Structure-Activity Relationships of a Series of \([\text{D-Ala}^2]\)Deltorphin I and II Analogues; \textit{in Vitro} Blood-Brain Barrier Permeability and Stability

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ABSTRACT

\([\text{D-Ala}^2]\)Deltorphins are enzymatically stable, amphibian heptapeptides that have a higher affinity and selectivity for \textit{delta}-opioid receptors than any endogenous mammalian compound known. This study investigated the \textit{in vitro} blood-brain barrier permeability, using primary bovine brain microvessel endothelium culture, and the resistance to enzymatic degradation, in mouse 15\% brain membrane homogenates and 100\% plasma, of \([\text{D-Ala}^2]\)deltorphin I, \([\text{D-Ala}^2]\)deltorphin II and several analogues. Derivatives were designed with the addition of N-terminal neutral and basic amino acids or with alterations of the amino acids present within the deltorphin sequences. The results indicated that the N-terminal sequence and the amino acids in position 4 and 5 are critical to deltorphin analogue BBB permeability and biological stability, \textit{i.e.}, \(t_{1/2}\) brain; 4.8 hr-\([\text{D-Ala}^2]\)deltorphin I; >15 hr-\([\text{D-Ala}^2,\text{Ser}^4,\text{D-Ala}^5]\)deltorphin. Although, no analogue was found to increase the BBB permeability coefficient (PC; \(\times 10^{-4}\) cm/min) of the parent compounds (\([\text{D-Ala}^2]\)deltorphin II, PC = 23.49 \pm 2.42) analogues were identified: \([\text{Arg}^0,\text{D-Ala}^2]^{13}\)deltorphin II, PC = 19.06 \pm 3.73 and \([\text{Pro}^{-1},\text{Pro}^0,\text{D-Ala}^2]\)deltorphin II, PC = 22.22 \pm 5.93; which had similar permeability coefficients, even though they had larger molecular weights and, in the case of the cationic prodrug, a significantly lower lipophilicity. These analogues provide directions in the development of future prodrugs for the treatment of pain and this study further clarifies the structure-activity relationship of the deltorphins.

Deltorphins are linear heptapeptides that are secreted from the skin glands of \textit{Phyllomedusa} amphibians and have a higher affinity and selectivity for \textit{delta} opioid binding sites than any other endogenous compound known (Kreil \textit{et al.}, 1989; Erspamer \textit{et al.}, 1989; Lazarus \textit{et al.}, 1996). Although the first deltorphin to be discovered, deltorphin A, has a significantly different sequence than \([\text{D-Ala}^2]\)deltorphin I or II, it does share a common N-terminus tripeptide sequence, H-Tyr-D-Xaa-Phe (table 1), and similar \textit{delta}-opioid receptor selectivity (Kreil \textit{et al.}, 1989). In contrast to the endogenous mammalian opioid peptides, which have the common N-terminus tetrapeptide sequence Tyr-Gly-Gly-Phe, the deltorphins are more stable in biological fluids (Kramer \textit{et al.}, 1991) and this can be partly related to the naturally occurring \(\text{D}-\)amino acid in position 2, the source of which is unknown (Marastoni \textit{et al.}, 1991). The deltorphins and their analogues are of considerable scientific interest because they have the potential to be used either as effective therapeutic tools against acute and chronic pain and/or in the further elucidation of the structure-activity relationships of \textit{delta}-opioid receptor agonists (Jiang \textit{et al.}, 1990; Lazarus \textit{et al.}, 1996).

It is thought that \textit{delta}-opioid analgesia is a centrally mediated event. Therefore only those opioids that can cross the (BBB) intact will be able to elicit a biological effect (Fredrickson \textit{et al.}, 1981; Shook \textit{et al.}, 1987). Thus an essential consideration in the design and development of opioid analgesics is their ability to cross the BBB and their resistance to enzymatic degradation in biological fluids. Our research group has designed several series of deltorphin derivatives either with alterations of the amino acids present within the peptide sequence or with additions of neutral and basic amino acids to the N-terminus toward these ends. \textit{In vitro} cultures of cerebral endothelial cells and time-course metabolism studies in brain membrane homogenates and plasma allow rapid assessment of the potential BBB permeability and metabolism of drugs (Weber \textit{et al.}, 1993; Brownson \textit{et al.}, 1994; Greene \textit{et al.}, 1996). The scope of our study was to

ABBREVIATIONS: BBB, blood-brain barrier; BMEC, brain microvessel endothelial cells; PC, permeability coefficient; RP-HPLC, reverse phase high-performance liquid chromatography; \(k^\prime\), capacity factor; Abu, \(\alpha\)-aminobutyric acid; HOBt, N-hydroxysuccinimide; BOP, benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium; TFA, trifluoroacetic acid.
TABLE 1

