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In vitro Biotransformation of Dynorphin A (1–17) Is Similar in Human and Rhesus Monkey Blood as Studied by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry[1]

JIM YU, EDUARDO R. BUTELMAN, JAMES H. WOODS, BRIAN T. CHAIT and MARY JEANNE KREEK

The Laboratory of Biology of Addictive Disease (J.Y., M.J.K.) and the Laboratory of Mass Spectrometry and Gaseous Ion Chemistry (B.T.C.), the Rockefeller University, New York, New York, and the Department of Pharmacology (E.R.B., J.H.W.), the University of Michigan, Medical School, Ann Arbor, Michigan

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ABSTRACT

Dynorphin A (1–17) [Dyn A (1–17)] is an endogenous opioid peptide. *In vitro* biotransformation of Dyn A (1–17) in human and rhesus monkey blood was studied by matrix-assisted laser desorption/ionization mass spectrometry. Biotransformation was observed to produce various opioid and nonopioid dynorphin A peptides. In this study, *in vitro* Dyn A (1–17) biotransformation at physiological temperature (37°C) was found to be very similar in human and rhesus monkey blood, although Dyn A (1–17) processing occurred at a faster rate *in vitro* in monkey blood than in human blood. One dominant pathway in this biotransformation was the slow removal of tyrosine at position one from Dyn A (1–17) to yield the dominant product, Dyn A (2–17). Further slow biotransformation of Dyn A (2–17) also occurred. Another major pathway of Dyn A (1–17) biotransformation is cleavage of the peptide linkage between Arg(6) and Arg(7) to produce the opioid peptide, Dyn A (1–6), and the nonopioid peptide, Dyn A (7–17). These two peptides had a short lifetime in blood, undergoing rapid biotransformation. Our results indicate that the rhesus monkey may be a good model for further *in vivo* pharmacological and neurobiological studies.

Studies of the endogenous neuropeptide Dyn A (1–17) have indicated that this peptide and its nonopioid congeners may attenuate the behavioral signs and measurements of the opioid withdrawal syndrome in morphine-dependent rodents and may be useful as a therapeutic agent for the treatment of opioid-dependent patients (Takemori *et al.*, 1992, 1993). It has also been shown that Dyn A peptides can modulate morphine tolerance in morphine-dependent mice (Hooke *et al.*, 1995a; Takemori *et al.*, 1992, 1993). Dyn A peptides have been demonstrated to have analgesic properties and may have potential application in the management of pain (Hooke *et al.*, 1995b; Smith and Lee, 1988). Preliminary studies of natural but shortened length Dyn A (1–13) in humans have shown that this peptide caused elevation in serum levels of prolactin, which reflects diminished dopaminergic tone in hypothalamus (Kreek *et al.*, 1993). Drugs of abuse such as cocaine are known to increase extracellular fluid dopamine levels in striatum and nucleus accumbens of rats (Maisonneuve and Kreek, 1994). Cocaine also alters the dynorphin system in rats: preprodynorphin mRNA is increased in the caudate putamen (Spangler *et al*, 1993), and *kappa* opioid receptor mRNA is decreased in the substantia nigra (Spangler *et al*, 1994, 1996). Thus dynorphin peptides may have therapeutic usefulness in management of cocaine dependency. Both opioid and nonopioid dynorphin peptides have been shown to alter one important aspect of immune function, natural killer cell activity (Bodner *et al.*, 1991). In recent studies, the nonopioid Dyn A (2–17) as well as the opioid Dyn A (1–17) have been shown to inhibit adenylyl cyclase activity in rat caudate putamen membranes in a concentration-dependent manner (Claye *et al.*, 1994, 1996a,b).

Isolation and characterization of the processing of dynorphin A have been studied in nervous system tissues including specific brain regions (Devi *et al.*, 1991; Devi and Goldstein, 1984; Goldstein *et al.*, 1979; Herman *et al.*, 1980; Lesie and Goldstein, 1982), in spinal cord (Silberring *et al.*, 1992) and rat liver cell cultures (Petanceska *et al.*, 1993). However, there is still relatively little information about the nature and dynamics of Dyn A biotransformation in blood (Chou *et al.*, 1993, 1994). The processing of Dyn A (1–17) produces various opioid and nonopioid peptides, which are potentially biologically active. To find a suitable model for invasive stud-

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ABBREVIATIONS: Dyn A, dynorphin A; TFA, trifluoroacetic acid; Arg, arginine; Tyr, tyrosine; MALDI-MS, mass spectrometer with a matrix-assisted laser desorption ion source; HPLC, high-performance liquid chromatography.

ies of the biotransformation of Dyn A (1–17), and to evaluate the potential therapeutic effects of this peptide, the rhesus monkey was chosen as a primate model. Comparative studies of *in vitro* biotransformation of Dyn A (1–17) at physiological temperature in rhesus monkey and in human blood were conducted. In this study, the newly developed, sensitive and specific technique of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was used to identify the biotransformation products of Dyn A (1–17) when added to freshly drawn human and to rhesus monkey blood, *in vitro*, and to elucidate the kinetics of the biotransformation processes.

Materials and Methods

Subjects. Blood was obtained from seven healthy volunteers, five men and two women with an average age of 32 years (range, 25–47 years). Blood was also obtained from three healthy adult female rhesus monkeys, average age 8.3 years (range, 7–10 years).

Chemicals. Dyn A (1–17) was synthesized by Chiron Mimotopes Peptide Systems (San Diego, CA), and made available by the National Institute on Drug Abuse (Rockville, MD). Dyn A (2–17) was synthesized by American Peptide Company (Sunnyvale, CA), and made available by Neurobiological Technologies Inc. (Richmond, CA). Dyn A (1–12) was purchased from Peninsula Laboratories, Inc. (Belmont, CA). Dyn A (1–6) was synthesized by Peninsula Laboratories, Inc. and made available by Neurobiological Technologies Inc. Somatostatin was purchased from Sigma Chemical Company (St. Louis, MO). Saline (0.9% NaCl) was from Abbott Laboratories (North Chicago, IL). α-Cyano-4-hydroxycinnamic acid was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). HPLC-grade acetonitrile was purchased from Burdick & Jackson (Muskegon, MI) and TFA from Fisher Scientific (Fair Lawn, NJ).

