MOLECULAR PHARMACOLOGY, 25:343–348

Site-Directed Alkylation of Multiple Opioid Receptors

II. Pharmacological Selectivity[1]

AVRAM GOLDSTEIN AND IAIN F. JAMES

Addiction Research Foundation and Stanford University, Palo Alto, California 94304

Received October 26, 1983; Accepted January 19, 1984

SUMMARY

A site-directed alkylating agent was used to inactivate one or more types of opioid receptor in two bioassay preparations in the presence of type-selective ligands as protectors of other opioid receptor types. Since the pharmacological potency of an agonist is decreased when the receptor type through which it acts has been inactivated, the method can be used to characterize the pharmacological selectivity of opioid agonists. All of the smaller opioid products of the enkephalin gene were found to be δ-selective in the mouse vas deferens, but BAM-12P, BAM-22P, and Peptide E were not. In the same tissue, β_c-endorphin was not μ-selective, but in the guinea pig ileum preparation it evidently combined with μ and κ receptors. The presence of functional ε receptors, however, could not be ruled out. The approach described here is applicable to any pharmacologically active receptors of which there are multiple types, and for which site-directed alkylating agents and type-selective protector ligands are available.

INTRODUCTION

With the recognition of multiple types of opioid receptors (e.g., μ, δ, κ, ε, σ) (1–3) came the need for reliable techniques to identify receptor types and to characterize the type selectivity of ligands. One useful technique is receptor inactivation, employing the site-directed alkylating agent β-CNA[2] (4). Our first use of the β-CNA technique was to inactivate or protect the μ and κ receptors in GPI (5), and similar studies were carried out subsequently with MVD (6). In both tissues, dynorphin A or dynorphin A-(1–13) protected κ receptors preferentially. β-CNA in the presence of appropriate protector ligands has proved effective in preparing neural membranes enriched in one type of opioid binding site (7), and we have used such preparations to generate binding selectivity profiles (i.e., ratios of equilibrium binding constants at the several types of binding site) for various opioid ligands (8, 9). β-CNA, which is a derivative of the antagonist naltrexone, is sufficiently site-directed toward opioid receptors for indiscriminate alkylation to be avoided. This offers the advantage that the same alkylating agent can be used not only for binding studies with membranes [where phenoxybenzamine (10) and *N*-ethylmaleimide (11) have also been employed] but for pharmacological studies as well, in which widespread alkylation would interfere nonspecifically with the tissue responses. For the specific purpose of inactivating μ receptors, the μ-directed alkylating agent FNA (12) offers an alternative to the technique described here.

To measure pharmacological selectivity we compare the potency of an agonist in untreated tissue and in tissue that has been exposed to β-CNA with protector added to preserve the functional capability of the desired receptor type(s). For example, in MVD, after δ protection (μ and κ inactivation), an agonist that produced its pharmacological effect initially through δ receptors would show little or no change in potency, whereas one that produced its effect through μ or κ receptors would show a decrease in potency. The magnitude of the potency shift can be a quantitative measure of pharmacological selectivity of the agonist for its preferred receptor type over its next-preferred type. We show here how the method can be used to characterize the pharmacological selectivity of an opioid agonist, and also to investigate the possible presence of a receptor type ordinarily obscured by agonist interaction with receptors of other types.

MATERIALS AND METHODS

GPI (13, 14) and MVD (15) were used as described elsewhere. EC_{50} is the agonist concentration required to produce half-maximal inhibi-

This work was supported by Grant DA-1199 from the National Institute on Drug Abuse.

[1] The preceding paper of this two-part series (see ref. 9) deals with Binding Selectivity.

[2] The abbreviations used are: β-CNA, β-chlornaltrexamine; GPI, guinea pig ileum myenteric plexus-longitudinal muscle preparation; MVD, mouse vas deferens; FNA, β-funaltrexamine; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DAGO, [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin.

