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DYNORPHIN A: INHIBITORY EFFECT ON OTHER OPIATE LIGAND BINDINGS IN THE MOUSE BRAIN

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Abstract—The opioid peptide dynorphin A antagonizes morphine-induced analgesia *in vivo* and inhibits opiate binding *in vitro*, although most of it is rapidly degraded under both conditions. The inhibitory effect was present even in tissue treated *in vivo* with dynorphin A and assayed *in vitro* without it. Shorter fragments of this peptide lacked these effects, indicating that the apparent potency did not result from a metabolite. Na^+ ion specifically reversed both agonist and antagonist binding from *in vitro* inhibition by dynorphin A. These results are discussed in terms of current opioid receptor theories.

Dynorphin A is a leu-enkephalin containing opioid peptide originated from the proenkephalin B [1]. It behaves like a potent agonist in isolated organ preparations sensitive to opioid where it inhibits the electrically induced twitching acting on a specific receptor sensitive to the narcotic antagonist naloxone [2]. In the mouse brain, binding experiments have demonstrated the affinity of this peptide for the μ, δ and κ opioid receptors [3, 4]; however, intraventricular administration to mice had very little or no analgesic effect [5]. A rapid destruction of the peptide might account for this lack of *in vivo* effect, but surprisingly dynorphin A is able to antagonize the morphine-induced analgesia in mice [6], suggesting the presence of the whole sequence or a potent metabolite in the brain for a significant length of time.

We have previously reported that the first thirteen amino acids of dynorphin A inhibit the binding of different opiates and opioid peptides to membrane preparations from mouse brain [3] at 37°, a temperature at which the metabolism rate of endogenous peptides is very high. In the present study we further examine the characteristics of the inhibitory effect of dynorphin A on the binding of the antagonist naloxone and opiate agonists at 37°. A comparative study was performed with shorter sequences of this peptide and other peptides originating from the common precursor, the proenkephalin B.

MATERIALS AND METHODS

Tritiated D-Ala^2-D-Leu^5-enkephalin (31 Ci/mmole) and ethylketocyclazocine (15–25 Ci/mmole) were purchased from New England Nuclear (Boston, MA). Tritiated dihydromorphine (70–85 Ci/mmole) and naloxone (40 Ci/mmole) were from Amersham (Arlington Heights, IL).

Male ICR mice (25 ± 2 g) were obtained from Simonsen Labs. (Gilroy, CA). The animals were killed by cervical dislocation and the brains (minus cerebellum and medulla) pooled. Binding studies were performed in crude synaptosomal (P_2) fractions. The samples containing 25 mM buffer-HEPES K^+ salt, pH 7.7, 1.4 mg protein and the tritiated ligand in a final volume of 2 ml were incubated at 37° in a shaking incubator for 30 min unless otherwise stated. Samples were filtered through Watman GP/B filters under 15 mmHg vacuum, and washed twice with 5 ml of ice-cold HEPES buffer 5 mM, pH 7.7 and counted in the presence of 10 ml of Liquiscint (National Diagnostics) in a Beckman LS-100C scintillation counter 24 hr later.

All binding assays were carried out in triplicate and the variability among the samples was less than 15% of the mean. The saturation experiments were performed with 14–24 concentrations of the radiolabelled ligand and the nonsaturable binding was determined as the remaining in the presence of 1 μM of the non-labelled form of the ligand. Protein was determined by the Lowry method [7] using BSA as standard.

In the isolated organ experiments, the vasa deferentia with the prostatic end sectioned as close as possible to the seminal vesicle were dissected from fat and seminal content, and placed in oxygenated Krebs buffer at room temperature. A single vas deferens was mounted in a 20 ml organ bath between two rings of platinum electrodes. Tissues were maintained at 37° in Krebs (NaCl 118.0, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 0.93, $NaHCO_3$ 24.0, glucose 11.0, tyrosine 0.3 mM), which was continuously bubbled with a mixture of O_2–CO_2 (95%–5%). The upper end of the vas deferens was tied with nylon thread and connected to a Grass force displacement transducer model FT 03 (Grass Instrument Co. Quincy, MA), which was connected to a model 7 polygraph (Grass Instruments Co). Contractions were recorded isometrically, a basal resting tension of 200 mg was applied and the tissues were allowed

