Miscellaneous Procedures. Mass spectrometric analyses of the stypoldione reaction products were carried out on a VG ZAB-2F spectrometer at high resolution, using positive chemical ionization. ^{1}H NMR spectroscopy was carried out by using either a Nicolet NT-300 (300 MHz) or a 360-MHz Oxford Magnetics–Nicolet 1180E spectrometer. IR and UV spectra were obtained with Perkin-Elmer Model 137 and Beckman Acta IV spectrophotometers, respectively. DTNB, iodoacetate, amino acids, glutathione, and the proteins used for this study, except for tubulin, were obtained from Sigma Chemical Co.

Acknowledgment. This work was supported by USPHS Grant NS13560 from the Institute of Neurological, Communicative Disorders, and Stroke (L.W.), by USPHS Grant 16956 from the Division of Child Health and Human Development (D.J.A.), by NOAA National Sea Grant College Program, Department of Commerce Grant NA808A-D00102 through the California Sea Grant College Program (R.S.J.), and by NOAA, Department of Commerce, Office of Sea Grant, No. 04-7-158-44121 (W.F.). We thank Herbert Miller for preparing bovine brain microtubule protein.

Registry No. 1, 71103-05-4; [^{3}H]-1, 103533-00-2; 2, 103532-99-6; 4, 103532-97-4; 5, 103532-98-5; $HS(CH_2)_2OH$, 60-24-2; L-Cys, 52-90-4; glutathione, 70-18-8; hexokinase, 9001-51-8; deoxyribonuclease I, 9003-98-9; alkaline phosphatase, 9001-78-9.

Opioid Agonist and Antagonist Bivalent Ligands. The Relationship between Spacer Length and Selectivity at Multiple Opioid Receptors

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Bivalent ligands containing the oxymorphamine or naltrexamine pharmacophores connected to spacers of varying length were synthesized and evaluated for their selectivity at μ, κ, and δ opioid receptors. The oxymorphamine bivalent ligands (1–8) behaved as μ agonists on the electrically stimulated guinea pig ileum longitudinal muscle preparation (GPI). The spacer that conferred peak agonist activity in these series contains a total of four glycyl units (n = 2). Binding studies with guinea pig brain membranes showed a qualitatively similar profile at μ receptors as a function of spacer length. Also, δ receptor selectivity increased as the spacer was lengthened. The naltrexamine bivalent ligands (9–13) effectively antagonized the μ receptor agonist morphine in the GPI at the same optimal spacer length (n = 2) as in the agonist series. However, the peak antagonism of ethylketazocine, a κ receptor agonist, occurred with the bivalent ligand 9 containing the shortest spacer (n = 0), and it was found that 9 is the most selective κ antagonist in the series. While receptor binding roughly parallels that of κ antagonist activity in the GPI, no correlation between binding and antagonist activity was observed at μ opioid receptors. The possible significance of these results is discussed.

Compounds that contain two pharmacophores joined through a connecting unit (spacer) have been termed "bivalent ligands".[1-3] A classic example of bivalent ligands is the bis onium cholinergic blocking agents.[4] Bivalent ligands that contain opioid pharmacophores have attracted interest as molecular probes for opioid receptors.[1-3,5-11] In this connection, they have been employed as an approach to obtaining ligands with high selectivity for a single receptor type in the presence of multiple opioid receptors.[3]

The expectation for such high selectivity was based on three assumptions:[1,2] (1) simultaneous interaction (bridging) of both pharmacophores with vicinal receptors occurs with greatest frequency when the spacer chain of the bivalent ligand is an optimal length, (2) bridging under such conditions is preferred over a univalent binding mode, and (3) different opioid receptor types may be characterized by different vicinal interreceptor relationships in the clustered state.

According to this model, the relationship between spacer length and the activity of the bivalent ligand should be parabolic if very short or very long spacers decrease the frequency of binding to vicinal receptors. Moreover, the structure–activity profile for different receptor types should differ if they possess different interreceptor distances.

The present paper describes the results of studies conducted to investigate the interaction of bivalent ligands 1–13 at opioid receptors. Opioid pharmacophores with agonist and antagonist activities were employed in order to compare the structure–activity–relationship profiles. The results of these studies are consistent with the bridging principle.

Design Considerations and Chemistry

The pharmacophores that we selected for this study are

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§University of Minnesota, Department of Pharmacology.

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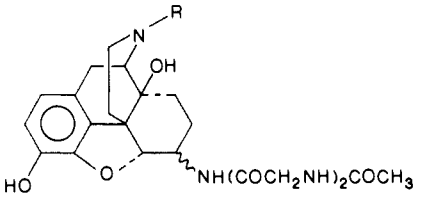
Table I. Physical Properties of Bis(glycyl)$_n$succinamide Spacers (**20**)

$CH_2CO(NHCH_2CO)_nOH$
$CH_2CO(NHCH_2CO)_nOH$

compd no.	n	mp, °C	R_f	purification method	% yield	formula[a]
20a	1	204	0.46[b]	*c*	46	$C_8H_{12}N_2O_6$
20b	2	251	0.43[d]	*e*	78	$C_{12}H_{18}N_4O_8$
20c	3	250	0.77[f]	*g*	77	$C_{16}H_{24}N_6O_{10}\cdot 0.5H_2O$
20d	4	274	0.52[d]	*h*	65	$C_{20}H_{30}N_8O_{12}$[i]

