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[D-Ala^2,(F$_5$)Phe^4]-Dynorphin$_{1-13}$-NH$_2$ (DAFPHEDYN): A Potent Analog of Dynorphin 1–13

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WALKER, J. M., D. H. COY, E. A. YOUNG, G. BALDRIGHI, S. F. SIEGEL, W. D. BOWEN AND H. AKIL. *[D-Ala^2, (F$_5$)Phe^4]-Dynorphin$_{1-13}$-NH$_2$ (DAFPHEDYN): A potent analog of dynorphin 1–13.* PEPTIDES **8**(5) 811–817, 1987.— Intracerebroventricular administration of the dynorphin analog, [D-Ala^2,(F$_5$)Phe^4]-dynorphin$_{1-13}$-NH$_2$ (DAFPHEDYN) in rats produced diuresis and profound analgesia. Both effects were antagonized by central administration of naltrexone or naloxone. Intravenous administration of 10, 25, and 50 mg/kg of DAFPHEDYN failed to induce diuresis. The increased potency of DAFPHEDYN was apparent from the failure of an equal dose of the parent compound (dynorphin 1–13) to produce diuresis and the failure of [D-Ala^2]-dynorphin$_{1-13}$-NH$_2$ to produce analgesia. Radioligand binding studies indicated the DAFPHEDYN retains the same degree of κ selectivity as the parent compound (dynorphin 1–13) though a drop in affinity occurred. DAFPHEDYN may be of significant interest because it retains the essential pharmacology of the parent compound and exhibits marked *in vivo* potency.

Dynorphin κ Analgesia Diuresis Opiate Naloxone Receptor

UNEXPECTEDLY, intraventricular administration of dynorphin produced few notable opiate effects [14, 16, 28, 31, 33, 35]. Such was the case for the enkephalins as well, probably in part because all these peptides exhibit rapid breakdown upon exposure to brain membranes [2, 7, 13, 16, 19–21, 23, 24, 30, 41]. [^{3}H]-dynorphin, injected intracerebrally, is converted to ^{3}H-tyrosine and des-Tyr-dynorphin with exceptional rapidity [41]. Therefore, most studies of the behavioral effects of intracerebral dynorphin may have actually been studies of potent nonopioid metabolite(s), especially des-Tyr-dynorphin [8, 14, 16, 17, 28, 29, 33, 35, 36]. One approach to inhibiting the metabolism of opioid peptides is the substitution of D-alanine in position 2 [3, 4, 27, 32, 34]. This modification, together with an (F$_5$)Phe^4 substitution, gave rise to an extremely potent analog of enkephalin which produced long lasting analgesia after either intracerebroventricular or systemic administration [5].

In considering the problem of enhancing the potency of dynorphin, the issue of κ receptor binding must be addressed. Previous work suggested that substitution of the Tyr^2 residue with D-amino acids changes the pharmacological properties of the resulting analog by differentially increasing μ binding compared to κ or δ [3]. On the other hand, D-amino acid substitution in the carboxyl region produced an analog with greatly enhanced κ receptor selectivity and high affinity. This analog, [D-Pro^{10}]-dynorphin$_{1-11}$ (and a monoiodo derivative), is very selective for κ receptors [10–12]. However, it is probably unsuitable for *in vivo* use because of the expected rapid cleavage of Tyr^1 discussed above.

In view of the desirability of a stable analog of dynorphin which retains the essential pharmacology of the natural peptide, we have investigated the binding and *in vivo* properties of [D-Ala^2,(F$_5$)Phe^4]-dynorphin$_{1-13}$-NH$_2$ (DAFPHEDYN). This compound exhibits a pattern of selectivity for κ, μ, and δ receptors which is very similar to that observed with dynorphin 1–13. DAFPHEDYN potently produces diuresis and profound insensitivity to thermal pain after intracerebroventricular microinjection. The diuretic effects are of particular interest because κ opiates are unique in causing this effect [18].