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Table 1. Specific ^{3}H-naloxone (1 nM) binding remaining at 37° (%)

Peptide (100 nM)		Time after peptide (min) 5	10	15	20	25	30	IC_{50} (n = 3), 0°
Dynorphin	1–5	80	100	—	—	—	—	30 μM ± 4.1
	1–6	94	85	82	77	70	60	5.7 μM ± 0.9
	1–7	91	91	98	—	—	—	1.7 μM ± 0.1
	1–8	98	104	—	—	—	—	1.0 μM ± 0.1
	1–9	46	73	79	82	—	—	300 nM ± 40
	1–10	45	67	64	90	—	—	97 nM ± 16
	1–11	10	13	18	30	38	53	10 nM ± 1.5
	1–12	16	17	28	38	53	65	100 nM ± 18
	1–13	17	23	27	28	38	44	15 nM ± 2.5
	1–17(A)	10	17	22	18	23	20	20 nM ± 3.0
Dynorphin	3–13	100	95	100	—	—	—	7.0 μM ± 1.1
	6–17	91	96	90	100	—	—	8.8 μM ± 1.5
	(1–5) + (6–17)	84	95	99	—	—	—	
Dynorphin (B)		54	73	76	85	89	96	30 nM ± 4.1
C-terminal		15	33	58	85	94	100	38 nM ± 6.0
α-Neo endorphin		92	112	98	100	—	—	50 nM ± 5.0
β-Neo endorphin		88	93	95	101	—	—	65 nM ± 4.2

^{3}H-Naloxone (1 nM) was preincubated with P_2 fractions for 30 min at 37°, time at which the steady state of equilibrium is largely reached. Peptides were added at time 0 (i.e. after 30 min preincubation) and binding was measured every 5 min. The experiment was repeated at least twice for every peptide. IC_{50} were determined by linear regression of the pseudo-linear part of the concentration–inhibition curve. The experiment was carried out at 0° for 3 hr. At this temperature the metabolism is minimal as determined in the mouse vas deferens assay (not shown).

to equilibrate for 60 min. Electrical field stimulation was used to excite the intramural nerves (Grass S4 stimulator, 0.2 Hz, 2 msec, supramaximal voltage).

RESULTS

The inhibitory effect of Dynorphin A and sequences as well as related opioid peptides derived from the proenkephalin B are studied on the naloxone binding. The IC_{50} of all these peptides competing for the binding was determined at 0° (Table 1), the temperature at which the metabolic rate of these peptides is minimal. Leu-enkephalin is the weakest, dynorphin A-(1–6), -(1–7) and -(1–8) are relatively better, -(1–9) and -(1–10) are intermediate in potency, -(1–11), -(1–13) and dynorphin A are the most potent peptides of the series. Sequences without the initial amino acids like -(3–13) or -(6–17) are very weak, inhibiting the naloxone binding. Dynorphin B and dynorphin B-29 and the neo-endorphins are similar in potency and are practically in the range of dynorphin A.

In Table 1 the way in which dynorphin A (100 nM) induced a potent inhibitory effect on the naloxone binding at 37° is shown, considering that the highest concentration of any metabolite originating from this dynorphin will not pass 100 nM. We selected this concentration for a similar study with the peptides derived from proenkephalin B and sequences of dynorphin A. -(1–13) was a potent inhibitor, but the inhibitory effect started to decrease sooner, -(1–11) and -(1–12) were similar in potency to dynorphin A at the first intervals but the recovery of naloxone binding took place faster. -(1–10) and -(1–9) were intermediate in potency, -(1–8), -(1–7) and -(1–5) were practically inactive. Dynorphin A-(1–6) showed a particular pattern, with potency increasing with time. This finding might indicate the appearance of some more potent metabolite than the -(1–6) itself. (3–13) and -(6–17) did very little. When leu-en-

Table 2. Inability of EKC to protect EKC site against dynorphin A inhibition

Pretreatment	^{3}H-EKC fmol/mg bound
Control	110
EKC (1 μM)	127
Dynorphin A (500 nM)	5
EKC (1 μM 10 min) then dynorphin A (500 nM)	5
Dynorphin A (500 nM 10 min) then EKC (1 μM)	5
EKC (1 μM) + dynorphin A (500 nM)	10

Mouse P_2 fractions were incubated for 20 min total at 37° following one of the described pretreatments. Afterwards, the samples were centrifuged six times in the original volume of buffer and resuspended for binding of ^{3}H-EKC (1 nM).