[a] C, H, N analyses were within 0.4% of theory. [b] EtOH–HOAc (3:1). [c] Crystallization from MeOH/H_2O. [d] EtOAc–EtOH–H_2O–HOAc (2:2:1:1). [e] Crystallization from H_2O. [f] EtOAc–MeOH–HOAc (8:2:1). [g] H_2O wash. [h] Hot EtOH digestion. [i] Analytical data were not obtained, but the compound was shown to be homogeneous by TLC and showed characteristic IR absorptions for acid and amide carbonyl stretching (1715, 1650, and 1550 cm^{-1}, respectively; KBr pellet) and NMR absorptions for succinyl and glycyl methylene protons (δ 2.40 and 3.76, respectively; in Me_2SO-d_6).

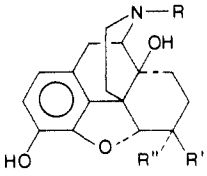
Table II. Monovalent Oxymorphamine and Naltrexamine Ligands



compd no.	R	C-6 config	R_f	mp, °C	purifn method	% yield	formula[a]
14	CH_3	α	0.31[b]	230 (base)	*c*	49	$C_{23}H_{30}N_4O_6\cdot HCl\cdot 0.5H_2O$
15	CH_3	β	0.35[d]	>250	*e*	30	$C_{23}H_{30}N_4O_6\cdot HCl\cdot 1.5H_2O$
16	$CH_2CH(CH_2)_2$	β	0.60[f]	251	*g*	52	$C_{26}H_{34}N_4O_6\cdot HCl\cdot 1.5H_2O$

[a] C, H, N analyses were within 0.4% of theory. [b] EtOH–MeOH–H_2O–NH_4OH (8:2:1:0.3). [c] Extraction into $CHCl_3$. [d] EtOAc–MeOH–NH_4OH (80:20:2). [e] Chromatography on silica gel. [f] EtOAc–EtOH–H_2O–NH_4OH (4:4:2:0.5). [g] Crystallization from EtOH.

derived from the opioid agonist oxymorphamine (**17**, **18**) and the antagonist naltrexamine (**19**). Both of these



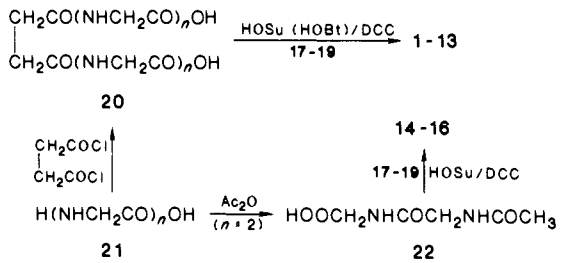
17: R = CH_3, R' = H, R'' = NH_2
18: R = CH_3, R' = NH_2, R'' = H
19: R = $CH_2CH(CH_2)_2$, R' = NH_2, R'' = H

pharmacophores have been employed in initial studies concerning the bivalent ligand concept. An important consideration in our synthetic approach was the ease of coupling the pharmacophores to the spacer and the minimization of incremental changes of partition coefficient upon lengthening the spacer. This was accomplished by employing glycyl units in the spacers. Symmetry was introduced into the spacers by means of a central succinyl group to which to glycyl units are linked (Table I). This symmetry facilitated the coupling of the amine function of the pharmacophores with the carboxyl groups of the spacer. The monovalent ligands **14**–**16** (Table II) were synthesized in order to factor out the contribution of the spacer itself to activity or to binding.

All of the bivalent compounds **1**–**13** were synthesized from the opioid pharmacophore bases **17**–**19** and the appropriate symmetrical spacers **20** by using standard peptide coupling procedures (Scheme I). The different spacers (**20**, n = 1–4) were prepared by reaction of 2 equiv of glycine homologue **21** with succinyl chloride under Schotten–Baumann conditions. Acidification of the reaction mixture precipitated the products in good yield (Table I). Similar conditions were used to prepare acetylglycylglycine from glycylglycine and acetic anhydride.

The coupling procedure employed both *N*-hydroxysuccinimide (HOSu) and *N*-hydroxyybenzotriazole (HOBt) to form intermediate active esters of the spacers, which

Scheme I



upon reaction with the opiate amines[12,13] **17**–**19** afforded the corresponding monovalent (Table II) or bivalent (Table III) ligands. In all cases where n = 2 or greater, satisfactory conversions were obtained. Where n = 0 or 1, a slower rate of conversion to the HOSu ester led to competitive side reactions that resulted in a much lower yield of the desired active ester. However, this was not a problem when HOBt was used. The initial procedures employed stepwise conversion to the intermediate HOSu ester and products, but the normally faster reaction using HOBt allowed the mixing of all reactants at the beginning. In order to maintain a homogeneous reaction solution, DMF was employed satisfactorily as the reaction medium. Only in the preparation of **4** was it necessary to employ Me_2SO.

Product purification methods ranged from washing the crude product with solvent to chromatography, depending on the nature and number of side products formed. In some cases digestion of the crude product in chloroform or acetone provided sufficient purity to allow isolation of the product as the base or its hydrochloride salt (after conversion in an appropriate solvent). More often, the crude product required chromatography on silica gel with use of relatively polar solvent systems, albeit with attendant lower yields.

