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Comparison of Dynorphin-Selective *Kappa* Receptors in Mouse Vas Deferens and Guinea Pig Ileum

Spare Receptor Fraction as a Determinant of Potency

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SUMMARY

Dynorphin inhibits electrically stimulated contractions of both mouse vas deferens and guinea pig ileum preparations *in vitro*. In both cases, a highly specific dynorphin-selective receptor appears to be implicated, but the peptide is 10-fold less potent in mouse vas deferens than in guinea pig ileum. The properties of the dynorphin-selective receptors in the two tissues have been compared in several ways: (a) by measurement of the potency of naloxone as an antagonist of dynorphin's effects; (b) by examining the structural features of the dynorphin molecule important for dynorphin receptor affinity; and (c) by comparison of the effect of amino acid deletions and substitutions in the dynorphin sequence on dynorphin potency. We found that with the exception of the apparent difference in sensitivity, the dynorphin receptor populations in the two tissues were not distinguishable. A feasible explanation for the potency difference would be the presence of a greater reserve of "spare" dynorphin receptors in guinea pig ileum than in mouse vas deferens. Treatment of mouse vasa deferentia with the irreversible opioid antagonist, β-chlornaltrexamine, resulted in a substantial reduction of the maximal response attainable with dynorphin. In contrast, an identical treatment of guinea pig ileum preparations resulted in a parallel shift to the right of the dynorphin dose-response curve, but had little effect on the maximal dynorphin response. We suggest that the greater potency of dynorphin in the ileum preparations results directly from a greater receptor reserve in this tissue; dynorphin needs to occupy a lower fraction of the receptor population in ileum than in vas deferens to produce a comparable effect.

INTRODUCTION

The opioid peptide dynorphin was recently isolated from porcine pituitary extracts. The form which predominates in the posterior lobe of the pituitary is a 17-amino acid peptide with the sequence Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln (1). This same peptide sequence has also been determined using dynorphin isolated from porcine duodenum (2). In the isolated guinea pig ileum longitudinal muscle-myenteric plexus preparation, dynorphin is the most potent opioid peptide known, producing a 50% inhibition of the electrically stimulated contractions of the tissue at a concentration of about 0.3 nM (3). However, in the isolated mouse vas deferens bioassay, while still quite potent, dynorphin is about 10-fold less potent than in the guinea pig ileum assay (2, 4). Dynorphin is only 2 or 3 times more potent than leucine-enkephalin in mouse vasa deferentia, in contrast to its 700-fold greater potency in guinea pig ileum (4).

Dynorphin has been shown to act in the guinea pig ileum through an opioid (i.e., naloxone-sensitive) receptor with different properties from the μ-receptor through which alkaloid opiates, such as normorphine, and small peptides, such as the enkephalins, preferentially act in this tissue (3–6). There is also evidence of opioid receptor heterogeneity in the isolated mouse vas deferens preparation. Using the technique of selective tolerance (7, 8), Wüster *et al.* (9) have shown that dynorphin is acting through an opiate receptor that is different from the morphine-selective μ-receptors and the enkephalin-selective δ-receptors in the mouse vas deferens. These results suggest that in mouse vas deferens, as in guinea pig ileum, there are opiate receptors with selective affinity for dynorphin. Further characterization has demonstrated that in both tissues the dynorphin-selective re-

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ceptors are likely to be identical with the κ-opiate receptor class (6, 10). However, because of the difference in potency of dynorphin in mouse vas deferens and guinea pig ileum it was of interest to compare the binding site topographies of the dynorphin-selective receptors in the two tissues. Our results demonstrate that the receptors in the two tissues are very similar; the greater potency of dynorphin in guinea pig ileum is probably attributable to the presence of a greater reserve of spare dynorphin-selective κ-receptors in this tissue.

EXPERIMENTAL PROCEDURES

Materials. Peptides of the dynorphin series were prepared or purchased as described in ref. 3; dynorphin (the hepatadecapeptide) was purchased from Peninsula Laboratories, Inc. (San Carlos, Calif.). Naloxone was a generous gift from Endo Laboratories (Garden City, N. Y.); normorphine was purchased from Applied Science (State College, Pa.); DADLE[3] was obtained from Bio-Search (San Rafael, Calif.); CNA was provided by Drs. P. S. Portoghese and A. E. Takemori (University of Minnesota); osmotic minipumps (No. 2001) were purchased from Alza Corporation (Palo Alto, Calif.). Peptides were diluted in methanol/0.1 N HCl, 1:1 (v/v), prior to use. Doses of less than 25 μl of this solvent in the 5-ml organ baths had no effect on the amplitude of contractions.