Dyn A processing and subsequent sample preparation. Blood was drawn from subjects into vacutainer tubes preconditioned with EDTA (Becton Dickinson, Rutherford, NJ). Typically, an 12.0-ml aliquot of freshly drawn blood was transferred to a 50-ml Falcon polypropylene tube (Becton Dickinson, Lincoln Park, NJ). After removal of 1.0 ml of blood for a control, 7.08 mg of Dyn A (1–17), dissolved in 500 μl of saline, was immediately added to the remaining blood (*i.e.*, 0.59 mg/ml or 196.4 μM). This dynorphin-containing blood was incubated in a water bath at 37°C (Bench Scale Equipment Co., Inc., Dayton, OH). In a comparative study, Dyn A (1–17) in human (n = 3) blood was also incubated at 25°C to relate these studies to previous studies from this laboratory (Chou *et al.*, 1993, 1994). Human (n = 7) blood samples were taken from the incubating tube at the following time points after the addition of Dyn A (1–17): 0, 5, 10, 15, 30, 45, 60, 90, 120, 180 and 240 min. Rhesus monkey (n = 3) blood samples were taken from the incubating tube at the time points: 0, 1, 2, 5, 8, 10, 15, 30, 40, 50 and 60 min. One milliliter of blood was used for each time point. Immediately after removal of the blood from the incubating tube, plasma was separated from the blood by centrifugation in a Sorvall RC-5B refrigerated (0–5°C) superspeed centrifuge (Sorvall Instruments, DuPont Company, Newtown, CT), at 4,000 rpm for 5 min. Then, somatostatin (100 μl of 315 μM or 0.516 mg/ml in 0.05% aqueous TFA) as the internal standard was added to 200 μl of plasma containing the biotransformed dynorphins and 1.8 ml of 1.0% TFA aqueous solution. The internal standard was used as the calibration reference for the sample filtration process and subsequent mass spectrometric analysis. However, the use of this internal standard would not allow for determination of whether or not Dyn A (1–17) or any biotransformation products were bound to blood cells and thus lost in the centrifugation step. The solution containing plasma, biotransformation products from Dyn A (1–17), somatostatin and TFA, was centrifuge-filtered with Centricon-SR3 concentrators (Amicon Inc., Beverly, MA), with molecular weight cutoff of 3,000 daltons. After filtration, 100 μl of the filtrate was evaporated in a Speed Vac concentrator (Savant Instruments, Farmingdale, NY) for 20 min to reduce the total volume to approximately 40 μl. Twenty microliters of the solution was then mixed with 10 μl of acetonitrile.

Mass spectrometry. Biotransformation products were identified and the kinetics of the biotransformation processes elucidated by a mass spectrometer consisting of a matrix-assisted laser desorption ion source, coupled with a linear time-of-flight mass analyzer (MALDI-MS). This instrument was constructed at the Rockefeller University. Details of the instrument have been described elsewhere (Beavis and Chait, 1989, 1990). For mass spectrometric measurement, plasma samples with acetonitrile (2:1) were mixed with matrix solution. Matrix solution was made by dissolving excess amount (5 mg/ml) of α-cyano-4-hydroxycinnamic acid in acetonitrile and 0.1% aqueous TFA (1:2, v/v) to make a saturated solution. Sample was mixed with matrix solution in a ratio of 1:2 (v/v). A 0.5-μl aliquot of sample matrix solution was applied to the mass spectrometer sample probe tip and allowed to evaporate to dryness in the air. (In the current instrument, the mass spectrometer sample probe tip allows for the simultaneous loading of 10 separate sample spots, each one 2 mm in diameter, allowing for rapid sequential determination of multiple samples.) The sample probe was inserted into the mass spectrometer vacuum system where solvents in the sample were completely removed. After 5 min, a working pressure of approximately 10^{-7} torr was achieved. To obtain adequate statistics, the results from 200 laser shots were added for each mass spectrum. In this study, one spectrum was obtained for each sample from each subject.

The MALDI-MS system was calibrated with a series of Dyn A peptides: Dyn A (1–17), Dyn A (2–17), Dyn A (1–12) and Dyn A (1–6). Serial dilution was performed to make 14 different concentrations of the peptide standards. A constant amount of internal standard (0.516 mg/ml or 7.88 nmol somatostatin) dissolved in 25 μl of 0.05% TFA was added to 500 μl of each concentration of the standard solutions (*i.e.*, 24.6 μg/ml somatostatin). Mass spectrometric measurements were carried out in triplicate for each standard solution. The ratio of the peak height of each Dyn A standard to the peak height of the internal standard and the standard error of the triplicate measurements were calculated.

Biotransformation kinetics of Dyn A peptides. The kinetics of Dyn A biotransformation *in vitro* in human and rhesus monkey blood was studied with MALDI-MS. Dyn A (1–17) was added to the freshly drawn blood to yield a concentration of 0.59 mg/ml. The blood was incubated at 37°C. Aliquots were withdrawn before and at specific time points after the addition of Dyn A (1–17). Mass spectrometric measurements were carried out with the internal standard, 31.5 nmol somatostatin, being added to 2.0 ml of the sample solution (*i.e.*, 24.6 μg/ml). The relative peak height of a Dyn A fragment to the peak height of the internal standard was used as a measure of the abundance of that fragment. The values of relative peak height of Dyn A (1–17) and its fragments, Dyn A (2–17), Dyn A (1–6) and Dyn A (7–17), were plotted *versus* the incubation time. The curve fitting was constructed with nonlinear curve-fitting routine with use of KaleidaGraph (version 3.0.5, Abelbeck Software, Reading, PA).

