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Pharmacological profiles of fentanyl analogs at μ, δ and κ opiate receptors

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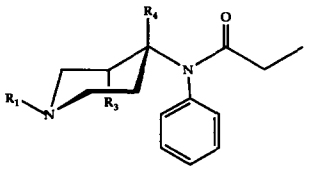
Receptor binding assays using [^{3}H]DAGO ([D-Ala^2,MePhe^4-Gly^5-ol]enkephalin) (μ), [^{3}H]DPDPE ([D-Pen^2,D-Pen^5]enkephalin) (δ) and [^{3}H]U-69593 (κ) were done in guinea pig whole brain membranes. Agonist activity was determined in norbinaltorphimine or β-funaltrexamine (β-FNA) treated guinea pig ileum (μ and κ, respectively) and β-FNA-treated mouse vas deferens (δ). The compounds with highest affinity were the most potent at the μ-receptor. The selectivity observed in the binding affinities was also found in in vitro activity. No correlation was found between μ-affinity and selectivity; the highest affinity analog, lofentanil, was found to be among the least selective, while another high affinity analog, R30490, was the most μ-selective. The results show that not all fentanyls are highly μ-selective, and could produce actions through δ- and κ-opiate receptors.

Opiates; Fentanyl; Lofentanil; Ileum; Vas deferens; (Receptor binding)

1. Introduction

Ever since the potent synthetic analgesic fentanyl (**1**, see fig. 1 for structures) (Janssen, 1965) was reported in the early sixties, the 4-anilidopiperidine class of opioids has been the subject of much interest. Over the years, many analogs with variations of the basic 4-anilidopiperidine structure have been synthesized, leading, in some cases, to compounds whose affinity for the opiate receptor and analgesic activity surpass that of fentanyl. While a large number of fentanyl analogs have been reported in the literature, knowledge of their pharmacology is surprisingly incomplete, and there is very little evidence for the widely accepted assumption that they are highly μ-selective analgesics. In fact, for carfentanil (**4**) and lofentanil (**5**) (Van Daele et al., 1976), recent studies, which probed binding to the μ- and δ-receptors in rat (Yeadon and Kitchen, 1988b; Titeler et al., 1989), human brain (Titeler et al., 1989) and in two cultured cell lines (Maloteaux et al., 1989) have led to a modification of this assumption. In these diverse systems, they were found to bind with significant affinity to both μ- and δ-receptors, with varying selectivity.

Fentanyl (**1**), sufentanil (**2**) (Niemegeers et al., 1976) and alfentanil (**3**) (Janssens et al., 1986) are used clinically as analgesics or analgesic/anesthetics, and carfentanil (**4**) (Van Daele et al., 1976), as an immobi-



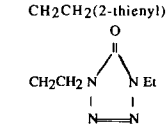
		R_1	R_3	R_4
1	fentanyl*	CH_2CH_2Ph	H	H
2	sufentanil	CH_2CH_2(2-thienyl)	H	CH_2OCH_3
3	alfentanil		H	CH_2OCH_3
4	carfentanil*	CH_2CH_2Ph	H	$COOCH_3$
5	lofentanil*	CH_2CH_2Ph	CH_3	$COOCH_3$
6	R30490*	CH_2CH_2Ph	H	CH_2OCH_3
7	N-methyl fentanyl*	CH_3	H	H
8	N-methyl carfentanil*	CH_3	H	$COOCH_3$

Fig. 1. Chemical structures of fentanyl analogs. * Those analogs which were included in this study. In previous reports, the Janssen internal code number, R32395, was used for N-methyl carfentanil.

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lizing agent in veterinary medicine (DeVos, 1978). The high analgesic potency of these compounds is counterbalanced by their profound respiratory depressant action. So far no antagonist has been found that can match the fentanyls in potency. In a promising development, a number of fentanyl analogs have been recently reported (Bagley et al., 1989; France et al., 1990) to have in vivo analgesic activity and to reverse morphine-induced respiratory depression. The origin of this behavior is not clear. The question of whether analgesia and respiratory depression are initiated through one type of receptor or different receptor subtypes, is still debated in the literature (Pasternak and Snyder, 1975; Lord and Waterfield, 1977; Wolozin and Pasternak, 1981; Ling et al., 1985).