Methods. Isolated vasa deferentia from 35- to 55-g Swiss-Webster mice obtained from Simonson (Gilroy, Calif.) were mounted in 5-ml organ baths at 36° in Mg^{2+}-free Krebs solution, essentially as described by Hughes *et al.* (11). Tissues were maintained under a resting tension of 200 mg and subjected to field stimulation through platinum electrodes inducing maximal contractions (80 V, 2.5 msec, 0.1 Hz). Tissues were allowed to equilibrate for at least 30 min, with changes of bathing fluid at 5- to 10-min intervals. The concentrations of opioids giving 50% inhibition of the electrically stimulated contractions, and sensitivity of the opioids to antagonism by naloxone, were determined as described by Chavkin and Goldstein (3). To allow computation of relative potencies, IC_{50} values for dynorphin- (1–13) and leucine-enkephalin were determined on each preparation.

The effect of removing the connective tissue sheath of the vas deferens on the potency of dynorphin- (1–13) was determined on two vasa deferentia. Pairs of vasa deferentia were removed from two untreated mice. One vas deferens from each mouse was carefully desheathed using fine forceps, prior to mounting in the organ bath; the other vas deferens in each pair served as a control tissue with intact sheath. The tension generated in response to electrical stimulation was not affected by the desheathing procedure.

Chronic treatment of mice with DADLE was carried out as described by Schulz *et al.* (8), using osmotic minipumps delivering DADLE solution at a rate of 1 μl/hr. Two pumps, each filled with 1 ml of DADLE solution (5.5 mg/ml in 0.9% NaCl solution) were implanted subcutaneously in each mouse under light ether anesthesia. After 6 days, the vasa deferentia were removed and mounted in Mg^{2+}-free Krebs solution containing 20 nM DADLE. The DADLE-treated tissue were maintained in this concentration of peptide throughout the experiment. Vasa deferentia from mice implanted for 6 days with minipumps containing saline alone were not different from untreated vasa deferentia in sensitivity to dynorphin-(1–13), leucine-enkephalin, or normorphine.

For comparisons of the effects of CNA (12), treatment on the sensitivity of mouse vas deferens and guinea pig ileum preparations to dynorphin, ileum and vas deferens preparations were set up in adjacent organ baths on the same day. Guinea pig ileum myenteric plexus-longitudinal muscle preparations were mounted in 5-ml organ baths as described by Goldstein and Schulz (13). After initial equilibration with frequent washing for 60 min, dose-response curves for dynorphin-(1–13) were obtained in each tissue. After washing, the tissues were then exposed to CNA, final bath concentration 3 nM, for 10 or 30 min. The CNA was washed from the tissues by three changes of the tissue bathing fluid, and the tissues were further washed by complete changes of the bathing medium at 10-min intervals for an additional 2 hr. Preliminary experiments indicated that all reversibly bound CNA was removed by this procedure, leaving an irreversible component of antagonism which was not reduced by more extensive washing. Dose-response curves for dynorphin-(1–13) were then redetermined in each tissue.

RESULTS

Antagonist potency of naloxone. Naloxone is a competitive antagonist of the effects of the opioids on the mouse vas deferens, producing a parallel shift to the right in the concentration-response curves for dynorphin-(1–13), leucine-enkephalin, and normorphine. The magnitude of this shift is thought to be characteristic of the type of receptor activated (14). In these experiments, the magnitude of the shift for each opioid after treatment of the strips with 100 nM naloxone for 20 min or longer has been measured. The potency shifts (i.e., ratio of opioid IC_{50} in presence of 100 nM naloxone to IC_{50} in absence of naloxone) for dynorphin, dynorphin-(1–13), leucine enkephalin, and normorphine are shown in Table 1. The observed potency shifts can be converted to estimates of the equilibrium constant of the antagonist-receptor interaction, if it is assumed that the effect of each agonist is mediated through a single receptor, in both the absence and presence of naloxone (14). In view of the evidence of multiple receptor types in this tissue, this assumption is questionable. Nevertheless, apparent naloxone K_e values have been computed from the potency shifts to facilitate comparison with previous reports. The estimated apparent naloxone K_e for normorphine of 3.6 nM is comparable to previous estimates in this tissue (15) and is consistent with the proposal that normorphine acts primarily through μ-receptors in mouse vas deferens. The estimated naloxone K_e values for dynorphin-(1–17), dynorphin-(1–13), and leucine enkephalin are about 20 nM. This value is also comparable with previous estimates (15) for leucine-enkephalin in this tissue, and comparable with the values reported for dynorphin-(1–17) and dynorphin-(1–13) in guinea pig ileum preparations (1, 3, 10).