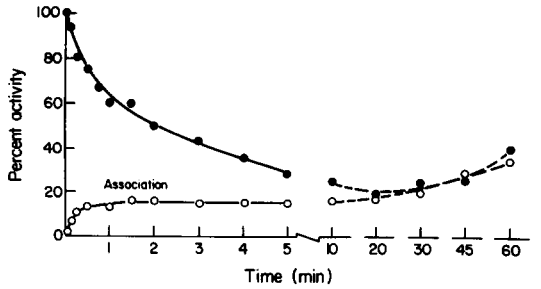

Fig. 1. Dynorphin A: Inhibitory effect on ^{3}H-EKC (1 nM) specific binding. ●, ^{3}H-EKC was equilibrated, 20 min at 37°, then dynorphin A (100 nM) was added and the specific binding expressed as percentage of the controls which received buffer instead of the peptide. ○, Samples at 37° containing ^{3}H-EKC plus dynorphin A (100 nM), received the tissue, the specific binding was then studied. The binding refers to the maximum reached at equilibrium without dynorphin A in the medium.

kephalin plus dynorphin-(6–17) were added together the effect was the same as the pentapeptide alone.

Other peptides from the proenkephalin B did not reproduce the action of dynorphin A. The most potent of them was dynorphin B-29 which started at a similar level of naloxone binding inhibition as dynorphin A, but the effect was recovered before 30 min. Dynorphin B was even weaker. The neoendorphins were also weak and the effect lasted 10 min.

The rapid metabolism *in vivo* of the dynorphin-(1–13) has been previously reported [8]. However, the persistence of the inhibition that we observed (see Table 1) led us to analyse the evolution of this effect when the "free" peptide is removed from the medium by centrifugation and subsequent resuspension of the membranes previously exposed to dynorphin A (Table 2). Considering that this peptide has been described as a potent kappa agonist [9–12], we selected a labelled opiate ligand with recognized kappa agonistic properties, the ethylketocyclazocine (EKC). ^{3}H-EKC binding was inhibited by the initial exposure of the membranes to the peptide. The addition of cold EKC before, after or simultaneously to dynorphin A did not modify the final inhibition of the labelled ligand binding. It is clear that EKC is not able to protect the opioid receptor from the action of dynorphin A; furthermore, the inhibition of dynorphin A is strong in nature and the interaction with the kappa receptor does not necessarily imply a strong binding as is evident by the reversibility of the binding after the exposure of the membranes to EKC alone. Comparative results were found when naloxone instead of EKC was studied together with dynorphin A (data not shown). It is evident that dynorphin is able to inhibit the opiate binding to brain membranes *in vitro* with high potency, even when most of the peptide was metabolized [13] or removed from the medium.

In order to obtain further information on the nature of the inhibition, we studied the time-dependent decrease in EKC binding as the result of the incubation with dynorphin A of brain membranes and the rate of ^{3}H-EKC association in the presence of the peptide (Fig. 1). If the inhibition was due to a metabolite, then the onset might exhibit a "lag" period. In addition in the presence of dynorphin A, the association of EKC with the opioid receptor would be predominant initially, in the absence of any metabolite, while the dissociation rate would, subsequently, increase. The inhibition began immediately (Fig. 1) and reached a maximum in about 10–20 min. The association also occurred almost immediately and reached a plateau in about 3 min, remaining at that level for 10–20 min. Further, the rate of association of EKC began to increase as the inhibition was reversing, 45 min later. All these findings suggest that dynorphin A, not a metabolite, could be responsible for these effects. Thus far, we have shown that at least 10 amino acids starting from the N-terminus tyrosine are required for this activity.

