Biochemical Pharmacology, Vol. 34, No. 1, pp. 25–30, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

RECEPTOR BINDING AND BIOLOGICAL ACTIVITY OF BIVALENT ENKEPHALINS

T. COSTA*, M. WÜSTER and A. HERZ

Department of Neuropharmacology, Max-Planck-Institut für Psychiatrie, Am Klopferspitz 18a, D-8033 Planegg-Martinsried, Federal Republic of Germany

and

Y. SHIMOHIGASHI, H.-C. CHEN and D. RODBARD

ERRB, and LTPB NICHD, National Institutes of Health, Bethesda, MD 20205, U.S.A.

(*Received* 9 *February* 1984; *accepted* 26 *June* 1984)

Abstract—Two series of dimeric enkephalin analogues were assayed for opioid activity in two isolated smooth muscle preparations: the guinea pig ileum (GPI) and the mouse vas deferens (MVD). Dimers have the general structure: X-$(CH_2)_n$-X, where X is H-Tyr-D-Ala-Gly-Phe-Leu-NH-(n = 0, 2, 4, 6, 8, 10, 12), for the first series of dimeric pentapeptide enkephalins (DPE_n), and H-Tyr-D-Ala-Gly-Phe-NH-(n = 2, 4, 6, 8, 12), for the series of dimeric tetrapeptide enkephalins (DTE_n). Comparison of biological activities with binding affinities revealed that: (1) the DPE series with n = 2–8 showed increased potency in the MVD assay relative to monomeric [D-Ala^2, Leu^5]enkephalinamide (DALEA); (2) there was an associated increase affinity for the delta receptor of rat brain or neuroblastoma-glioma hybrid cells. (however, the relative potencies were higher in the MVD assay than predicted on the basis of binding affinities); (3) the DTE series also showed an increase in delta receptor affinities and MVD potencies relative to DALEA, for n = 2–12; (4) for the DTE series, the increase in MVD activities was less than that expected on the basis of delta binding affinity; (5) for both the DPE and DTE series, activities in the GPI assay and mu-receptor affinities were highly correlated: as the length of the methylene bridge increased from 2 to 12, there was a progressive loss of activity in both assays, with a similar pattern for DPE and DTE.

Two selected dimers and their corresponding monomers were also assayed for antinociceptive activity *in vivo*: results were consistent with GPI and μ-binding but not with MVD and δ-binding. Two alkylamide analogs of penta- and tetrapeptide monomers, representing the monomer with the attached spacer of the most active dimers, were also assayed in biological and binding assays. Comparison of these compounds with the corresponding dimers suggest that the changes in activities and selectivities induced by dimerization are not a spurious effect of the presence of an akylamide derivative of the carboxy terminal of enkephalin but rather may represent a specific effect due to the bivalent nature of the ligands.

Considerable synthetic work has recently addressed the preparation of bivalent ligands for the opiate receptor. In attempts to develop new analogs of increased affinity, selectivity and biological potency, and in attempts to demonstrate that opioid receptors can be clustered and crosslinked, opioid-peptides [1–6], opiate alkaloids [2, 7–8] and benzomorphans [9] have been dimerized by a variety of strategies. In several cases the resulting compounds have shown significant increases in potencies compared to their monomeric counterparts.

In at least two cases [4, 8] the increases in potencies were selective for specific classes of opioid receptors and dependent on the length of the chain (spacers) connecting the two ligands. Thus it is possible that "bridging" of pairs of receptors by bivalent opioids may occur for an optimal spacer length and this "optimum" might be a characteristic of the membrane organization of different classes of opioid receptors.

How much of the increase in receptor affinity produced by bridging is translated into biological activity, however, is an open question. In principle, it should depend on the molecular mechanism underlying the coupling between receptor occupation and generation of the effect at the cellular level.

In previous studies we have described the receptor binding activities of two series of enkephalin dimers; both series consist of two molecules of an enkephalin analog linked by spacers of methylene units increasing in even steps from 0 to 12.

