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Acetalins: Opioid receptor antagonists determined through the use of synthetic peptide combinatorial libraries

C. T. DOOLEY*, N. N. CHUNG†, P. W. SCHILLER†, AND R. A. HOUGHTEN*‡

*Torrey Pines Institute for Molecular Studies, San Diego, CA 92121; and †Laboratory of Chemical Biology and Peptide Research, Clinical Research Institute of Montreal, Montreal, PQ, Canada H2W 1R7

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ABSTRACT **A synthetic peptide combinatorial library made up of 52,128,400 hexapeptides, each having an acetyl group at the N terminus and an amide group on the C terminus, was screened to find compounds able to displace tritiated [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin from μ opioid receptor binding sites in crude rat brain homogenates. Individual peptides with μ receptor affinity were found using an iterative process for successively determining the most active peptide mixtures. Upon completion of this iterative process, the three peptides with the highest affinity were Ac-RFMWMT-NH_2, Ac-RFMWMR-NH_2, and Ac-RFMWMK-NH_2. These peptides showed high affinity for μ and κ_3 opioid receptors, somewhat lower affinity for δ receptors, weak affinity for κ_1 receptors, and no affinity for κ_2 receptors. They were found to be potent μ receptor antagonists in the guinea pig ileum assay and relatively weak antagonists in the mouse vas deferens assay. These peptides represent a class of opioid receptor ligands that we have termed acetalins (*acetyl* plus enkeph*alin*).**

Development of opioid compounds with high specificity for each opioid receptor type (μ, δ, or κ) and subtype continues to be an important goal in opioid pharmacology. The three receptor types possess analgesic properties; however, the type of pain inhibited and the secondary functions of the receptors have been shown to differ among the three receptor types. The μ receptor has generally been regarded as the receptor type associated with pain relief and has been shown to be potent in regulating thermal pain (1). Nonanalgesic effects mediated by the μ receptor include respiratory depression (for review, see ref. 2), inhibition of intestinal motility (3), antidiuresis (4), suppression of the immune system (5), and (most importantly for therapeutic considerations) physical dependence (6). The δ receptor is also associated with thermal analgesia (1, 7), but with reduced effects on respiration (8) and addiction (9, 10). The κ receptor, in contrast, is most potent in the mediation of analgesia in response to pain induced by chemical stimuli (11, 12). It has also been shown to induce diuresis (13), food intake (14), and sedation (15, 16) and to regulate neuroendocrine synthesis and/or release (for review, see ref. 12). The κ receptor has a much reduced potential for dependence (6) but has been shown to be associated with dysphoric (17) and psychomimetic (18) effects. Such differences in receptor function encourage the search for drugs that produce analgesia without deleterious side effects.

Both receptor-specific opioid agonists and antagonists are useful pharmacological tools and have potential as therapeutic agents. Specific antagonists are required for the determination of effects mediated by specific receptor types and subtypes. In recent years, considerable progress has been made in the development of selective opioid receptor peptide ligands having agonist or antagonist properties. These were determined using various design strategies, including substitution of natural and nonproteinogenic amino acids, conformational restriction, and the bivalent ligand approach (for reviews, see refs. 19 and 20).

The recent development of peptide libraries allows for a more systematic approach for the determination of additional peptide ligands (21–29). Tens to hundreds of millions of peptide sequences can now be rapidly screened to determine peptide sequences that strongly interact with receptors, antibodies, etc. While the various library approaches each have their own specific merits, synthetic peptide combinatorial libraries (SPCLs) (21, 22, 30) differ from other peptide libraries in that the peptide mixtures are not support-bound and thus can be used directly in solution with any assay system. For receptor binding studies, the SPCL approach offers the advantage of not being limited to studies in which pure soluble receptors are available.