Because of the lack of knowledge of the mechanism and site of action of the fentanyl analogs and their continued and potential usefulness as therapeutic agents, the need is emerging for a renewed effort to investigate and interpret the structure/activity relationship for the fentanyl class of compounds with the dual goal of developing an antagonist of matching potency and a fentanyl analog devoid of respiratory side effects.

In an effort to investigate the structure/activity relationship for the fentanyl class of compounds, we have determined the binding affinities and in vitro activities at the μ-, δ- and κ-opioid receptors, of six fentanyl analogs shown in fig. 1. Affinity and selectivity of each compound for the μ-, δ- and κ-receptors was determined in competitive binding studies using [^{3}H]DAGO ([D-Ala^2,MePhe^4,Gly^5-ol]enkephalin) (μ), [^{3}H]DPDPE ([D-Pen^2,D-Pen^5]enkephalin) (δ) and [^{3}H]U-69593 (κ). Subsequently, the pharmacological activity of the compounds at the three receptors was assessed in the following bioassays: guinea pig ileum (GPI) with norbinaltorphimine (norBNI) (μ) (Portoghese et al., 1987), or β-funaltrexamine (β-FNA) (κ) (Ward et al., 1986) and mouse vas deferens (MVD) with β-FNA (δ) (Ward et al., 1982).

The compounds selected for experimental studies include three potent analgesics which differ only in the R_4 substituent of the piperidine ring: fentanyl (**1**) (Janssen, 1965), carfentanil (**4**) (Van Daele et al., 1976), and R30490 (**6**) (Van Bever et al., 1976), the parent compound of sufentanil. For one of these, carfentanil, we have also included the most potent of its 3-methyl derivatives, lofentanil (**5**) (Van Daele et al., 1976). For two of these analogs, fentanyl and carfentanil, the corresponding compounds with an N-methyl group replacing the N-phenethyl group were also included: N-methyl fentanyl (**7**) and N-methyl carfentanil (R32395, **8**), which have been reported to be, respectively, inactive (Casy et al., 1969) and twice as potent as morphine (Van Daele et al., 1976) in eliciting analgesia. The results of these studies are being used in a theoretical study to identify and characterize the steric and electronic properties of the molecule, such as dipole moments, atomic charges, etc., that modulate the affinity of the fentanyls at the μ-receptor (Cometta-Morini et al., in press).

2. Materials and methods

2.1. Materials

The following compounds were kindly provided by NIDA: U-69593, DAGO, DPDPE, normorphine, [^{3}H]DAGO and [^{3}H]DPDPE. [^{3}H]U-69593 was purchased from Amersham (Arlington Heights, IL). Lofentanil, R30490 and R32395 were generous gifts of Janssen Pharmaceutica (Belgium). Carfentanil was purchased from Wildlife Laboratories (Fort Collins, CO). Fentanyl was purchased from Sigma (St. Louis MO), β-FNA and norBNI from Research Biochemicals Inc. (Natick, MA). N-methyl fentanyl was synthesized in the laboratory of Dr. A.F. Casy of the University of Bath, using previously described methods (Casy et al., 1969). All other chemicals were from standard commercial sources.

2.2. Receptor binding assays

2.2.1. Preparation of membranes

Frozen whole guinea pig brains (including cerebella) (Pel Freeze, Rogers, AR) were thawed and homogenized with a polytron homogenizer in 40 volumes of 50 mM Tris HCl (pH 7.4 at 25°C, assay buffer) (Lahti et al., 1985) and centrifuged at 20000 $\times g$ for 10 min. The pellet was rehomogenized and centrifuged 2 additional times. For the binding assays, the membranes were suspended in assay buffer to a tissue concentration of 10 mg wet weight per ml.