In the first series (DPE_n) the analogue is the pentapeptide Tyr-D-Ala-Gly-Phe-Leu-$CONH_2$, in the second series (DTE_n), it is the tetrapeptide Tyr-D-Ala-Gly-Phe-$CONH_2$. We have assayed the biological activities *in vitro* of the two series of dimers in two isolated preparations: the longitudinal muscle-myenteric plexus of the guinea-pig ileum and the mouse vas deferens. The first contains only μ- and κ-receptors, the second contains δ-, μ- and κ-receptors [10]. Opioid receptor heterogeneity, even in single tissues, complicates the interpretation of data in terms of receptor selectivity when comparing the relative potencies of drugs on guinea-pig ileum and mouse vas deferens. The principal difference between both tissues, however, is the presence of δ-receptors in the mouse vas deferens. Thus, in the case of opioids selective for the δ-type of receptor, a comparison of potencies on guinea-pig ileum and

* To whom correspondence should be addressed.

on mouse vas deferens is indicative of the degree of preference for δ-receptors.

The dimers in the two series with the most interesting profile of *in vitro* activity were also tested in an antinociceptive assay employing intracerebroventricular administration to rats.

The purpose of the present study was to compare receptor affinities and biological activities of enkephalin dimers.

MATERIALS AND METHODS

Peptides and drugs. Dimeric penta- and tetrapeptides were synthesized as described previously [6]. Three additional enkephalin analogs were prepared for this study, and their synthesis will be reported in detail elsewhere. These include: [D-Ala^2, Leu^5] enkephalin ethyl amide (DALEA-C_2). [D-Ala^2, des-Leu^5]enkephalin dodecane amide (DAPHA-C_{12}) and the dimer (H-Tyr-D-Ala-Gly-Phe-Leu-NH-)$_2$ designated DPE_0 to emphasize the absence of a methylene crosslinking bridge.

[D-Ala^2, Leu^5]enkephalin amide (DALEA)* and [D-Ala^2, des-Leu^5]enkephalin amide (DAPHA) were obtained from Peninsula Labs (Belmont, CA), and [D-Ala^2, D-Leu^5]enkephalin (DADLE) from Bachem (Basel). Normorphine was purchased from Bio Products (Brussels, Belgium).

Electrically stimulated smooth muscle preparations. For the guinea-pig ileum assay (GPI), animals (males, 300–400 g) were decapitated and the longitudinal muscle of the ileum, with the adherent myenteric plexus, was prepared [13]. The strips were mounted in a 5 ml bath in Krebs–Ringer bicarbonate solution and field-stimulated (60 V, 0.5 msec, 0.1 Hz) as described [14]. Two hours of equilibration with frequent changes of the bath fluid were allowed before any peptide testing.

For the mouse vas deferens assays (MVD), vasa deferentia from NMRI mice (25–30 g) were prepared [15] and set up in identical organ baths, but with a modified Krebs–Ringer solution [16] stimulated with the same parameters used above at a resting tension of 50 mg.

Data were expressed as percentage of the control responses to electrically evoked twitch tension before addition of the drug to the bath. The concentration required to produce a half-maximal effect (EC_{50}), was calculated from two test concentrations producing approximately 30 and 70% inhibition, respectively, in the same preparation. Standard compounds (DADLE for the MVD, and normorphine for GPI) were assayed in each preparation to permit estimation of relative potencies.

Antinociceptive assay. Tests for antinociceptive activity were performed as described by Bläsig and Herz [17]. Briefly, rats (Sprague–Dawley, 200 g) were prepared for intracerebroventricular administration (i.c.v.) of compounds by chronically implanting a guide cannula into the right anterior horn of the lateral ventricle 5 days prior to testing. Peptides were administered as saline solution (10 μl) and antinociceptive activity was measured by a "vocalization" test. Rectangular electrical pulses of increasing intensity were applied to the tail root and the pain threshold was defined as the electrical stimulus (μA) which elicited vocalization.

Data analysis. Log_{10} values of the EC_{50}s in each smooth muscle preparation were used for calculation of the mean values and the 95% confidence limits. Binding data were analysed by the computer program LIGAND [18] to calculate the receptor affinity, or with the computer program ALLFIT [19] to obtain the half-maximal inhibitory concentration (IC_{50}) of each peptide.

RESULTS

The structures and the biological activities (EC_{50}s) of the dimeric enkephalins are summarized in Tables 1 and 2. In Figs. 1 and 2 data are displayed as relative potencies of the dimers compared with their monomer analogs. It is useful to examine each series separately.