0026-895X/84/030343-06$02.00/0

tion of the electrically stimulated muscle twitch. We have found that in GPI (although not in MVD) as much as 70% of the over-all variance in EC_{50} estimates is due to the variance between tissue preparations. Accordingly, we routinely compute the potency of a given agonist relative to that of standard normorphine or dynorphin A measured on the same preparation. Since pharmacological log dose-response curves tend to be linear throughout their useful portions, the frequency distribution of sample EC_{50} estimates is expected to be Gaussian on the logarithmic concentration axis. Therefore, we compute EC_{50} or relative potency as a geometric mean rather than an arithmetic mean, and confidence limits are obtained from the standard error of the mean computed in units of log concentration. We compute *potency shift* (i.e., the ratio of EC_{50} after β-CNA treatment to EC_{50} initially) on a preparation-by-preparation basis when practicable, as a logarithmic difference, then obtain the geometric mean and confidence limits from these individual shifts. Data are rounded to two significant figures. After all initial assays are completed, any protector ligand is added to the tissue bath, followed 1 min later by β-CNA, and the incubation is continued for an additional 20 min at 37°. The bath fluid is changed repeatedly at 10-min intervals to remove residual β-CNA and protector ligand until twitch responses return to normal amplitude (typically 30 min for MVD, 90 min for GPI); then testing proceeds. The functional integrity of δ receptors is preserved at low concentrations of β-CNA without need for a protector ligand; nevertheless, for uniformity we refer to such δ receptors, too, as "protected." To demonstrate effectiveness of the inactivation and protection, we include as controls in each experiment a standard agonist selective for each protected receptor type (a negligible potency shift being required) and also a standard agonist selective for each inactivated receptor type (a large potency shift being required). Naloxone K_e was determined in GPI by the method of Kosterlitz and Watt (16). All reagents were Baker reagent-grade or equivalent. Sources of compounds used were as follows: β-CNA, a gift from Drs. P. S. Portoghese and A. E. Takemori; morphine sulfate, Merck; levorphanol tartrate, a gift from Hoffmann-La Roche; naloxone hydrochloride, a gift from Endo Laboratories; [Leu]enkephalin, Beckman; [Met]enkephalin and DADLE, Biosearch; dynorphin A, dynorphin A-(1–13), dynorphin B, peptide F, and morphiceptin, Peninsula Laboratories; β_c-endorphin, Pierce Chemical Company; Tyr-c[D-Lys-Gly-Phe], a gift from Dr. P. Schiller; DAGO, a gift from Dr. D. Romer, Sandoz Ltd.; all other peptides, Bachem.

RESULTS

For which receptor type(s) do the enkephalin gene products have pharmacological selectivity in MVD? In Table 1A are illustrated the results of experiments in which δ receptors were protected, while μ and κ were inactivated, in order to assess the pharmacological selectivity in MVD of several opioid peptides derived from pro-enkephalin (17). Normorphine and dynorphin A (selective μ and κ agonists, respectively) had large potency shifts. The other dynorphin and neoendorphin peptides showed distinctly smaller potency shifts than dynorphin A itself, but more than [Leu]enkephalin. It is interesting that the actual molar potencies for activating the intact δ receptors in treated vas were about the same for dynorphin B and dynorphin A-(1–8) as for dynorphin A; thus, the larger potency shift with dynorphin A is attributable to its higher initial potency at the κ receptors and its consequent greater pharmacological κ selectivity. In sharp contrast to the potency shifts of normorphine and dynorphin A, there was virtually no shift with [Leu]enkephalin or with the [Met]enkephalin penta-, hepta-, or octapeptides. However, there were small but definite potency shifts with the longer enkephalin peptides BAM-12P, BAM-22P, and Peptide E. These results indicate that all of the shorter enkephalin gene products interact preferentially with δ receptors in MVD, but the longer ones do not.

To determine the receptor type for which the longer enkephalin peptides are selective, we used the highly μ-selective [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAGO) (18) to obtain μ protection in addition to δ protection. If these peptides were μ-selective, their potency shifts should now be greatly reduced or abolished, but if they were κ-selective, there should be little or no change in their potency shifts. Table 1B shows that, as expected, the potency shift for normorphine was nearly abolished. In contrast, the potency shifts for the dynorphins and neoendorphins were but little changed. Among the eight enkephalin peptides, there was no instance in which the potency shift was reduced by protection of μ receptors (compare Table 1A and 1B), indicating that none of these agonists is μ-selective in MVD. These results suggest that unless they act through some other, unspecified, receptor type, the larger enkephalin peptides BAM-12P, BAM-22P, and Peptide E may be selective (but only weakly so) for κ over δ in MVD. This interpretation might be tested directly by repeating all of the experiments of Table 1 in κ-protected preparations, provided that suitable conditions could be found for overcoming the relative resistance of δ receptors to inactivation by β-CNA.