Earlier studies in this laboratory have shown that the SPCL approach can be used for the rapid determination of peptides that bind strongly to μ opioid receptors (31, 32). In these initial studies, an SPCL composed of peptides having a free amino group at the N terminus, when used in conjunction with an iterative selection process, enabled the determination of individual peptides that inhibited binding of tritiated [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAMGO) to μ receptors in crude rat brain homogenates. The most effective peptides found were related to the naturally occurring enkephalins and had activities in the range of 20–40 nM. In the present study, an N-terminal acetylated SPCL, used successfully in a variety of earlier studies for the identification of antigenic determinants (21, 22, 30, 33, 34) and for the development of antimicrobial peptides (21, 22, 35), has been employed in the determination of additional peptide ligands found to be potent inhibitors of DAMGO binding at the μ receptor. The peptide library used in this study is made up of 400 mixtures, each composed of 130,321 hexapeptides (19^4). In total, the library contains 52,128,400 hexapeptides (400 × 130,321). The library can be represented by the formula Ac-$O_1O_2X_3X_4X_5X_6$-NH_2, in which the first two positions (O_1 and O_2) are individually defined using the 20 natural L-amino acids (i.e., AA, AC, AD, . . . , YV, YW, YY). The remaining four positions (X_3, X_4, X_5, and X_6) consist of equimolar mixtures of 19 of the 20 natural L-amino acids (cysteine omitted). The 400 mixtures making up this SPCL were screened for their ability to inhibit the specific binding of ^{3}H-labeled DAMGO to μ receptors in crude rat brain homogenates. The most active mixtures found from the initial screening were further defined in an iterative selection process, which sequentially defined the four mixture positions

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Abbreviations: SPCL, synthetic peptide combinatorial library; DAMGO, [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin; MVD, mouse vas deferens; GPI, guinea pig ileum; NIDA, National Institute of Drug Abuse.

‡To whom reprint requests should be addressed at: Torrey Pines Institute for Molecular Studies, 3550 General Atomics Court, San Diego, CA 92121.

(i.e., $O_1O_2O_3XXX\text{-}NH_2$, $O_1O_2O_3O_4XX\text{-}NH_2$, $O_1O_2O_3O_4O_5X\text{-}NH_2$, and $O_1O_2O_3O_4O_5O_6\text{-}NH_2$) (36). The iterative process for one of these mixtures is described here. The μ, δ, κ_1, κ_2, and κ_3 receptor binding affinities of three of the individual peptides thus determined and their antagonist potencies in the guinea pig ileum (GPI) and mouse vas deferens (MVD) bioassays are presented.

MATERIALS AND METHODS

Preparation of SPCL. Assembly of the SPCL and preparation of mixtures for the iterative process have been described in detail (21, 22). XXXX-resin was prepared using a process of division, coupling, and recombination of individual resins. The XXXX-resin was then divided into 400 equal portions (each containing 250 mg). Two individual amino acids, O_1 and O_2, were added using simultaneous multiple peptide synthesis (37), an adaptation of Merrifield's solid-phase method (38). After acetylation, deprotection, and cleavage from the resins, each of the 400 peptide mixtures was extracted with water to yield a final peptide concentration of 1–3 mg/ml.

HPLC and Purification. Analytical HPLC was carried out using a Beckman–Altex model 421 HPLC system and dual model 110a pumps with a Vydac (Hesperia, CA) C_{18} column (25 cm × 4.6 mm) and Hitachi 100-20 spectrophotometer. Chromatograms were recorded and peak heights were integrated on a Shimadzu CR3A Chromatopac integrator. Individual peptides were purified using a Waters Milliprep 300 preparative HPLC modified with a Gilson model 232 preparative autosampler and Foxy fraction collector. Pure fractions (determined using analytical HPLC) were pooled and lyophilized.

Opioid Receptor Binding Assay. Preparation of rat brain membranes and the receptor binding assay were carried out as described (32). Each tube in the screening assay contained 0.5 ml of membrane suspension, 7 nM ^{3}H-labeled DAMGO [specific activity, 36 Ci/mmol (1 Ci = 37 GBq), obtained from National Institute of Drug Abuse (NIDA) repository, prepared by Multiple Peptide Systems (San Diego)], a peptide mixture (0.08 mg/ml), and 50 mM Tris·HCl (pH 7.4) in a total volume of 0.65 ml. Competition curves were generated using serial dilutions of the peptide mixtures. IC_{50} values were determined for active mixtures using the software GRAPHPAD (ISI, San Diego).

GPI and MVD Bioassays. The GPI (39) and MVD (40) bioassays were carried out as reported in detail elsewhere (41, 42). A logarithmic dose–response curve was obtained with [Leu^5]enkephalin for each ileum and vas preparation, and the IC_{50} value was determined. K_e values for antagonists were determined from the ratio of IC_{50} values obtained with [Leu^5]enkephalin in the presence and absence of a fixed antagonist concentration (43).