Pentapeptide series

Figure 1 shows the relative potencies for dimeric

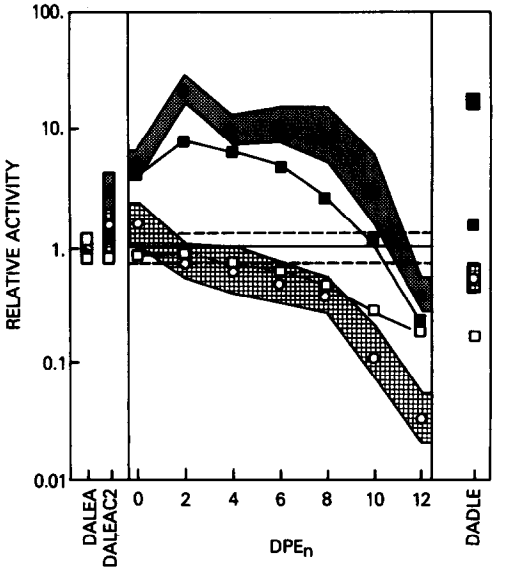

Fig. 1. Biological activities of dimeric enkephalins (pentapeptide series) as compared to receptor binding affinities. Data are displayed relative to the monomer (DALEA), for the mouse vas deferens and guinea-pig ileum. Hatched areas indicate confidence limits. Data for binding activity vs ^{3}H-DADLE and ^{3}H-naloxone are shown for comparison. The number of experiments performed are given in Table 1.

* Abbreviations: DPE, dimeric pentapeptide enkephalin; DTE, dimeric tetrapeptide enkephalin; DADLE, [D-Ala^2, D-Leu^5]enkephalin; DALEA, [D-Ala^2, Leu^5]enkephalin amide; DAPHA, [D-Ala^2, des-Leu^5]enkephalin amide; MVD, mouse vas deferens; GPI, guinea-pig ileum; ED_{50}, drug concentration required to produce a half-maximal effect in the *in vivo* studies; ED_{50}, drug concentration required to produce a half-maximal effect in the *in vivo* studies; EC_{50}, drug concentration necessary to produce a half-maximal inhibition of the electrically induced muscle contractions of the isolated guinea-pig ileum and mouse vas deferens preparation; IC_{50}, drug concentration necessary to reduce specific trace binding to 50%.

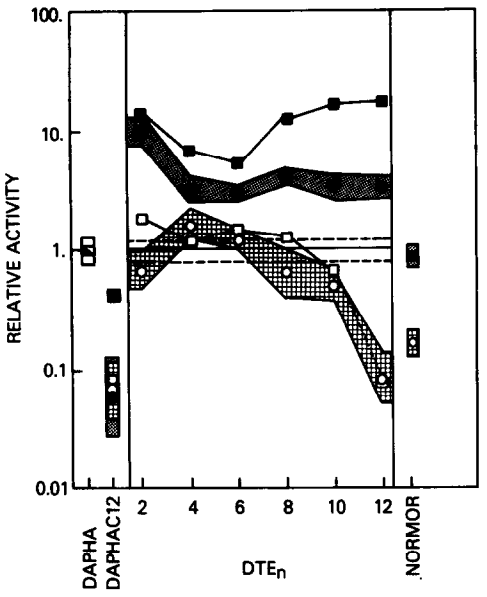

Fig. 2. Biological activities and receptor binding affinities of dimeric enkephalins (tetrapeptide series). Data are given relative to the monomer (DAPHA). For further explanation refer to Fig. 1.

pentapeptide enkephalins (DPE_n) in the electrically stimulated myenteric plexus-longitudinal muscle of the guinea-pig ileum (GPI), and in the electrically stimulated mouse vas deferens (MVD).

Under the present assay conditions, the monomer DALEA displayed an EC_{50} of 16 nM in the GPI and about 9 nM in the MVD (Table 1). The affinities of DALEA in ^{3}H-naloxone (μ-receptor) and ^{3}H-DADLE (δ-receptor) binding assays are almost identical (data not shown), consistent with the established fact that this peptide does not discriminate between δ- and μ-receptors [21]. In contrast, the peptide DADLE, which differs from DALEA principally by virtue of its free carboxyl group (substitution of D-Leu for L-Leu in position 5 affording relative protection from carboxypeptidase without sacrifying the carboxy terminal group), shows an ED_{50} in the MVD (0.52 nM) much lower than in the GPI (29 nM), and its affinity for the δ-receptor in a binding assay is higher than for the μ-receptor [20]. Thus, the monomer DALEA is an enkephalin analog which has lost at least some of its "enkephalin character" [21, 22] as shown by a low ratio of the ED_{50}s in GPI and MVD (less than 2) and identical affinities for μ- and δ-receptors in binding assay.