[3] The abbreviations used are: DADLE, D-Ala^2-D-Leu^5-enkephalin; CNA, β-chlornaltrexamine.

TABLE 1

Naloxone antagonism of opioids in mouse vas deferens

DADLE treatment, and the method of computation of the potency shifts induced by 100 nM naloxone, are described in the text. The K_e estimates have been calculated from the mean potency shift estimates, from the relationship K_e = (antagonist concentration)/(potency shift − 1) (14). This assumes that each agonist acts through a single class of receptors (see comments in text). *n* Indicates the individual vasa deferentia used in each determination.

Opioid	Control vasa deferentia		DADLE-treated vasa deferentia	
	Naloxone-induced potency shift ± SEM (*n*)	K_e	Naloxone-induced potency shift ± SEM (*n*)	K_e
		nM		*nM*
Normorphine	29 ± 6.9 (12)	3.6	25 ± 2.9 (13)	4.2
Leucine-enkephalin	5.2 ± 0.42 (24)	24	17 ± 1.7 (18)	6.2
Dynorphin-(1–13)	7.1 ± 1.5 (22)	16	8.1 ± 2.1 (18)	14
Dynorphin-(1–17)	6.5 ± 0.5 (9)	18	ND[a]	ND

[a] ND, Not determined.

The similar naloxone sensitivities of dynorphin and leucine-enkephalin could indicate that both opioids act through the same receptor population in mouse vas deferens. To test this possibility, the effect of DADLE pretreatment of the vas deferens on the sensitivity of these two peptides to antagonism by naloxone has been determined. As previously reported by Wüster *et al.* (9), we found that pretreatment with DADLE substantially reduced the potency of leucine-enkephalin but did not affect the potencies of normorphine or dynorphin-(1–13). Because naloxone does not produce a withdrawal contraction in morphine or DADLE pretreated mouse vas deferens preparations (7, 8), it was possible to obtain an estimate of the potency shifts for the opioids in the DADLE-tolerant tissues (Table 1). The naloxone-induced shifts in the concentration-response curves for normorphine and dynorphin-(1–13) were not affected by the DADLE pretreatment. However, leucine-enkephalin became significantly more sensitive to naloxone antagonism after DADLE treatment. The estimated K_e value for leucine-enkephalin after DADLE treatment suggests that the peptide now acted through μ-receptors and implies that the δ-receptors had been selectively inactivated. The failure of DADLE treatment to affect dynorphin-(1–13) sensitivity to naloxone antagonism suggests that this peptide does not act through δ-receptors in mouse vasa deferentia, despite the similarities in the potency shifts induced by naloxone against dynorphin-(1–13) and leucine-enkephalin.

Potencies of dynorphin fragments; effect of D-Ala² substitution. To compare the dynorphin receptors in mouse vas deferens with those in guinea pig ileum, we tested the potencies of a series of dynorphin fragments. If the receptor binding site topographies are identical, then the effect on potency of amino acid substitutions and deletions should be similar. Two series of dynorphin peptides were examined, the first having progressive COOH-terminal deletions and the second having D-alanine substituted for Gly² in several dynorphin fragments. The effects of each of these modifications has been determined previously in the guinea pig ileum assay (3).

The IC_{50} values for dynorphin fragments in which the COOH-terminal amino acids were sequentially removed, starting from dynorphin-(1–13), are listed in Table 2. In this series, dynorphin-(1–13) had the highest potency, but differences in potency through the series were relatively small: leucine-enkephalin was only 2- to 3-fold less potent than dynorphin-(1–13), in contrast to guinea pig ileum, where it was 500- to 700-fold less potent (3, 4).

The considerable activity of the shorter dynorphin fragments on δ-receptors in mouse vasa deferentia precluded a direct comparison of the relative potencies of the series of peptides in mouse vas deferens and guinea pig ileum. However, after DADLE pretreatment, the δ-receptors are effectively inactivated (see above, and ref. 8), and the resulting preparations are now comparable to guinea pig ileum in that functional μ- and dynorphin-selective opioid receptors are present, but δ-receptors, although present, are not functional (16).