2.2.2. General assay method

Membranes (1 ml) were incubated in triplicate with 0.5 nM [^{3}H]DAGO, [^{3}H]DPDPE or 1.0 nM [^{3}H]U-69593 and 15 concentrations of unlabeled drug in a total volume of 2 ml at 25°C until equilibrium was reached (Tiberi and Magnan, 1990). The incubation times, determined experimentally, were 3, 4 or 1 h for [^{3}H]DAGO, [^{3}H]DPDPE and [^{3}H]U-69593, respectively. Nonspecific binding was determined in the presence of 1 μM unlabeled ligand (DAGO, DPDPE or U-69593) or WIN 44441-3. The reaction was terminated by filtration (Brandel cell harvester) through glass fiber filters (Whatman GF/B, presoaked in 0.1% polyethylenimine) followed by three, 5 ml washes with ice cold buffer. Radioactivity bound to the filters was quantitated by liquid scintillation with ReadySafe (Beckman) after 12 h at room temperature.

2.2.3. Data analysis

Data obtained from competitive binding assays were analyzed by a modified version (Toll et al., 1984) of the program LIGAND (Munson and Rodbard, 1980), which calculates the binding affinities and receptor densities using weighted nonlinear, least squares regression analysis. The program constructs models of binding according to the law of mass action and determines receptor binding affinities and capacities that best fit experimental data to these models.

2.3. In Vitro model tissue studies

2.3.1. Preparation of tissue

Guinea pig ilea tissues were prepared as described by Kosterlitz et al. (1970). A section of intestine was removed from male albino guinea pigs (300–450 g) (Simonsen, Gilroy, CA), flushed with aerated (95% O_2–5% CO_2) modified Krebs buffer at 37°C (118 mM NaCl, 25 mM $NaHCO_3$, 11 mM glucose, 4.7 mM KCl, 2.45 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$) and the longitudinal muscle layer separated from the underlying tissue. Each strip of tissue was mounted in a 10 ml tissue bath. A resting tension of 0.7–1.0 g was applied to the tissues, which were then stimulated with single 0.9 ms pulses of supramaximal voltage at a rate of 0.1 Hz. Tissues were allowed to equilibrate for at least 40 min before each assay.

The mouse vas deferens tissue were prepared as described by Schulz et al. (1979). The vasa deferentia were removed from male Swiss-Webster mice (30–40 g) (Simonsen Labs, Gilroy, CA) and the tissues were mounted in the same fashion as the GPI in 10 ml tissue baths containing $MgSO_4$-free Krebs buffer. The baths were maintained at 31°C and aerated as above. A resting tension of 0.2 g was applied to each tissue. The tissues were stimulated with sets of twin pulses of supramaximal voltage applied at a rate of 10 Hz. The twin pulse trains were in turn applied for a duration of 220 ms at a rate of 0.1 Hz. Tissues were allowed to equilibrate for 30 min.

After preparing and equilibrating, highly receptor-specific opiate antagonists were introduced to the bath with the aim of reducing all tissue response to that mediated through one subtype (μ, δ and κ) only. Effectiveness of receptor blockage treatment was assessed by measuring the effect of identical doses of selective agonists before and after receptor blockage.

β-FNA (200 nM), administered as a single injection into the tissue baths, was used to inactivate μ-receptors in both GPI (Ward et al., 1982) and MVD (Ward et al., 1986) assays. GPI were incubated with β-FNA for 60 min and then washed until no further recovery was observed (Ward et al., 1982), after which, the maximum acceptable inhibition produced by 20 nM DAGO was 10% while a dose of 4 nM U-69593 was required to produce at least 30% inhibition.

MVD were incubated with β-FNA for 30 min and then washed at 5 min intervals for 60 min (Ward et al., 1986). Five minutes after injection of β-FNA, electrical stimulation was discontinued for 20 min and then restored for the remainder of the assay. After washing, an injection of 200 nM DAGO should cause no more than a 30% reduction in twitch strength. Response to a dose of 10 nM DPDPE should be $\geqslant 25\%$.

The κ antagonist norBNI (20 nM) in Krebs solution was used to antagonize κ-receptors in the GPI (Portoghese et al., 1987). Tissues were preincubated in norBNI–Krebs for 30 min, after which the response to 20 nM DAGO was expected to be at least 25% inhibition, while the maximum allowable level of inhibition by 4 nM U-69593 was 10%.