Through which receptor type(s) do [Met]enkephalin and β-endorphin act? In MVD, we preserved δ and κ while inactivating μ receptors with β-CNA. Now an agonist that acts through μ receptors should show a potency shift, whereas an agonist that acts through δ or κ should not. Table 2 shows the expected large potency shifts for the μ agonists morphine and levorphanol, both of which display greater μ selectivity than normorphine. Morphiceptin was also strongly μ-selective, although not very potent, as we found in binding studies (8, 9). The cyclic peptide Tyr-c[D-Lys-Gly-Phe] (19) was selective for μ receptors, but not strongly so. As expected, [Leu]enkephalin showed virtually no potency shift, nor did the κ agonists dynorphin A and dynorphin A-(1–8).

It has been proposed, although with little supporting evidence, that while [Leu]enkephalin preferentially activates δ receptors, [Met]enkephalin shows preference for μ receptors (20). The data of Table 2 show, however, that the potency of [Met]enkephalin was very little changed by inactivation of μ receptors, a decisive demonstration that—at least in MVD—this peptide does not have pharmacological μ-selectivity.

Although β-endorphin was the first of the larger opioid peptides to be characterized (21, 22), its receptor selectivity properties have not yet been fully clarified. Table 2 shows that β_c-endorphin did not display a significant potency shift; thus, it is certainly not selective for μ over δ or κ in MVD. There is evidence that β-endorphin has its own unique receptor, ϵ, which is present and mediates a pharmacological effect in rat vas deferens (23, 24) and which is presumably present in brain as well (25). Our result suggests that, if ϵ receptors mediate the effects of β-endorphin in MVD, they share with δ receptors a

TABLE 1

Potency shifts in mouse vas deferens after selective protection of δ receptors

EC_{50} values were obtained with MVD preparations. For A, tissue was treated with 10 nM β-CNA for 20 min, washed for 30 min, and EC_{50} values were determined again. Under these conditions, even in the absence of a protecting δ ligand, μ and κ receptors are inactivated preferentially by β-CNA. For B, DAGO was present at 3 μM during exposure to β-CNA in order to protect μ receptors. Data are geometric means (and 95% confidence limits) for eight independent experiments with each agonist, except in B, where $N = 32$ for normorphine, [Leu]enkephalin, and dynorphin A. EC_{50} is agonist concentration producing half-maximal inhibition of the electrically stimulated muscle twitch, computed as described under Materials and Methods. Potency Shift is the geometric mean of the set of ratios of EC_{50} values after β-CNA treatment to those before β-CNA treatment in the same tissue preparations; this mean value is not mathematically the same as the ratio of the group means of the EC_{50} values. For sources of compounds used, see Materials and Methods.

Agonist	A. Selective protections of δ receptors only		B. Selective protection of δ and μ receptors	
	EC_{50} before β-CNA	Potency shift after β-CNA	EC_{50} before β-CNA	Potency shift after β-CNA
	nM		*nM*	
Normorphine	520 (250–1100)	60.0 (31–120)	630 (480–820)	3.5 (2.6–4.8)
[Leu]enkephalin	20 (11–38)	1.1 (0.70–1.6)	19 (17–21)	1.7 (1.3–2.1)
[Met]enkephalin	10 (6.0–18)	1.8 (1.2–2.6)	17 (14–22)	1.8 (1.2–2.6)
[Met]enkephalin-Arg-Phe	39 (31–50)	1.3 (0.91–1.9)	21 (15–30)	3.8 (2.9–5.0)
[Met]enkephalin-Arg-Gly-Leu	230 (170–300)	1.3 (0.96–1.8)	63 (45–89)	4.3 (3.1–6.0)
BAM-12P	230 (160–330)	3.2 (2.4–4.2)	53 (43–66)	3.6 (2.5–5.2)
BAM-22P	79 (50–120)	4.9 (3.8–6.3)	480 (310–720)	3.3 (2.3–4.8)
Peptide E	73 (52–100)	4.7 (3.8–5.8)	180 (140–230)	3.5 (3.0–4.3)
Peptide F	270 (200–360)	2.4 (2.0–2.7)	360 (290–440)	1.9 (1.3–2.9)
Dynorphin A	6.3 (4.0–9.7)	34.0 (24–46)	12 (10–16)	19.0 (15.0–24.0)
Dynorphin A-(1–8)	57 (36–91)	3.8 (2.5–5.8)	120 (84–190)	2.2 (1.7–2.6)
Dynorphin B	39 (22–70)	5.8 (3.8–8.9)	66 (52–84)	3.6 (2.7–4.8)
α-Neoendorphin	12 (8.8–18)	5.8 (4.5–7.4)	15 (12–18)	1.9 (1.4–2.5)
β-Neoendorphin	16 (12–19)	3.4 (2.9–3.8)	17 (15–19)	3.4 (2.4–4.6)

relative resistance to inactivation by β-CNA under the conditions of our experiments.