The relative potencies of the dynorphin fragments were therefore redetermined in DADLE-pretreated preparations and compared with their previously determined relative potencies in guinea pig ileum (Table 3). Leucine-enkephalin, dynorphin-(1–6), and dynorphin-(1–7) were substantially less potent after DADLE pretreatment. The potencies of intermediate length fragments were less greatly reduced, and the potencies of the fragments 1–10

TABLE 2

Potencies of dynorphin peptides in mouse vas deferens

IC_{50} is the geometric mean computed from complete log concentration-inhibition curves from *n* vasa. The 95% confidence limits were calculated from the standard deviations of the log IC_{50} values. The amino acid sequence of dynorphin-(1–17)—OH is reported in ref. 1. Dynorphin-(1–5)—OH is leucine-enkephalin. Dynorphin-(1–13)—OMe is the methyl ester of dynorphin-(1–13)—OH.

Peptide	IC_{50}	95% Confidence limits	*n*
	nM		
Dynorphin-(1–17)—OH	7.30	2.91–11.7	9
Dynorphin-(1–13)—OMe	3.14	1.53–6.44	8
Dynorphin-(1–13)—OH	4.21	3.67–4.82	135
Dynorphin-(1–12)—OH	35.7	26.0–49.1	18
Dynorphin-(1–11)—OH	9.93	7.54–13.1	25
Dynorphin-(1–10)—OH	49.8	44.1–56.2	10
Dynorphin-(1–9)—OH	19.0	16.2–22.1	21
Dynorphin-(1–8)—OH	52.7	32.4–85.9	10
Dynorphin-(1–7)—OH	12.2	9.13–16.3	17
Dynorphin-(1–6)—OH	149	103–216	23
Dynorphin-(1–5)—OH	11.9	11.0–12.9	137

TABLE 3

Potencies of dynorphin peptides in DADLE-treated mouse vas deferens

IC_{50} values and 95% confidence intervals were calculated as described in Table 2. Relative potency values were corrected for variance between vasa deferentia by comparing the IC_{50} values of dynorphin-(1–13) (as 100%) with the IC_{50} values of each peptide determined on the same vas deferens preparation. Relative potencies are therefore not necessarily identical with the ratios of mean IC_{50} values, since not all peptides were tested on each vas deferens. Relative potencies of dynorphin peptides in guinea pig ileum were taken from ref. 3. *n* Indicates the number of vasa deferentia in each group. *Q* Is the ratio of the mean IC_{50} values in DADLE-treated vasa deferentia to the mean IC_{50} values in control vasa deferentia (from Table 2), and provides a measure of the degree of of tolerance for each peptide.

Peptide	IC_{50}	*n*	95% Confidence limits	*Q*	Relative potency	Relative potency in guinea pig ileum
	nM					
Dynorphin-(1–13)	5.99	32	4.50–7.96	1.4	100	100
Dynorphin-(1–12)	13.8[a]	6	9.34–20.3	0.39	30	9.4
Dynorphin-(1–11)	10.2	14	6.25–16.5	1.0	62	64
Dynorphin-(1–10)	75.6[a]	6	54.2–106	1.5	5.4	2.2
Dynorphin-(1–9)	213[a]	14	113–402	11	3.3	1.8
Dynorphin-(1–8)	213[a]	6	150–302	4.0	1.9	3.1
Dynorphin-(1–7)	231[a]	14	174–308	19	2.5	2.0
Dynorphin-(1–6)	2500[a]	12	2090–2990	17	0.17	0.09
Dynorphin-(1–5)	973[a]	32	807–1170	82	1.0	0.20

[a] Log IC_{50} values significantly different from log IC_{50} values in control vasa deferentia (see Table 2; *t*-test, $p < 0.01$). Dynorphin-(1–5) is leucine-enkephalin.

through 1–13 were only slightly affected by the treatment. It seems probable therefore that a significant component of the activities in untreated vasa deferentia of the fragments shorter than fragment 1–10 is attributable to an interaction with the δ-receptor, whereas the larger fragments act almost exclusively through the dynorphin-selective receptor.

There was a close correlation between the potencies of the dynorphin fragments in the DADLE-treated mouse vas deferens and in guinea pig ileum preparations ($R^2 = 0.982$). In both tissues, dynorphin-(1–12) was less potent than dynorphin-(1–13) and dynorphin-(1–11). Similarly, dynorphin-(1–6) was less potent than dynorphin-(1–7) and leucine-enkephalin. In guinea pig ileum, these effects were attributed to the inappropriate placement of the terminal—COOH group in dynorphin-(1–12) and dynorphin-(1–6) (3); a similar explanation may apply to mouse vas deferens. Significant decrements in potency also occurred on removal of the basic residues in positions 11 and 7 in both tissues [note that dynorphin-(1–7) was significantly more potent than leucine-enkephalin]. A specific interaction of these basic residues with the dynorphin receptor of guinea pig ileum has been proposed (3).