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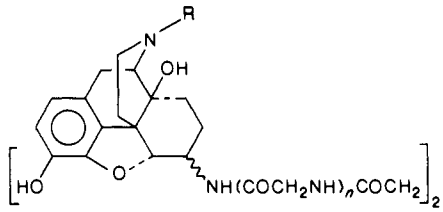
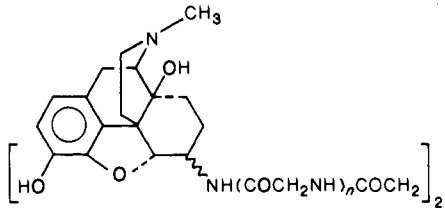
Table III. Physical Properties of Bisoxymorphamine and Bisnaltrexamine Oligoglycylsuccinamides



compd no.	R	C-6 config	*n*	mp, °C	R_f	% yield	purifn method	formula[a]
1	CH_3	α	0	110	0.43[b]	60	*c*	$C_{38}H_{46}N_4O_8 \cdot 4H_2O$
2	CH_3	α	1	160	0.55[b]	60	*c*	$C_{42}H_{52}N_6O_{10} \cdot 5H_2O$
3	CH_3	α	2	244 dec	0.07[d]	28	*e*	$C_{46}H_{58}N_8O_{12} \cdot 2HCl \cdot 5H_2O$
4	CH_3	α	3	165	0.54[f]	65	*c*	$C_{50}H_{64}N_{10}O_{14} \cdot 4H_2O$
5	CH_3	β	0	>250	0.26[g]	67	*h*	$C_{38}H_{46}N_4O_8 \cdot 2HCl \cdot 2H_2O$
6	CH_3	β	1	>250	0.21[i]	32	*h*	$C_{42}H_{52}N_6O_{10} \cdot 2HCl \cdot 2.75H_2O$
7	CH_3	β	2	>270	0.45[j]	21	*h*	$C_{46}H_{58}N_8O_{12} \cdot 2HCl \cdot 5H_2O$
8	CH_3	β	3	>250	0.18[k]	37	*h*	$C_{50}H_{64}N_{10}O_{14} \cdot 2HCl \cdot 4H_2O$
9	$CH_2CH(CH_2)_2$	β	0	>275	0.26[l]	83	*h*	$C_{44}H_{54}N_4O_8 \cdot 2HCl \cdot 3H_2O$
10	$CH_2CH(CH_2)_2$	β	1	>275	0.30[m]	30	*n*	$C_{48}H_{60}N_6O_{10} \cdot 2HCl \cdot 2H_2O$
11	$CH_2CH(CH_2)_2$	β	2	235 dec	0.69[b]	48	*n*	$C_{52}H_{66}N_8O_{12} \cdot 2HCl \cdot 3H_2O$
12	$CH_2CH(CH_2)_2$	β	3	>270	0.23[k]	36	*n*	$C_{56}H_{72}N_{10}O_{14} \cdot 2HCl \cdot 3H_2O$
13	$CH_2CH(CH_2)_2$	β	4	248 dec	0.43[o]	28	*n*	$C_{60}H_{78}N_{12}O_{16} \cdot 2HCl \cdot 4H_2O$

[a] C, H, N analyses were within 0.4% of theory. [b] EtOAc–MeOH–H_2O–NH_4OH (8:8:8:1). [c] $CHCl_3$ digestion. [d] EtOAc–MeOH–H_2O–NH_4OH (10:10:10:1). [e] Crystallization from *p*-PrOH. [f] EtOAc–MeOH–H_2O–NH_4OH (8:8:8:3). [g] Et_2O–MeOH–NH_4OH (80:20:1). [h] Chromatography on silica gel. [i] EtOAc–MeOH–NH_4OH (70:30:2). [j] EtOAc–MeOH–H_2O–NH_4OH (75:20:5:2). [k] EtOAc–MeOH–H_2O–NH_4OH (75:24:6:2). [l] Me_2CO–Et_2O–NH_4OH (80:20:1). [m] EtOAc–MeOH–NH_4OH (80:20:2). [n] Crystallization from Me_2CO–H_2O. [o] EtOAc–EtOH–H_2O–NH_4OH (4:4:2:0.5).

Table IV. Opioid Agonist Potencies in the GPI



compd no.	*n*	C-6 config	IC_{50}, nM[a]	potency ratio[b]	rel potency[c]
1	0	α	30.2 ± 8.7	4.6 ± 0.8	2.4
2	1	α	22.0 ± 5.0	4.0 ± 0.6	2.1
3	2	α	3.9 ± 0.6	36.0 ± 6.6	18.9
4	3	α	25.1 ± 5.2	5.5 ± 0.7	2.9
14 (monomer)		α	66.8 ± 11.7	1.9 ± 0.3	1.0
5	0	β	15.7 ± 3.4	4.3 ± 0.3	1.9
6	1	β	19.2 ± 4.1	5.8 ± 0.8	2.5
7	2	β	6.3 ± 0.7	8.0 ± 1.6	3.5
8	3	β	12.6 ± 0.3	3.9 ± 0.4	1.7
15 (monomer)		β	45.9 ± 6.1	2.3 ± 0.3	1.0

[a] The results represent the mean of at least three replicate experiments. [b] Ratio of morphine IC_{50} to compound IC_{50} on same tissue. [c] Bivalent potency ratio divided by monovalent potency ratio.