The amino acid sequence of the endogenous amphibian deltorphins

<table>
<thead>
<tr>
<th>Deltorphin</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>A/dermenkephalin</td>
<td>Tyr-γ-Met-Phe-His-Leu-Met-Asp-NH₂</td>
</tr>
<tr>
<td>C/β-Ala³/deltorphin I</td>
<td>Tyr-γ-Ala-Phe-Asp-Val-Val-Gly-NH₂</td>
</tr>
<tr>
<td>B/β-Ala³/deltorphin II</td>
<td>Tyr-γ-Ala-Phe-Glu-Val-Val-Gly-NH₂</td>
</tr>
</tbody>
</table>

investigate the in vitro BBB permeability and stability of β-Ala³deltorphin analogues, together with their parent compounds, using the methods outlined above and to identify the best possible drug candidate(s) for clinical nociceptive pain management as well as furthering our knowledge of the structural requirements necessary for biological effects.

Methods

In Vitro BBB (BMEC)

The in vitro BBB model uses primary cultures of bovine BMEC (Audus and Borchardt, 1986; Weber et al., 1993). Briefly, isolated brain microvessels were seeded (50,000 cells/cm²) onto a tissue culture dish that had been precoated with rat tail collagen and fibronectin and contained 25-mm Costar Nucleopore polycarbonate membrane filters (10 µm; Costar Corp., Cambridge, MA). After the cells had grown to confluence (12–14 days), the membrane filters plus the confluent monolayer were moved into the center of a Side-Bi-Side diffusion chamber (Crown Glass Co., Sommerville, NJ), which contained 3 ml of the physiological assay buffer (NaCl 122.0 mM; KCl 2.6 mM; MgSO₄ 1.2 mM; NaHCO₃ 25.0 mM; K₂HPO₄ 0.4 mM; CaCl₂ 1.4 mM; d-glucose 10 mM; HEPES 10 mM) at 37°C. At time zero, the test drug, together with a membrane impermeant marker, [¹⁴C]sucrose (0.44 Ci/mmol; NEN Research Products, Boston, MA), was added to the donor chamber and 200-µl samples were taken from the receiver chamber at 0, 15, 30, 60, 90 and 120 min. An equal volume of assay buffer was added to the receiver chamber to replace that removed. A 200-µl sample was also taken from the donor chamber at time 0 and 120 min. An equivalent volume of acetonitrile-water (v/v, 50/50) was added to each sample and the samples stored at −40°C until use. Background leakiness was monitored and corrected for by determining the levels of [¹⁴C]sucrose in the samples via scintillation spectrometry (efficiency 93% for ¹⁴C; Beckman LS 5000 TD counter, Beckman Instruments Inc., Fullerton, CA). Control studies confirmed that the passage of [¹⁴C]sucrose across collagen- and fibronectin-coated membrane filters without the BMEC monolayers was significantly higher than filters with BMEC monolayers (P < .01).

Passage of the test solute across the in vitro BBB monolayer was determined by RP-HPLC analysis of the samples, as described below, and was expressed in the form of a PC. This was calculated by means of the following equation:

\[ PC = X(A \times t \times C_D) \]

where PC is the apparent permeability coefficient in cm/min, X is the amount of substance in moles in the receptor chamber after correction for sampling and paracellular passage based on sucrose levels at time t in minutes, A is the diffusion area (i.e., 0.636 cm²) and C_D is the concentration of substance in the donor chamber in mol/cm³ (C_D remains >90% of the initial value over the time of the experiments). Data points collected at times 60, 90, and 120 min were used to compute the apparent permeability coefficients.

In Vitro Stability Incubations

The effect of both brain membrane-associated and plasma enzymes on the degradation of the test compounds was examined over a period of 5 hr at 37°C as previously described (Davis and Culling-Berglund, 1985; Weber et al., 1992).

Preparation of brain membranes and plasma. Adult ICR male mice (25–30 g) were anesthetized (sodium pentobarbital; 80 mg/kg) and blood collected from the abdominal aorta with a heparinized syringe. The blood was left overnight at 4°C and then centrifuged for 20 min at 20,000 × g. The plasma/ supernatant was then removed and stored at −40°C. Twice-washed membranes were prepared from whole brain minus cerebellum as previously described by Gillespie et al. (1992) and were resuspended in 50 mM Tris buffer to a final protein concentration of 7 mg/ml and stored at −40°C until use. The protein content of the suspension was confirmed by the Folin-Lowry procedure (Lowry et al., 1951).