Results

Dyn A (1–17) *in vitro* biotransformation in human blood. Dyn A (1–17) was biotransformed into a variety of Dyn A fragments upon incubation at 37°C in human blood. Table 1 summarizes the biotransformation products of Dyn A (1–17) detected in seven human subjects. The major biotransformation products were identified to be Dyn A (2–17), Dyn A (1–6) and Dyn A (7–17). These products were further processed into minor products: Dyn A (2–17) to Dyn A (3–17) and Dyn A (4–17); Dyn A (1–6) to Dyn A (2–6); Dyn A (7–17) to

TABLE 1

Biotransformation products of Dyn A (1–17) *in vitro* processing in rhesus monkey and human blood by matrix-assisted laser desorption/ionization mass spectrometry

Monkey (n = 3)	Human (n = 7)
(2–17)	(2–17)
(3–17)	(3–17)
(4–17)	(4–17)
(1–6)	(1–6)
	(2–6)
(7–17)	(7–17)
(8–17)	(8–17)
(9–17)	(9–17)
	(11–17)
	(8–15)
(9–15)	(9–15)
(1–12)	(1–12)
(2–12)	
(4–12)	
(1–11)	(1–11)
	(4–11)

Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln
1 5 10 15

Dyn A (8–17), Dyn A (9–17) and Dyn A (11–17). Other minor biotransformation products, such as Dyn A (8–15), Dyn A (9–15), Dyn A (1–11), Dyn A (1–12) and Dyn A (4–11), were also detected in blood of some human subjects. Figure 1, a and b, are two mass spectra of Dyn A fragments detected after 120 min incubation from two different subjects.

Dyn A (1–17) *in vitro* biotransformation in rhesus monkey blood. Biotransformation patterns identified in rhesus monkey blood were similar to those identified in human blood. Table 1 summarizes the results of studies of Dyn A (1–17) biotransformation *in vitro* in blood from three rhesus monkeys. The major biotransformation products were identified to be Dyn A (2–17), Dyn A (1–6) and Dyn A (7–17). Differences from Dyn A (1–17) *in vitro* biotransformation in human blood exist in formation of minor products. For example, Dyn A (2–12) and Dyn A (4–12) were detected in rhesus monkey blood, but not in human blood. On the other hand, Dyn A (2–6), Dyn A (11–17), Dyn A (8–15) and Dyn A (4–11) were detected in blood of some human subjects but not in