2.3.2. General assay procedure

All compounds were allowed to incubate in the tissue baths until full effect was reached, and then washed at regular intervals (10 min for GPI, 5 min for MVD) until pre-dosage twitch strengths were recovered or no further change was observed. If tissue recovery of at least 75% of original twitch strength could not be achieved, the tissue was discarded. Dose–response curves were constructed from at least three points, but as many data points as possible were gathered. All points used in the calculations fell between 10 and 90% inhibition.

2.3.3. Calculation of IC_{50}

A plot of the % of pre-dosage contraction strength versus log dose was constructed for each compound. From the linear regression, the IC_{50} was calculated as that dose which produced 50% inhibition. The values reported are means ± S.E.M.

3. Results

Receptor binding assays were carried out under equilibrium conditions. The time required to reach equilibrium was determined experimentally for each radioligand (not shown). Nonspecific binding was typically 6, 15 and 25% of the total [^{3}H]U-69593, [^{3}H]DAGO and [^{3}H]DPDPE binding, respectively. All data was best fit to a one-site model for each receptor. Table 1 shows the K_i for the six fentanyl analogs in order of decreasing μ-receptor affinity. While all compounds, with the exception of the very low affinity N-methyl fentanyl, bind with highest affinity to the μ-receptor, they have different μ-selectivities, due to differences in binding affinities at δ- and κ-receptors. However, the rank order of affinities is the same for all receptors. The N-methyl derivatives displayed much

TABLE 1

Opiate receptor binding affinities and selectivities of fentanyl analogs

	K_i (nM)			Selectivity	
	μ [a]	δ [b]	κ [c]	μ/δ	μ/κ
Lofentanil	0.023 ± 0.004	0.24 ± 0.02	0.60 ± 0.05	10	26
Carfentanil	0.024 ± 0.004	3.3 ± 0.04	43 ± 4	138	1792
R30490	0.09 ± 0.01	23 ± 3	63 ± 6	256	700
Fentanyl	1.2 ± 0.2	180 ± 18	290 ± 24	150	242
N-Methyl carfentanil	42 ± 6	3000 ± 300	6900 ± 800	71	164
N-Methyl fentanyl	18000 ± 3000	11000 ± 1000	27000 ± 3000	0.6	1.5

Inhibition of [a] [^{3}H]DAGO, [b] [^{3}H]DPDPE and [c] [^{3}H]U-69593 binding to guinea pig whole brain membranes was determined as described in Materials and methods. K_i values were calculated using LIGAND. Values are means ± S.E. for three to four assays. [d] The selectivity was calculated as follows: μ/δ, $K_i(\delta)/K_i(\mu)$; μ/κ, $K_i(\kappa)/K_i(\mu)$.

lower affinities than their N-phenethyl counterparts. Among the analogs, lofentanil was the least and R30490, the most μ-selective. The enhanced μ-selectivity of R30490 and the diminished selectivity of lofentanil were due to the modulation of binding affinity at δ- and κ-receptors. Although their affinity at the μ-receptor was similar, R30490 displayed much lower δ- and κ-affinities than lofentanil.

The ability of each of the compounds to inhibit electrically stimulated muscle contractions through each of the opiate receptors was assayed in the GPI (μ- and κ-receptors) and the MVD (δ-sites). Preliminary assays using MVD revealed the presence of μ-receptors in relatively low concentration (not shown). Because of the high affinity and variable μ-selectivity of the fentanyl analogs (table 1), it was necessary to ensure that μ-receptors were sufficiently blocked to accurately measure the activity at the κ- (GPI) and δ- (MVD) receptors. Blocking was achieved by pretreating with β-FNA which irreversibly inactivates the μ-receptor by alkylation (Portoghese et al., 1980). In order to accurately measure the μ-activity, the κ-receptors in the GPI were reversibly blocked by norBNI (Portoghese et al., 1987), which was present throughout the assay system.

