of this incubation were removed at several designated time points. Actual observation periods for individual peptides differed according to the stability of the peptides and ranged from 10 to 180 min. Actual time points are shown in Fig. 1 and were selected from preliminary experiments according to the stability of the individual compounds. Thus, a difference in the number time points for the selected derivatives was due to a flexible sampling procedure. The incubation was then terminated by adding an equal volume of the blocking solution, which was made up of 5% aq. $ZnSO_4$ solution, Me-OH, and ACN (5:3:2) and served to precipitate proteins, in order to clean-up the plasma and terminate metabolic activity before injection into HPLC. The mixture was vortexed and centrifuged at 10,000 × *g* for 3 min, then 100 μl of clear supernatant was directly injected into the HPLC system.

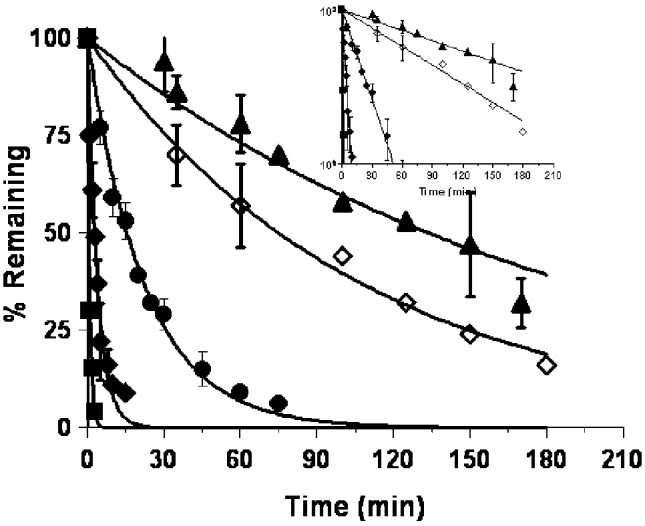

Fig. 1. Stability of Dyn A(1-13) derivatives in human plasma: Dyn A(1-13) (■), Dyn A(1-13) amide (●), [D-Tyr^1]-Dyn A(1-13) amide (▲), and [N-Me-Tyr^1]-Dyn A(1-13) amide (♦)

Enzyme Inhibition Studies

Enzyme inhibition studies were used to determine the metabolic route of Dyn A(1-10), Dyn A(1-10) amide, [N-Met-Tyr^1]-Dyn A(1-13) amide, and [D-Tyr^1]-Dyn A(1-13) amide in human plasma *in vitro*. To determine the half-life of a dynorphin peptide in the presence of a specific enzyme inhibitor, the enzyme inhibitor was mixed with human plasma at 37°C and allowed to equilibrate for 5 min before the dynorphin peptide was added.

The following inhibitor concentrations were used in the final incubation mixture in order to block the indicated enzymes: L-leucinethiol (10 μM, aminopeptidases), GEMSA (10 μM, carboxypeptidases), and captopril [100 μM, angiotensin converting enzyme (ACE)]. Samples were then analyzed by HPLC after stopping the enzymatic activity, as described above.

HPLC

The HPLC system consisted of a ZORBAX column (Agilent, Palo Alto, CA, USA) (300-SB-C18, 4.6 × 150 mm with a particle size of 5 μm), and UV detection at a wavelength of 210 nm. The mobile phase was made up of two solvent systems: solvent A consisted of 5% acetonitrile in water with 0.5% pentanesulfonic acid and 0.03% trifluoroacetic acid, whereas mobile phase B consisted of 20% water in acetonitrile with 0.5% pentanesulfonic acid and 0.03% trifluoroacetic acid. A linear gradient was run from 75% A to 55% A in 20 min at a flow rate of 1.0 ml/min. As controls, blank human plasma and aqueous solutions of 100 μg/ml of Dyn A(1-13) derivatives were injected into the HPLC system. As stability tests followed only the relative decrease in peak areas of the