The effects of D-Ala^2 substitution on the potencies of selected dynorphin fragments is presented in Table 4. D-Ala^2 substitution has been shown to increase the potency of leucine-enkephalin in the mouse vas deferens (17). In general, D-Ala^2 substitution increased the potencies of shorter members of the series [dynorphin-(1–5) through dynorphin-(1–7)] from 4- to 6-fold. The substitution had little effect on the potencies of intermediate-length fragments, but reduced the potency of dynorphin-(1–13) by more than 50%. Wüster *et al.* (18) have previously reported that D-Ala^2 substitution reduces the potency of dynorphin-(1–13) in DADLE-treated mouse vas deferens preparations. The potency of dynorphin at the dynorphin-selective receptor in guinea pig ileum myenteric plexus is also adversely affected by D-alanine substitution for Gly^2 in dynorphin peptides (3). The results suggest that D-Ala^2 substitution enhances the affinities of the shorter dynorphin fragments at δ-receptors but reduces the potency of dynorphin-(1–13) at dynorphin-selective receptors. Collectively, these results emphasize

TABLE 4

Effects of D-Ala^2 substitution on the potencies of dynorphin peptides in mouse vas deferens

IC_{50} values and 95% confidence limits were calculated as in Table 2. The ratios, IC_{50}, Gly^2 peptide/IC_{50}, D-Ala^2 peptide, were calculated from the mean IC_{50} values for each peptide. In the case of dynorphin-(1–13), D-Ala^2-dynorphin-(1–13)—NH_2 was compared with dynorphin-(1–13)—OMe.

Peptide	IC_{50} of D-Ala^2 peptide	95% Confidence limits	*n*	Ratio: IC_{50}, Gly^2/IC_{50}, D-Ala^2
	nM			
Dynorphin-(1–13)—NH_2	8.68[a]	7.11–10.6	8	0.36
Dynorphin-(1–11)—OH	7.17	4.83–10.6	8	1.4
Dynorphin-(1–10)—OH	19.0[b]	6.18–58.2	7	2.6
Dynorphin-(1–7)—OH	2.14[b]	1.88–2.45	4	5.7
Dynorphin-(1–6)—OH	34.3[b]	9.12–129	4	4.3
Dynorphin-(1–5)—OH	2.38[b]	1.87–3.04	8	5.0

[a] Log IC_{50} value for the D-Ala^2 peptide was significantly higher than the log IC_{50} value for the Gly^2 peptide ($p < 0.01$).

[b] Log IC_{50} values for the D-Ala^2 peptide were significantly lower than the log IC_{50} values for the Gly^2 peptide (*t*-test; $p < 0.01$).

the similarities in the receptors with which dynorphin interacts in the two tissues.

Other potential determinants of dynorphin potency in guinea pig ileum and mouse vas deferens. The dynorphin-(1–13) IC_{50} in mouse vas was 10- to 20-fold higher than in guinea pig ileum, in confirmation of previous estimates (4). The mouse vas deferens has a thick connective-tissue sheath through which peptides and drugs must diffuse in order to affect transmitter release from the nerve terminals. Since dynorphin-(1–13) is known to be strongly adsorbed to membrane surfaces (19), it is possible that the sheath of the vas deferens presents a more formidable barrier to dynorphin diffusion than the connective tissue surrounding the exposed ganglia of the myenteric plexus in guinea pig ileum. However, in two preparations in which the connective tissue sheath was carefully dissected off, the IC_{50} for dynorphin-(1–13) (2.9, 4.7 nM) was little different from the values obtained in the paired control vasa deferentia (4.2, 5.0 nM). There was also little difference in leucine-enkephalin in IC_{50} values (desheathed, 10, 18 nM; intact, 11, 16 nM). It seems unlikely that the presence of the connective-tissue sheath could account for the difference in dynorphin-(1–13) potency in mouse vas deferens and guinea pig ileum.

The difference in absolute potency of dynorphin in mouse vas deferens and guinea pig ileum might be explained by a difference in the number of spare receptors for dynorphin in the two tissues. An excess of receptors above the minimum required for 100% agonist response ensures that the fractional occupancy required for 50% of maximal effect is less than 0.5; the agonist IC_{50} (the concentration required for 50% of maximal effect) is lower than its K_D (the concentration required for 50% occupancy of the receptors). The larger the spare receptor fraction, the lower the ratio of IC_{50} to K_D becomes. Thus a greater excess of dynorphin-selective receptors in guinea pig ileum than in mouse vas deferens would confer greater absolute potency even though the receptors in each tissue had equal affinity for dynorphin.