The evolution of the inhibitory effect of Leu-enkephalin (Fig. 2), dynorphin A-(1–13) (Fig. 3) and dynorphin A (Fig. 4) were studied throughout. Because of the higher stability of the antagonist binding, naloxone was selected as labelled ligand in the following experiments. We also obtain an index of the free form of the peptide remaining in the incubation medium using the mouse vas deferens bioassay.

Leu-enkephalin inhibited the naloxone binding, but unlike dynorphin A, the inhibition was rather incomplete and disappeared as the peptide was degraded (Fig. 2). When dynorphin A-(1–13) was studied, a long-lasting inhibition was found (Fig. 3); however, the addition of NaCl reversed the inhibitory effect almost instantaneously. The same effect was induced by NaCl when dynorphin A was used. Furthermore, KCl 100 mM, $MgCl_2$ 5 mM or $MmCl_2$ 5 mM cannot substitute NaCl for this effect (Fig. 5), LiCl 100 mM was less potent than NaCl, but more than KCl. These results suggest a rather specific Na effect. GPP(NH)P did not modify dynorphin A inhibition at concentrations below 1 μM,

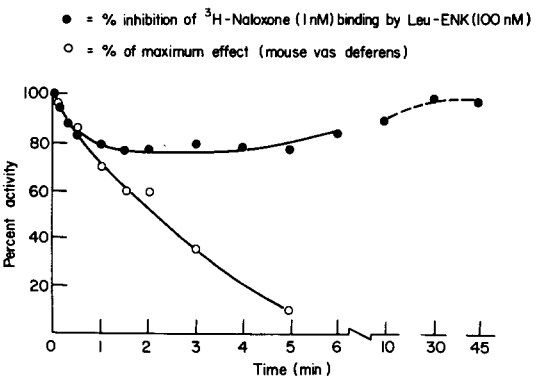

Fig. 2. Leu-enkephalin: Inhibitory effect on ^{3}H-naloxone (1 nM) specific binding. ●, ^{3}H-Nx was equilibrated, 15 min at 37°, then leu-enkephalin (100 nM) was added. The specific binding is percentage of control which received buffer instead of peptide. ○, Samples receiving Leu-enkephalin (100 nM) without ^{3}H-Nx were studied in the mouse vas deferens assay. The amount of pentapeptide at the different time intervals was determined through its dose–response curve and then expressed as percent of the initial concentration added.

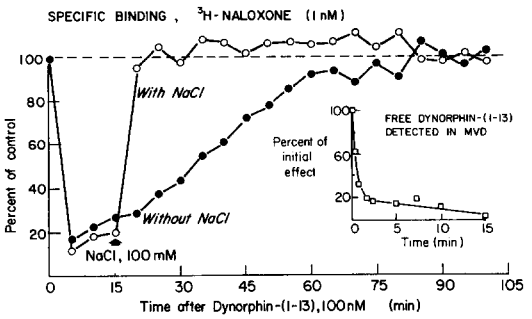

Fig. 3. Time course of the inhibitory effect of dynorphin A-(1–13) on the ^{3}H-naloxone (1 nM) specific binding. Antagonistic action of the sodium ion. ●, The effect of dynorphin A-(1–13) (100 nM) was studied as in Fig. 2. ○, Mouse brain membranes were equilibrated with naloxone for 20 min at 37°. At zero time the peptide (100 nM) was added to the samples and 15 min later received NaCl 100 mM or HEPES buffer. The binding was refered to the samples without dynorphin. □, Free dynorphin was determined by the same procedure as described in Fig. 2.

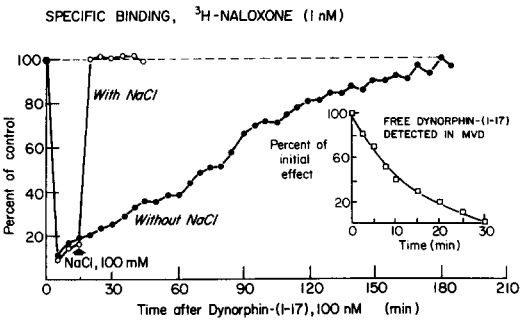

Fig. 4. Time course of the inhibitory effect of dynorphin A on the ^{3}H-naloxone (1 nM) specific binding. The experimental procedure is identical to that described in Fig. 3.