METHOD

Drugs and Peptides

DAFPHEDYN was synthesized by one of us (D.H.C.) using solid phase methods generally as discussed previously [4]. Dynorphin, dynorphin$_{1-13}$, [D-Ala^2]-dynorphin$_{1-13}$, and

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[D-Ala^2]-dynorphin$_{1-13}$-NH$_2$ were purchased from Peninsula Laboratories, Belmont, CA. Naloxone HCl and naltrexone HCl were generously provided by DuPont, Glenholden, PA.

Receptor Binding

The selectivity of the various dynorphin-like peptides for μ, δ and κ receptors was estimated using radioligand binding to rat and guinea pig brain membranes. The labelling ligands were ^{3}H-Tyr-D-Ala-Gly-NMe-Phe-Gly-ol (DAGO) (1 nM) for μ receptors, ^{3}H-[D-Ser^2,Leu^5,Thr^6]-enkephalin (DSLET) (1 nM) for δ receptors and ^{3}H-bremazocine (0.2 nM) in the presence of 100 nM DAGO and DSLET for κ receptors.

The methods for the binding experiments were described in previous publications [39,40)]. Briefly then, rat and guinea pig brains minus cerebellum were homogenized with a Brinkman polytron at a concentration of 50 mg tissue per ml of 0.05 M TRIS buffer pH 7.4. The preparation was preincubated at 37° for 40 minutes. It was then centrifuged at 30,000×g for 20 minutes and resuspended in 0.05 M TRIS buffer containing 0.2% bovine serum albumin (BSA) (faction V, Sigma). After a second incubation for 40 minutes at 25°, various concentrations of the unlabelled peptides were incubated with the various tritiated ligands in a final volume of 0.5 ml at 30 mg/ml tissue concentration. Incubations were carried out at 4° for 60 minutes. Finally, bound ligands were separated by filtration over Whatman GF/B glass fiber filters using the 0.05 M TRIS buffer. Nonspecific binding was estimated from tubes containing 1 μM unlabelled ligand. The means of the samples in this condition were subtracted from values collected in competition experiments with the various test compounds to derive an estimate of the specific binding. The experiment was repeated three times.

The K_d values of the tritiated ligands were determined in separate experiments from the same pools of rat and guinea pig brain. Scatchard analyses were performed using a computer program developed by Munson and Rodbard [25]. The analysis was simplified by the single component linear fits characteristic of the labelled ligands used in the experiments. The IC_{50} and the K_d values were then used to construct K_i values for the individual test compounds.

Surgical and Injection Procedures

At least one week before testing, cannulas were implanted in the left lateral ventricles of 114 Sprague-Dawley rats (250–375 grams) as described previously [34]. The cannulas were constructed from 24 ga thinwall steel hypodermic tubing which were sharpened and beveled at the tips. Under sodium pentobarbital anesthesia, ventricular cannulas were stereotaxically placed 1 mm posterior to bregma, 1.5 mm lateral to the midline, and 4.1 mm below the skull surface (lambda and bregma at same DV). Dental acrylic and stainless steel screws were used to secure the cannulas.

A microsyringe equipped with a 31 ga needle was used for injections. The needle extended 0.5 mm beyond the tip of the cannula. Injections were conducted over a period of one minute in a volume of 5 μl.

Analgesia Testing

Analgesia was measured by the tail flick test of D'Amour and Smith [6]. The apparatus consisted of an adjustable heat source which could be directed onto the tail of the rat. Application of power to the heat source started a solid state timer which in turn was stopped by withdrawal of the tail, allowing the light from the heat source to activate a photocell. A digital readout to tenths of a second was thereby provided for the latency to tail-withdrawal which is the index of pain responsiveness.

Before testing, the output of the heat source was adjusted to obtain tail-withdrawal latencies between 2.5 and 4 sec. Tail flick latencies were then measured every 3 min for a baseline period of 15 min. Next the peptide or control solution was microinjected in a volume of 5 μl, and testing continued at 3 min intervals for a minimum of 30 minutes or until tail flick latencies had returned to within 30% of their baseline values on three consecutive trials.