The presence of spare receptors can be demonstrated by the use of irreversible antagonists; in the presence of spare receptors an irreversible antagonist shifts the agonist dose-response curve to the right until the excess receptor population is occupied by antagonist. At this point further receptor occupation by the irreversible antagonist results in a reduction in maximal agonist response (20). CNA has been shown to antagonise the effects of dynorphin-(1–13) irreversibly in guinea pig ileum preparations (5). We have therefore compared the effects of CNA treatment on dose-response curves for dynorphin-(1–13) in guinea pig ileum and mouse deferens preparations (Fig. 1). Exposure of guinea pig ileum preparations to 3 nM CNA for up to 30 min resulted in a substantial shift to the right in the position of the dynorphin-(1–13) dose-response curve, with an IC_{50} ratio (mean IC_{50} after CNA treatment/mean IC_{50} before CNA treatment) of 70, after 10 min of exposure, and 120 after 30 min of exposure. Despite the large shift in the dose-response curve, substantial inhibitions of the electrically stimulated contractions of the tissue could still be obtained with sufficient concentration of dynorphin-(1–13).

In contrast, in mouse vas deferens preparations, exposure to the same CNA solution for the same period of time produced a substantial reduction in the maximal inhibition attainable with very high concentrations of dynorphin-(1–13). After a 10-min exposure, the maximal dynorphin-induced inhibition of electrically stimulated tissue contractions was 40%; after a 30-min exposure, maximal inhibition was 24%. The difference in effect of CNA treatment on the maximal response to dynorphin in the two tissues did not appear to result from a lower sensitivity of the ileum dynorphin-selective receptors to CNA. If the CNA-induced shift in dose-response curve is estimated from the rising phase of the partial curve obtained after 10 min of CNA treatment in mouse vas

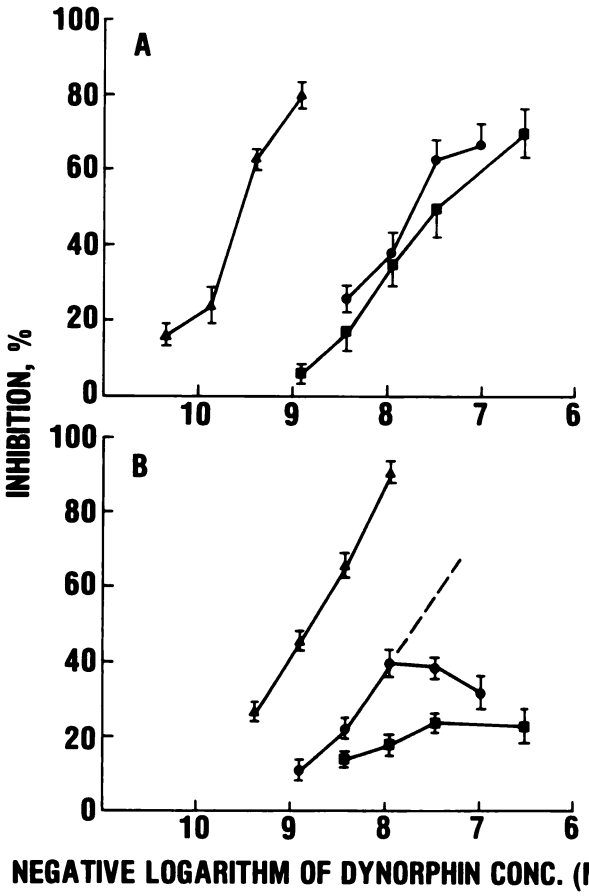

FIG. 1. *Effects of treatment of guinea pig ileum (A) and mouse vas deferens (B) preparations with CNA on responses to dynorphin-(1–13)*

Pairs of ileum and vas preparations were set up in adjacent organ baths. After initial determination of sensitivity to dynorphin-(1–13), the tissues were exposed to CNA, final bath concentration of 3 nM, for 10 min or 30 min. The tissues were washed for 2 hr by repeated changes of the bathing medium. The sensitivity to dynorphin-(1–13) was then redetermined. Mean values (± standard error of the mean) of inhibition of electrically stimulated contractions of the tissue are plotted against the negative logarithm of the nominal bath concentration of dynorphin-(1–13). Initial dynorphin sensitivity (▲): guinea pig ileum, n (number of preparations) = 6; mouse vas deferens; n = 8. After CNA exposure for 10 min (●): guinea pig ileum, n = 3, mouse vas deferens, n = 4. After CNA exposure for 30 min (■): guinea pig ileum, n = 3; mouse vas deferens, n = 4. The *dashed line* is an extrapolation of the rising phase of the dynorphin-(1–13) dose-response curve after 10 min of CNA treatment, drawn to permit an estimate of the dose-response curve shift induced by this treatment in mouse vas deferens (see text).

deferens (*dashed line*, Fig. 1), it is found that a 10-min exposure to CNA resulted in more than a 4-fold greater shift in the guinea pig ileum preparations without any clear reduction in maximal effect. The results support the hypothesis that there is a greater excess of dynorphin-selective receptors in guinea pig ileum than in mouse vas deferens.