Incubations. The time course of metabolism was investigated by incubating the plasma or brain membrane homogenates with 100 µM test compound at 37°C for 0, 60, 120, 180 and 300 min. If the test peptide was found to have a half-life less than 60 min, incubations were performed using time intervals of 0, 7.5, 15, 22.5 and 30 min. After incubation an equal volume of acetonitrile was added and the samples were vortexed before being kept on ice for a few minutes. Samples were further diluted 1:1 with 0.5% acetic acid to prevent any enzymatic degradation not stopped by the addition of the acetonitrile and centrifuged at 13,000 × g for 15 min. The supernatants were collected and frozen at −40°C until analyzed by RP-HPLC as described below. The half-life of the test compounds was calculated via linear regression analysis of percentage of recovery vs. time (Tallarida and Murray, 1987).

HPLC analysis. Both stability and BMEC samples were analyzed by a RP-HPLC system consisting of a Waters Associates WISP 712 Autoinjector (Waters Associates, Milford MA), Perkin Elmer Binary LC pump 250 and LC-15 UV Detector (210 nm; Perkin Elmer, Norwalk, CT), Hewlett-Packard 3396A Integrator (Hewlett-Packard Co., Avondale, PA) and a Vydac 218TP54 column (4.6 × 250 mm; Vydac Hesperia, CA) or an Inertsil ODS-2 µm column (4.6 × 150 mm; Metachem Technologies Inc., Torrance CA) as previously described by Davis (1990). Samples were eluted using a linear gradient (10–30, 0–30 or 5–25% in 30 min) of acetonitrile vs. 0.1M NaH₂PO₄ (pH 2.4) at 1.5 ml/min at 37°C. [Ala¹,Pro₀,D-Ala²]deltorphin II, [Pro⁻¹,Pro⁻²,D-Ala²]deltorphin II and [Ala⁻¹,Abu₀,D-Ala²]deltorphin II were eluted using a linear gradient of 10 to 30% acetonitrile versus 0.1M NaH₂PO₄ (pH 7.4) at 2 ml/min at 37°C and a Zorbax Pro-10/300 column (4.6 × 250 mm; Du Pont, Wilmington, DE).

As a measure of lipophilicity the conditions (10–30% acetonitrile vs. 0.1M NaH₂PO₄ (pH 2.4) in 30 min; Inertsil ODS-2 µm column) and system described above were used to determine a k' for each of the test compounds.

Capacity factor = k' = (tᵣ − t₀)/tᵣ

where tᵣ is the retention time of the retained peak and t₀ is the retention time of an unretained peak (Weber et al., 1993).

Peptide Synthesis

All the β-Ala³deltorphin analogues were synthesized by the solid phase technique with BOP/HOBt mediated Fmoc strategy and using a Rink amide p-methylenzhydrylamine resin [0.56 mmol/g Nova-biochem (La Jolla, CA)]. The protected amino acids were purchased from Advanced ChemTech (Louisville, KY) or Bachem (Torrance, CA). The side chain protecting groups were Boc for Lys, Pmc for Arg and tert-butyl for Ser, Gln and Tyr. After incorporation of all the amino acids the N-terminal Fmoc group was removed and the resin was washed several times and dried in a vacuum desiccator. The side-chain protected peptidyl resin was treated with a mixture of TFA/phenol/water (90:5:5), 10 ml/g peptide resin, for 120 min at ambient temperature. The resin was filtered off and the filtrate was concentrated in vacuo. The peptide was precipitated with anhydrous ether, collected by centrifugation and washed several times with ether. The peptide was dissolved in acetic acid-water and lyophilized. The crude peptide was purified by preparative RP-HPLC as previously reported (Mischka et al., 1994). All the peptides synthesized
had a purity of more than 95% as measured by RP-HPLC analysis (Hewlett-Packard model 1090 monitored at 222, 256 and 278 nm) and gave satisfactory fast atom bombardment-mass spectrometry spectra.

Chemicals

[δ-Ala2]deltorphin I and II were supplied by Multiple Peptide Systems (San Diego, CA) under the direction of the National Institute on Drug Abuse. All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

In vitro BBB. The in vitro BBB permeability measured in the form of a PC for all the test compounds was summarized in table 2. HPLC analyses revealed that the 120-min receiver and donor chamber samples eluted as a single peak, that had the same retention time as the reference sample, not exposed to the monolayer, taken at 0 min. Thus these PC values do represent the passage of intact peptide across the BMEC monolayer. Further control experiments confirmed that the PC of the test analogues was related to their ability to freely cross the BMEC monolayer and not their ability to bind to the walls of the glass diffusion chambers and/or the polycarbonate filter membranes. The PC determined for each compound were compared to each other by analysis of variance coupled with the Newman-Keuls test (table 3). The BBB passage of [δ-Ala2]deltorphin II, which had one of the highest PC values, and [δ-Ala2,Gln4,d-Val5]deltorphin, which had one of the lowest PC values, can be seen in figure 1A and illustrates the range of values determined from these in vitro studies, as well as the linearity of passage observed the 120-min experimental time period (table 2).