In the GPI, dimers with spacers of 0 and 2 carbon units (Fig. 1, central panel) show no significant changes in relative potency compared with monomer DALEA.

Further increases in the chain length resulted in progressive loss of potencies. The dimers DPE_4, DPE_6, and DPE_8 exhibit, respectively, 65, 50 and 40% of the monomer activity. Spacers of 10 or 12 methylene units resulted in a still more pronounced loss of potency, retaining only 10% and 3.5% of residual activity, respectively.

In the MVD, the pentapeptide dimers were significantly more potent than the monomeric DALEA. The most active compound, DPE_2, was 20-fold more potent than DALEA (Fig. 1). Spacers of 4, 6 and 8 methylene units produce a consistent increase in potency of a factor of ten. Further extension of the methylene bridge to $n = 10, 12$ resulted in a

Table 1. Biological activity (EC_{50}) of dimeric enkephalin analogs. Pentapeptide series

Compound	Abbreviation	GPI (nM)	c.l.	MVD (nM)	c.l.
H-Tyr-D-Ala-Gly-Phe-Leu-NH_2	DALEA	16.2 (10)	11.9–22	8.8 (17)	6.7–11.6
H-Tyr-D-Ala-Gly-Phe-Leu-NH-CH_2-CH_3	DALEA-C_2	10.5 (3)	5–20	2.9 (3)	2 –4.1
H-Tyr-D-Ala-Gly-Phe-D-Leu-OH	DADLE	28.6 (12)	21–39	0.52 (35)	0.46–0.58
	DPE_0	10.5 (3)	6.8–15.2	1.79 (6)	1.35–2.4
	DPE_2	21.5 (10)	15.5–29.8	0.40 (19)	0.30–0.53
H-Tyr-D-Ala-Gly-Phe-Leu-NH	DPE_4	25.5 (7)	16–41	0.90 (14)	0.68–1.2
$(CH_2)_n$	DPE_6	33.2 (7)	22.3–49.4	0.82 (12)	0.59–1.13
H-Tyr-D-Ala-Gly-Phe-Leu-NH	DPE_8	42.8 (7)	30.2–60.7	0.99 (7)	0.56–1.74
	DPE_{10}	127.8 (6)	75–217	2.99 (7)	1.48–6.05
	DPE_{12}	478 (6)	291–784	22.6 (7)	16.3–31.5

Data represent mean values and confidence limits (c.l.) The number of experiments performed is given in parenthesis.

Table 2. Biological activity (EC_{50}) of dimeric enkephalin analogs. Tetrapeptide series

Compound	Abbreviation	GPI (nM)	c.l.	MVD (nM)	c.l.
H-Tyr-D-Ala-Gly-Phe-NH_2	DAPHA	89 (11)	69–117	126 (11)	102–156
H-Tyr-D-Ala-Gly-Phe-NH-$(CH_2)_{11}CH_3$	DAPHA-C_{12}	1276 (6)	748–2179	2158 (4)	942–4941
	DTE_2	130 (6)	91–186	12 (11)	10–16
	DTE_4	56 (6)	41–77	39 (11)	31–50
H-Tyr-D-Ala-Gly-Phe-NH	DTE_6	74 (6)	63–86	43 (11)	38–50
$(CH_2)_n$	DTE_8	138 (3)	89–214	31 (11)	27–36
H-Tyr-D-Ala-Gly-Phe-NH	DTE_{10}	180 (5)	140–232	37 (11)	29–49
	DTE_{12}	1033 (8)	629–1695	38 (7)	31–46

Data represent mean values and confidence limits (c.l.). The number of experiments performed is given in parenthesis.

progressive loss of activity. Even DPE_0 showed a fivefold increase in potency over the monomer.

DALEA-C_2 the ethyl amide analog of DALEA, serves as a control to evaluate the effects of the alkylation of DALEA. This compound is 3 times more potent than DALEA but 7 times less potent than DPE_2 in the MVD assay.

In the GPI assay, DALEA-C_2 is not significantly more potent than DALEA. Thus the hydrophobicity introduced with the spacer contributes only slightly to the 20-fold increase in MVD activity of DPE_2.