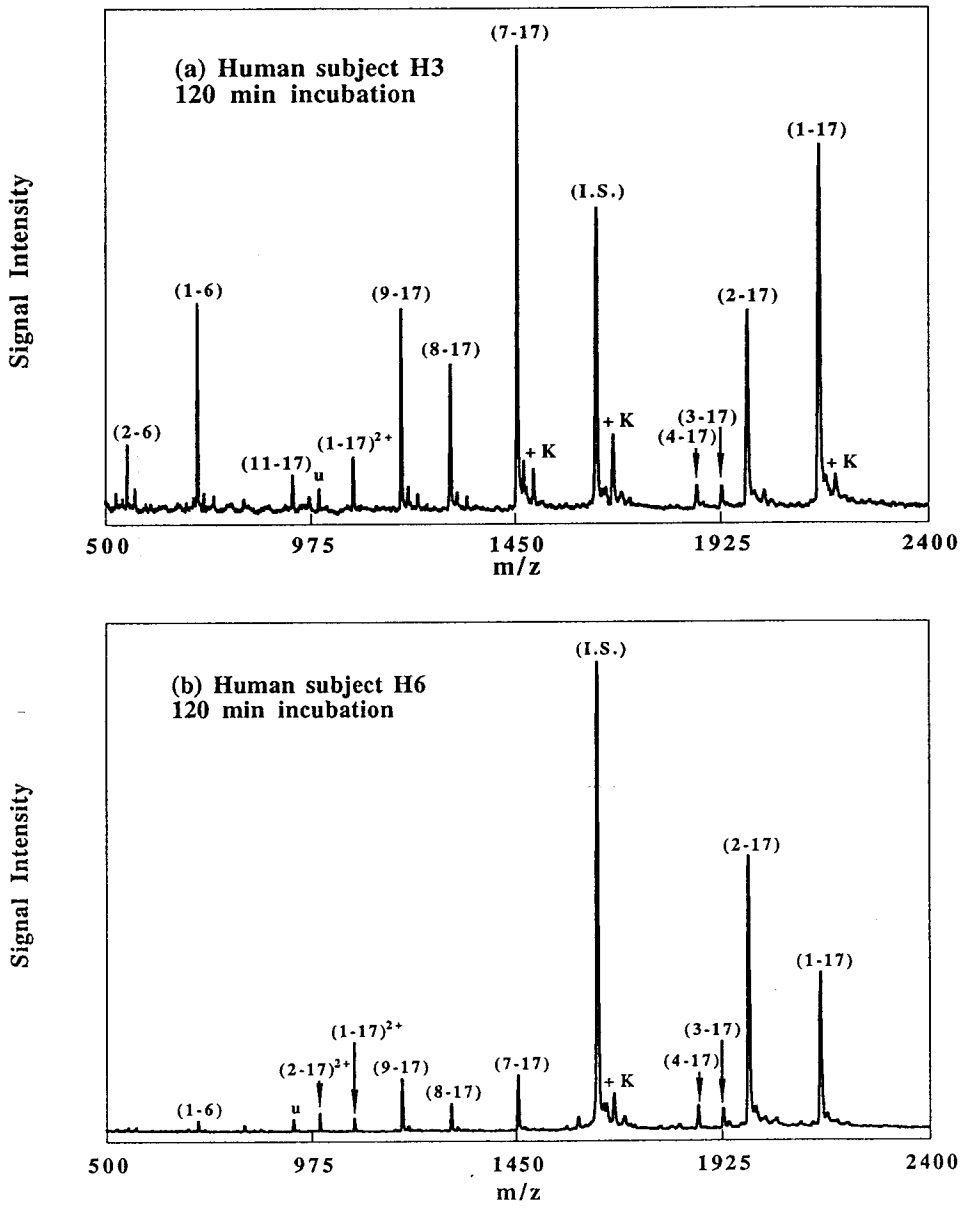

Fig. 1. Matrix-assisted laser desorption/ionization mass spectra of Dyn A (1–17) (0.59 mg/ml or 196.4 μM) *in vitro* biotransformation, incubation at 37°C for 120.0 min, in freshly drawn blood of human subjects H3 (a) and H6 (b). The numbers refer to Dyn A fragments; the notation of 2+, doubly charged ions; I.S., the internal standard, somatostatin (31.5 nmol or 0.516 mg/ml); u, unidentified peaks; +K, signal corresponding to potassium adduct species; +Cu, signal corresponding to copper adduct species.

blood of rhesus monkey subjects. Figures 2, a and b, are mass spectra of Dyn A (1–17) *in vitro* biotransformation in rhesus monkey blood after 15 min incubation from two different subjects.

Calibration of the mass spectrometric system. The mass spectrometric system was calibrated with a mixture of four standard Dyn A peptides. Over the range of 0.0 to 3.3 pmol peptide applied to the sample probe tip, two linear sections of the relative peak height *versus* the amount of peptide used were obtained. These curves had different slopes as may be seen in figure 3. Most of the relative peak height values observed in this study fell into the concentration range corresponding to less than 1.0 pmol (fig. 3a). In the concentration range between 0.0 and 1.0 pmol, which is the range of the amounts of peptides applied to the sample probe tip in this study, the linear correlation coefficient values for Dyn A (1–17), Dyn A (2–17), Dyn A (1–12) and Dyn A (1–6) were 0.95, 0.97, 0.96 and 0.89, respectively. In this concentration range, all the slopes were significant at least at .0005 level with F values equal or larger than 30.8.

The kinetics of *in vitro* Dyn A (1–17) biotransformation in human and rhesus monkey blood. *In vitro* biotransformation kinetics of Dyn A (1–17) were studied by MALDI-MS. The mass spectrometric system was calibrated with four Dyn A peptides, Dyn A (1–17), Dyn A (2–17), Dyn A (1–12) and Dyn A (1–6). The calibration curves are shown in figure 3. Kinetics of Dyn A peptide biotransformation in human and rhesus monkey blood is shown in figures 4 and 5.

Temperature effect on the *in vitro* biotransformation of Dyn A peptides. The effect of incubation temperature on the biotransformation of Dyn A peptides was studied with human blood of three subjects, at 25°C incubation, the temperature of the initial study in our laboratory (Chou *et al.*, 1993, 1994), and compared with the incubation temperature of 37°C used in the present studies (fig. 6). Biotransformation of Dyn A (1–17) followed the same profile when incubated in human blood at 25°C and 37°C, although more Dyn A (1–17) remained intact in blood at 25°C after 240 min (fig. 6). At 25°C, Dyn A (2–17) was still being formed in blood, whereas at 37°C, the formation of Dyn A (2–17) had pla-