DISCUSSION

In guinea pig ileum, dynorphin-(1–13) interacts preferentially with κ-opioid receptors to inhibit the muscle contractions induced by electrical stimulation of the cholinergic nerve terminals (3, 6, 10). Dynorphin-(1–13) also inhibits the electrically stimulated contractions of mouse vas deferens preparations (4). However, its absolute potency in mouse vas is roughly 10-fold lower than in guinea pig ileum (4). It was therefore of interest to compare the properties of the receptors through which dynorphin peptides can act in the two tissues. The features of the dynorphin molecule which facilitate activation of the dynorphin-selective receptor in guinea pig ileum have been reported previously (3).

Measurement of the antagonist potency of naloxone against dynorphin-(1–13) in mouse vas deferens suggested that this peptide did not act through μ-receptors in this tissue. The observed apparent K_e value of about 20 nM would be compatible with an interaction either at δ-receptors (leucine-enkephalin, i.e., dynorphin-(1–5), had a naloxone K_e value of 24 nM), or at κ-receptors of the type found in guinea pig ileum, which are also relatively insensitive to naloxone (3, 4). Two results suggest that dynorphin-(1–13) does not activate δ-receptors to any significant extent in mouse vas deferens. First, D-Ala^2 substitution, which was previously shown to increase the activity of opioid peptides at δ-receptors (17), reduced the potency of dynorphin-(1–13) on mouse vasa deferentia. Second, pretreatment of mice with the selective δ-receptor ligand, DADLE, substantially reduced the potency of dynorphin-(1–5), but did not significantly reduce the potency of dynorphin-(1–13), as previously reported (8). The results suggest that in mouse vas deferens, as in guinea pig ileum, dynorphin-(1–13) inhibits transmitter release through receptors with selective affinity for dynorphin.

However, a direct comparison of the relative potencies of the series of dynorphin fragments from 1–13 to 1–5 in mouse vas deferens with their relative potencies in guinea pig ileum would be misleading. The effects of D-Ala^2 substitution, and of DADLE pretreatment, indicate that the shorter dynorphin fragments produce a significant part of their activity through a δ-receptor interaction. Thus the potencies of dynorphin-(1–5), -(1–6), and -(1–7) were significantly enhanced by D-Ala^2 substitution, and considerable tolerance to these peptides was apparent after DADLE pretreatment. Interaction δ-receptors, which are not present in functionally active form in guinea pig ileum (15, 16), complicates the interpretation of dynorphin potencies in mouse vas deferens.

The ability of DADLE pretreatment of the mouse, followed by incubation of the vasa deferentia in DADLE-containing solution, to reduce greatly the potencies of agonists acting through the δ-receptor provided an opportunity to measure the interactions of the dynorphin fragments with opioid receptors in ileum and vas deferens under comparable conditions. DADLE pretreatment increased the IC_{50} of leucine-enkephalin by about 80-fold. Its activity in the presence of DADLE was much more sensitive to antagonism by naloxone than in untreated vasa deferentia. The estimated naloxone K_e value in the presence of DADLE was comparable to that expected for opioids acting through the μ-receptor. This result suggests that the δ-receptors in the DADLE-treated vasa deferentia were effectively inactivated, and also indicates that leucine-enkephalin has a higher affinity for μ-receptors than for dynorphin-selective κ-receptors in mouse vas deferens. The presence of 20 mM DADLE in the bathing fluid of the pretreated vasa deferentia apparently does not affect the quantitative estimates of the naloxone-induced potency shifts for ligands acting preferentially at μ- or κ-receptors; the potency shifts for normorphine and dynorphin-(1–13) were essentially unaffected by the DADLE treatment.