Male Sprague-Dawley rats were prepared as above and used to test the analgesic potency of 5 μg (n=8), 10 μg (n=7), 20 μg (n=11), and 40 μg (n=6) of DAFPHEDYN. Each animal received only one injection. Separate groups of 12 animals were tested with either the immediate parent, [D-Ala^2]-dynorphin$_{1-13}$-NH$_2$ (n=6) at a dose of 40 μg, or saline (n=6).

The ability of naloxone to antagonize the analgesic effects of DAFPHEDYN was tested using subcutaneous and ICV injections of the antagonist. In order to test the effects of systemically administered naloxone, animals were treated with either 10 mg/kg naloxone HCl or an equal volume of saline (n=14). Ten minutes later DAFPHEDYN (40 μg ICV) was injected. The effects of centrally administered naloxone were tested by mixing 10 μg naloxone HCl with 40 μg DAFPHEDYN and coadministering them ICV in 5 μl (n=7). These animals were compared to a separate group of rats (n=7) treated with 40 μg DAFPHEDYN ICV.

Measurement of Diuresis

For the investigation of the effects of DAFPHEDYN on diuresis, rats underwent surgery for placement of lateral ventricular cannulas using the procedures described above. After a recovery period of at least one week, the rats were microinjected with saline (n=4), DAFPHEDYN 0.125 μg (n=5), 0.5 μg (n=5), 2 μg (n=7), or dynorphin$_{1-13}$ 2 μg (n=6) and placed in metabolism cages for a two hour period during which the urine was collected. At the end of the period urine volume was recorded.

Naltrexone, rather than naloxone, was used for studies of opiate antagonism of the diuretic effects of DAFPHEDYN because it exhibits a longer duration of action. Rats were either injected subcutaneously (2 mg/kg) 10 minutes before DAFPHEDYN administration (n=8), or naltrexone (20 μg) was mixed with 2 μg DAFPHEDYN and microinjected (n=7). As above, the rats were then placed in metabolism cages for a period of two hours.

Studies of the ability of DAFPHEDYN to induce diuresis after intravenous administration were carried out using 100 to 120 gram male Sprague-Dawley rats. The rats were restrained and DAFPHEDYN was injected into the lateral tail vein at 10 mg/kg (n=2), 25 mg/kg (n=2), or 50 mg/kg (n=2). The number of subjects was kept to a minimum because of the large amounts of the analog necessary for these experiments. Following injection, the rats were placed in metabolism cages for 2 hours as above, and any changes in the behavior of the animals were also noted.

Histology

After the experiments were completed, animals were sacrificed by an overdose of sodium pentobarbitol and injected ICV with a 2% solution of pontamine sky blue dye in

TABLE 1

	Dynorphin		DAFPHEDYN		$Dynorphin_{1-13}$		D-Ala^2-$dynorphin_{1-13}$-NH_2	
	Rat	Guinea Pig	Rat	Guinea Pig	Rat	Guinea Pig	Rat	Guinea Pig
	A							
	K_i values (±SEM) of various dynorphin fragments and analogs in rat and guinea pig brain							
(κ) ^{3}H-Bremazocine	0.17 ± 0.1	0.34 ± 0.04	1.2 ± 0.9	1.3 ± 0.8	0.15 ± 0.1	0.05 ± 0.03	1.9 ± 0.4	1.5 ± 0.03
(μ) ^{3}H-DAGO	1.3 ± 0.3	1.5 ± 1.1	2.0 ± 0.1	16.4 ± 1.7	0.4 ± 0.1	0.55 ± 0.2	1.1 ± 0.4	1.5 ± 0.2
(δ) ^{3}H-DSLET	5.8 ± 1.1	12 ± 0.8	50 ± 5.4	21 ± 3.6	5.4 ± 0.8	6.0 ± 2.1	2.7 ± 0.9	3.2 ± 0.6