mother compounds on a given day, only limited validation experiments were performed for Dyn A(1-13) as the model compound. Calibration curves for Dyn A1-13 with concentrations ranging from 1 to 100 μg/ml were established in a mixture of plasma and blocking solution. Calibration curves were linear with r^2 values always greater than 0.994 (n = 5). Variability in the slopes was less than 8% (n = 5). The general linearity of the assays was also supported by previous reports on the detailed evaluation of dynorphin derivatives, all of which showed linear calibration curves (12). Variability of Dyn A(1-13) quality control samples (10 μg/ml, n = 3, lower range of the assay) was less than 6%. Thus, the lower limit of quantification was at least 10 μg/ml (10% of the starting concentration). Recovery for the precipitation procedure was between 99 and 112% (n = 3). After establishing the linearity and recovery of the assay, stability tests were performed without calibration curves, as long as the signal of the starting concentration (t = 0) was in agreement with values determined during the validation.

Mass Spectrometry

Identification of the metabolites of the tested Dyn A(1-10) and Dyn A(1-13) derivatives in enzyme inhibition studies was performed by mass spectrometry (MS). Prior to analysis, 1-ml fractions were collected off the HPLC system using a Gilson FC 203B fraction collector (Middleton, WI, USA). Subsequently, they were evaporated to dryness under vacuum; the residue was reconstituted in 100 μl of methanol (0.1% trifluoroacetic acid). The respective fractions corresponding to metabolite peaks were consequently assayed by matrix assisted laser desorption mass spectrometry (MALDI) using a Vestec LaserTec instrument at the Protein Chemistry Core Facility, Interdisciplinary Center for Biotechnology Research, University of Florida.

Animal Studies

Animals. Animal studies were performed close to a protocol described by Lee and co-workers (22), but differed in the procedure to induce tolerance (morphine injections instead of pellets).

Male Sprague-Dawley rats (250–300 g) were housed two per cage in a temperature and humidity-controlled environment and fed *ad libitum*. The University Institutional Animal Care and Use Committee of University of Florida approved all experiments.

Induction of Tolerance. Rats were made tolerant to morphine by injecting 10 mg/kg morphine sulfate twice a day (9 a.m., 9 p.m.) for 5 days subcutaneously.

Catheter Implantation. Directly after the last dose of morphine, animals were anesthetized with a combination of ketamine (30 mg/kg) and xylazine (6 mg/kg) for implantation of a jugular catheter. The catheter [silicone tubing, size 0.025′ i.d. (0.64 mm i.d.) and 0.047′ o.d. (1.19 mm o.d.) from Helix Medical, Inc, Carpinteria, CA, USA] was inserted to the level of the right atrium and the distal end was passed through a rat jacket (Lomir Biomedical Inc., Baltimore, MD, USA) so the animal cannot reach and damage the catheter.

Drug Administration. Six hours after the last morphine injection, the rats were administered dynorphin (5 μmol/kg in a volume of 0.5 ml) or saline (0.5 ml) intravenously through the jugular catheter. The time between the anesthesia for implantation of a jugular catheter and the dynorphin treatment was judged sufficient, especially as animals were fully awake and responsive before dynorphin administration. In addition, active treatment and saline animals were treated identically, but differed significantly in the tail flick results (see "Results," below) with placebo injections being identical to pre-morphine baseline evaluations on day 1.

Measurement of Tolerance. The degree of tolerance was measured using the radiant heat tail-flick test (Tail Flick Analgesia Meter, 0570-001L, Columbus Instruments Int. Corp., Columbus, OH, USA) (23). For the measurements of tail-flick latencies, the rats were held in dark Plexiglas boxes with the tail positioned on tail-flick apparatus for radiant heat stimulation. The tail-flick response was elicited by applying the heat stimulus to the dorsal part of the tail. The heat stimulus was adjusted to yield baseline latencies of 2 to 3 s (lamp intensity was 10), with a 12-s cut-off time to prevent tail damage. The baseline measure was determined by using the median of three tail-flick tests separated by approximately 20 s. Tail-flick latencies were also measured before and 1 h after dynorphin administration.