The results from these in vitro tissue assays are shown in table 2, with the analogs in the same order as in table 1. It should be noted that in norBNI-treated GPI, the tissue was slow to recover from the administration of $\geqslant 0.3$ nM lofentanil, the average maximum recovery being 58.4 ± 12.8% (n = 8) with variable wash times from 1 to 2 h. Therefore, each concentration was assayed using different tissue. As expected from their binding profiles, all of the analogs had the highest activity at the μ-receptor. The rank order and selectivity of activation of each receptor parallels that of binding affinity.

The one anomaly was N-methyl fentanyl. Despite its low binding affinity at μ- and κ-receptors (table 1), it had high nanomolar agonist activity in the GPI which had been treated with either β-FNA and norBNI (table 2). However, this action could not be reversed by

TABLE 2

Inhibition of electrically stimulated muscle contractions in guinea pig ileum and mouse vas deferens by fentanyl analogs.

	IC_{50} (nM) [a]			Selectivity [e]	
	μ [b]	δ [c]	κ [d]	μ/δ	μ/κ
Lofentanil	0.18 [f]	1.0 ± 0.2	1.3 ± 0.3	5.6	7.2
Carfentanil	0.019 ± 0.002 (3)	17 ± 8	59 ± 28 (3)	895	3100
R30490	0.15 ± 0.08	1200 ± 700	3900 ± 1400	8000	26000
Fentanyl	3.6 ± 0.8	700 ± 480 (3)	760 ± 460	194	208
N-Methyl carfentanil	450 ± 97	18.4% @ 20 μM (3)	27.7% @ 20 μM (3)		
N-Methyl fentanyl	310 ± 73 [g]	No activity at 10 μM	450 ± 110 [g]		

[a] IC_{50} values were determined as described in Materials and methods. Unless otherwise noted in parentheses, n = 4. [b] μ: (GPI + 20 nM norBNI); [c] δ: (MVD + 200 nM β-FNA); [d] κ: (GPI + 200 nM β-FNA). [e] The selectivity was calculated as follows: μ/δ, $IC_{50}(\delta)/IC_{50}(\mu)$; μ/κ, $IC_{50}(\kappa)/IC_{50}(\mu)$. [f] Because the recovery from inhibition was 58.4 ± 12.8% (n = 8) after 1 to 2 h of washing, the responses to each dose were assessed using different tissues. The IC_{50} was calculated from the average inhibition at each dose. [g] Not reversible by naloxone or norBNI (see table 3).

TABLE 3

Reversibility of μ- and κ-agonist activity in guinea pig ileum.

	% inhibition [a] (agonist only)	% inhibition [b] (agonist + antagonists)
DAGO (20 nM)	36 ± 12 (8) [c]	0 (8)
U-69593 (10 nM)	64 ± 11 (8)	0 (8)
Fentanyl (3 nM)	50 ± 9 (4)	0 (4)
N-Methyl-fentanyl (400 nM)	54 ± 8 (4)	37 ± 16 (4)

[a] % inhibition of electrically stimulated muscle contractions in the presence of the indicated concentrations of agonist. [b] % inhibition produced by the agonist, in the presence of 200 nM naloxone and 20 nM norBNI. [c] (Number of determinations).

naloxone (200 nM) or norBNI (20 nM), indicating that this effect was not mediated through opiate receptors (table 3).

4. Discussion

The results obtained represent the first in vitro characterization of a series of fentanyl analogs at μ-, δ- and κ-receptors, using both binding affinity and activity in model tissues. The goal of this work was to provide a more complete data set which could be used as a basis for subsequent theoretical studies, to determine the molecular requirements for binding affinity and selectivity within this class of compounds.

All of the compounds reported here have been previously characterized for their in vivo activity (Casy et al., 1969; Van Bever et al., 1976; Van Daele et al., 1976), some have known μ-binding affinity, against [^{3}H]fentanyl (Leysen et al., 1977; Leysen et al., 1978; Leysen and Laduron, 1978) or [^{3}H]DAGO (Yeadon and Kitchen, 1988a), and a few have been investigated for δ-receptor binding using [^{3}H]DPDPE (Yeadon and Kitchen, 1988a). However, only fentanyl has been previously evaluated at all three receptors, with binding at the κ-receptor assayed using [^{3}H]EKC (ethylketocyclazocine) in the presence of μ- and δ-blocking agents (Magnan et al., 1982).