Pharmacology

GPI and MVD Preparations. Members of the agonist and antagonist series of bivalent ligands (**1–13**) and the corresponding monovalent ligands (**14–16**) were evaluated on the guinea pig ileum longitudinal muscle[14] (GPI) and mouse vas deferens[15] (MVD) preparations. All ligands were incubated for 30 min prior to testing, and their potencies were compared with those of the monovalent analogues having a spacer of intermediate length. These data (Tables IV, V) are expressed as relative potencies with the potencies of monovalent ligands (**14–16**) set equal to unity.

In order to rule out the possibility that the peptide spacers were cleaved at significant rates by enzymatic hydrolysis, two bivalent ligands (**11**, **13**) were incubated with the GPI for periods of 5 and 10 min before testing for antagonism to morphine. The fact that the morphine IC_{50} values in the presence of 10 nM **11** or **13** did not differ significantly from the results obtained after 30 min of incubation indicates that significant hydrolysis was not a factor within the time frame of the testing.

Members of the α-oxymorphamine agonist series (Table IV) all were more potent than morphine. None of the compounds possessed demonstrable antagonist activity. In the α series (**1–4**), the greatest potency was associated with bivalent ligand **3**, which was 18.9-fold greater than its monovalent analogue **14**.

The β-oxymorphamine series (Table IV) exhibited agonist potencies in the same range as members of the α series, and peak activity was observed with **7**. However, the potency enhancement was only 3.5 times greater than that of its monomer **15**.

In order to determine whether or not the agonist effect of **1–8**, **14**, and **15** were mediated principally through mu opioid receptors, we evaluated these ligands on GPI

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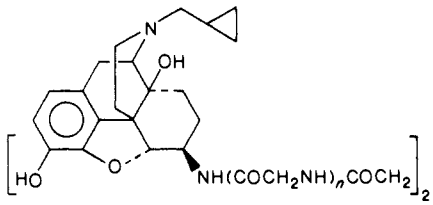
Table V. Opioid Antagonist Potencies in GPI



compound no.	n	morphine IC_{50} ratio[a]	rel potency[b]	EK IC_{50} ratio[a]	rel potency[b]	selectivity ratio[c]
9	0	27.6 ± 6.5	5.1	30.4 ± 9.6	25.3	1.1
10	1	57.4 ± 18.9	10.6	22.3 ± 4.7	18.6	0.4
11	2	181.0 ± 34.3	33.5	17.0 ± 2.4	10.7	0.2
12	3	61.4 ± 23.4	11.4	8.1 ± 2.4	6.8	0.1
13	4	40.7 ± 2.4	7.5	7.2 ± 1.2	6.0	0.2
16 (monomer)		5.4 ± 0.6	1.0	1.2 ± 0.1	1.0	0.2

[a] The IC_{50} of the morphine of ethylketazocine (EK) in the presence of the antagonist (10 nM) divided by the control IC_{50} (same preparation without antagonist). [b] IC_{50} in presence of bivalent ligand divided by IC_{50} in presence of monovalent ligand. [c] EK IC_{50} ratio divided by morphine IC_{50} ratio.

Table VI. Receptor Affinities of Opioid Agonists for μ, κ, and δ Opioid Receptors in Guinea Pig Brain Membranes

	receptor affinity: K_i,[a] nM						selectivity ratio
compound no.	μ	rel affinity	κ	rel affinity	δ	rel affinity	μ κ δ
6α series							
1	50 ± 10	3.6	500 ± 45	12.8	100 ± 45	4.6	1:0.10:0.48
2	44 ± 7	4.1	590 ± 118	10.8	110 ± 10	4.5	1:0.07:0.40
3	14 ± 3	12.9	460 ± 78	13.8	50 ± 2	9.6	1:0.03:0.28
4	34 ± 3	5.3	1000 ± 128	6.5	26 ± 4	18.4	1:0.03:1.3
14 (monomer)	180 ± 41	1	6400 ± 262	1	480 ± 32	1	1:0.03:0.38
6β series							
5	23 ± 3	2.7	180 ± 10	25.1	200 ± 52	3.5	1:0.13:0.11
6	18 ± 1	3.5	200 ± 43	22.0	86 ± 17	8.1	1:0.09:0.21
7	10 ± 0.3	6.3	94 ± 1	46.7	27 ± 4	25.9	1:0.11:0.37
8	22 ± 2.9	2.9	270 ± 38	16.1	42 ± 4	16.6	1:0.08:0.52
15 (monomer)	63 ± 7	1	4400 ± 531	1	700 ± 84	1	1:0.01:0.09

[a] Data are the mean ± SE of three to six experiments in duplicate. [b] Monomer K_i divided by bivalent K_i. Binding was performed in the presence of 100 mM NaCl.

preparations that were depleted of functional μ receptors by treatment with β-funaltrexamine (β-FNA).[16] Large shifts in the concentration–response curves were obtained in this preparation, with IC_{50} values that were increased by 10–30-fold or with greatly reduced maxima (25–50% at 1–10 μM) than those in the untreated preparation. This suggests that the agonist activity of the bivalent ligands **1–8** and the corresponding monomers (**14**, **15**) is highly μ selective.