The pharmacological selectivity of β-endorphin was further studied in GPI. Here μ and κ receptors are known to be present, each type with a large spare receptor fraction (6, 26). If δ receptors are also present, they must be relatively sparse, since enkephalins, which act potently upon δ receptors in MVD, clearly act through μ receptors in GPI (2). For β_c-endorphin in GPI, in eight preparations, we found that $IC_{50} = 52$ nM (95% confidence interval 24–114), about the same potency as normorphine. Naloxone K_e against β_c-endorphin was 12 ± 3 nM (mean ± SEM), whereas the comparable value against normorphine was 3.2 ± 0.2 nM. Naloxone K_e against dynorphin A or dynorphin A-(1–13) in GPI is 25–30 nM (27). Thus, the result with β_c-endorphin is ambiguous. It could represent a mixed activation of μ and another receptor type (such as κ) that is less sensitive to naloxone. Alternatively, it could represent activation of ε receptors, which (in rat vas deferens) have a naloxone K_e value similar to that found here (28).

We treated GPI with β-CNA in the presence and absence of various protecting concentrations of β_c-endorphin, then determined the potency shifts for normorphine, dynorphin A, and β_c-endorphin as agonists. Differential protection of the agonist potency of β_c-endorphin would have been evidence for occupancy of receptors other than μ or κ. Table 3 shows that at every concentration β-endorphin reduced the potency shifts for all three agonists to essentially the same extent. Evidently, β-endorphin can occupy μ and κ receptors in this tissue.

DISCUSSION

This investigation illustrates the use of the β-CNA technique to assess the pharmacological selectivity of agonists. We showed that several peptide products of the enkephalin gene displayed pharmacological selectivity for δ receptors in MVD; these included [Leu]enkephalin, [Met]enkephalin, and the COOH-terminally extended [Met]enkephalin-Arg-Phe and [Met]enkephalin-Arg-Gly-Leu, but not the longer extended peptides. BAM-12P, BAM-22P, and Peptide E contain Arg^6-Arg^7 in common with dynorphins A and B, and also arginine at position 10; and Peptide F contains Lys^6-Lys^7 but no basic residue at position 10 or 11. We have shown previously that arginine-7 (but not arginine-6) confers some degree of κ selectivity in the dynorphins and that this selectivity is greatly enhanced by a basic residue at position 10 or 11 (8, 29). Accordingly, it is of interest that (with the possible exception of Peptide F) these [Met]enkephalin-containing peptides, as judged by potency shifts (Table 1), were not δ-selective. That they displayed similar small potency shifts whether only δ receptors or both δ and μ receptors had been protected suggests that in MVD they preferentially activate κ receptors. Thus—at least for this tissue—we have to abandon the attractive idea that all of the main products of the enkephalin gene are δ-selective, as the main products of the dynorphin gene are κ-selective (27).

When we inactivated μ receptors while protecting both δ and κ in MVD, large potency shifts were observed for known μ agonists. Among these was morphiceptin (30), which contains the NH_2-terminal sequence of β-caso-

TABLE 2

Potency shifts after selective protection of δ and κ receptors (μ inactivation) in MVD

For each agonist, estimates of EC_{50} were obtained on vasa from the same animal, one treated with 3 nM β-CNA in the presence of 100 nM dynorphin A ("treated vas"), the other with 100 nM dynorphin A alone ("control vas"). This procedure takes advantage of the fact, noted earlier, that in MVD a low concentration of β-CNA has little or no effect on the potency of [Leu]enkephalin or [Met]enkephalin, whereas it produces a large potency shift for normorphine. Thus, since dynorphin A protected the κ receptors, μ receptors were selectively inactivated. Data are geometric means and 95% confidence limits, as in Table 1, for the number of independent experiments given under *N*. For meanings of EC_{50} and potency shift, see legend to Table 1. Here mean potency shifts and confidence intervals are computed from the individual potency shifts for pairs of vasa from the same animal. Some μ agonists did not inhibit muscle twitch of β-CNA-treated vas by as much as 50% even when used at highest practical concentrations, presumably because nearly all μ receptors were inactivated. In these cases, EC_{50} values are listed as greater than the highest concentration tested, and potency shift is stated as greater than the lower 95% confidence limit. For sources of compounds used, see Materials and Methods.