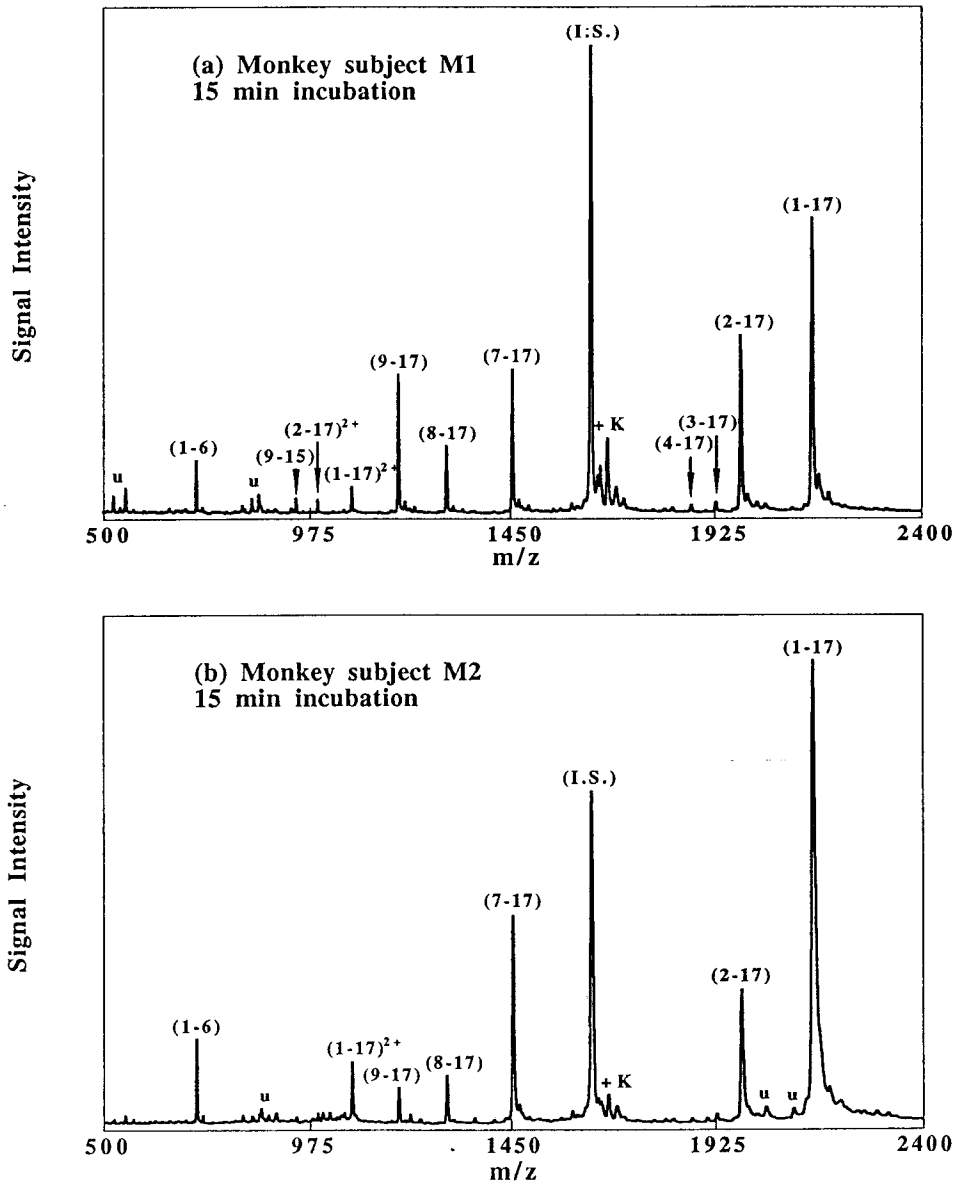

Fig. 2. Matrix-assisted laser desorption/ionization mass spectra of Dyn A (1–17) (0.59 mg/ml or 196.4 μM) *in vitro* biotransformation, incubation at 37°C for 15.0 min, in freshly drawn blood of rhesus monkey subjects M1 (a) and M2 (b). Other details are as in figure 1.

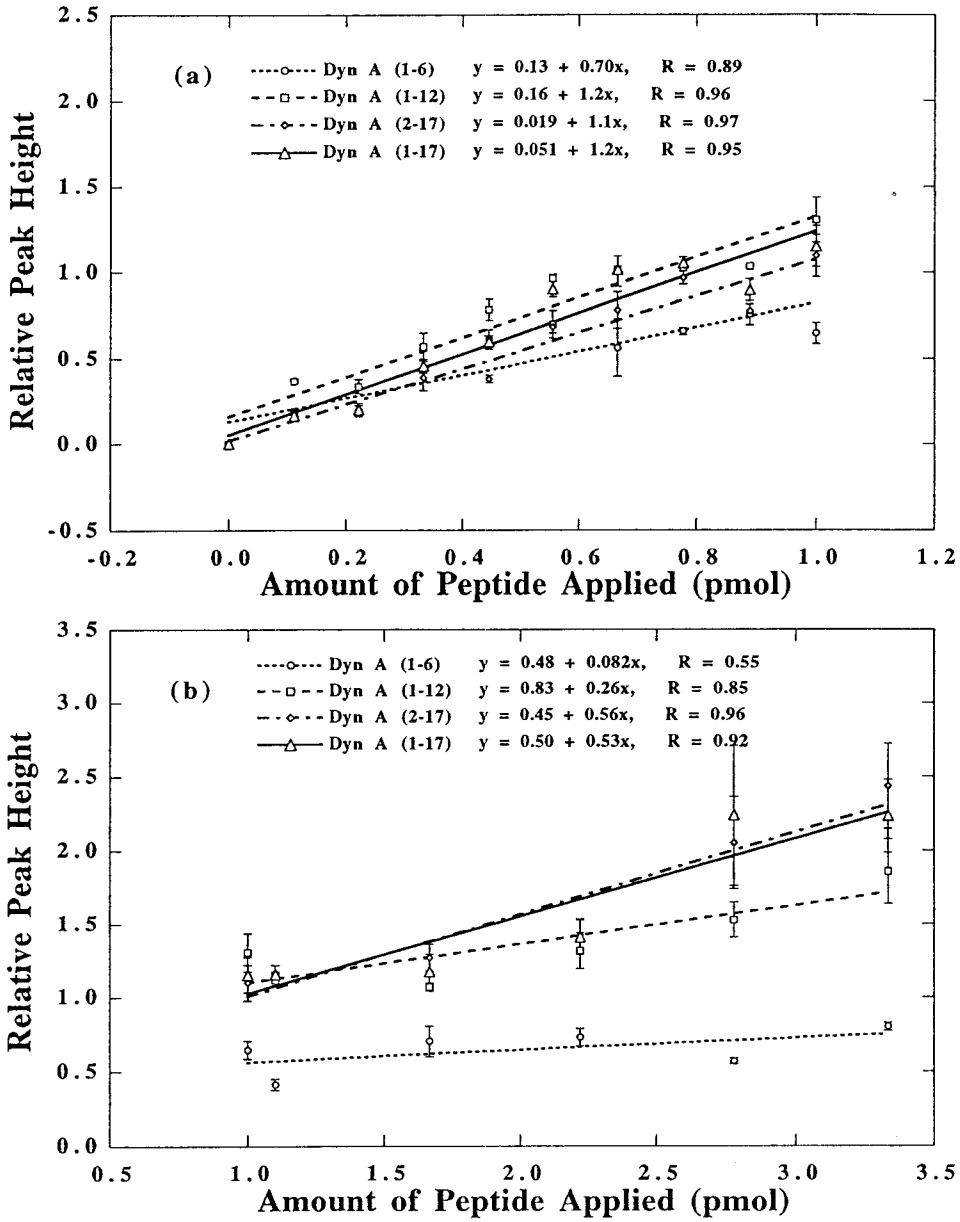

Fig. 3. Linear curve fit for the calibration of the mass spectrometric system with the amount of peptide in the concentration range of 0.0 (a) to 1.0 pmol and 1.0 to 3.3 pmol (b). Error bars represent the standard error of the mean of triplicate measurements for each standard solution. Relative peak height is the ratio of peak height of a Dyn A fragment to the peak height of the internal standard.

teaued within 240 min. The formation of Dyn A (7–17) was relatively slow at 25°C (fig. 6); by contrast, at 37°C, Dyn A (7–17) was formed within an hour of incubation and rapidly disappeared.