B

K_i values from above expressed as ratios: (κ:μ:δ) normalized for κ equal to one

Rat	Guinea Pig	
1:8:34	1:4:35	Dynorphin
1:2:42	1:13:16	DAFPHEDYN
1:3:36	1:11:120	$Dynorphin_{1-13}$
1:0.6:1.4	1:1:2	D-Ala^2-$dynorphin_{1-13}$-NH_2

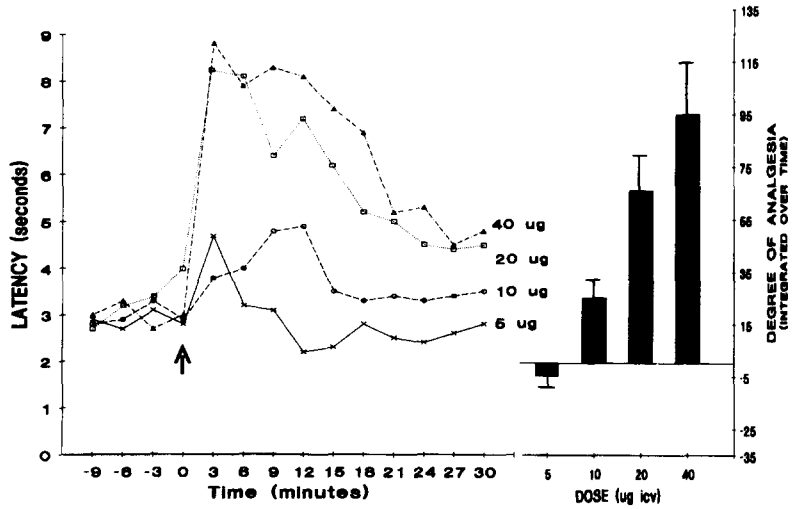

FIG. 1. Left: Effect of various doses of DAFPHEDYN on the tail flick latency of rats after intracerebroventricular injection (arrow). Right: Same data plotted as Simpson's approximation to the integral of the change from baseline mean.

physiological saline. Brains were fixed by the perfusion of 10% formalin through the heart. The brain was cut with a scalpel and the ventricular system was checked for the presence of dye.

RESULTS

Receptor Binding

DAFPHEDYN potently displaces a variety of selective ligands from their binding sites in rat and guinea pig brains. As shown in Table 1A, the K_i was found to be 1.2 nM for the rat brain at κ receptors. Although this value reflects a ten-fold loss of potency compared to $dynorphin_{1-13}$, it is still well within the range of potent opiates.

Table 1B illustrates the ratios of the K_i values at μ, κ, and δ sites normalizing the κ binding to one. Dynorphin and $dynorphin_{1-13}$ show highest affinity for κ receptors, 3 to 11-fold less affinity for μ receptors, and 33 to 120-fold lower affinity for δ receptors in rats and guinea pigs respectively. Amidation and the D-Ala^2 substitution cause a marked loss of selectivity resulting in an analog that is almost equipotent at the various receptor types. Further substitution of (F_5)Phe^4, however, shifts the ratios back to values closely resembling the profile of $dynorphin_{1-13}$.

Analgesia

DAFPHEDYN induces profound analgesia in a dose-dependent manner as shown in Fig. 1. Analysis of variance indicated a highly significant effect across the doses of the analog, $F(3,28)=6.72$, $p=0.0015$. The dose-relatedness of the effect is readily observed in Fig. 1 (right) which plots a math-