RESULTS

The 400 peptide mixtures in the SPCL ($Ac\text{-}O_1O_2XXXX\text{-}NH_2$) were assayed to determine their ability to inhibit the binding of ^{3}H-labeled DAMGO to crude rat brain homogenates (Fig. 1). Eighty percent of the mixtures inhibited <50% of DAMGO binding at the concentration screened (0.08 mg/ml). IC_{50} values for mixtures that showed the greatest inhibition in the initial screening were determined. The most effective inhibitors of tritiated DAMGO binding were found to be mixtures containing arginine at the first (N-terminal) position (Table 1). $Ac\text{-}RWXXXX\text{-}NH_2$, with an IC_{50} value of 2128 nM, and $Ac\text{-}RFXXXX\text{-}NH_2$, with an IC_{50} value of 2347 nM, were found to have the greatest ability to inhibit ^{3}H-labeled DAMGO binding (the difference between these two IC_{50} values was not significant). $Ac\text{-}FRXXXX\text{-}NH_2$ (IC_{50} = 3034 nM) and $Ac\text{-}WRXXXX\text{-}NH_2$ (IC_{50} = 6153 nM) were the most effective mixtures with amino acids other than arginine at the

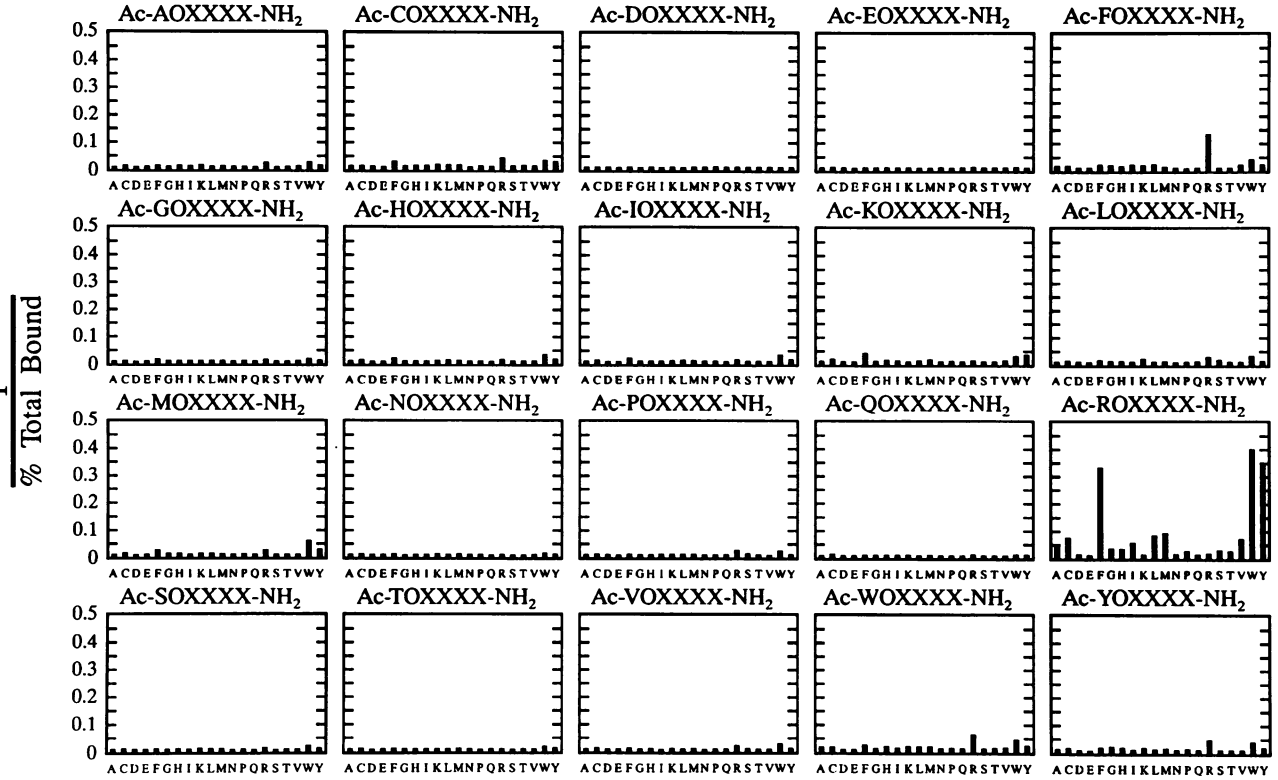

FIG. 1. Initial screening of the SPCL ($Ac\text{-}OOXXXX\text{-}NH_2$) for the ability to inhibit the binding of [^{3}H]DAMGO to crude rat brain homogenates. Individual bar graphs are segregated by the first amino acid (O_1), with individual bars in each graph representing the 20 amino acids making up the second position (O_2). The *y* axis represents the reciprocal of the percentage of [^{3}H]DAMGO bound.