Discussion

Several previous studies have been carried out using a truncated analog, Dyn A (1–13). In 1979, Goldstein *et al.* reported the observation of the rapid degradation of Dyn A (1–13) by well-washed rat brain membranes. The same group carried out a separate study on the degradation of Dyn A (1–13) by membrane-bound enzymes in rat brain minus cerebellum (Leslie and Goldstein, 1982), with ^{125}I-labeled and nonlabeled peptide. Two metabolites were identified by a chromatographic method: [^{125}I]tyrosine, arising from cleavage of Tyr(1) from the N terminus of the peptide; and [^{125}I]Dyn A (1–12), arising from cleavage of Lys(13) from the C terminus of the peptide. The authors reported no major discrepancy between the degree of degradation of iodinated and noniodinated peptide.

Later studies with Dyn A (1–17) were carried out in rat and guinea pig brain membranes *in vitro* and with cannula implantation into the periaqueductal gray *in vivo* (Young *et al.*, 1987). Two chromatographic methods (a rapid molecular sieving and reversed-phase HPLC) were used for product identification. The major product identified was Dyn A (2–17), with no extensive C-terminal cleavage of [^{3}H]Dyn A (1–17) under the study conditions. They did observe a small but consistent peak of radioactivity that comigrated on HPLC with Leu-enkephalin. Without a technique for more specific peptide identification, it was suggested that conversion of [^{3}H]Dyn A (1–17) to smaller active opiate forms was not a major pathway of degradation.

In other studies, enzymatic cleavage of Dyn A (1–17) at the Arg(6)-Arg(7) peptide linkage was extensively studied and reported (Nyberg *et al.*, 1985; Silberring *et al.*, 1992). The

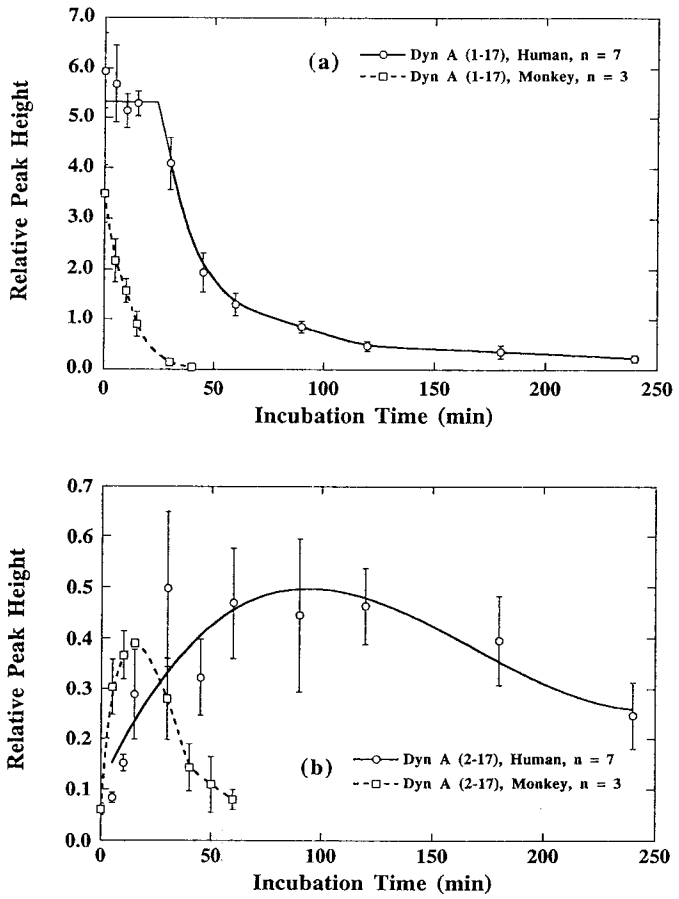

Fig. 4. Kinetics of Dyn A (1–17) (a) and Dyn A (2–17) (b) *in vitro* biotransformation in human and in rhesus monkey blood. Dyn A (1–17) (0.59 mg/ml or 196.4 μM) was added to freshly drawn blood. The blood was incubated at 37°C. The measurements were an average of data obtained from seven human subjects and three rhesus monkey subjects. Error bars represent the standard error of the mean for each time point.

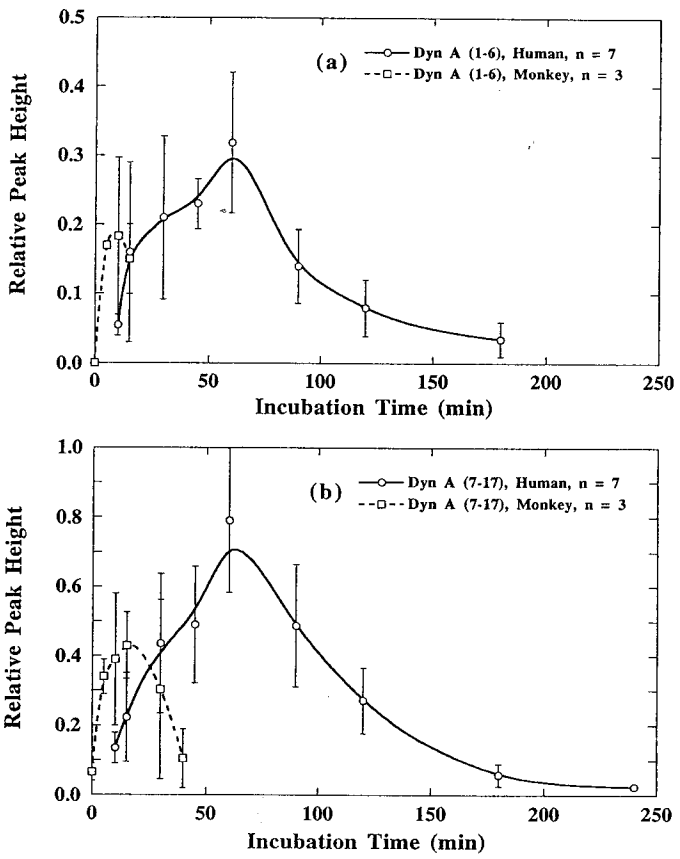

Fig. 5. Kinetics of Dyn A (1–6) (a) and Dyn A (7–17) (b) *in vitro* biotransformation in human and in rhesus monkey blood. Other details are as in figure 4.

enzyme, termed "Dyn A-converting enzyme," was purified from human cerebrospinal fluid and human spinal cord tissue. Reversed-phase HPLC in conjunction with UV detection was used to separate the enzymatic digestion products. The separated fragments were analyzed by fast atom bombardment mass spectrometry to identify the products. Results obtained with the partially purified enzyme indicated that Dyn A (1–6) was the major biotransformation product. Other products identified included Dyn A (7–17), Dyn A (8–17), Dyn A (6–17) and Dyn A (1–7).