Data Analysis

As degradation of all peptides followed pseudo-first order kinetics (see Fig. 1), half-lives of Dyn A(1-10) and Dyn A(1-13) derivatives were estimated by fitting the areas under the peak (AUP) obtained by HPLC at various time points for each Dyn A(1-13) derivative to a first-order one-compartment model with Cp_0 representing the starting peak area, which was set as 100%:

$$Cp = Cp_0 \times e^{-ke.t}$$

$$t_{1/2} = \frac{0.693}{k_e}$$

The fraction (F) describing the role of a specific enzyme in the overall metabolism of a Dyn peptide is calculated from the half-lives of a given peptide in the presence ($t_{0.5inh}$) or absence ($t_{0.5deg}$) of the enzyme inhibitor:

$$F = \left[1 - \frac{t_{0.5deg}}{t_{0.5inh}}\right]$$

The data of antinociception obtained by tail-flick test was expressed as a percent of maximum possible effect (MPE%) (24), which is calculated: $[(T_1 - T_o)/(T_2 - T_0)] \times 100$, where T_0 and T_1 were pre-drug latencies on day 1 and post-drug latencies on day 5, whereas T_2 was the cut-off time (12 s). The difference between the cut-off time (T_2) and the animal's baseline reaction time (T_0) was taken as 100% effect (25). Unpaired Student's *t* test was used to analyze the statistical difference between the dynorphin treated and control group.

RESULTS

In Vitro Metabolism and Enzyme Inhibition Studies

The half-lives of the tested Dyn peptides are listed in Table I. Dyn A(1-10) has a half-life of 1.3 min, whereas Dyn A(1-13) showed a half-life of 0.9 min in human plasma, which agrees well with previous studies that had demonstrated a half-life of 0.85 min for Dyn A(1-13) in human plasma (12). Amidation of Dyn A(1-10) increased the half-life to 3.3 min.,

Table I. Half-Lives Obtained from Enzyme Inhibition Studies for Modified Dynorphin (Dyn) Peptides

Enzyme inhibitor	Half-life (min ± SD) Dyn A(1–10)	Half-life (min ± SD) Dyn A(1–10) amide	Half-life (min ± SD) Dyn A(1–13)	Half-life (min ± SD) Dyn A(1–13) amide	Half-life (min ± SD) [D-Tyr^1]-Dyn A(1–13) amide	Half-life (min ± SD) [N-Me-Tyr^1]-Dyn A(1–13) amide
None	1.3 ± 0.2 (n = 3)	3.3 ± 0.8 (n = 8)	0.9 ± 0.1 (n = 3)	23.8 ± 5.1 (n = 10)	70.2 ± 10.7 (n = 4)	130.1 ± 14.3 (n = 8)
L-leucinethiol (10 μM)	8.2 (84%)*	44.0 ± 7.8 (92.5%)*	0.80 (0%)*	84.4 ± 34.8 (71.8%)	119.1 ± 33.9 (41.1%)*	123.2 ± 17.1 (0%)*
Aminopeptidase	(n = 2)	(n = 7)	(n = 1)	(n = 12)	(n = 4)	(n = 6)
GEMSA (10 μM)	1.4 ± 0.3 (7%)*	4 ± 0.3 (17.5%)*	4.4 (80%)*	21.4 ± 2.4 (0%)*	86.8 ± 12.4 (19.1%)*	151.5 ± 31.0 (14.1%)*
Carboxypeptidase	(n = 4)	(n = 3)	(n = 1)	(n = 4)	(n = 4)	(n = 3)
Captopril (ACE) (100 μM)	1.6 ± 0.2 (19%)* (n = 4)	4.7 ± 0.2 (29.8%)* (n = 3)	0.7 ± 0.1 (0%)* (n = 3)	23.5 ± 3.1 (0%)* (n = 3)	165.0 ± 11.2 (57.5%)* (n = 4)	240.3 ± 11.0 (45.9%)* (n = 3)

* The fraction (F in %) describing the role of a specific enzyme in the overall metabolism.

whereas amidation of the C-terminus of Dyn A(1-13) resulted in a half-life of 24 min in human plasma. Results of the enzyme inhibition studies are listed in Table I.