Table 1. Inhibition of [^{3}H]DAMGO by peptide mixtures derived from the acetylated SPCL

Ac-ROXXXX-NH_2	IC_{50}, nM	Ac-RFOXXX-NH_2	IC_{50}, nM	Ac-RFMOXX-NH_2	IC_{50}, nM	Ac-RFMWOX-NH_2	IC_{50}, nM	Ac-RFMWMO-NH_2	IC_{50}, nM
Ac-RMXXXX-NH_2	2128 ±513	Ac-RFMXXX-NH_2	723 ±252	Ac-RFMWXX-NH_2	174 ±18	Ac-RFMWMX-NH_2	30 ±5	Ac-RFMWMK-NH_2	5 ±3
Ac-RFXXXX-NH_2	2347 ±571	Ac-RFLXXX-NH_2	1252 ±490	Ac-RFMSXX-NH_2	541 ±68	Ac-RFMWVX-NH_2	54 ±6	Ac-RFMWMT-NH_2	5 ±1
Ac-RTXXXX-NH_2	7906 ±2236	Ac-RFIXXX-NH_2	1432 ±398	Ac-RFMAXX-NH_2	595 ±95	Ac-RFMWQX-NH_2	83 ±23	Ac-RFMWMR-NH_2	6 ±1
Ac-RYXXXX-NH_2	8164 ±2673	Ac-RFXXXX-NH_2	2701 ±707	Ac-RFMGXX-NH_2	641 ±73	Ac-RFMWXX-NH_2	106 ±7	Ac-RFMWMS-NH_2	7 ±3
Ac-RVXXXX-NH_2	8380 ±1056	Ac-RFWXXX-NH_2	3009 ±278	Ac-RFMIXX-NH_2	730 ±87	Ac-RFMWIX-NH_2	108 ±7	Ac-RFMWMG-NH_2	10 ±1
Ac-RMXXXX-NH_2	11060 ±2189	Ac-RFFXXX-NH_2	3031 ±704	Ac-RFMVXX-NH_2	790 ±54	Ac-RFMWYX-NH_2	125 ±4	Ac-RFMWMN-NH_2	11 ±3
Ac-RAXXXX-NH_2	13836 ±969	Ac-RFGXXX-NH_2	4484 ±1255	Ac-RFMMXX-NH_2	870 ±128	Ac-RFMWLX-NH_2	180 ±23	Ac-RFMWMQ-NH_2	12 ±3
Ac-RGXXXX-NH_2	14555 ±3534	Ac-RFYXXX-NH_2	7204 ±1369	Ac-RFMRXX-NH_2	882 ±107	Ac-RFMWRX-NH_2	187 ±7	Ac-RFMWMA-NH_2	15 ±4
Ac-RCXXXX-NH_2	16823 ±2794	Ac-RFHXXX-NH_2	7296 ±955	Ac-RFMXXX-NH_2	907 ±100	Ac-RFMWAX-NH_2	204 ±5	Ac-RFMWMV-NH_2	17 ±7
Ac-RIXXXX-NH_2	19629 ±6022	Ac-RFNXXX-NH_2	7377 ±4658	Ac-RFMCXX-NH_2	915 ±12	Ac-RFMWIX-NH_2	210 ±15	Ac-RFMWMY-NH_2	17 ±4
Ac-RHXXXX-NH_2	24134 ±6388	Ac-RFRXXX-NH_2	7980 ±432	Ac-RFMHXX-NH_2	927 ±45	Ac-RFMWGX-NH_2	313 ±42	Ac-RFMWMM-NH_2	18 ±4
Ac-RLXXXX-NH_2	28578 ±7222	Ac-RFCXXX-NH_2	8792 ±1942	Ac-RFMTXX-NH_2	1048 ±52	Ac-RFMWTX-NH_2	321 ±26	Ac-RFMWML-NH_2	20 ±3
Ac-RPXXXX-NH_2	31391 ±6888	Ac-RFQXXX-NH_2	11519 ±1691	Ac-RFMNXX-NH_2	1169 ±110	Ac-RFMWSX-NH_2	327 ±53	Ac-RFMWMH-NH_2	20 ±5
Ac-RSXXXX-NH_2	38263 ±22941	Ac-RFTXXX-NH_2	11561 ±794	Ac-RFMIXX-NH_2	1310 ±155	Ac-RFMWWX-NH_2	327 ±21	Ac-RFMWMP-NH_2	24 ±7
Ac-RQXXXX-NH_2	109327 ±25720	Ac-RFAXXX-NH_2	12972 ±1624	Ac-RFMQXX-NH_2	1375 ±188	Ac-RFMWHX-NH_2	348 ±17	Ac-RFMWMX-NH_2	24 ±6
Ac-RRXXXX-NH_2	116347 ±29932	Ac-RFKXXX-NH_2	14003 ±147	Ac-RFMYXX-NH_2	1468 ±146	Ac-RFMWFX-NH_2	378 ±177	Ac-RFMWMW-NH_2	46 ±13
Ac-RNXXXX-NH_2	127342 ±36372	Ac-RFSXXX-NH_2	14145 ±1642	Ac-RFMDXX-NH_2	1908 ±13	Ac-RFMWNX-NH_2	454 ±4	Ac-RFMWMI-NH_2	67 ±27
Ac-REXXXX-NH_2	133230 ±33324	Ac-RFPXXX-NH_2	23895 ±6044	Ac-RFMFXX-NH_2	2004 ±136	Ac-RFMWPX-NH_2	616 ±66	Ac-RFMWMF-NH_2	68 ±20
Ac-RDXXXX-NH_2	146864 ±84802	Ac-RFVXXX-NH_2	30282 ±19881	Ac-RFMLXX-NH_2	2166 ±220	Ac-RFMWEX-NH_2	795 ±9	Ac-RFMWMC-NH_2	79 ±14
Ac-RKXXXX-NH_2	194501 ±61212	Ac-RFDXXX-NH_2	44588 ±7648	Ac-RFMPXX-NH_2	2626 ±204	Ac-RFMWDX-NH_2	952 ±143	Ac-RFMWME-NH_2	96 ±9
		Ac-RFEXXX-NH_2	77533 ±4311	Ac-RFMEXX-NH_2	3912 ±44	Ac-RFMWCX-NH_2	3185 ±369	Ac-RFMWMD-NH_2	109 ±16