Tetrapeptide series

Figure 2 shows the relative potencies of dimeric tetrapeptide enkephalins (DTEn) in the GPI and MVD assays.

The monomer DAPHA shows little differences in the ED_{50} values in the GPI (89 nM) and in the MVD (126 nM). This is in contrast to its binding assays [5] where the tetrapeptide-amide shows 10-fold selectivity for the μ-receptor (cf. Table 3). As a control, normorphine, which shows an even higher μ-selectivity in binding assays [17], has an EC_{50} in the GPI seven times lower than in the MVD. Dimerization of DAPHA produces, again, different results in the GPI and in the MVD.

In the MVD assays, dimeric tetrapeptides are consistently more potent than the monomer. The most active compound is DTE_2 (relative activity = 10) while DTE_4, DTE_6, DTE_8, DTE_{10} and DTE_{12} show only a 3- or 4-fold increase in potency compared to DAPHA.

In the GPI, DTE_n with spacers of 2, 4, 6, 8 and even 10 methylene units are nearly equipotent with the monomer: the most potent appears to be DTE_4 (1.6-fold more potent than DAPHA), and the least potent is DTE_{10} (50% of monomer activity). In contrast, a spacer of 12 carbons produces, instead, a sharp fall in GPI activity; DTE_{12} retains only 8% of monomer activity. This is in marked contrast to the retention of potency by DTE_{12} in the MVD and δ-binding assays. The monomeric dodecane amide congener of DAPHA, DAPHA-C_{12}, shows a corresponding drop in potency suggesting that the dodecane spacer is responsible for the abrupt decrease in potency observed for DTE_{12}.

Comparison of bioactivity with binding activity

Figures 1 and 2 display the affinities of dimers, previously determined [5, 6] in ^{3}H-DADLE binding assay and in ^{3}H-naloxone binding assay normalized to the corresponding affinities of the monomers (DALEA, DAPHA). This allows a direct comparison of biological potency and binding activity for μ- and δ-receptors (Fig. 2 A, B). The nominal "δ/μ- selectivity" may be seen as the vertical distance between MVD and GPI or between ^{3}H-DADLE and ^{3}H-naloxone binding.

For the pentapeptide series (Fig. 1) there is excellent agreement between GPI activity and μ-binding. In both assays there is a progressive and generally parallel decrease in potency compared to the monomer with increasing spacer length. However, DPE_{10} and DPE_{12} show a more pronounced drop in biological potency than in binding affinity. MVD and δ-binding activities are also generally parallel. Here the relative enhancement of bioactivity appears significantly greater than the relative increase in binding affinity for all the dimers except DPE_0.

For the tetrapeptide series, MVD activity and ^{3}H-naloxone binding were also coincident, with only a minor discrepancy for DTE_2. In contrast, activities in δ-binding are consistently higher than in the MVD and the discrepancy appears to be a systematic function of the spacer length: while DTE_2 has a 14-fold increase in binding and a 10-fold increase in bioassay relative potency, DTE_{12}, is 15 times as potent as DAPHA for δ-receptor binding but only 3 times in the MVD assay (Fig. 2). DAPHA-C_{12} showed similar discrepancies; while its activity in μ-binding and GPI was decreased to the same extent, δ-binding activity was reduced substantially less than MVD potency.

The effect of the addition of an alkyl spacer to the monomer is shown in Table 3. Addition of the ethyl spacer to DALEA produces a 30% increase in activity in the δ-binding assay, a 20% decrease in μ-binding assay, but a 300% increase in MVD and a 60% enhancement in GPI activities. The attachment of the dodecane moiety to DAPHA causes 66% and 16% reductions in delta and μ-binding, and comparable decreases in MVD and in GPI.

Thus for both pentapeptide and tetrapeptide amides, the alkyl spacer affects bioactivity to a greater extent than affinity.