In the MVD preparation, all of the α-oxymorphamine derivatives behaved as feeble agonists in that they were approximately $^1/_{100}$ the potency of [D-Ala^2,D-Leu^5]enkephalin (DADLE) (data not shown). Their weak agonist activity relative to the δ-selective ligand DADLE make these data of dubious significance. For this reason, members of the β-oxymorphamine series were not tested on this preparation.

Members of the β-naltrexamine series (**9–13**, **16**) were evaluated as antagonists of morphine (μ-selective agonist) and ethylketazocine (EK) (κ-selective agonist) on the GPI preparation (Table V). The antagonist potencies are expressed relative to that of the monovalent ligand **16** in this series. Bivalent ligand **11** (n = 2) showed a peak 33.5-fold enhancement of morphine antagonism relative to its monomer (**16**), whereas maximal antagonism of EK (25-fold) in this series was seen with the ligand having the shortest spacer (**9**, n = 0). The agonist potencies of these bivalent ligands were very weak or not apparent at 1 μM. The one compound (**10**) that behaved as an agonist at this concentration gave maximal effect of 35%.

The bivalent antagonists **9–13** were not very effective in antagonizing the agonist effect of DADLE on the MVD preparation. In this regard, an antagonist concentration of 1 μM afforded IC_{50} ratios of only 10–20, with relative potencies varying only by a factor of 2–3 (data not shown). These MVD data are in dramatic contrast with the results obtained on the GPI with this series. Thus, this combination of opiate pharmacophore and peptide spacer does not appear to be well-suited for the study of delta receptors.

Binding Studies. In order to complement the pharmacologic data, the affinities of compounds **1–16** were determined on guinea pig brain membranes prepared as described previously.[17] Binding at μ receptors was evaluated with [^{3}H]naloxone, δ receptors with [^{3}H]DADLE in the presence of sufentanil, and κ receptors with [^{3}H]EK in the presence of sufentanil and DADLE. The binding data are expressed as K_i values, and they also are tabulated as relative affinities, with the affinities of the monovalent ligands **14–16** set equal to unity (Tables VI and VII).

The structure–binding relationship of members of the α- and β-oxymorphamine series (Table VI) had profiles that correlated very well (r = 0.99 and 0.96, respectively) with their agonist rank-order activity at μ receptors in the GPI preparation (Table IV). In both series, the greatest affinity for μ receptors occurred with the bivalent ligands that contain spacers with a total of four glycyl units (n =

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Table VII. Receptor Affinities of Opioid Antagonists for μ, κ, and δ Opioid Receptors in Guinea Pig Brain Membranes

compound no.	receptor affinity: K_i,[a] nM						selectivity ratio
	μ	rel affinity	κ	rel affinity	δ	rel affinity	μ κ δ
9	1.7 ± 0.5	0.8	1.2 ± 0.2	15.0	12 ± 1	3.2	1:1.4:0.14
10	2.3 ± 0.5	0.6	2.3 ± 0.1	7.8	7.8 ± 0.7	4.9	1:1.0:0.29
11	1.5 ± 0.1	0.9	2.3 ± 0.3	7.8	4.9 ± 0.4	7.8	1:0.65:0.31
12	1.6 ± 0.03	0.9	2.5 ± 0.2	7.2	6.4 ± 0.6	5.9	1:0.64:0.25
16 (monomer)	1.4 ± 0.1	1	18 ± 2	1	38 ± 2	1	1:0.08:0.04

[a] See Table VI for further information. Binding was performed in the presence of 100 mM NaCl.

2). These ligands (1–8) bound to κ sites with considerably less affinity than to μ. However, bivalency enhanced up to 47-fold the binding at κ receptors for all of the ligands (1–8) relative to the monomers.

Affinity for δ receptors in the α series (1–4) appeared to differ from that found in the β series (5–8) in that the former exhibited a continuous increase as a function of spacer length, while the latter displayed a maximum affinity at an intermediate-size (n = 2) spacer (**6**). In both series, the relative selectivity for binding to δ, as opposed to μ or κ, receptors was best when the bivalent ligand contained six glycines (n = 3).

The β-naltrexamine ligands (**9–13, 16**) showed no significant change in μ receptor affinity (Table VII). This is in contrast to the substantial μ antagonist activity enhancement that was observed on the GPI in this series. On the other hand, it appears that binding and antagonism parallel each other at κ receptors (r = 0.84). The bivalent ligand **9** with the shortest spacer (n = 0) was most selective toward κ receptors. Binding at δ receptors was greatest for **11**, which contains four glycyl units (n = 2).

Discussion

The fact that the agonist potencies and affinities of the bivalent ligands (1–8) are greater than double those of the corresponding monovalent agonists (**14, 15**) suggests that simultaneous occupation of vicinal recognition sites occurs. This is consistent with an "optimal" spacer length in a series that allows bridging of the bivalent ligand between such sites.

It is likely that the frequency of bridging to vicinal sites is considerably less than optimal for a number of reasons. Thus, even when the spacer is of sufficient length to permit bridging, the binding of the second pharmacophore in the univalently bound ligand may not be as effective as that of a monovalent interaction with the vicinal recognition site. This is because the translational–rotational mobility of the tethered, unbound pharmacophore may be sufficiently impaired by the conformational restriction of the spacer. Under such circumstances, the adverse effects on "docking" and binding of the second pharmacophore to the vicinal recognition site would give rise to affinity enhancements that are considerably less than would be expected on theoretical grounds. For these reasons, the linear chain length of a spacer cannot be employed as a measuring device for estimating intersite distance. At best, it represents the maximum between vicinal recognition sites.