Agonist	*N*	EC_{50} in control vas	Potency shift in treated vas
		nM	
Normorphine	21	410 (270–610)	35.0 (26–48)
Morphine	4	650 (230–1800)	>57.0
Levorphanol	4	120 (59–220)	>310.0
Morphiceptin	4	1900 (1300–2800)	>36.0
Tyr-c[D-Lys-Gly-Phe]	8	32 (24–43)	6.4 (4.5–8.9)
[Leu]enkephalin	16	8.1 (6.2–10)	1.2 (1.0–1.4)
[Met]enkephalin	6	2.0 (1.2–3.2)	1.8 (1.4–2.3)
Dynorphin A	6	5.5 (2.7–11)	2.2 (1.2–3.9)
Dynorphin A-(1–8)	12	77 (48–110)	1.2 (0.79–1.8)
β_c-endorphin	12	59 (40–86)	1.7 (1.2–2.3)

TABLE 3

Potency shifts in GPI after protection with β_c-endorphin

EC_{50} values were obtained with GPI; see Materials and Methods for details. The concentration of β-CNA was 30 nM. Data are geometric means (and 95% confidence limits) of the potency shifts, based on four independent preparations for each concentration of β_c-endorphin as protector.

Concentration of β_c-endorphin as protector	Potency shift for test agonist		
	Normorphine	Dynorphin A	β_c-Endorphin
μM			
0	27.0 (12.0–59.0)	38.0 (16.0–90.0)	23.0 (11.0–48.0)
0.3	9.7 (6.1–16.0)	20.0 (10.0–40.0)	14.0 (7.0–26.0)
1.0	7.7 (3.6–17.0)	14.0 (5.9–34.0)	9.4 (4.7–19.0)
3.0	1.9 (0.79–4.4)	4.7 (1.3–16.0)	2.2 (1.3–3.7)

morphin found in milk (31). Morphiceptin is the only naturally derived peptide agonist we have found with significant pharmacological selectivity for μ receptors. A modest degree of μ selectivity has been reported recently for the proenkephalin-derived peptide metorphamide (32). Neither [Met]enkephalin nor β-endorphin is a selective μ agonist in MVD.

An interesting finding was that, after inactivation of κ receptors in MVD, dynorphin A was much less potent than the enkephalins, whereas in untreated MVD it was—as shown very early (33)—about 3 times more potent. This demonstrates very clearly that dynorphin A is not an indiscriminately nonselective agonist with high potency, as proposed by Iversen (34), but owes its high potency in the vas (as in GPI) to its selectively high affinity for κ receptors.

We investigated whether GPI contains functional ε receptors, in addition to its well-known complement of μ and κ, by using as protector the putative ε ligand β-endorphin. No differential protection of β-endorphin potency as compared with normorphine or dynorphin A potency could be achieved. However, it is very difficult to rule out the existence of a minor receptor type. Even high affinity of an agonist for a preferred minor receptor type could be outweighed by the potency-enhancing effect of a large μ or κ receptor reserve (see below). Thus, a minor type could be present but not have the opportunity to be activated by its selective agonist, if a full biological response is elicited through the superabundant μ or κ receptors. To unmask a minor receptor type would require inactivating virtually all of the μ and κ receptors, but none of the receptors of interest. The problem is similar to that of demonstrating the presence of cryptic δ receptors in GPI. There is evidence for the presence of δ binding sites (35); furthermore, a small number of functional δ receptors has been observed in morphine-tolerant GPI (36) and after destruction of μ receptors by β-FNA (37). With the availability of highly selective δ protectors and agonists, such as the cyclic penicillamine derivatives of enkephalin introduced by Mosberg *et al.* (9, 38), it may be possible to demonstrate functional δ receptors by the technique described here. However, a complement of cryptic receptors (ε or δ) could be present and functional at a small number of synapses, yet be undetectable by our method.