Analgesic effect of dimeric enkephalin

A vocalization test [17] was used to assess the analgesic activity of DPE_2 and DTE_{12} (Table 4). The pentapeptide dimer DPE_2 produces an analgesic effect virtually identical to DADLE. The two peptides were almost equipotent on a molar basis and the duration of the effect was approximately the same. The tetrapeptide dimer DTE_{12} was inactive up to the highest dose tolerated by the animals, in

Table 3. Comparison of binding activity and bioactivity of enkephalin analogs (relative to DALEA)

Peptide	Binding assay δ	Binding assay μ	Binding assay δ/μ	Bioassay MVD	Bioassay GPI	Bioassay MVD/GPI
DALEA	1	1		1	1	
DALEA-C_2	1.3	0.82	1.6	3	1.6	1.9
DADLE	1.4	0.20	7	17	0.56	30.3
DAPHA	0.065	0.58	0.11	0.071	0.16	0.44
DAPHA-C_{12}	0.021	0.48	0.43	0.0042	0.013	0.32
DPE_2	8	0.75	10.6	23	0.75	30.6
DTE_{12}	0.84	0.045	19	0.23	0.015	15.3

Table 4. Antinociceptive activities of opioids after i.c.v. injection in rats

Opioid	ED_{50} (nmoles) ± S.E.M.
DADLE	3.5 ± 0.2 (5)
DALEA	15.0 ± 2.1 (4)
DPE_2	6.0 ± 0.7 (4)
DAPHA	13.0 ± 1.8 (3)
DTE_{12}	>25 (4)

Numbers in parenthesis indicate number of experiments.

contrast with the corresponding monomer DAPHA which induced a strong analgesic effect.

DISCUSSION

The data presented in this study indicate that: (1) dimerization of enkephalin analogs can produce ligands with enhanced biological activity compared to the monomeric congeners, consistent with the increased receptor affinity; (2) the increases in activity and affinity are limited to the δ-type of opiate receptor, irrespective of the selectivity of the monomeric amide analog corresponding to dimers. For both series of dimeric enkephalins μ-activity and affinity were either little changed or significantly decreased, depending on the spacer length.

(1) *Binding affinity and biological activity in isolated preparations*

For both series of dimeric enkephalins, there was a close correspondence between potencies in GPI and in the ^{3}H-naloxone binding assay (Figs. 1 and 2). This was in contrast with the obvious discrepancies between potencies in MVD and δ-binding assays. The magnitude of the increases in potency produced by dimerization (dimer/monomer ratios) in the two assays were discordant for both series of peptides: relative binding activity was lower than relative biological activity for the pentapeptides, while the opposite was observed for the tetrapeptides.

The discrepancy is also related to the spacer chain length: for the pentapeptide series, where a dimer without spacer is available, DPE_0 was the only compound in which bioactivity and binding affinity were are almost coincident. For the tetrapeptide series, the divergence in the two assays appears a function of the spacer length, being smaller for the shorter and larger for the longer spacers.

The discrepancy between binding and bioassay activities of dimers may be due in part to the different extent to which the hydrophobicity introduced with the spacer affects the two assays: bioassay seems to be much more sensitive to changes in hydrophobicity than binding assays. This was tested for one compound of each series: the DPE_2–DALEA-C_2 pair for the pentapeptide dimers and the DTE_{12}–DAPHA-C_{12} pair for the tetrapeptide series. In the first case, DPE_{12} exhibits a 21-fold increase over DALEA in the bioassay but only an eightfold increase in the binding assay. However, ethylamide substitution of DALEA (spacer addition) to create DALEA-C_2 produces a threefold increase in potency in the MVD but an insignificant (1.3-fold) increase in δ-binding. Thus, we estimate that the net increase due to the bivalency of DPE_2 is 7-fold for the bioassay and 6.1-fold for the binding assay—quite consistent results.

In the second case, the dimer DTE_{12} compared to DAPHA showed a 13-fold increase in binding activity but only a threefold increase in bioactivity. However, the introduction of the long dodecane spacer in DAPHA-C_{12} produced a moderate (threefold) decrease of delta affinity and a more pronounced (17-fold) drop of bioactivity. Thus, the net relative effect attributable to bivalency of DTE_{12} is a 40–50-fold increase in both cases.

The importance of the hydrophobicity for dimers with a long spacer is also apparent from the characteristics of the twitch inhibition produced by these compounds. In both GPI and MVD assays, the onset time of the effect of DPE_{12}, DPE_{10}, DAPHA-C_{12}, DTE_{12}, DTE_{10} was always slower than for shorter chain dimers, the dose–response curves were flat and the inhibition of the twitch could not be easily reversed by extensive, repeated washing, although it remained fully naloxone-reversible.