Whether the vicinal recognition sites are both functional opioid receptors or merely a single opioid receptor and an accessory binding site is not entirely clear. However, recent studies with bivalent ligands that contain antipodal opiates have indicated that the sites vicinal to μ receptors prefer the active (−)-opiate to the inactive (+)-opiate.[18] Consequently, it appears likely that, at least for μ receptors, both vicinal sites are of the opioid type when n = 2. Whether or not these sites are identical has not been established.

In regard to the α-oxymorphamine series (1–4), peak potency and binding to μ receptors occurred when four glycyl units (n = 2) were in the spacer. According to our model, this would suggest that the bridging frequency to vicinal sites should be optimal at this length. The decline in potency and binding at longer spacer length is presumed to be due to the much larger containment volume of the free pharmacophore attached to the univalently bound bivalent ligand. This results in a lower effective concentration of the tethered, unbound pharmacophore in the vicinity of the unoccupied recognition sites. However, at very long spacer lengths the second pharmacophore should behave more like a monovalent ligand, as bridging between distal recognition sites would become more frequent. Additionally, unfavorable entropic factors could contribute to a lowering of the affinity at long spacer length. The decline in agonist potency and binding of **4** is consistent with this interpretation.

Because of the low activity of the oxymorphamine congeners at κ receptors in the GPI and at δ receptors in the MVD, no comparison of κ or δ opioid agonist potencies with affinities could be made. However, the observation that all of the bivalent ligands 1–8 possessed affinities that were substantially greater than the corresponding monovalent analogues (**14, 15**) is consistent with bridging between vicinal recognition sites. Interestingly, binding selectivity for κ receptors appears to be greatest for the shortest spacer, an effect that was considerably more apparent in the β-naltrexamine series (vide infra). On the other hand, δ selectivity increased upon lengthening the spacer to n = 3 (**4, 8**). These changes in selectivity as a function of spacer length may reflect different organizations of vicinal recognition sites among different opioid receptor types.

Of particular interest is the contrast between the structure–activity and structure–affinity profiles for ligands in the β-naltrexamine series. The difference between μ antagonist activity and affinity is striking in that the 34-fold potency increase for **11** was not paralleled by an increased in binding. In fact, no significant change in affinity was observed for any of the bivalent antagonists. Similar results on μ binding were obtained with use of [^{3}H]dihydromorphine and [^{3}H]naloxone in the absence of NaCl. This absence of correlation is unexpected, since antagonist interactions with receptors generally are regarded to be less complex than those of agonists because of the intrinsic activity or efficacy component in the latter. The significance of these results is unclear, particularly in view of the recent report that vicinal recognition sites stereoselectively interact with both opioid pharmacophores of **11**.[18] This profound difference between activity in the GPI and μ affinity in guinea pig brain may mean that the μ receptor systems in these tissues differ. Another possibility is that the brain membranes contain antagonist binding sites that are disrupted during the homogenization.

In contrast to the total absence of correlation between antagonist potency and affinity at μ receptors, the κ and

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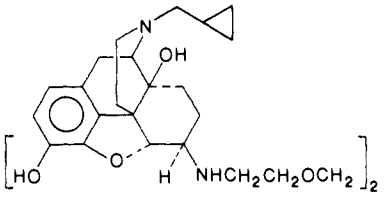
δ antagonist activity profiles in this series (**9–13**) paralleled affinity at these receptors. The substantial difference between the μ and κ structure–activity profiles in the GPI is of significance because it demonstrates that a short spacer ($n = 0$) confers high κ selectivity. This is not unique to this series, as it has been reported[1,3] that the bivalent ligand TENA (**23**), a selective κ antagonist, contains a more



23

flexible spacer, which could facilitate bridging. These results suggest that the vicinal sites that recognize κ-selective bivalent ligands are organized differently from one another than those that recognize μ- or δ-selective ligands. However, it is uncertain whether such recognition sites represent vicinal κ receptors or a κ receptor and an accessory subsite.

Conclusions

The results of this study demonstrate the viability of the bivalent ligand approach to conferring selectivity to opioid ligands. The fact that the greatest selectivity change from μ to κ opioid receptors is conferred when the spacer is shortened ($n = 0$) suggests that these receptor systems are organized differently from one another. The high κ selectivity of the bivalent antagonist TENA (**23**) is in harmony with this view, particularly since **9** and TENA both have the shortest spacers in their respective series.

As there is good correlation between μ agonist activity and binding as a function of spacer length (**1–8**) and an absence of such correlation in the μ antagonist series (**9–13**), it seems likely that the μ sites that mediate opioid agonism are different from those that are involved in antagonism. The absence of such correlation may signify a fundamental difference between μ agonist and antagonist recognition sites. This is consistent with recent studies in the GPI that indicate the possibility of discrete recognition sites for μ agonists and μ antagonists.[19]

Experimental Section

Melting points were determined in open capillary tubes by using a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses were performed by M-H-W, Phoenix, AZ, and are within 0.4% of the theoretical values unless otherwise specified. TLC data were obtained with use of using Analtech silica gel plates. IR spectra were obtained with a KBr pellet on a Perkin-Elmer 281 infrared Spectrometer. NMR spectra were recorded at ambient temperature on a JEOL 90-MHz or Nicolet 300-MHz spectrometer Me_2SO-d_6 as solvent and Me_4Si as internal standard. All reagents and solvents were reagent grade and were used without purification. Abbreviations employed are DCC, dicyclohexylcarbodiimide; HOSu, *N*-hydroxysuccinimide; HOBt, *N*-hydroxybenzotriazole; DCU, dicyclohexylurea; Me_2SO, dimethyl sulfoxide; DMF, dimethylformamide; EK, ethylketazocine; and DADLE, [D-Ala^2,D-Leu^5]enkephalin.