In the present studies, a variety of Dyn A fragments were formed and identified after addition of Dyn A (1–17) to freshly drawn human blood. Two major biotransformation pathways were identified during *in vitro* biotransformation of Dyn A (1–17). One major pathway is the slow cleavage of the amino-terminal tyrosine to yield Dyn A (2–17). Dyn A (2–17) was found to be the major nonopioid biotransformation product. Dyn A (2–17) was further biotransformed into Dyn A (3–17) and Dyn A (4–17); however, this latter conversion occurred very slowly. Another major biotransformation pathway is the cleavage of the peptide linkage between Arg(6) and Arg(7) to produce the opioid peptide Dyn A (1–6) and the nonopioid peptide Dyn A (7–17). Both of these biotransformation products were further processed into minor products: Dyn A (1–6) to Dyn A (2–6); and Dyn A (7–17) to Dyn A (8–17), Dyn A (9–17) and Dyn A (11–17) (fig. 1). Two types of enzymes are thought to be mainly responsible for Dyn A peptide biotransformation: aminopeptidase(s) (Benuck *et al.*, 1984), which cleaves off amino acid residues from the N terminus of the peptides; and endopeptidase(s) such as dynorphin-converting enzyme (Nyberg *et al.*, 1985; Silberring *et al.*, 1992), which cleaves at paired basic amino acid residues, such as Arg(6)-Arg(7). Enzyme-catalyzed hydrolysis of the peptides in the blood might also contribute to the cleavage of peptide linkage. In the present study, the MALDI-MS technique allows for simultaneous detection of most of the biotransformation products at a given time point. By monitoring the metabolic profile of Dyn A biotransformation along the time course of the incubation, the appearance and disappearance of a specific peptide fragment can be followed; based on such observations, it was concluded that the biotransformation of Dyn A peptides mainly occurred as successive processing.

Biotransformation of Dyn A (1–17) *in vitro* in rhesus monkey blood and in human blood was found to be very similar. Two major biotransformation pathways were identified for Dyn A (1–17) *in vitro* processing in rhesus monkey blood: cleavage of N-terminal Tyr(1) to form Dyn A (2–17) and cleavage between Arg(6) and Arg(7) to form Dyn A (1–6) and Dyn A (7–17). The major biotransformation products, Dyn A (2–17) and Dyn A (7–17), were further processed to form minor products: Dyn A (2–17) to Dyn A (3–17) and Dyn A (4–17), Dyn A (7–17) to Dyn A (8–17) and Dyn A (9–17).

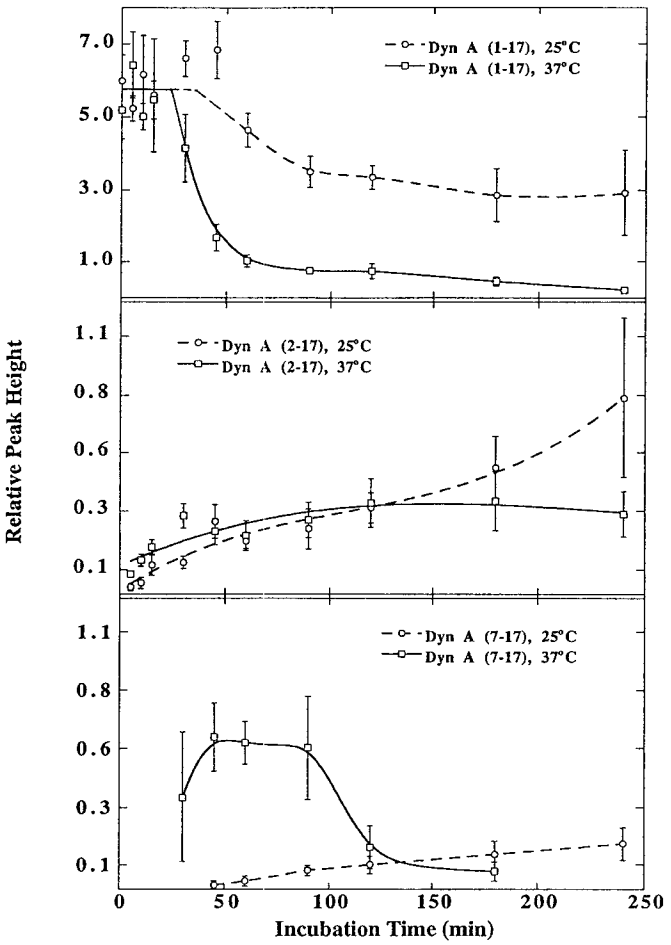

Fig. 6. Temperature effect on the biotransformation kinetics of Dyn A peptides. The measurements were an average of data obtained from three human subjects. Error bars represent the standard error of the mean for each time point. It should be noted that the scales vary for the three peptide fragments reflecting their different concentrations.