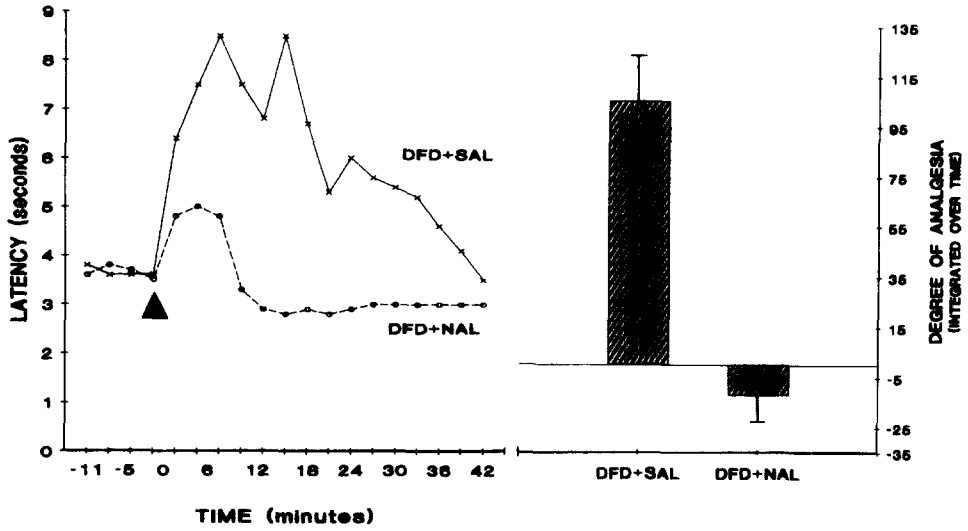

FIG. 2. Left: Effect of naloxone (10 μg ICV) coadministered with DAFPHEDYN (40 μg ICV) on mean tail flick latency over time. Naloxone significantly reduced the analgesic efficacy of DAFPHEDYN. Right: Same data plotted as Simpson's integrals of tail flick latency over time.

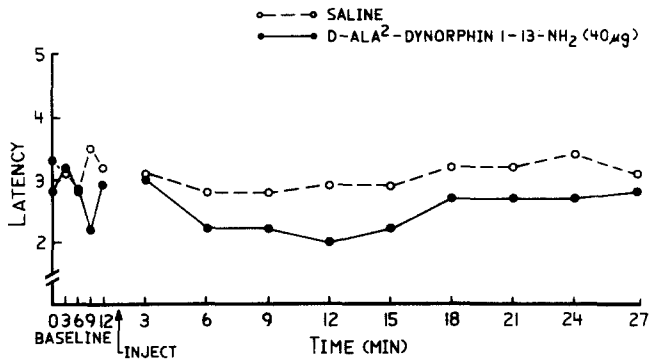

FIG. 3. Tail flick latency before and after treatment with [D-Ala^2]-dynorphin$_{1-13}$-NH_2 (ICV 40 μg). This compound, the immediate parent of DAFPHEDYN, fails to produce analgesia. ○ Saline, ● [D-Ala^2]-dynorphin$_{1-13}$-NH_2.

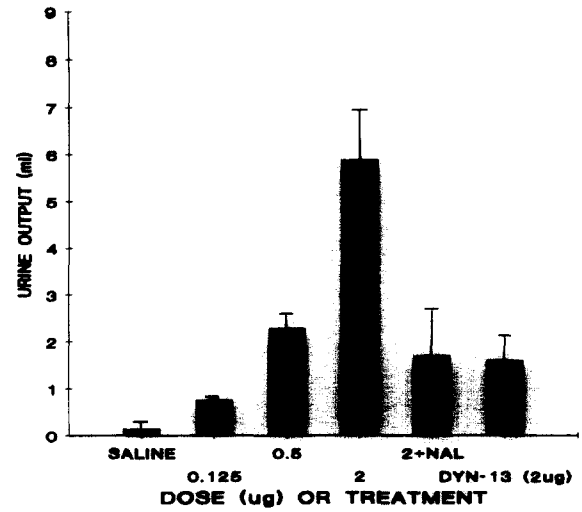

FIG. 4. Mean urine output collected for two hours after ICV administration of saline, various doses of DAFPHEDYN, DAFPHEDYN (2 μg) with natrexone (20 μg) (2+NAL), or dynorphin$_{1-13}$ (2 μg) (DYN13).

ematical approximation of the integral of the post injection tail flick latency adjusted for the baseline average.