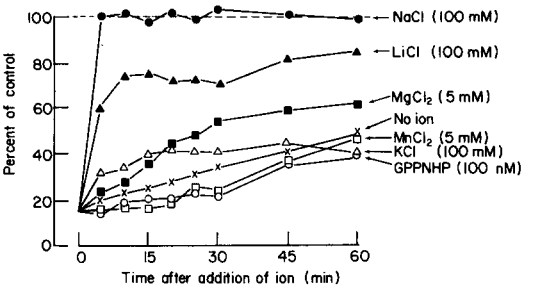

Fig. 5. Ionic and GPP(NH)P effect on the inhibition of ^{3}H-naloxone (1 nM) specific binding induced by dynorphin A (100 nM). Samples containing ^{3}H-naloxone were equilibrated for 15 min at 37°, then dynorphin A (100 nM) was added to half of the samples. After 15 min the ion or GPP(NH)P was added to all the samples (time 0) and the specific binding of samples containing ^{3}H-Nx (1 nM), dynorphin A and the ion or GPP(NH)P, refered to the ones without dynorphin A. The ionic or GPP(NH)P concentrations selected did not reduce ^{3}H-naloxone binding in more than 10%.

at 1 mM 40% of naloxone binding was then detectable (data not shown).

Scatchard of ^{3}H-naloxone binding with or without NaCl during the incubation revealed that Na ion increased the antagonist binding (Fig. 6), as it is known [14]. Therefore it was of interest to see whether this reversal of dynorphin A inhibition on naloxone binding by Na^+ was not merely due to the increase of naloxone binding. ^{3}H-naloxone binding was performed in the presence or absence of NaCl (Table 3). The tissues were preincubated with NaCl or KCl. Even when the subsequent ^{3}H-naloxone binding was conducted in the absence of salt, the tissue preincubated with NaCl showed higher binding than that preincubated in KCl. Tissues preincubated with NaCl did not increase the binding to the opioid receptor, when NaCl was added during the binding assay, while it was so if the preincubation was in KCl.

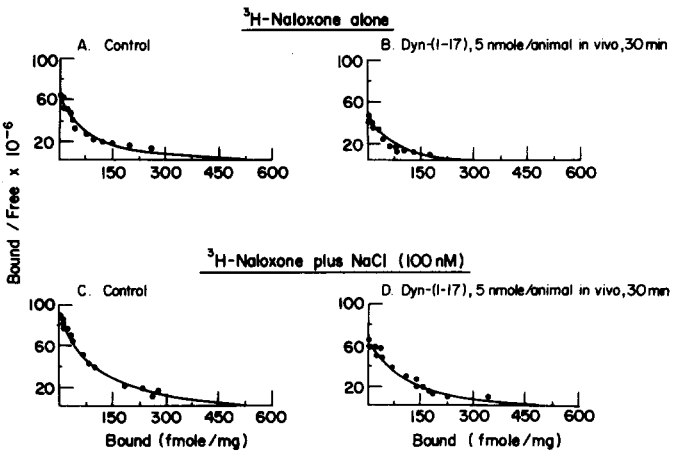

Fig. 6. Sodium effect on ^{3}H-naloxone binding to tissues obtained from mice pretreated or not with dynorphin A. *in vivo*. Animals were administered 5 nmole/mouse, icv. After 30 min P_2 fractions were obtained and ^{3}H-Nx binding was performed at 37° for 30 min with or without NaCl 100 mM in the medium. The result is compared with control tissues from mice that only received saline. At least 14 concentrations of the labelled naloxone were utilized in these saturation experiments.