Pharmacological selectivity differs in principle from *binding selectivity*, which concerns relative affinities of ligands for receptor types (8, 9). Potency depends upon an agonist's affinity for a receptor, its intrinsic activity (39) [or "efficacy" (40)], and the receptor reserve (40, 41). In a single tissue, affinity and intrinsic activity may vary independently from agonist to agonist. Receptor reserve (and possibly affinity and intrinsic activity) may vary between tissues, even for the same agonist-receptor pair. The pharmacological selectivity of an opioid agonist can only be stated for a specified tissue, meaning that the agonist produces its effect in that tissue through preferential activation of a particular receptor type. In another tissue it might be selective for a different receptor type, as exemplified by the enkephalins, which are δ-selective in MVD but μ-selective in GPI. Moreover, an agonist need not even produce its biological effect through the same receptor type at which it binds with highest affinity; i.e., pharmacological selectivity need not correspond to binding selectivity. Thus, comparing potencies of several agonists in a single tissue or of a single agonist in several tissues cannot, in general, yield valid information about agonist selectivity. An exception arises when a receptor type is abundant in one tissue but

scarce or absent in another, as with δ receptors in MVD and GPI, respectively. An agonist with a high degree of δ selectivity will, indeed, have a high ratio of potency in MVD to potency in GPI (42), but MVD is not in any sense a special "δ tissue." Moreover, a low ratio of potency in MVD to potency in GPI does not necessarily indicate μ selectivity [as suggested by Kosterlitz *et al.* (42)] but is ambiguous, since both μ and κ receptors are abundant in both tissues (16, 26). The β-CNA technique described here has the merit of providing unambiguous information about the pharmacological selectivity of an opioid agonist in a given tissue.

Protection of a given receptor type can provide evidence that a certain agonist is or is not selective for that type. If there is no significant potency shift, while other agonists do show a shift, one may conclude that the agonist in question is selective for the protected receptor type. If there is a shift, while agonists known to be selective for the protected type do not display a shift, one may conclude that the agonist was selective for some unprotected receptor, and the magnitude of the shift suggests the degree of selectivity. The receptor type in question can only be identified conclusively by protecting it and abolishing the potency shift.

For testing unknown substances, inactivation rather than protection of the receptor type of interest offers an advantage. After protection, a substance that causes a non-opioid pharmacological effect mimicking that of opioids would continue to do so, thus falsely appearing to be a selective ligand of the protected receptor. Some additional test of opioid specificity, such as naloxone blockade, would then be required. After receptor inactivation, however, such a non-opioid compound will be distinguished from a selective agonist of the inactivated receptor, since it will not display a potency shift. This procedure could obviously be used to search for an endogenous ligand with high selectivity for the μ opioid receptor.

ACKNOWLEDGMENTS

We thank Patricia Lowery for expert technical assistance. Drs. P. S. Portoghese and A. E. Takemori furnished the β-CNA, without which this investigation could not have been carried out; we are deeply grateful for their generosity in making this important reagent available.