IC_{50} values for peptide mixtures, Ac-ROXXXX-NH_2, from the SPCL and for peptides defining the third, fourth, fifth, and sixth positions in the iterative process as indicated. The IC_{50} value of the peptide mixture found most effective in the previous mixture is boxed for comparison.

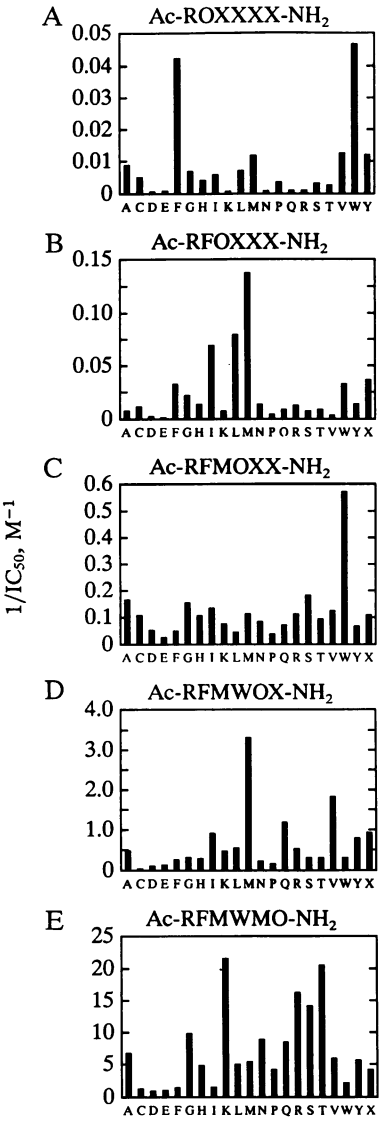

FIG. 2. Reciprocal of IC_{50} values obtained for peptide mixtures, Ac-ROXXXX-NH_2, from the SPCL (*A*), and for peptide mixtures defining the third (*B*), fourth (*C*), fifth (*D*), and sixth (*E*) positions in the iterative process. Individual bars are labeled on the *x* axis by the 20 amino acids (single-letter code) used to define the particular position (O). The most effective peptide mixture from the previous iterative step is labeled X.