Capacity factors. The lipophilicity of the test compounds was measured in the form of a k determined from the HPLC retention times (table 2). [δ-Ala2, Ser4,d-Val3]deltorphin and [δ-Ala2,Gln4,d-Val5]deltorphin had similar capacity factors and were among the most lipophilic of all the compounds tested. [Arg1,Arg2,δ-Ala2]deltorphin and [Lys1,Lys2,δ-Ala3]deltorphin also had similar capacity factors to each other, but of all the compounds tested they were the least lipophilic (table 2). Figure 1b illustrates the relationship between permeability coefficient and capacity factor and as can be seen the permeability coefficients determined for most of the [δ-Ala2]deltorphins increase with lipophilicity. However, four analogues have much lower PCs that would be predicted from their capacity factor and they are [δ-Ala2, Ser4,d-Val3]-, [δ-Ala2,Gln4,d-Val5]-, [δ-Ala2,Gln4,d-Ala5]-, and [Lys1,δ-Ala3]-deltorphin II.

In vitro stability. Table 2 also summarizes the half-lives determined for all the test compounds in 100% mouse plasma and twice washed 15% mouse brain membrane homogenate. The percent recovery of intact [δ-Ala2]deltorphin I and II in both brain membrane homogenate and plasma was plotted as a function of time in Figure 2A and B, respectively. Although both [δ-Ala2]deltorphin I and II had half-lives, several of their analogues were much more resistant to in vitro degradation, for example [δ-Ala2,Ser4,d-Val3]deltorphin (fig. 3). In contrast, the cationic analogues and the proline- and α-aminoctylic acid-containing analogues, had much shorter half-lives (table 2) and it was possible to plot their in vitro conversion to [δ-Ala2]deltorphin II in both matrices (figs. 4 and 5). The degradation of [Arg1,Arg2,δ-Ala3]-, [Lys1,Lys2,δ-Ala3]- and [Abu1,Abu2,δ-Ala3]-deltorphin II, involved the formation of [Arg2,δ-Ala3]-, [Lys2,δ-Ala3]- or [Abu2,δ-Ala3]-deltorphin II, respectively, as well as [δ-Ala2]deltorphin II (fig. 4). However, the degradation of the proline-containing analogues involved the direct formation of the parent compound by cleavage of the N-terminal dipeptide sequence (fig. 5). The peaks for each compound were identified from the retention times determined for the relevant standards (fig. 6).

Discussion

A systematic screening of the in vitro BBB permeability and biological stability of [δ-Ala2]deltorphin I and II and their analogues was undertaken to determine the potential of these compounds as clinical analgesics and to further clarify the structure-activity relationships of delta-opioid agonists. This study revealed that even though several delorphin analogues had BBB permeability coefficients similar to the parent compounds and warrant further study, there was no

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Molecular Weight</th>
<th>Permeability Coefficient</th>
<th>Capacity Factor</th>
<th>Stability (Half-Lives hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± S.E.M. cm/min *10⁻⁴</td>
<td>k'</td>
<td>Brain</td>
</tr>
<tr>
<td>1. [δ-Ala2]deltorphin I</td>
<td>768.9</td>
<td>22.73 ± 0.22</td>
<td>11.54</td>
<td>4.8</td>
</tr>
<tr>
<td>2. [δ-Ala2]deltorphin II</td>
<td>782.9</td>
<td>23.49 ± 2.42</td>
<td>11.29</td>
<td>9.8</td>
</tr>
<tr>
<td>3. [δ-Ala2, Ser4,d-Val3]deltorphin</td>
<td>740.9</td>
<td>11.72 ± 0.30</td>
<td>12.57</td>
<td>&gt;15</td>
</tr>
<tr>
<td>4. [δ-Ala2, Ser4,d-Ala3]deltorphin</td>
<td>712.8</td>
<td>14.07 ± 2.82</td>
<td>9.22</td>
<td>&gt;15</td>
</tr>
<tr>
<td>5. [δ-Ala2, Gln4,d-Val5]deltorphin</td>
<td>781.9</td>
<td>6.07 ± 0.81</td>
<td>12.38</td>
<td>&gt;15</td>
</tr>
<tr>
<td>6. [δ-Ala2, Gln4,d-Ala5]deltorphin</td>
<td>753.9</td>
<td>7.77 ± 1.41</td>
<td>9.22</td>
<td>&gt;15</td>
</tr>
<tr>
<td>7. [Arg1,Arg2,δ-Ala2]deltorphin</td>
<td>1275.4</td>
<td>16.12 ± 2.10</td>
<td>8.50</td>
<td>10 min</td>
</tr>
<tr>
<td>8. [Arg1,δ-Ala2]deltorphin</td>
<td>1059.2</td>
<td>19.06 ± 3.73</td>
<td>9.36</td>
<td>16 min</td>
</tr>
<tr>
<td>9. [Lys1,δ-Ala3]deltorphin</td>
<td>1219.4</td>
<td>14.07 ± 2.82</td>
<td>8.21</td>
<td>7 min</td>
</tr>
<tr>
<td>10. [Lys2,δ-Ala3]deltorphin</td>
<td>1032.1</td>
<td>8.53 ± 0.58</td>
<td>9.28</td>
<td>27 min</td>
</tr>
<tr>
<td>11. [Ala1,Pro0,d-Ala3]deltorphin</td>
<td>952.0</td>
<td>17.38 ± 1.01</td>
<td>11.95</td>
<td>52 min</td>
</tr>
<tr>
<td>12. [Pro1,Pro0,d-Ala3]deltorphin</td>
<td>970.8</td>
<td>22.22 ± 5.93</td>
<td>12.41</td>
<td>43 min</td>
</tr>
<tr>
<td>13. [Abu1,Abu2,d-Ala3]deltorphin</td>
<td>935.3</td>
<td>29.30 ± 1.70</td>
<td>13.08</td>
<td>10 min</td>
</tr>
</tbody>
</table>
analogue that had a significantly higher ability to cross the in vitro BBB (table 3). In fact, both \([D-Ala^2]deltorphin\) I and II had significantly higher PC values compared to the majority of the analogues (table 2). A 150-fold difference between in vivo and in vitro values for drugs that cross the BBB via lipid mediation has been observed (Pardridge et al., 1990).