Bis(oligoglycyl)succinamides of α- and β-Oxymorphamine and β-Naltrexamine (1–13). The opiate base (**17**, **18**, or **19**) (1.1 mmol) was mixed with a solution of HOBt (or HOSu) (1.1 mmol) and the bis(glycyl)$_n$succinamide (**20**, $n = 0$–4) (0.5 mmol) in DMF or Me_2SO (10 mL). DCC (227 mg, 1.1 mmol) in 1 mL of DMF was added, and the reaction mixture was stirred at 23 °C for 1 day. Completion of reaction was verified by TLC, and the DCU that had precipitated was removed by filtration. Alternatively, the bis(glycyl)$_n$succinamide was allowed to react with HOSu (1.1 mmol) and DCC (1.1 mmol) in DMF (10 mL) for 48 h at 23 °C, and then, following removal of precipitated DCU, the opiate base (**17**, **18**, or **19**) was added and stirring continued for an additional day. Following removal of the solvent from the reaction products either by dilution with Et_2O or by evaporation in vacuo, the product mixture was purified by digestion in $CHCl_3$ or CH_3CN/EtOAc or by dry column chromatography on silica gel with use of EtOAc–MeOH–H_2O–NH_4OH of various composition as the development and elution solvent. The purified base was isolated as a powder or converted to the hydrochloride salt, which usually precipitated from acetone, MeOH–Et_2O, or acetone–H_2O. All compounds exhibited characteristic IR amide carbonyl stretching (1645–1660 and 1530–1550 cm^{-1}). Characteristic NMR absorptions for the succinyl and glycyl methylene, opiate C-5, and secondary amide protons were observed at δ 2.32–2.58, δ, 3.68–3.73, 4.38–4.78, and 7.98–8.30, respectively.

Bis(oligoglycyl)succinamides (20, n = 1–4). To a solution of the oligoglycine (**21**, n = 1–4) (33 mmol) in 22 mL of 1.5 N NaOH at 0 °C was added a solution of succinyl chloride (15 mmol) in acetone (2 mL) over a 60-min period. During the addition, the pH was maintained between 10 and 11 by frequent addition of 1.5 N NaOH. The mixture was stirred for an additional 30 min and then acidified to pH 1 with 1 N HCl. The precipitated product was isolated on a filter and washed with water. Recrystallization of the product was made from H_2O or MeOH–H_2O. The compounds exhibited characteristic IR carbonyl and NMR proton absorptions for the succinyl and glycyl moieties.

***N*-(Acetylglycyl)glycinamides of 6α- and 6β-Oxymorphamine and 6β-Naltrexamine (14–16).** To a solution of *N*-(acetylglycyl)glycine[20] (261 mg, 1.5 mmol) and HOSu (190 mg, 1.6 mmol) in DMF (20 mL) was added DCC (330 mg, 1.6 mmol), and the mixture was stirred at 23 °C for 24 h. Precipitated DCU was removed by filtration. To the filtrate was added the amine base (**17**, **18**, or **19**) (1.5 mmol), and this mixture was stirred at room temperature for another day. After concentration of the reaction solution to $^1/_4$ volume, it was diluted with Et_2O to precipitate the crude product base. This was purified by fractional crystallization of the hydrochloride salt or by chromatography on silica gel with use of EtOAc–MeOH–NH_4OH (80:20:1) and crystallization of the hydrochloride salt. The compounds gave IR amide carbonyl absorptions and NMR proton absorptions consistent with their structures.

Binding Studies. Brains were dissected from Hartley guinea pigs (250–300 g) that were decapitated. Brain membranes were prepared by the method described previously.[17] One-milliliter aliquots of the membrane preparation containing about 1 mg of protein were incubation with unlabeled drugs, radiolabeled ligand, and Tris·HCl containing NaCl in a final volume of 2 mL with a final concentration of 100 mM NaCl and 50 μg of bacitracin per mL in 50 mM Tris·HCl, pH 7.4. Samples were incubated at room temperature for 45 min and quickly filtered through Whatman GF/C glass-fiber filters under negative pressure and washed three times each with 5 mL ice-cold Tris buffer. Nonspecific binding of [^{3}H]naloxone, [^{3}H]DADLE, and (−)-[^{3}H]EK was determined in the presence of 10 μM naloxone, DADLE, and EK, respectively. Bound radioactivity was determined by liquid scintillation counting in 5 mL of Liquiscent (National Diagnostics, Somerville, NJ). Protein was determined according to the method of Lowry et al.[21] [^{3}H]Naloxone (48.6 Ci/mmol), [^{3}H]DADLE (39.5 Ci/mmol), and [^{3}H]EK (24.8 Ci/mmol) were obtained from NEN Research Products (Boston, MA). The following final concentrations of labeled ligands were used: 0.5 nM [^{3}H]naloxone (μ binding); 0.4 nM [^{3}H]DADLE in the presence of 4 nM sufentanil (δ binding); 1 nM (−)-[^{3}H]EK in the presence of 500 nM DADLE and 20 nM sufentanil (κ binding). Under the experimental conditions, the apparent K_d values for [^{3}H]naloxone, [^{3}H]DADLE, and (−)-[^{3}H]EK were 0.98, 0.64, and 0.62 nM, respectively.[22] IC_{50} values were calculated from log–logit plots. Apparent K_i values