Dyn A (2–17) was the major biotransformation product both in human and rhesus monkey blood. Although it does not bind to opioid receptors (Walker *et al.*, 1982), Dyn A (2–17) has been shown to produce a profile of pharmacological activities capable of potent physiological effects, in part similar to those of the full-length opioid peptide, Dyn A (1–17). Dyn A (2–17) was shown to stimulate many of the pathophysiological consequences of Dyn A (1–17) such as motor and electrophysiological effects when administered to rats (Walker *et al.*, 1982). Like Dyn A (1–17), Dyn A (2–17) administered through a microdialysis probe placed in rat hippocampus caused a dose-related increase in the extracellular levels of excitatory amino acids, glutamate and aspartate (Faden, 1992). Dyn A (2–17) was shown to be as effective as the active opioid Dyn A (1–17) to suppress naloxone-precipitated withdrawal jumping in morphine-dependent mice (Takemori *et al.*, 1993). Comparative studies of Dyn A (2–17) with the opioid-active but shortened form Dyn A (1–13) indicated that both peptides were effective in inhibiting the expression of physical dependence on morphine in mice after acute, high-dose exposure and chronic exposure. Both Dyn A (1–13) and Dyn A (2–17) effectively suppressed the expression of chronic opiate tolerance (Hooke *et al.*, 1995a). Dyn A (2–17) was shown to have antinociceptive effect in the writhing assay, similar to Dyn A (1–13) when injected to mice by the intravenous, intrathecal, intracerebroventricular and intraperitoneal routes (Hooke *et al.*, 1995b). Studies have indicated that the behavioral effects of Dyn A (1–13) were generally unaffected by naloxone (Walker *et al.*, 1980). It was suggested that within Dyn A (1–17), two biological active sequences existed, one opiate and the other nonopiate, both of which were able to produce significant behavioral activity (Walker *et al.*, 1982). The physiological effects of dynorphin peptides appear to be mediated by both opioid and nonopioid mechanisms. In the processing of Dyn A (1–17), the formation of Dyn A (2–17) as the major biotransformation product preserved some of the physiological activities of the precursor peptide.

We observed a significant degree of variation among individual subjects, both human and rhesus monkey, in the biotransformation pathways and formation of biotransformation products. These observations are demonstrated in figure 1, a and b, for human subjects, and in figure 2, a and b, for rhesus monkey subjects.

Although biotransformation of Dyn A (1–17) in human and rhesus monkey blood was observed to follow similar biotransformation pathways (table 1), Dyn A (1–17) was much more rapidly biotransformed in rhesus monkey blood than in human blood. In human blood, Dyn A (1–17) gradually disappeared with incubation time, but still could be detected in the blood of some subjects at 240 min incubation. By comparison, Dyn A (1–17) rapidly disappeared in rhesus monkey blood (fig. 4a). Dyn A (2–17), the major nonopioid biotransformation product, was the long-lasting Dyn A fragment both in human and rhesus monkey blood (fig. 4b). Compared with the biotransformation kinetics in rhesus monkey blood, Dyn A (2–17) formed more slowly in human blood but lasted much longer. It still could be detected in human blood at 240 min incubation.

In both human and rhesus monkey blood, Dyn A (1–6) and Dyn A (7–17) were formed during the incubation of Dyn A (1–17), but rapidly disappeared. Dyn A (1–6) and Dyn A (7–17) disappeared within 180 min of incubation in human blood and 45 min in rhesus monkey blood (fig. 5). Although there was a considerable variation in the kinetics of formation of Dyn A (1–6) and Dyn A (7–17) among individual subjects, human and rhesus monkey alike, a strong correspondence was observed between the kinetics of appearance and disappearance of Dyn A (1–6) and of Dyn A (7–17) (fig. 5).

Studies were also carried out at a lower incubation temperature (25°C) to relate the present results with those obtained from previous studies in this laboratory. The lower incubation temperature was observed to decrease the biotransformation rate of Dyn A peptides in blood. A temperature dependence in Dyn A (1–13) biotransformation by membrane-bound rat brain enzyme was previously reported (Leslie and Goldstein, 1982), with the rate of metabolism reduced by incubation at 0°C.

Rhesus monkeys as a primate model have been used in many behavioral studies of the effects of Dyn A (1–13). The effects of Dyn A (1–13) were studied in morphine-dependent rhesus monkeys (Aceto *et al.*, 1982). These studies indicated that Dyn A (1–13) suppressed withdrawal signs in a dose-related manner within 30 min. In non-morphin-dependent monkeys, Dyn A (1–13) did not produce typical morphine

effects. Studies also showed that Dyn A (1–13) effects were significantly decreasing at 90 min. In another study (Butelman *et al.*, 1995), it was found that intravenously administered Dyn A (1–13) also produced thermal antinociception in rhesus monkeys. This effect was blocked by opioid antagonists. The antinociceptive action of Dyn A (1–13) (3.2 mg/kg) was present for approximately 30 min after administration. Dyn A peptides, both the shortened Dyn A (1–13) and the natural full-length Dyn A (1–17), can be biotransformed in a biological system into both opioid and also nonopioid Dyn A fragments, some of which have opioid-like activities (Chou *et al.*, 1993, 1994). It is, therefore, instructive to study the biotransformation of Dyn A peptides with primate models like rhesus monkeys for comparison with biotransformation of Dyn A peptides in humans.

In conclusion, Dyn A (1–17) *in vitro* biotransformation in human and rhesus monkey blood was found to be similar. Two major biotransformation pathways were identified: one is the cleavage of Tyr(1) to form Dyn A (2–17); the other is the cleavage between Arg(6) and Arg(7) to form Dyn A (1–6) and Dyn A (7–17). These major biotransformation products are further processed to form minor biotransformation products. A considerable variation was observed in both the pattern and rate of the formation of minor biotransformation products among individual subjects, human and rhesus monkey alike. These results suggest that rhesus monkey may be a good model for further *in vivo* pharmacological and neurobiological studies, and for studies leading to the development of new pharmacotherapeutic agents.

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Send reprint requests to: Jim Yu, Ph.D., The Rockefeller University, Box 171, 1230 York Avenue, New York, NY 10021.