All compounds which display significant affinity bind with highest affinity to the μ-receptor and have the greatest agonist activity initiated through this receptor. However, the compounds studied showed a wide range of μ-affinities and selectivities, clearly illustrating that while the anilidopiperidine moiety itself is necessary for μ-affinity, it is the presence and nature of the ring substituents which modulate the affinity at δ- and κ-receptors, and hence μ-selectivity.

Another striking result is that, comparing the pairs of compounds differing in only an N-phenethyl versus an N-methyl substituent, the N-methyl analogs had greatly diminished affinity as compared to its N-phenethyl counterpart. This reduced affinity of the N-methyl derivatives is unique to this class of opioids and suggests important differences in μ-receptor recognition for the fentanyl family of analogs.

It has been suggested that within the fentanyl class of compounds, as the affinity of the compounds increases at the μ-receptor, its μ/δ selectivity decreases (Yeadon and Kitchen, 1988b). From the results presented here, this conclusion clearly cannot be drawn. Although the highest affinity analog, lofentanil, was the least selective, we have shown carfentanil to bind with very high affinity to the μ-receptor and to be as μ/δ selective as fentanyl. Moreover, R30490, which has 10-fold higher affinity than fentanyl at the μ-receptor, is the most μ-selective of the compounds tested.

Comparison of the receptor binding results reported here with previous studies with respect to μ/δ selectivity indicates that the extent of receptor subtype selectivity can vary depending on the species used. A recent report compared the binding affinities of a series of fentanyl analogs at the μ- and δ-receptors in rat and marmoset brain membranes (Yeadon and Kitchen, 1988a). At the μ-receptor, the binding affinities were similar in the two tissues. However, the affinities for the δ-receptor were variable, leading to differences in the calculated selectivity of carfentanil. In rat membranes, this compound was nonselective between μ- and δ-receptors, yet in marmoset membranes, it had 20-fold higher affinity for the μ-receptor. Our current data (table 1) showed an even greater μ/δ selectivity in guinea pig brain membranes. Although the μ-selectivity of these compounds varied between species, the rank order of binding affinities remained the same.

Our results also point to a possible difference in dissociation rates of lofentanil from the different opioid receptors. In a previous study, lofentanil was radiolabeled in an effort to study the origin of its long-lasting analgesic activity (duration > 24 h) with a potency 6000 × morphine, an effect that could not be explained by its enhanced affinity alone (Gommeren and Leysen, 1982). It was found to dissociate very slowly, a behavior initially assumed to be characteristic of binding to all receptor subtypes. However, our model tissue studies suggest that this effect might be true only for the μ-receptor. It was observed that for β-FNA-treated GPI (κ) and MVD (δ), there was essentially complete recovery within 90 min. However, for norBNI-treated GPI, the average maximum recovery of the μ-receptor after administration of lofentanil was 58.4 ± 12.8% (n = 8) (see table 2).

The GPI and MVD model tissue systems have not been widely used to characterize the activity of fentanyl analogs. Of the compounds included in this study, only fentanyl has been previously assayed using these systems, primarily demonstrating its μ-activity (Magnan et al., 1982; Essawi and Portoghese, 1983; Hayes et al., 1985). The reported IC_{50} for fentanyl in untreated

GPI (3.45 nM) (Essawi and Portoghese, 1983) is similar to our reported μ-activity in GPI with norBNI pretreatment (3.6 nM) (table 2), consistent with the relatively high μ/κ selectivity for activation found for this compound.

In general, the rank order of activity at each receptor parallels that of binding affinity. However, if we use the ratio of affinity to activity as a qualitative measure, there is a wide range of ability of agonists to activate the μ-receptor with carfentanil > R30490 > fentanyl > lofentanil > N-methyl carfentanil, with a difference of two orders of magnitude between carfentanil and its N-methyl analog. All compounds which were μ-selective in binding were found to be even more selective in activity. Lofentanil was relatively nonselective in each case, while R30490, the most selective in receptor binding had even greater selectivity for activation of the μ-receptor. R30490 is thus an ideal candidate as a template for probing the molecular determinants of μ-recognition and activation.

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