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were calculated from the equation $K_i = IC_{50}/[1 + (L/K_d)]$, where L is the concentration of radioligand and K_d is its dissociation constant.[23] Values represent the mean ± SE of 3–6 experiments in duplicate.

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Registry No. 1, 103616-11-1; 2, 103616-12-2; 3, 97168-80-4; 4, 103616-13-3; 5, 103617-34-1; 6, 103616-14-4; 7, 103616-15-5; 8, 103616-16-6; 9, 103499-93-0; 10, 103499-94-1; 11, 97134-60-6; 12, 103499-95-2; 13, 103499-96-3; 14, 97073-83-1; 15, 103499-97-4; 16, 97073-85-3; 17, 98634-03-8; 18, 72782-04-8; 19, 67025-97-2; 20 (n = 0), 110-15-6; 20a, 102817-61-8; 20b, 97073-86-4; 20c, 103499-98-5; 20d, 103499-99-6; 21a, 56-40-6; 21b, 556-50-3; 21c, 556-33-2; 21d, 637-84-3; 22, 5687-48-9; $ClCO(CH_2)_2COCl$, 543-20-4.

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Synthesis and Biological Activity of Analogues of β-Chlornaltrexamine and β-Funaltrexamine at Opioid Receptors

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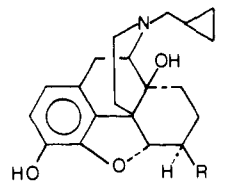
β-Chlornaltrexamine and β-funaltrexamine analogues 4–7 with different length "arms" to which an electrophilic moiety is attached were synthesized in an effort to obtain affinity labels that would selectively and irreversibly block specific opioid receptor types and subtypes. One of the compounds, **4**, was a potent, irreversible blocker of opioid receptors in the guinea pig ileum and mouse vas deferens preparations. The results of this study suggest that nucleophiles that are remote from the recognition locus are capable of alkylation by reactive electrophiles.

The nonequilibrium opioid receptor antagonists, β-chlornaltrexamine[1] (β-CNA) **1** and β-funaltrexamine[2] (β-FNA) **2**, have found wide use as tools in opioid research.[3] β-CNA is capable of alkylating at least three types of opioid receptors, and β-FNA is known to be highly selective for μ receptors. These ligands are effective both in vitro and in vivo.

Extensive structure–activity relationship studies of ligands related to **1** and **2** have revealed that the selectivity of covalent binding of opioid receptors is dependent on two consecutive recognition steps.[4] Thus, the ligand must have sufficient receptor affinity (primary recognition), and its electrophile must also be capable of reacting with a proximal receptor-based nucleophile in a selective fashion (secondary recognition).

This concept led to the development of ligands (e.g., **3**)



1, $N(CH_2CH_2Cl)_2$

2, $NHCO-C(H)=C(H)-CO_2CH_3$

3, $NHCO-CH_2N$ (succinimide)

that could irreversibly and selectively block one μ receptor subtype in the presence of other μ subtypes.[5] Our working hypothesis in this approach was that very closely related recognition sites (e.g., μ opioid receptor subtypes) possess a high degree of homology at their recognition locus, but considerably less homology as the distance from this locus is increased. The present study describes our effort to explore this approach further.

Design Rationale and Chemistry. In order to orient the electrophilic moiety in the ligand with receptor-based nucleophiles that may be distal to the recognition locus of the opiate pharmacophore, an oligoglycine "arm" was employed. Thus, the nitrogen mustard compound **4** is a β-CNA (**1**) analogue with a glycyl spacer, and the fumaramate esters (**5–7**) similarly can be viewed as being related to β-FNA.

Compound **4** was prepared by condensing the bromoacetamide **9**[5] with diethanolamine to yield **10**, which was treated with trioctylphosphine and CCl_4 to effect the replacement of OH by Cl.

Synthesis of the fumaramate esters **5–7** involved the mixed anhydride-mediated coupling of *N*-carbobenzyloxyglycine or its oligomer to β-naltrexamine[6] (**8**) to afford intermediates **11a–c**. Catalytic hydrogenolysis to oligomers **12a–c**, followed by coupling with fumaric acid monomethyl ester, afforded the desired compounds **5–7**.

Pharmacology. Compounds **4–7** were tested on the electrically stimulated longitudinal muscle of the guinea pig ileum[7] (GPI) and on the mouse vas deferens[8] (MVD) (Tables I and II, respectively).

In the GPI, compound **4** was a full, reversible agonist and possessed 8-fold greater agonist activity than morphine. All of the other members (**5–7**) in the series were partial agonists and exhibited a maximal response of about 45%. They appear to have varying degrees of reversible antagonist activity at a concentration of 20 nM. The ir-

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