Naloxone, administered intracerebroventricularly, significantly reduced the analgesic effects of DAFPHEDYN. This effect, which is illustrated in Fig. 2, was confirmed statistically, $t(6)=4.59$, $p<0.005$. As reported by others, difficulties were encountered when naloxone was administered subcutaneously [15]. Naloxone (10 mg/kg, SC) failed to significantly antagonize the analgesic effects of DAFPHEDYN. This was suggested by analysis of variance which revealed a significant effect of DAFPHEDYN, $F(2,12)=5.69$, $p=0.0018$, but no significant loss of efficacy in the presence of SC naloxone.

As shown in Fig. 3, the immediate parent compound, [D-Ala^2]-dynorphin$_{1-13}$-NH_2, failed to produce any measurable analgesia in the tail flick test at the highest dose used for DAFPHEDYN, $t(11)=1.07$, nonsignificant.

Diuresis

DAFPHEDYN caused dose-dependent diuretic effects. This action, illustrated in Fig. 4, was confirmed by one way analysis of variance across the various doses of the analog, $F(4,20)=5.22$, $p<0.005$. The increased potency of DAFPHEDYN was apparent in a comparison with dynorphin 1–13. As shown in Fig. 4, DAFPHEDYN was considerably more potent than the parent compound at the same dose, $t(11)=2.44$, $p=0.03$.

When administered intracerebroventricularly, naltrexone reduced the diuretic effect of DAFPHEDYN. As suggested by the means shown in Fig. 4, this action is statistically significant, $t(12)=2.54$, $p=0.026$. As with the studies of analgesia, naltrexone failed to show statistically significant antagonism at 2 mg/kg SC, 10 minutes prior to injection of DAFPHEDYN, $t(13)=1.53$, nonsignificant. There was, nevertheless, a marked drop in the mean urine output under

these conditions (mean DAFPHEDYN alone=5.89 ml, with naltrexone (2 mg/kg SC)=3.25 ml).

Intravenous administration of DAFPHEDYN failed to evoke significant diuresis. Although the number of subjects was small, the lack of effect was readily apparent from the means of urine output (ml): saline: 0.55; DAFPHEDYN: 10 mg/kg: 0.0, 25 mg/kg: 0.30, 50 mg/kg: 0.69. The appearance of a slight effect was not statistically significant by one way analysis of variance across the drug doses, $F(3,6)=2.41$, ns. Likewise, the urine output after 2 μg DAFPHEDYN ICV was 8 times greater than after 50 mg/kg intravenously.

Animals treated with the highest dose of DAFPHEDYN intravenously did exhibit marked but transient behavioral change. These included a profound lethargy and lack of movement for a period of one or two minutes immediately after the injection.

DISCUSSION

DAFPHEDYN and dynorphin$_{1-13}$ (the parent compound) exhibit similar patterns of receptor selectivity as judged by the K_i values obtained in competition experiments. It seems possible that other substitutions might well increase the κ selectivity further. Nevertheless, results from both rat and guinea pig brains are strongly indicative of a κ receptor preference in DAFPHEDYN, in spite of a ten-fold drop in binding affinity. This drop in binding affinity is apprently compensated for by increased resistance to metabolism because DAFPHEDYN exhibits considerable *in vivo* potency.

DAFPHEDYN potently induces diuresis and analgesia which can be reversed by naloxone or naltrexone. The increase in potency is reminiscent of a similar increase observed with a fluorinated analog of [D-Ala^2,Met^5]-enkephalin-NH_2 [5]. However, the enkephalin and dynorphin analogs differ in that the enkephalin analog produced analgesia that was very susceptible to naloxone. The weak actions of systemically administered naloxone with DAFPHEDYN are consistent with difficulties encountered by others when opiate agonists were administered ICV [15]. It has also been found that kappa opiates are quite resistant to naloxone [22,38] and that dynorphin may exhibit increased potency because it disassociates from its receptors very slowly [9]. Conceivably, a combination of these factors gave rise to the lack of significant antagonism by systemically administered naloxone. Nevertheless, naloxone and naltrexone were effective when co-administered with DAFPHEDYN, which strongly supports the notion that DAFPHEDYN acted through opiate receptors.