The finding that hydrophobicity in opiate ligands strongly influences their apparent potencies *in vitro* is not new. Earlier studies [23] have shown that physico-chemical factors like hydrophobicity can affect biological potency in isolated preparations and perturb the comparison with receptor binding affinities. Portoghese *et al.* [7] have also stressed the important role that hydrophobic spacers may have in determining the activity of a bivalent ligand.

A more complex level of comparison between binding assays and biological ones involves the pattern of receptor discrimination as predicted by the two pairs of tests. Amidation of the C-terminal carboxyl group in enkephalin analogs eliminates the δ-preference of the peptides in both biological [20, 22] and binding assays [20, 24]. However, the mechanisms of the loss of selectivity is different in the two assay systems: loss in δ-potency with little changes in μ-activity for biological assays, but loss in μ-activity with no changes in δ-potency for binding assays.

In contrast, dimers of the pentapeptide series become δ-selective compared to the monomer DALEA through an identical mechanism for both biological and binding assays: a selective increase in δ-activities. The shift in selectivity is not a non-specific effect of the spacer, as evident by comparing DALEA/DALEA-C_2/DPE_2 (Table 3) and it is, therefore, mainly due to bivalency.

(2) *Antinociceptive properties*

For the analogs of monomeric enkephalins, there is a general agreement that GPI and not MVD bioactivities are highly correlated with the analgesic potencies [22, 25]. The present data, although limited to few peptides, are consistent with those findings: On a molar basis, DADLE and DPE_2 show very similar activities in the vocalization test, in agreement with their relative activities on GPI but not on the MVD.

DAPHA is analgesic [26], but its dimer DTE_{12} produces no effect. Again this is at variance with δ-assays, where the dimer is more potent than the monomer, but parallels the situation in μ-assays,

where DTE_{12} exhibits a dramatic loss of activity in respect to DAPHA.

(3) *Is the increase in activity due to dimerization* per se?

It is likely that the overall macroscopic affinity of a ligand for its receptor is the result of multiple, weaker interactions between a number of chemical groups on the ligand's molecule and corresponding subsites present on the receptor [27]. Chemical modification of the ligand may increase the number of such interactions, thereby increasing the affinity. An extreme case of increase in potential interactions if offered by a bivalent ligand, i.e. in which two active molecules are linked by a spacer. Bivalency, *per se*, might be expected to produce a twofold enhancement in affinity, because of the statistical advantage in association rate. However, if the spacer connecting the two units is long enough to permit simultaneous binding with two adjacent receptor molecules, the enhancement in affinity should be much larger. The exact magnitude of such increase cannot be predicted since several unknown factors are involved (e.g. changes in steric hindrance, flexibility of the spacers, mobile receptors and membrane fluidity, receptor isomerization states, etc.) The affinity for a dimer crosslinking two receptors might be anywhere between a minimal value, about two fold that of the monomer, and a maximal value equal to the square of the affinity constant. If receptors for the ligand are heterogeneous and differ in their topological organization on the membrane, it is possible that "bridging" of receptors might be selective for a single type of them.

An increase in selectivity, along with affinity, are features predictable for bivalent ligand able to bind adjacent receptors; both these features have been observed for enkephalin dimers [5, 6]. New aspects emerge from the data presented here: (a) Most of the increase in activity and receptor preference observed for the dimers are a result of the presence of the second peptide in the molecule, not a non-specific effect of the spacer; (b) spacers influence monomer activities to a different extent depending on the receptor class and the assay type; (c) the increase in affinity for the dimer is linearly related to a corresponding enhancement in biological activity, suggesting that no changes in efficacy are introduced by bivalency.

Apart from the possibility that altered physicochemical characteristics of the dimeric ligands may be involved in some of the observed effects, several specific mechanisms can theoretically explain the gain in activity and selectivity of dimeric enkephalins. These include: (a) both the enkephalin moieties of the dimer occupy independent δ-sites, and this produces high affinity and δ-preference, but the hydrophobic spacer perturbs the effect of inter-receptor distance; (b) one of the enkephalin moieties occupies the receptor, but the second one binds in a distorted way to a neighbouring receptor site. This produces a limited increase in activity and the changes in selectivity differ for the tetra- and penta-peptides; (c) the δ-, but not the μ-receptor "surface" might contain several "copies" of enkephalin binding sites, thus dimers have more affinity (and preference) for δ-sites because they can establish more points of interaction, but μ-binding is impaired by progressively longer spacers.