first position. The iterative selection process carried out for the mixture Ac-RFXXXX-NH_2 is reported here. Iterations for other initially less active cases will be reported elsewhere.

Twenty peptide mixtures, each made up of 6859 hexapeptides (19^3), were synthesized to define the third position of Ac-RFXXXX-NH_2. These mixtures are represented by Ac-RFOXXX-NH_2 (i.e., Ac-RFAXXX-NH_2 through Ac-RFYXXX-NH_2). Three mixtures had binding affinities greater than that of Ac-RFXXXX-NH_2: Ac-RFMXXX-NH_2 (IC_{50} = 723 nM), Ac-RFLXXX-NH_2 (IC_{50} = 1252 nM), and Ac-RFIXXX-NH_2 (IC_{50} = 1432 nM). The remaining positions of Ac-RFMXXX-NH_2 (i.e., X_4, X_5, and X_6) were then defined in sequential order in a similar manner. Binding affinities (IC_{50}, mean ± SEM) of the peptide mixtures found for each step of the screening and selection process are shown in Table 1; improvements in binding inhibition made at each iterative step are illustrated in Fig. 2.

Of the 20 mixtures making up Ac-RFMOXX-NH_2 (each composed of 361 peptides), 8 exhibited binding affinities

Table 2. Affinity of acetalins for three opioid receptors

Peptide	Receptor	IC_{50}, nM	K_i, nM	STD ratio, K_iSTD/K_i	Hill slope
Ac-RFMWMT-NH_2	μ	1.7	0.8	1.38	1.0
Ac-RFMWMK-NH_2	μ	1.9	0.4	2.75	1.0
Ac-RFMWMR-NH_2	μ	1.1	0.5	2.20	1.2
Ac-RFMWMT-NH_2	δ	3.4	0.9	0.333	1.2
Ac-RFMWMK-NH_2	δ	24.7	5.6	0.054	0.8
Ac-RFMWMR-NH_2	δ	32.4	7.4	0.041	1.1
Ac-RFMWMT-NH_2	κ_1	2411	1108	0.006	0.9
Ac-RFMWMK-NH_2	κ_1	853	392	0.002	0.8
Ac-RFMWMR-NH_2	κ_1	494	227	0.003	0.7
Ac-RFMWMT-NH_2	κ_2	>10,000	<0.0001		
Ac-RFMWMK-NH_2	κ_2	>10,000	<0.0001		
Ac-RFMWMR-NH_2	κ_2	>10,000	<0.0001		
Ac-RFMWMT-NH_2	κ_3	1.0	0.6	0.5	1.0
Ac-RFMWMK-NH_2	κ_3	0.7	0.4	0.8	0.9
Ac-RFMWMR-NH_2	κ_3	2.6	1.4	0.21	1.1

Guinea pig brain suspension [1.8 ml, 6.7 mg (wet weight) tissue per ml in Tris·HCl (pH 7.7)] was incubated for 1 hr at 25°C with 100 μl of radioligand and 100 μl of peptide (10^{-5}–10^{-11} M). Membranes were labeled (≈1 nM) with [^{3}H]DAMGO, cyclic [^{3}H][D-Pen^2,D-Pen^5]enkephalin (DPDE) (where Pen is penicillamine), [^{3}H]U 69,593, [^{3}H]bremazocine in the presence of 100 nM DAMGO, [D-Ser^2,D-Leu^5]enkephalin-Thr (DSLET), and U 69,593, and [^{3}H]naloxone benzoylhydrazone in the presence of 100 nM U 69,593 for μ, δ, κ_1, κ_2, and κ_3 receptors, respectively. Nonspecific binding was determined using 1 μM DAMGO, Cl-DPDPE, or U 69,593 for μ, δ, and κ_1 receptors, respectively, or 10 μM bremazocine and naloxone benzoylhydrazone (κ_2 and κ_3 receptors). This data was provided by NIDA (Contract 271-89-8159). STD, standard.