Enzyme inhibition studies for Dyn A(1-10) and Dyn A(1-13) suggested that the metabolic pathways differed between the peptides, with the majority of Dyn A(1-10) being metabolized by aminopeptidases, while the majority of Dyn A(1-13) was metabolized by carboxypeptidases Metabolism of Dyn A(1-13) amide was shifted to leucinethiol sensitive enzymes (aminopeptidases). The carboxypeptidase inhibitor GEMSA had little effect on the metabolism of Dyn A(1-13) amide (F = 0%); thus, amide substitution at the C-terminus of Dyn A(1-13) seems to have a profound protective effect against carboxypeptidase attack on that site.

Captopril, the specific ACE inhibitor, had no effect on the metabolism of Dyn A(1-13) (F = 0%) or Dyn A(1-13) amide (F = 0%), suggesting that amidation did not result in an activity gain by ACE. Contrarily, amidation of Dyn A(1-10) was unable to fully block attack from GEMSA and captopril sensitive enzymes.

The shift to the aminopeptidase pathway of degradation was also confirmed for Dyn A(1-13) amide by mass spectrometrical analysis of the main metabolite fraction collected by HPLC. Dyn A(1-13) amide was incubated in plasma, analyzed by HPLC and the main peaks collected. Main metabolites were identified as Dyn A(2-13) amide, Dyn A(3-13) amide, and Dyn A(4-13) amide. As for Dyn A(1-10) amide, the major metabolic fragment identified was Dyn A(2-10) amide with some identification of ACE attack. This corroborates results obtained from enzyme inhibition studies which point to a major role for aminopeptidases in the metabolism of both Dyn A(1-10) amide and Dyn A(1-13) amide.

Further stabilization of Dyn A(1-13) amide was attempted by replacing L-Tyr^1 with D-Tyr^1 or N-methylation of the N-terminal Tyr residue in the structure of Dyn A(1-13) resulted in half-lives of 70 and 130 min, respectively (Fig. 1., Table I).

The results of the enzyme inhibition studies for the N and C-terminally stabilized peptides are summarized in Table I. Enzyme inhibition studies for [D-Tyr^1]-Dyn A(1-13) amide suggested that modification at the N-terminal reduced attack but not fully prevented attack by aminopeptidases with about half of the slow metabolism being occurring at the C-terminal and by captopril sensitive enzymes (Table I). However, as indicated by the leucine-thiol experiments, N-terminal attack could not be fully prevented for [D-Tyr^1]-Dyn A(1-13) amide (F = 41.1%). The involvement of the N-terminal metabolism on the fate of [N-Met-Tyr^1]-Dyn A(1-13) amide was however reduced, with the majority of the metabolism being affected by captopril and GEMSA. Thus, N-methylation of Tyr^1 was a more effective modification than replacement of L-Tyr^1 with D-Tyr^1 in protecting against aminopeptidase attack. The carboxypeptidase inhibitor GEMSA had little effect on the metabolism of [N-Met-Tyr^1]-Dyn A(1-13) amide (F = 14.1%), or [D-Tyr^1]-Dyn A(1-13) amide (F = 19.1%), mirroring the results for Dyn A(1-13)amide and supporting that amide substitution at the C-terminus of Dyn A(1-13) seems to have a profound protective effect against carboxypeptidase attack on that site. Captopril resulted in a significant slowing down in the metabolism of [D-Tyr^1]-Dyn A(1-13) amide (F = 57.5%) and [N-Met-Tyr^1]-Dyn A(1-13) amide (F = 45.9%), which

demonstrates the important role ACE plays in the metabolism of these derivatives.

No metabolites were detected on HPLC for the two stable derivatives, [D-Tyr^1]-Dyn A(1-13) amide and [N-Met-Tyr^1]-Dyn A(1-13) amide, which might be due to the degradation rates of those metabolites being greater than their rates of formation. However, upon using the enzyme inhibitor captopril to determine the metabolic contribution of ACE, significant formation of [D-Tyr^1]-Dyn A(1-6) and [N-Met-Tyr^1]-Dyn A(1-6) was observed, as shown by HPLC and MS results.