Studies with enkephalin dimers in which one of the two peptides is modified with structural changes known to reduce the affinity should be helpful to clarify these aspects.

Acknowledgement—We are grateful to Th. Gratemayer for excellent technical assistance. This work was supported by Deutsche Forschungsgemeinschaft, Bonn.

REFERENCES

1. D. H. Coy, A. J. Kastin, M. J. Walker, R. F. McGivern and C. A. Sandman, *Biochem. biophys. Res. Commun.* **83**, 977 (1978).
2. E. Hazum, K.-J. Chang, H. J. Leighton, O. W. Lever and P. Cuatrecasas, *Biochem. biophys. Res. Commun.* **104**, 347 (1982).
3. A. W. Lipkowski, A. M. Konecka and I. Sroczynska, *Peptides* **3**, 697 (1982).
4. T. Costa, Y. Shimohigashi, S. Matsuura, H.-C. Chen and D. Rodbard, in *Peptides* (Eds. D. H. Rich and E. Gross), p. 625. Raven Press, New York (1982).
5. Y. Shimohigashi, T. Costa, H.-C. Chen and D. Rodbard, *Nature, Lond.* **297**, 333 (1982).
6. Y. Shimohigashi, T. Costa, S. Matsuura, H.-C. Chen and D. Rodbard, *Molec. Pharmac.* **21**, 558 (1982).
7. M. Erez, A. E. Takemori and P. S. Portoghese, *J. med. Chem.* **25**, 847 (1982).
8. P. S. Portoghese, G. Ronsisvalle, D. L. Larson, C. B. Yim, L. M. Sayre and A. E. Takemori, *Life Sci.* **31**, 1283 (1982).
9. H. Merz and E. May, personal communication.
10. M. Wüster, R. Schulz and A. Herz, *Biochem. Pharmac.* **30**, 1883 (1981).
11. K.-J. Chang, B. R. Cooper, E. Hazum and P. Cuatrecasas, *Molec. Pharmac.* **16**, 91 (1979).
12. P. Y. Law, D. S. Hom and H. H. Loh, *Molec. Pharmac.* **23**, 26 (1983).
13. H. W. Kosterlitz, R. J. Lydon and A. J, *Br. J. Pharmac.* **39**, 398 (1970).
14. R. Schulz and A. Goldstein, *J. Pharmac. exp. Ther.* **183**, 404 (1972).
15. J. Hughes, H. W. Kosterlitz and F. M. Leslie, *Br. J. Pharmac.* **53**, 371 (1975).
16. R. Schulz, M. Wüster, H. Krenss and A. Herz, *Molec. Pharmac.* **18**, 395 (1980).
17. J. Bläsig and A. Herz, *Naunyn-Schmiedeberg's Arch. Pharmac.* **294**, 297 (1976).
18. P. J. Munson and D. Rodbard, *Analyt. Biochem.* **107**, 220 (1980).
19. A. DeLean, P. J. Munson and D. Rodbard, *Am. J. Physiol.* **235**, E97 (1978).
20. H. W. Kosterlitz, J. A. H. Lord, S. J. Paterson and A. A. Waterfield, *Br. J. Pharmac.* **68**, 333 (1980).
21. L. E. Robson and H. W. Kosterlitz, *Proc. R. Soc. Lond. Biol. Sci.* **205**, 425 (1979).
22. A. Z. Rónai, L. P. Berzétei, J. I. Székely, E. Miglécz, J. Kurgys and S. Bajusz, *Eur. J. Pharmac.* **69**, 263 (1981).
23. M. Wüster and A. Herz, in *Opiates and Endogenous Opioid Peptides*, p. 447. Elsevier, Amsterdam (1976).
24. Y. Shimohigashi, M. L. English, C. H. Stammer and T. Costa, *Biochem. biophys. Res. Commun.* **104**, 583 (1982).
25. G. Gacel, M.-C. Fournié-Zaluski, E. Fellion and B. P. Roques, *J. med. Chem.* **24**, 1119 (1981).
26. W. H. McGregor, L. Stein and J. D. Belluzzi, *Life Sci.* **23**, 1371 (1978).
27. A. DeLean, P. J. Munson and D. Rodbard, *Molec. Pharmac.* **15**, 60 (1979).