REFERENCES

1. Martin, W. R., C. G. Eades, J. A. Thompson, R. E. Huppler, and P. E. Gilbert. The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog. *J. Pharmacol. Exp. Ther.* **197**:517–532 (1976).
2. Lord, J. A. H., A. A. Waterfield, J. Hughes, and H. W. Kosterlitz. Endogenous opioid peptides: multiple agonists and receptors. *Nature (Lond.)* **267**:495–499 (1977).
3. Chang, K. J., and P. Cuatrecasas. Multiple opiate receptors: enkephalins and morphine bind to receptors of different specificity. *J. Biol. Chem.* **254**:2610–2618 (1979).
4. Portoghese, P. S., D. L. Larson, J. B. Jiang, T. P. Caruso, and A. E. Takemori. Synthesis and pharmacologic characterization of an alkylating analogue (chlornaltrexamine) of naltrexone with ultralong-lasting narcotic antagonist properties. *J. Med. Chem.* **22**:168–173 (1979).
5. Chavkin, C., and A. Goldstein. Demonstration of a specific dynorphin receptor in guinea pig ileum myenteric plexus. *Nature (Lond.)* **291**:591–593 (1981).
6. Cox, B. M., and C. Chavkin. Comparison of dynorphin-selective kappa receptors in mouse vas deferens and guinea pig ileum: spare receptor fraction as a determinant of potency. *Mol. Pharmacol.* **23**:36–43 (1983).
7. James, I. F., C. Chavkin, and A. Goldstein. Preparation of brain membranes containing a single type of opioid receptor highly selective for dynorphin. *Proc. Natl. Acad. Sci. U. S. A.* **79**:7570–7574 (1982).
8. Goldstein, A., and I. F. James. The dynorphin opioid peptides and the kappa opioid receptor, in *Mechanism of Drug Action* (T. P. Singer and R. N. Ondarza, eds.). Academic Press, New York, in press (1984).
9. James, I. F., and A. Goldstein. Site-directed alkylation of multiple opioid receptors. I. Binding selectivity. Mol. Pharmacol. **25**:337–342 (1984).
10. Robson, L. E., and H. W. Kosterlitz. Specific protection of the binding sites of D-Ala^2-D-Leu^5-enkephalin (δ-receptors) and dihydromorphine (μ-receptors). *Proc. R. Soc. Lond. Biol. Sci.* **205**:425–432 (1979).
11. Smith, J. R., and E. J. Simon. Selective protection of stereospecific enkephalin and opiate binding against inactivation by *N*-ethylmaleimide: evidence for two classes of opiate receptors. *Proc. Natl. Acad. Sci. U. S. A.* **77**:281–284 (1980).
12. Ward, S. J., P. S. Portoghese, and A. E. Takemori. Improved assays for the assessment of κ- and δ-properties of opioid ligands. *Eur. J. Pharmacol.* **85**:163–170 (1982).
13. Kosterlitz, H. W., and A. A. Waterfield. In vitro models in the study of structure-activity relationships of narcotic analgesics. *Annu. Rev. Pharmacol. Toxicol.* **15**:29–48 (1975).
14. Goldstein, A., and R. Schulz. Morphine-tolerant longitudinal muscle strip from guinea-pig ileum. *Br. J. Pharmacol.* **48**:655–666 (1973).
15. Hughes, J., H. W. Kosterlitz, and F. M. Leslie. Effect of morphine on adrenergic transmission in the mouse vas deferens: assessment of agonist and antagonist potencies of narcotic analgesics. *Br. J. Pharmacol.* **53**:371–381 (1975).
16. Kosterlitz, H. W., and A. J. Watt. Kinetic parameters of narcotic agonists and antagonists, with particular reference to *N*-allyl-noroxymorphine (naloxone). *Br. J. Pharmacol. Chemother.* **33**:266–276 (1968).
17. Noda, M., Y. Furutani, H. Takahashi, M. Toyosato, T. Hirose, S. Inayama, S. Nakanishi, and S. Numa. Cloning and sequence analysis of cDNA for bovine adrenal pre-proenkephalin. *Nature (Lond.)* **295**:202–206 (1982).
18. Gillan, M. G. C., and H. W. Kosterlitz. Spectrum of the μ-, δ- and κ-binding sites in homogenates of rat brain. *Br. J. Pharmacol.* **77**:461–469 (1982).
19. DiMaio, J., T. M. D. Nguyen, C. Lemieux, and P. W. Schiller. Synthesis and pharmacological characterization in vitro of cyclic enkephalin analogues: effect of conformational restraint on opiate receptor selectivity. *J. Med. Chem.* **25**:1432–1438 (1982).
20. Snyder, S. H. A multiplicity of opiate receptors and enkephalin neuronal systems. *J. Clin. Psychiatry* **43**:9–12 (1982).