Although, the ability of most of the \([D-Ala^2]deltorphin\) analogues to cross the in vitro BBB would appear to be dependent on lipophilicity (fig. 1b), it is possible that a saturable transport system is involved. Saturable transport systems have previously been identified for the passage of opioid pentapeptides across the BBB (Zlokovic et al., 1989; Williams et al., 1996).

\([D-Ala^2, Gln^4]deltorphin\) and \([D-Ala^2, Ser^4]deltorphin\) were developed to explore the topographical requirements for delta-opioid receptor ligands (Misicka et al., 1991). Interestingly, neither the amidation of the \(\beta\)-carboxyl groups of \([Glu^4] \ (D-Ala^2, Gln^4)deltorphin\) nor the replacement of a \(\beta\)-carboxyl group with a hydroxyl group (\([D-Ala^2, Ser^4]deltorphin\) significantly reduce the \(\delta\)-opioid potency of \([D-Ala^2]deltorphin\) I and II (Misicka et al., 1991). However, amidation does enhance \(\mu\) receptor affinities by 2.5- to 12-fold (Lazarus et al., 1991; Salvadori et al., 1991) and it is thought that the negative charge of the glutamic or aspartic acid residue in the C-terminal "address" domain of deltorphin is primarily responsible for \(\delta\)-selectivity (Melchiorri et al., 1991; Salvadori et al., 1991; Bryant et al., 1993), by preventing \(\mu\)-site recognition and binding (Lazarus et al., 1991; Sagan et al., 1992).

Similarly, receptor binding studies indicate that \([D-Ala^2, Ala^5]deltorphin\) II maintains the high \(\delta\)-affinity of the parent compound, although decreasing the \(\delta\)-selectivity due to an increase in \(\mu\)-affinity (Sasaki et al., 1991; Salvadori et al., 1991; Nikiforovich and Hruby, 1990).

\([D-Ala^2, Gln^4, D-Val^5]\) and \([D-Ala^2, Gln^4, D-Ala^5]\)deltorphin are analogues in which the acidic side chain at position 4 has been replaced by a neutral, amide moiety. Additionally, in \([D-Ala^2, Gln^4, D-Ala^5]\)deltorphin, the lipophilic Val5 residue of the natural peptide has been replaced by the much less lipophilic, \(D\)-alanine. Although, the \(\delta\)-opioid binding affinity of analogues 3, 4, 5 and 6 has not been determined, based on the previous discussed studies, which found that similar alterations in positions 4 or 5 does not alter \(\delta\)-opioid binding affinity, it is assumed that these analogues maintain the high \(\delta\)-opioid potency of the parent compound. It is of considerable interest that \([D-Ala^2, Gln^4, D-Val^5]\) and \([D-Ala^2, Gln^4, D-Val^5]\)
Ala\(^2\)-deltorphin have similar BBB PCs even though their lipophilicities, as measured by capacity factors, were different (fig. 1B). Furthermore, the in vitro BBB permeabilities of [d-Ala\(^2\),Ser\(^4\),D-Val\(^5\)]- and [d-Ala\(^2\),Ser\(^4\),d-Ala\(^5\)]-deltorphin were also not different from each other, in contrast to their capacity factors (fig. 1B). Although, the differences in permeability between analogues 3 and 4 and analogues 5 and 6 did not attain statistical significance, the patterns produced suggest that a smaller molecular size and the presence of a hydroxyl group vs. amidation at the side chain in position 4, may contribute to increased BBB permeability. This group of [d-Ala\(^2\)]deltorphin analogues (analogues 3, 4, 5 and 6) had significantly lower permeability coefficients when compared to either [d-Ala\(^2\)]deltorphin I or II (table 3). This result also indicates the importance of the amino acid in position 4 and possibly an L-isomer in position 5. It must be considered that the low in vitro BBB permeability observed for the serine\(^4\) analogues of [d-Ala\(^2\)]deltorphin may reflect an inability to exit from brain-to-blood and may explain the 1.4-fold more potent antinociception of intracerebroventricular administered [d-Ala\(^2\),Ser\(^4\)]deltorphin when compared to [d-Ala\(^2\)]deltorphin II (Horan et al., 1993).