Table 3. ^{3}H-Naloxone binding in tissues treated with dynorphin A

^{3}H-naloxone (nM)	Preincubated with KCl (100 mM) Sp. binding, fmol/mg protein in No NaCl	KCl (100 mM) Sp. binding, fmol/mg protein in NaCl (100 mM)	NaCl (100 mM) Sp. binding, fmol/mg protein in No NaCl	NaCl (100 mM) Sp. binding, fmol/mg protein in NaCl (100 mM)
0.1	10.6	15.1	16.1	17.4
0.4	38.9	59.0	59.5	62.3
0.7	67.8	101.0	96.5	108.3
1.0	114.0	132.3	146.9	154.3
4.0	290.4	307.5	414.8	358.8
7.0	435.6	496.0	611.4	578.0

Samples of P_2 fraction were preincubated with dynorphin A (100 nM) for 15 min at 37° and then with either NaCl or KCl 100 mM for an additional 15 min. After preincubation, the tissue was centrifuged and the pellet resuspended for naloxone binding.

^{3}H-Naloxone binding was performed with or without NaCl 100 mM in the incubation medium.

This finding indicated that the reversal of dynorphin inhibition has already occurred during the preincubation of the membranes with the Na ion.

To determine whether the preincubation of Na^+ with the tissue would also change the agonist binding, the P_2 fraction was preincubated with dynorphin A, then NaCl 100 mM was added. After removal of the ion ^{3}H-dihydromorphine, ^{3}H-D-Ala^2-D-Leu^5-enkephalin and ^{3}H-EKC bindings were all increased (Table 4). Since Na^+ has already been described as inhibiting the agonist binding, we present evidence that Na has an effect on dynorphin inhibition independent of any effect on the ligand binding.

NaCl is present *in vivo*, so it was of interest to study whether the membranes from *in vivo* treated animals still present a sodium effect. P_2 fractions were obtained from mice treated with 5 nmole of the peptide (icv), and ^{3}H-naloxone binding was performed with and without NaCl in the medium (Fig. 6). The results showed that the administration of dynorphin A *in vivo* resulted in a lower naloxone binding *in vitro*. Surprisingly, however, when NaCl was added to the incubation the binding of naloxone in both control and peptide-treated membranes was increased. This result indicates that the sodium present *in vivo* does not antagonize the inhibitory action of dynorphin A at the opioid receptor level.

Table 4. Effect of NaCl preincubation on the inhibitory effect of dynorphin A on agonist bindings

^{3}H-ligand (nM)		Preincubation with Buffer Sp. binding, fmol/mg protein	NaCl (100 mM) Sp. binding, fmol/mg protein
Dihydromorphine	0.4	1.2	5.2
	0.8	4.1	8.4
	2.0	7.7	17.1
D-Ala^2-D-Leu^5-enk	0.8	6.8	19.0
Ethylketociclazocine	0.4	11.2	27.0
	0.8	20.1	45.8
	2.0	55.5	98.1

Samples of tissue were preincubated with dynorphin A (100 nM) for 15 min at 37° and then with buffer HEPES pH 7.7 or NaCl 100 mM for 15 min more at 37°, then centrifuged and the pellet was resuspended in buffer as described in Methods section.

DISCUSSION

Dynorphin has potent opioid actions. It inhibits the electrically stimulated twitching in the guinea pig ileum [2] and mouse vas deferens. However, dynorphin, unlike most opiates, is not analgesic when given by the icv [5] or sc routes, and antagonizes morphine-induced analgesia [5, 6]. In *in vitro* opioid receptor binding assay, dynorphin A-(1–13) was found to inhibit dihydromorphine, D-Ala^2-D-Leu^5-enkephalin, naloxone and ethylketocyclazocine binding [3]; the same inhibition of *in vitro* binding was found when dynorphin A-(1–13) was given icv *in vivo* [15]. Although dynorphin A-(1–13) has been reported to be metabolized rapidly [8], its inhibitory action, whether with *in vivo* or *in vitro* treatment was rather long-lasting [3, 15], the maximum inhibition persisted long after the free peptide was degraded or removed by centrifugation. Recently we have found that dynorphin A is able to distinguish between its receptor in the mouse vas deferens and guinea pig ileum [16]; its inhibitory effect is rapidly reversed by washing in the first but it requires several to recover the initial twitching in the second. Since the mediation of the kappa type of opioid receptor in both organs is well documented [17, 18], we have to admit that kappa receptors are somehow different in characteristics. The presence of isoreceptors for the opioid receptor, as has been suggested [19], could well explain this apparent inconsistency. If this is so, the kappa subtype present in the brain would be closer in properties to the one in the ileum.