Pharmacodynamic Studies

The *in vivo* activity of the most stable derivative, [N-Met-Tyr^1]-Dyn A(1-13) amide, was tested in morphine dependent following a procedure described by Lee and co-workers (22). We observed a significant increase in tail flick latencies at the 1 h time point as compared to the saline treated group after dynorphin administration and this difference between the treated and the control group was significant ($p < 0.01$). Figure 2 shows the results of [N-Met-Tyr^1]-Dyn A(1-13) amide administration at 1 h to the rats. This result confirms that the most stable derivative, the [N-Met-Tyr^1]-Dyn A(1-13) amide successfully suppressed tolerance in morphine dependent rats.

DISCUSSION

The effectiveness of chemical modifications to enhance the stability of a peptide depends mainly on the metabolic pathway and the specific enzymes involved. Amidation was more effective in enhancing the stability of Dyn A(1-13) than Dyn A(1-10). Earlier studies, which are confirmed here, have shown that up to 80% of Dyn A(1-13) is metabolized by carboxypeptidases, whereas other enzymes such as ACE are unable to attack the C-terminal (12). Therefore, it is not surprising that amidation of the C-terminus completely blocks attack at that site. As for Dyn A(1-10), it is believed to be metabolized primarily by aminopeptidases and ACE (Table I). Because ACE still shows significant activity toward Dyn A(1-10) amide, it could be concluded that ACE is insensitive to amidation of the peptide substrate, which agrees with previous studies that showed purified ACE preparations retained activity toward amidated LHRH (26) and amidated substance P (27). However, it needs to be mentioned that captopril at high concentrations (100 μM) can inhibit carboxypeptidase N (28), which would result in the overestimation of the contribution of ACE to the overall metabolism if captopril would be assumed to be ACE specific. Though the smaller increase in half-life upon amidation was not the reason for making the decision to not further concentrate on N-terminal stabilized Dyn A(1-10), amidation of Dyn A(1-10) was not able to fully block C-terminal enzymatic attack, whereas amidation of Dyn A(1-13) was able to fully stop C-terminal truncation. This was the reason to further concentrate on N-terminal stabilized Dyn A(1-13) amide derivatives.

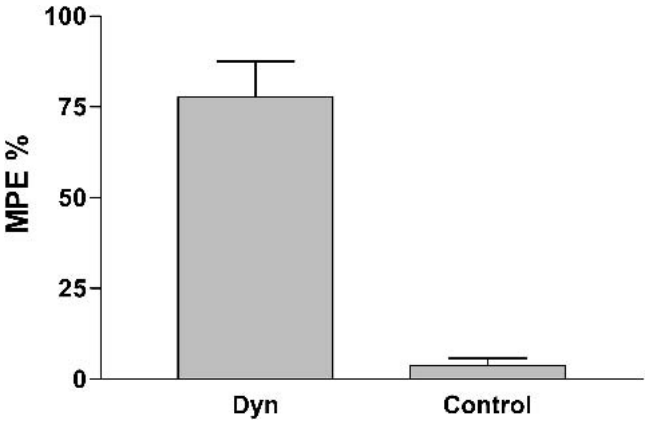

Fig. 2. Effect on MPE % at 1 h after i.v. administration of N-Met-Tyr^1 Dyn A(1-13) amide or saline to morphine-dependent rats (n = 8).

The *in vitro* metabolism studies indicate a marked improvement in the half-life of Dyn A(1-13) upon end-capping the N- and C-termini, as noted for [N-Met-Tyr^1]-Dyn A(1-13) amide and [D-Tyr^1]-Dyn A(-13) amide. Though [D-Tyr^1]-Dyn A(-13) amide still showed leucin-thiol sensitive degradation, enzyme inhibition studies for [N-Met-Tyr^1]-Dyn A(1-13) amide showed no appreciable metabolism by amino- and carboxy-peptidases, as noted by the effectiveness of the modifications in protecting against those enzymes (Table I). The sum of F values for any peptide should ideally add up to no more than 100%. However, it is noted that the sum of F values is significantly less than 100% for Dyn A(1-13) amide (71.8%) and [N-Me-Tyr^1]-Dyn A(1-13) amide (70%). This is likely due to contributions to the metabolism by other metabolic enzymes, such as endopeptidases which were unaccounted for.