When the relative potencies of the dynorphin fragments in DADLE-treated mouse vas deferens and in guinea pig ileum were compared, a close correlation was observed ($R^2 = 0.982$). As in guinea pig ileum (3), the basic amino acid residues, arginine-7 and lysine-11, were clearly important in facilitating dynorphin-like activity, and lysine-13 may make a relatively minor contribution. The similarity with dynorphin action in guinea pig ileum also extends to the disadvantageous effect of locating a free terminal—COOH group at residues 6 or 12 [as in dynorphin-(1–6) and dynorphin-(1–12)]. Furthermore, D-Ala^2 substitution reduced the potency of dynorphin-(1–13) in mouse vas deferens, as in guinea pig ileum. These observations emphasize the similarities in the properties of the dynorphin-selective receptors in the two tissues. Our results do not bear directly on the suggestion that there are subtypes of κ-receptor in mouse vas deferens (21).

Despite the apparent similarities of the dynorphin-selective receptors in guinea pig ileum and mouse vas deferens, the absolute potency of dynorphin in the two tissues is different; dynorphin-(1–13) was about 10-fold less potent in mouse vas deferens. This difference could result from the presence of a greater diffusional barrier for the highly adsorbed dynorphin-(1–13) in the vas deferens. Nevertheless, the potency of this peptide was not affected by removal of the connective tissue sheath of the vas deferens.

An alternative explanation of the higher potency of dynorphin in guinea pig ileum is the presence of a greater excess of dynorphin-selective receptors in this tissue. This hypothesis is supported by the experiments with the irreversible opioid antagonist, CNA (12). CNA treatment substantially reduced the maximal inhibition attainable with dynorphin-(1–13) in mouse vas deferens preparations, whereas maximal inhibition was little affected by an identical treatment of guinea pig ileum preparations. This difference was not the result of a lower sensitivity to CNA of dynorphin receptors in the ileum, since the shift to the right of the dynorphin dose-response curve induced by CNA treatment was at least as great in the ileum as in vas deferens preparations. The simplest

explanation of these observations is the presence of a greater reserve of dynorphin receptors in ileum than in vas deferens; dynorphin therefore needs to occupy a greater proportion of the receptors in mouse vas deferens than in guinea pig ileum to produce a comparable effect.

According to the occupancy assumption of ligand-receptor interaction developed by Clark (22) and Gaddum (23), the response of a system is proportional to the fraction of receptors occupied by an agonist. The maximal effect of an agonist is assumed to require full saturation of the receptors. In some cases, however, the maximal response can apparently be achieved at less than full receptor occupancy (20). In this situation, a fraction of the total potentially functional pool of receptors is in reserve; this fraction was termed "spare" by Stephenson (24). The consequences of the occurrence of a receptor reserve have been discussed by van Rossum and Ariens (25).

The fraction of spare receptors in a system is controlled by many factors, and may vary for different agonists acting on the same receptor population, since it will be affected by agonist efficacy (24). The presence or absence of a receptor reserve is not an absolute for any tissue, but depends on the particular physiological and experimental circumstances. Thus the existence of a receptor reserve can only be demonstrated for a particular tissue under defined experimental conditions. Factors affecting the presence or absence of a receptor reserve in electrically stimulated, isolated smooth-muscle preparations will include (but are not limited to) the absolute numbers of receptors in a tissue, the efficiency of receptor coupling to the primary effector system, the coupling efficiencies at all subsequent steps in the chain of events between receptor activation and inhibition of muscle contraction, and the presence or absence of other variable inputs regulating the response to electrical stimulation. The present study concerns two *in vitro* preparations suspended in media of different ionic compositions and subjected to different stimulation conditions. The question of whether there are spare receptors in these tissues *in vivo* is not addressed. However, the possibility that the sensitivity to opiates and endogenous opioids can be regulated by changes in the fraction of spare receptors is raised.

Since dynorphin is thought to be a κ-selective agonist (6, 10, 20), the difference in its potency in the two tissues suggests that there are fewer spare κ-receptors in mouse vas deferens than in guinea pig ileum. This is supported by the obervations of Hutchinson *et al.* (26), who found that κ-selective opioids in the benzomorphan series are also 10-fold less potent in the mouse vas deferens than in guinea pig ileum. However, the role of receptor excess in determining the relative potencies of agonists in different tissues is probably not restricted to κ-opioid receptors. The higher potencies of μ-opioid receptor ligands in guinea pig ileum than in mouse vas deferens (15) may also result from a greater excess of μ-receptors in ileum than in vas deferens.[4] Our studies emphasize the importance of receptor density as a determinant of potency. Although affinity remains a critical determinant, since receptor occupation must occur for effects to be observed, substantial increments in potency result if tissues generate an excess of receptors above the minimum required for full agonist effect.

[4] B. M. Cox and C. Chavkin, unpublished observations.

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