Pro-drugs were developed as site-directed chemotherapeutic agents against malignant tumors (Carl, 1983), but they are now used to overcome the cellular barriers that prevent drug delivery to certain organ systems (Pardridge, 1991; Bodor et al., 1992). Both [d-Ala\(^2\)]deltorphin I and II have a higher affinity for delta receptors than dermenkephalin (Erspamer et al., 1989) and [d-Ala\(^2\)]deltorphin II is the most selective natural delta opioid receptor agonist currently available (Jiang et al., 1990; Bryant et al., 1993). Thus the pro-drugs in table 2 were based on the parent compound, [d-Ala\(^2\)]deltorphin II. Previous studies have shown that cationization can improve the central nervous system entry of peptides (Van Deurs et al., 1989; Griffin and Giffels, 1982; Kumagai et al., 1987). This is related to the presence of anionic sites on the endothelial cell membranes of the BBB (Vorbrodt, 1989) and the ability of cationized molecules to use absorptive-mediated endocytosis to cross the BBB (Kumagai et al., 1987; Terasaki et al., 1989; Shimura et al., 1991). In our study one group of potential pro-drugs (analogues 7, 8, 9 and 10) was developed to improve on the ability of the parent compounds to cross the in vitro BBB by cationization. However, except for the analogue, [Arg\(^0\),d-Ala\(^2\)]deltorphin II, cationization significantly decreased the ability of the parent compound to cross the in vitro BBB (table 3). This result is most likely due to the increased molecular size and decreased lipophilicity associated with the cationic amino acid additions to the N-terminus of [d-Ala\(^2\)]deltorphin II (table 2; fig. 1B). The similar PC value for [Arg\(^0\),d-Ala\(^2\)]- and [d-Ala\(^2\)]-deltorphin II, in contrast to [Lys\(^0\),d-Ala\(^2\)]deltorphin, is possibly related to the more polycationic nature of arginine versus lysine. Although, the lower permeability coefficients of the analogues 7 and 9, would appear to dispute this explanation. Thus, it is likely that the BBB permeability of these cationized pro-drugs reflects a balance between their ionic nature,
molecular weight and lipophilicity. It must be noted that opioid peptides that carry a net positive charge show mu receptor preference, whereas neutral and negatively charged peptides preferentially interact with the delta receptor site (Salvadori et al., 1991). Lipophilicity would also appear to play a role in the similarity of the PCs observed for the proline- and alpha-aminobutyric acid-containing analogues (11, 12 and 13) compared to [D-Ala²]deltorphin II (parent compound) co-migrated with the relevant standards.

Fig. 4. In vitro conversion of [Lys¹,Lys²,D-Ala³]deltorphin II in (A) 15% mouse brain membranes and (B) 100% plasma to [Lys²,D-Ala³]deltorphin II and to [D-Ala³]deltorphin II. As [Lys¹,Lys²,D-Ala³]deltorphin II (potential pro-drug) was degraded, [Lys²,D-Ala³]deltorphin II and [D-Ala³]deltorphin II (parent compound) were formed. Results were expressed as percent mean degradation ± S.E.M.; n = 3 for each time-point. The peaks for [Lys¹,Lys²,D-Ala³]deltorphin II, [Lys²,D-Ala³]deltorphin II and [D-Ala³]deltorphin II co-migrated with the relevant standards.

Fig. 5. In vitro conversion of [Ala¹,Pro²,D-Ala³]deltorphin II in (A) 15% mouse brain membranes and (B) 100% plasma to [D-Ala³]deltorphin II. As [Ala¹,Pro²,D-Ala³]deltorphin II (potential pro-drug) was degraded, [D-Ala³]deltorphin II (parent compound) was formed. Results were expressed as percent mean degradation ± S.E.M.; n = 3 for each time-point. The peaks for the pro-drug and [D-Ala³]deltorphin II co-migrated with the relevant standards.