21. Bradbury, A. F., D. G. Smyth, C. R. Snell, N. J. M. Birdsall, and E. C. Hulme. C fragment of lipotropin has a high affinity for brain opiate receptors. *Nature (Lond.)* **260**:793–795 (1976).
22. Cox, B. M., A. Goldstein, and C. H. Li. Opioid activity of a peptide, β-lipotropin-(61-91), derived from β-lipotropin. *Proc. Natl. Acad. Sci. U. S. A.* **73**:1821–1823 (1976).
23. Lemaire, S., J. Magnan, and D. Regoli. Rat vas deferens: a specific bioassay for endogenous opioid peptides. *Br. J. Pharmacol.* **64**:327–329 (1978).
24. Schulz, R., M. Wuster, and A. Herz. Pharmacological characterization of the ε-opiate receptor. *J. Pharmacol. Exp. Ther.* **216**:604–606 (1981).
25. Hammonds, R. G., N. Ling, and D. Puett. Interaction of tritiated β-endorphin with rat brain membranes. *Anal. Biochem.* **114**:75–84 (1981).
26. Chavkin, C., and A. Goldstein. Reduction in opiate receptor reserve in morphine tolerant guinea pig ilea. *Life Sci.* **31**:1687–1690 (1982).
27. James, I. F., W. Fischli, and A. Goldstein. Opioid receptor selectivity of dynorphin gene products. *J. Pharmacol. Exp. Ther.* **228**:88–93 (1984).
28. Gillan, M. G. C., H. W. Kosterlitz, and J. Magnan. Unexpected antagonism in the rat vas deferens by benzomorphans which are agonists in other pharmacological tests. *Br. J. Pharmacol.* **72**:13–15 (1981).
29. Chavkin, C., and A. Goldstein. Specific receptor for the opioid peptide dynorphin: structure-activity relationships. *Proc. Natl. Acad. Sci. U. S. A.* **78**:6543–6547 (1981).
30. Chang, K. J., A. Killian, E. Hazum, P. Cuatrecasas, and J. K. Chang. Morphiceptin: a potent and specific agonist for morphine (μ) receptors. *Science (Wash. D. C.)* **212**:75–77 (1981).
31. Henschen, A., F. Lottspeich, V. Brantl, and H. Teschemacher. Novel opioid peptides derived from casein (β-casomorphins). II. Structure of active components from bovine casein peptone. *Hoppe-Seyler's Z. Physiol. Chem.* **360**:1217–1224 (1979).
32. Weber, E., F. S. Esch, P. Bohlen, S. Paterson, A. D. Corbett, A. T. McKnight, H. W. Kosterlitz, J. D. Barchas, and C. J. Evans. Metorphamide: isolation, structure, and biologic activity of an amidated opioid octapeptide from bovine brain. *Proc. Natl. Acad. Sci. U. S. A.* **80**:7362–7366 (1983).
33. Goldstein, A., S. Tachibana, L. I. Lowney, M. Hunkapiller, and L. Hood. Dynorphin-(1–13), an extraordinarily potent opioid peptide. *Proc. Natl. Acad. Sci. U. S. A.* **76**:6666–6670 (1979).
34. Iversen, L. L. Yet another opioid peptide? *Nature (Lond.)* **299**:578–579 (1982).
35. Leslie, F. M., C. Chavkin, and B. M. Cox. Opioid binding properties of brain and peripheral tissues: evidence for heterogeneity in opioid ligand binding sites. *J. Pharmacol. Exp. Ther.* **214**:395–402 (1980).
36. Gintzler, A. R., and J. A. Scalisi. Physiological analysis of δ opioid receptors in the guinea pig myenteric plexus. *Life Sci.* **31**:2363–2366 (1982).
37. Gintzler, A. R., and D. Hyde. Unmasking myenteric delta receptors. *Life Sci. [Suppl. I]* **33**:323–325 (1983).
38. Mosberg, H. I., R. Hurst, V. J. Hruby, K. Gee, H. I. Yamamura, J. J. Galligan,

and T. F. Burks. Bis-penicillamine enkephalins possess highly improved specificity toward δ opioid receptors. *Proc. Natl. Acad. Sci. U. S. A.* **80**:5871–5874 (1983).
39. Ariens, E. J., J. M. Van Rossum, and P. C. Koopman. Receptor reserve and threshold phenomena. I. Theory and experiments with autonomic drugs tested on isolated organs. *Arch. Int. Pharmacodyn.* **127**:459–478 (1960).
40. Nickerson, M. Receptor occupancy and tissue response. *Nature (Lond.)* **178**:697–698 (1956).
41. Stephenson, R. P. A modification of receptor theory. *Br. J. Pharmacol.* **11**:379–393 (1956).
42. Kosterlitz, H. W., J. A. H. Lord, S. J. Paterson, and A. A. Waterfield. Effects of changes in the structure of enkephalins and of narcotic analgesic drugs on their interactions with μ- and δ-receptors. *Br. J. Pharmacol.* **68**:333–342 (1980).

Send reprint requests to: Dr. Avram Goldstein, Addiction Research Foundation, 701 Welch Road, Palo Alto, Calif. 94304.