The *in vivo* effects of DAFPHEDYN appear to be mediated, at least in part, through κ receptors. Previous work has shown that μ and κ opiates exhibit different effects on urine output. Kappa-selective opiates have diuretic effects, whereas μ selective opiates generally have antidiuretic effects [18]. The findings that DAFPHEDYN prefers κ receptors *in vitro* and produces diuretic effects *in vivo* that are susceptible only to relatively high doses of naloxone or naltrexone all support the conclusion that the *in vivo* effects of DAFPHEDYN involve κ receptors.

It is certainly conceivable that some effects of DAFPHEDYN are mediated by μ receptors. In particular, the effects on tail flick after central administration are not expected for κ agonists, although we have found moderate increases in tail flick latency after ICV administration of a κ alkaloid, U50,488h (unpublished data). The μ activity of DAFPHEDYN is sufficient to assume that the analgesic effects could be μ related, in spite of the only weak effects of systemically-administered naloxone. Since the relative affinity of DAFPHEDYN for μ, κ and δ receptors so closely resembles that of the natural parent compound, it seems possible that dynorphin itself can give rise to analgesia when released from central neurons. Thus, although the precise mechanism of action of DAFPHEDYN in analgesia is presently unknown, these analgesic effects offer some support to the possibility that centrally released dynorphin might cause thermal analgesia.

In view of the above, it is rather surprising that the immediate parent compound [D-Ala^2]-dynorphin$_{1-13}$-NH_2 failed to produce analgesia after central administration. This failure occurred in spite of an increase in μ binding and a presumed higher metabolic stability. The effects of this compound are reminiscent of past failures of dynorphin to produce thermal analgesia and suggestions that dynorphin [9] or its des-Tyr nonopiate counterpart [36] might exhibit physiological or pharmacological antagonism of morphine. Viewed as a whole, the picture regarding central thermal analgesia with dynorphin is very interesting but complex. More work is needed to resolve competing hypotheses which variously involve concepts of κ analgesia, μ agonist effects of dynorphin, μ antagonist effects of dynorphin, or antagonism of some μ effects by biologically active nonopioid dynorphin metabolites.

Although direct evidence is lacking, it appears that DAFPHEDYN shows increased resistance to enzymatic degradation. In past studies of homologous opioid peptides [4, 27, 32] including dynorphin 1–9 [26], D-Ala^2 substitution and blocking of the C-terminal by amidation were found to reduce enzymatic degradation. In those cases, as in the present case, an increase in behavioral potency was found. In addition, using methods previously described [41], we found that DAFPHEDYN is a poor inhibitor of ^{3}H-dynorphin breakdown (unlike many dynorphin fragments), suggesting that it is a poor substrate for enzyme(s) which typically cleave dynorphin (unpublished data). Unfortunately, these factors do not seem to bestow systemic potency to DAFPHEDYN beyond transient changes which could result from peripheral vascular effects. Direct studies of the breakdown of DAFPHEDYN are still needed to be sure that the increased activity *in vivo* is not due in part to the formation of potent fragments of DAFPHEDYN through enzymatic activity.

To our knowledge DAFPHEDYN represents the first stable analog of dynorphin that retains a dynorphin-like binding profile. This is of importance because although other stabilized analogs of dynorphin may exhibit increases in potency, they are compromised by exhibiting marked changes from the parent in their pharmacological profiles. Furthermore, although there are other compounds which exhibit a greater degree of κ selectivity, DAFPHEDYN may be of interest because it is closely related to dynorphin structurally and pharmacologically, yet its *in vivo* potency is markedly enhanced. This suggests that DAFPHEDYN could form the basis for future analogs of significant interest. For example, a combination of the present modifications with C-terminal substitutions such as D-Pro^{10} might eventually give rise to a stable peptide analog having great κ selectivity. In addition, further study of fluorinated analogs of dynorphin may be of significance because of possible applications involving nuclear magnetic resonance or positron emission tomography.

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