greater than that of Ac-RFMXXX-NH_2. The peptide mixture with the highest affinity in this series (Ac-RFMWXX-NH_2; IC_{50} = 174 nM) was found to displace ^{3}H-labeled DAMGO three times more readily than the next most potent mixture, Ac-RFMSXX-NH_2 (IC_{50} = 541 nM) and five to six times more readily than Ac-RFMXXX-NH_2. Upon defining the fifth position, Ac-RFMWOX-NH_2 (19 peptides in each mixture), three mixtures exhibited inhibiting capabilities greater than that of Ac-RFMWXX-NH_2: Ac-RFMWMX-NH_2 (IC_{50} = 30 nM), Ac-RFMWVX-NH_2 (IC_{50} = 54 nM), and Ac-RFMWQX-NH_2 (IC_{50} = 83 nM). For the final iteration, in which all six positions were defined (Ac-RFMWMO-NH_2), 14 peptides had inhibiting capabilities greater than that of Ac-RFMWMX-NH_2. The four most potent peptides had IC_{50} values below 10 nM: Ac-RFMWMK-NH_2 (IC_{50} = 5 nM), Ac-RFMWMT-NH_2 (IC_{50} = 5 nM), Ac-RFMWMR-NH_2 (IC_{50} = 6 nM), and Ac-RFMWMS-NH_2 (IC_{50} = 7 nM). The IC_{50} value determined for unlabeled DAMGO was 7 nM.

The importance of the N-terminal acetyl and C-terminal amide groups was investigated. Affinities for three peptides synthesized without an acetyl moiety on the N terminus were RFMWMT-NH_2 (IC_{50} = 1799 ± 178 nM), RFMWMK-NH_2 (IC_{50} = 1041 ± 71 nM), and RFMWMR-NH_2 (IC_{50} = 630 ± 92 nM). Affinities of three peptides synthesized with a carboxyl group at the C terminus and an acetyl group at the N terminus were Ac-RFMWMT-COOH (IC_{50} = 352 ± 73 nM), Ac-RFMWMK-COOH (IC_{50} = 164 ± 25 nM), and Ac-RFMWMR-COOH (IC_{50} = 276 ± 51 nM).

The affinities of Ac-RFMWMR-NH_2, Ac-RFMWMK-NH_2, and Ac-RFMWMT-NH_2 for μ, δ, κ_1, κ_2, and κ_3 receptors were determined in specific binding assays (Table 2).

Table 3. Opioid antagonist potencies (K_e values) of peptides in the GPI and MVD assays

Peptide	K_e, nM	
	GPI	MVD
Ac-RFMWMT-NH_2	24.8 ± 6.5	>1000
Ac-RFMWMK-NH_2	2.53 ± 0.41	955 ± 201
Ac-RFMWMR-NH_2	2.92 ± 0.62	326 ± 31

Values were determined against [Leu^5]enkephalin as agonist.

These three peptides showed no affinity for κ_2 receptors at the highest concentration tested (10 μM) and weak affinity for κ_1 receptors with IC_{50} values of 494 nM, 853 nM, and 2410 nM, respectively. These three peptides, however, displayed high affinities for the κ_3 receptor, with respective IC_{50} values of 2.6 nM, 0.7 nM, and 1.0 nM. Affinities of the three peptides were also high for the μ receptor with IC_{50} values of 1.1 nM, 0.9 nM, and 1.7 nM, respectively. The peptides had somewhat lower affinities for the δ receptor with IC_{50} values of 32.4 nM, 24.7 nM, and 3.4 nM, respectively. Ac-RFMWMR-NH_2 was the only peptide that displayed even modest selectivity for μ receptors [$K_i(\mu)/K_i(\kappa_3)/K_i(\delta)$ ratio of 1:3:15]. Ac-RFMWMK-NH_2 had a $K_i(\mu)/K_i(\kappa_3)/K_i(\delta)$ ratio of 1:1:14, and Ac-RFMWMT-NH_2 had close to equal affinities for the μ, κ, and δ receptors [$K_i(\mu)/K_i(\kappa_3)/K_i(\delta)$ ratio of 1:1:1]. Differences in IC_{50} values found in this laboratory (Table 1) and those obtained at NIDA (Table 2) may be due to species differences, rat tissue vs. guinea pig tissue, or to the lower concentration of labeled DAMGO used in the NIDA study.