The results also point to the involvement of captopril sensitive enzymes in the metabolism of those derivatives in human plasma. Hence, any further improvement in the stability of Dyn A(1-13) derivatives should include modifications that will protect against such attack. It has been reported that the introduction of a proline, glutamate or aspartate residue on the C-terminus inhibits ACE activity (26,29).

The metabolism of Dyn A(1-13) amide and [N-Met-Tyr^1]-Dyn A(1-13) amide seems to involve action by endopeptidases. This was indicated in part by MS analysis of degradation of [N-Met-Tyr^1]-Dyn A(1-13) amide and [D-Tyr^1]-Dyn A(-13) amide in the presence of captopril, which demonstrated the formation of Dyn A(1-6) fragments. These studies do not clearly demonstrate that [N-Met-Tyr^1]-Dyn A(1-13) amide and [D-Tyr^1]-Dyn A(-13) amide are directly converted into Dyn A(1-6) derivatives, as the mass spectrometrical analysis of visible HPLC peaks only indicate that under captopril these derivatives are visible during HPLC analysis whereas not in the absence of captopril. It does, however, indicate that captopril is blocking the major metabolic pathway for these Dyn A(1-6) metabolites resulting in their accumulation. This is supported by studies that have shown ACE to very potently inactivate Leu-enkephalin-Arg^6 by cleavage of the Phe^4-Leu^5 bond (30). The increase in stability of the Dyn A(1-13) derivatives under captopril indicated also that captopril sensitive enzymes are responsible for the metabolism of the parent compounds. Further studies need to clarify whether modifications of the cores will be able to block ACE or endopeptidase attack. Examples of dynorphin peptides with modified cores include: [D-Pro^{10}]-Dyn A(1-11) (31), [Phe^1, D-Ala^8, D-Pro^{10}]-Dyn A(1-11) amide (31), [Cys^5, Cys^{11}]-Dyn (1–11) amide (32), and [N-Me-Tyr^1, N-Me-Arg^7, D-Leu^8]-Dyn A(1-8) ethyl amide (33). The majority of these

peptides were designed to interact with the opioid κ-receptor; therefore, the active N-terminal pentapeptide sequence was maximally preserved. Because the core of Dyn A(1-13) is responsible for its ability to modulate tolerance and withdrawal (32), any future attempt to develop more stable Dyn A(1-13) derivatives that retain the activity of Dyn A(1-13) must take care not to introduce any deleterious modifications into the core which would result in loss of the desirable activity.

The second part of this study was interested in evaluating whether modifications made in the molecules were able to retain effects modulating tolerance and dependence. Pharmacokinetic studies, although certainly of interest were not performed, as pharmacodynamic studies answered the question whether the derivatives maintained the pharmacological activity. These studies were only performed with the most stable derivative. Our limited studies confirmed that [N-Met-Tyr^1]-Dyn A(1-13) amide retained activity. This was in agreement with results of Lee and co-workers, who showed that N and C-terminal truncations did not result in a loss of antinociceptive activity.

In conclusion, the chemical modifications of Dyn A(1-13) used in this study, specifically, amidation of the C-terminus, replacement of L-Tyr^1 with a D-Tyr^1 at the N-terminus, and N-methylation of Tyr^1 have shown to be quite effective in protecting against carboxypeptidases and aminopeptidases in human plasma. [N-Met-Tyr^1]-Dyn A(1-13) amide proved to be the most stable derivative with a half-life of more than 2 h and suppressed the tolerance in the morphine-dependent rat model.

Further work is needed to assess the potential use of those derivatives in the management in the management of other non-opioid-mediated actions as suppression of opiate tolerance and withdrawal.

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