Dynorpin A inhibition at 37° was rather specific and at least 11 amino acid residues starting from the N-terminus are required for the action. Although dynorphin A contains Leu-enkephalin at its N-terminus, this peptide was weak, inhibiting naloxone binding, and the effect diminished as the peptide was metabolized. Extension of the pentapeptide at its carboxyl end up to eight amino acids did not increase its potency, -(1–9) and -(1–10) displayed higher potency. -(1–11), -(1–12) and -(1–13) were more

potent and in a similar range. The fact that -(1–13) inhibition is shorter than that of -(1–17) may be due to differences either in affinity or resistance to the enzymatic cleavage.

The carboxyl-terminus -(6–17) was inactive. Furthermore, administering both -(1–5) and -(6–17) fragments together did not result in any significant inhibition, again suggesting that the complete peptide was the active component for this inhibitory effect. Other peptides such as the neoendorphins were also weak, dynorphin B and -B-29 were more active but far from dynorphin A potency. However, at 0° the potency was similar, suggesting differences in the metabolism. A similar situation might account for other peptides. Dynorphin-(1–8) has been described as a selective kappa ligand in guinea pig [11, 20] and mouse [4] brain, but at 37° and in this experimental dosing, its rapid metabolism does not allow the peptide to work. This result is in agreement with the findings reported by Corbett *et al.* [11], who described a much higher metabolism for the shorter sequences of dynorphin.

The present data seems to rule out convincingly the possibility that a metabolite of dynorphin A is responsible for its inhibitory action. We are left with three alternative explanations: (i) dynorphin dissociates very slowly from the receptor, remaining bound and active after the free ligand is degraded; (ii) dynorphin induces a long-lasting change in the receptor, persisting after its dissociation and degradation; and (iii) free dynorphin remaining becomes concentrated in a microenvironment around the receptor. We cannot yet distinguish between these possibilities, though the first seems the most consistent with the slow dissociation rate found in this study and would account for its apparent non-competitive effect in binding studies [3].

We have reported that inhibition of opioid binding by dynorphin-(1–13) was long lasting under both *in vivo* and *in vitro* conditions [3, 15]. Therefore, it was surprising to observe that Na ion can rapidly reverse the inhibition. It is possible, however, that *in vivo* another ion such as Mg^{2+} or Ca^{2+} balances the Na^+ effect. This ion-specific effect resembled the so-called "Na effect" on opiate binding [14]. Our results showed that within 10 min of the addition of Na^+, naloxone binding was completely restored, and the effect persists after the ion has been removed. This increase in opiate binding was not only for antagonist, since agonists binding were all enhanced. However, since Na^+ has an additional effect on the agonist binding, we have only assayed the agonist binding in those Na^+ treated membranes without the presence of the ion.

The "Na effect" on ligand binding has been observed not only for opioid receptors but other receptors such as muscarinic [21]. This suggests that the ion either may exert some rather general effect on the membrane which has an effect on specific receptors, or the presence of a specific locus for the ion in more than one receptor type. In any case the "Na effect" of dynorphin A may result from the removal of the peptide from the binding site, thus increasing the binding of the labelled ligand. Consistent with this, researchers have reported that an endogenous ligand or inhibitor may be removed if the tissue is preincubated in NaCl [22]; however, this inhibitor has never been identified. We have examined and discarded other endogenous ligands such as the enkephalins and beta-endorphin [9] and peptides derived from proenkephalin A (manuscript in preparation). Our study suggests that among all the opioid peptides described up to date, dynorphin A is the best candidate as the endogenous ligand susceptible to be removed by the incubation in presence of Na ion *in vitro*.

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