In the μ-receptor-representative GPI assay, Ac-RFMWMR-NH_2 and Ac-RFMWMT-NH_2 were found to be potent μ antagonists of [Leu^5]enkephalin, with K_e values of 2.53 nM and 2.92 nM, respectively. They were, however, >100 times less potent as δ antagonists against [Leu^5]enkephalin in the MVD assay (K_e = 955 nM and 326 nM, respectively) (Table 3). In comparison with the latter two peptides, Ac-RFMWMK-NH_2 had an ≈10 times lower μ antagonist potency in the GPI assay and showed no δ antagonist activity at concentrations up to 1 μM in the MVD assay. The weak δ antagonist potencies of these compounds are in agreement with the fact that they have relatively lower affinities for δ receptors than for μ receptors. Qualitative and quantitative differences observed between the receptor binding data and the bioassay results may reflect the existence of different μ and δ receptor subtype populations in rat brain and in the isolated tissue preparations.

DISCUSSION

In the present study, an SPCL composed of >52 million hexapeptides, when linked with an iterative selection and

enhancement process, enabled the discovery of a class of acetylated peptide sequences capable of displacing ^{3}H-labeled DAMGO from its receptor binding sites. The peptides found in the current study are not listed in the Registry produced by Chemical Abstracts Service obtained through The Scientific and Technical Information Network as known peptides or as part of known protein sequences. We have termed these peptides "acetalins" due to their opioid binding characteristics and the presence of the N-terminal acetyl group (acetalins = *acet*ylated enkeph*alins*).

The presence of the acetyl group on the N terminus is critical for the binding of these peptides, since analogues lacking the acetyl moiety exhibited very weak binding (IC_{50} in the micromolar range). An amide group on the C terminus is also preferred for binding, with a 10- to 30-fold decrease in affinity when the amide group was replaced by a carboxyl group (IC_{50} = 100–300 nM).

In each of the iterative steps, defining a position with an aspartic acid or glutamic acid residue resulted in very weak activity. Mixtures containing either of these residues were always among the least active of the 20 peptides in an iteration. This observation is in agreement with reports that the presence of an aspartic acid or glutamic acid residue in deltorphin infers high selectivity for the δ receptor, apparently by inhibiting binding to the μ receptor (44, 45).

The three peptides Ac-RFMWMT-NH_2, Ac-RFMWMK-NH_2, and Ac-RFMWMR-NH_2 are opioid receptor ligands with high affinity for μ and κ_3 receptors. They show negligible preference for μ over κ_3 receptors. It has been suggested, however, that the κ_3 receptor subtype can be considered an isoform of the μ receptor (12). The concentration of tritiated DAMGO used in the assay is expected to occupy the κ_3 receptors (12). This concentration potentially reduces the ability to discriminate between μ and κ_3 sites. The concentration of label used, however, was constrained by the limitations of the filtration system employed (22). These compounds, therefore, appear not to be highly selective for a single opioid receptor type or subtype. It is interesting to note that the acetalins have high affinity for μ and κ_3 receptors, even though their N-terminal group is acetylated. A positively charged N-terminal amino group is considered to be critical for binding to opioid receptors and is generally thought to be involved in an electrostatic interaction with a negatively charged receptor moiety. It is possible that the positively charged side chain of the Arg^1 residue in these peptides plays a role similar to the N-terminal α-amino functionality in classical opioid peptides in opioid receptor binding.

In a separate study, an N-acetylated C-terminal amidated SPCL composed entirely of D-amino acids was examined. We were able to identify potent inhibitors of DAMGO composed solely of D-amino acids. The peptides derived from this all D-amino acid library had sequences that contained similarities to, but differed from, those found in the present study. The most potent of the sequences identified was Ac-D-Arg-D-Phe-D-Trp-D-Ile-D-Asn-D-Lys (IC_{50} = 16 nM; unpublished data). The current report of the potent N-acetylated peptide sequences describes the use of peptide libraries to determine additional sequences that bind to a membrane receptor. The acetalins, though potent inhibitors of binding at the μ, δ, and κ_3 receptor sites, must be considered lead compounds.

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