Our study also confirmed the inherent stability of [D-Ala³]deltorphin I and II in both 15% mouse brain membrane homogenate and 100% mouse plasma (fig. 2), which had previously been observed in membrane homogenates and synaptosomal membrane fractions prepared from rat brains (Erspamer et al., 1989; Marastoni et al., 1991; Horan et al., 1993; Sasaki et al., 1994). As mentioned in the introduction, this resistance to enzymatic degradation is partially related to the presence of the D-isomer of alanine in position 2 (Erspamer et al., 1989), which prevents cleavage by aminopeptidases, but may also be related to the presence of the C-terminal amide, which is known to protect against carboxypeptidases (Moss, 1995). Furthermore, deltorphin I and II analogues containing D-alanine at position 2 possess greater antinociceptive properties than their corresponding isomers containing L-alanine (Ji et al., 1995). It is thought that the presence of amino acids branched at the beta-carbon atom or with a bulky side chain at residue 5 (i.e., Val of [D-Ala³]deltorphin I and II) is important for enzymatic sta-
bility, with [D-Ala²,Ala⁵]deltorphin II being readily degraded in a rat brain synaptosomal membrane fraction by neprilysin (neutral endopeptidase; EC 3.4.24.11) (Sasaki et al., 1994). Furthermore, [D-Ala²,Ser⁴]deltorphin II has been shown to have a shorter half-life in rat brain membrane homogenate than [D-Ala³]deltorphin II (Horan et al., 1993). Thus, in this study it is likely that the extremely long half-lives observed for the [D-Ala³]deltorphin analogues, 3, 4, 5 and 6 (table 2), are due to the presence of the D-isomer of either valine or alanine in position 5 protecting the peptide against neprilysin and possibly angiotensin converting enzyme (EC 3.4.15.1) activity (Sasaki et al., 1994; Waters et al., 1996). It must be noted that neprilysin and ACE are present in brain (Dauch et al., 1993) and ACE is present in cerebral microvessels and plasma (Shibanoki et al., 1991; Brownson et al., 1994). Another potential cleavage site in [D-Ala³]deltorphin I and II is likely to be the Phe-Asp or Phe-Glu bond by metalloendopeptidase 24.15 (Mentlein and Dahms, 1994; Waters et al., 1996), which is found heterogenously distributed in brain (Dauch et al., 1993), but in contrast to neprilysin, is predominately a cytosolic enzyme (Dahms and Mentlein, 1992).

Proline-containing peptide sequences are conformationally constrained and therefore resistant to common proteinases, but easily degraded by proline-specific proteinases (Mentlein, 1988; Vanhoof et al., 1995). Dipeptidyl aminopeptidase IV (EC 3.4.14.5) is a membrane protease associated with the metabolic barrier function of the cerebral microvessels (Schnabel et al., 1992; Brust et al., 1994) and is evenly distributed throughout the brain (Dauch et al., 1993) and is present in high concentrations in serum (Mentlein et al., 1993b). It degrades endogenous peptides, such as substance P, by liberating dipeptides with proline or alanine adjacent to the aminoterminus (Kato et al., 1978; Mentlein et al., 1993a). However, the optimal substrates for its action are peptides having a N-terminal dipeptide sequence containing α-aminobutyric acid in the penultimate position (Bongers et al., 1992). Thus our research group developed proline- and α-aminobutyric acid-containing analogues with the aim of producing potential [D-Ala³]deltorphin pro-drugs that were protected against nonspecific N-terminal degradation and could be specifically cleaved to form the active parent compound at the BBB. Table 2 shows that this series of pro-drugs were relatively rapidly degraded in the 15% brain membrane homogenate and 100% plasma when compared to their parent compound, [D-Ala³]deltorphin II. Interestingly, both [Ala⁻¹,Pro⁰,D-Ala²]deltorphin and [Pro⁻¹,Pro⁰,D-Ala³]deltorphin had longer half-lives in brain membrane homogenate compared to plasma (table 2) and this might be related to the presence of aminopeptidase P (EC 3.4.11.9) in plasma, which releases N-terminal amino acids from sequences with a penultimate proline residue (Ahmad and Ward, 1992; Checler, 1993; Brownlees and Williams, 1995; Vanhoof et al., 1995).

Figure 4 illustrates the degradation of the cationized prodrug, [Lys²⁵¹,Lys⁰,D-Ala²]deltorphin II and its in vitro conversion to [Lys⁰,D-Ala²]deltorphin II and [D-Ala³]deltorphin II. The enzymes likely to be responsible for the degradation of the cationic peptides in table 2 are aminopeptidase M (EC 3.4.1.12), B (EC 3.4.11.6) and MII. Aminopeptidase M has a broad substrate specificity affecting numerous peptides (Palmieri et al., 1985), although aminopeptidase B and MII exhibit a specificity for N-terminal basic amino acids (Checler, 1993). Both aminopeptidase M and B are found in
high concentrations throughout murine brain (Dauch et al., 1993) and aminepeptidase M is also highly concentrated in cerebral microvessels (Churchill et al., 1987; Schnabel et al., 1992) and plasma (Shibanoki et al., 1991). The faster degradation of the cationic peptides in brain membrane homogenate compared to plasma suggests the presence of differing aminepeptidase concentrations in these two matrices and may be due, at least in part, to the observed absence of aminepeptidase MII in rat serum and plasma (McLellan et al., 1988; Shibanoki et al., 1991).

The potential pro-drugs (analogues 7–13) all show a relatively rapid cleavage of their N-terminal additions (table 2), which is essential because the N-terminal tripeptide sequence (H-Tyr-Glu-Xaa-Phe) is a structural requirement of the type II β-turn that appears to be critical for receptor binding of deltorphin (Salvadori et al., 1993). The deltorphin analogues containing the arginine-arginine, lysine-lysine or α-aminobutyric acid dipeptide sequences were cleaved to form [Arg<sup>2</sup>, α-Ala<sup>3</sup>], [Lys<sup>2</sup>, α-Ala<sup>3</sup>]- or [Abu<sup>2</sup>, α-Ala<sup>3</sup>]-deltorphin II, respectively, followed by a further degradation step to form [d-Ala<sup>2</sup>]-deltorphin II (fig. 4). This is in sharp contrast to the proline-containing pro-drugs that appeared to directly form [d-Ala<sup>2</sup>]-deltorphin II by cleavage of the N-terminal dipeptide sequence (fig. 5). Therefore, these in vitro stability results confirm that aminepeptidase M, with its broad specificity, is not capable of cleaving a proline peptide bond, i.e., Ala-Pro or Pro-Pro of analogues 11 and 12 (Mentlein, 1988), but can cleave the bond between an α-aminobutyric acid dipeptide sequence. These results would also confirm the presence of a dipeptide-cleaving peptide in both brain and plasma, which is responsible for the degradation of the proline-containing pro-drugs. Furthermore, the shorter half-lives in brain membrane homogenate for the cationic and the α-aminobutyric acid-containing pro-drugs compared to the proline-containing pro-drugs may be related to the higher concentration of aminepeptidase M vs. dipeptidyl peptidase IV in the mouse brain (Schnabel et al., 1992). Although the shorter half-lives in the plasma for the proline-containing analogues compared to the cationic pro-drugs would suggest that the higher concentration of aminepeptidase M vs. dipeptidyl peptidase IV in this matrix is not responsible for this result (Schnabel et al., 1992). However, this observation may reflect the presence of aminepeptidase P in plasma (Ahmad and Ward, 1992).

Structure-activity studies have suggested that the deltorphins are similar to other peptide hormones (Schwyzer, 1986), in that they have two distinct proximal regions, which confer specific attributes to the peptide; a N-terminal “message” domain that defines biological responsiveness and a C-terminal “address” domain that influences binding affinities for a specific receptor type (Sagan et al., 1989a; 1989b; Portoghese, 1989; Misicka et al., 1991; 1994). Our study would also appear to indicate that the N-terminal sequence and the amino acids in position 4 and 5 are important in the ability of these deltorphin analogues to cross the in vivo BBB and in their resistance to enzymatic degradation in biological fluids. Although, no [d-Ala<sup>2</sup>]-deltorphin analogue was found to increase the BBB permeability of the parent compounds, analogues were identified, [Arg<sup>2</sup>, α-Ala<sup>3</sup>]-, [Ala<sup>2</sup>, Pro<sup>3</sup>, α-Ala<sup>3</sup>], [Pro<sup>1</sup>, Pro<sup>3</sup>, α-Ala<sup>3</sup>]- and [Abu<sup>1</sup>, Abu<sup>3</sup>, α-Ala<sup>3</sup>]-deltorphin II, which had similar permeability coefficients even though they had larger molecular weights and, in the case of the cationic pro-drug, a significantly lower lipophilicity. Thus these analogues provide directions in the development of [d-Ala<sup>2</sup>]-deltorphin drugs for the alleviation of pain and this study further clarifies the structure-activity relationship of [d-Ala<sup>2</sup>]-deltorphin I and II, in terms of in vivo BBB permeability and biological stability.

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References


Horo, P. J., Wild, K. D., Misiek, A., Lipkowska, A., Haeseth, R. C., Heubt, V.