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μ-, δ- and κ-opioid receptor-mediated inhibition of neurotransmitter release and adenylate cyclase activity in rat brain slices: studies with fentanyl isothiocyanate

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We investigated the effects of [D-Ala^2,D-Leu^5]enkephalin (DADLE), [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAGO), [D-Pen^2,D-Pen^5]enkephalin (DPDPE) (0.01-1 μM) and bremazocine (0.001-0.3 μM) on the electrically evoked release of radiolabelled neurotransmitters and on the dopamine (DA)-stimulated cyclic AMP efflux from superfused rat brain slices. The differential inhibitory effects of these agonists on the evoked neurotransmitter release indicate that the opioid receptors mediating presynaptic inhibition of [^{3}H]noradrenaline (NA, cortex), [^{14}C]acetylcholine (ACh, striatum) and [^{3}H]DA (striatum) release represent μ, δ and κ receptors, respectively In agreement with this classification, preincubation (60 min) of the slices with the δ-opioid receptor-selective irreversible ligand, fentanyl isothiocyanate (FIT, 0.01-1 μM), antagonized the inhibitory effects of DADLE and DPDPE on striatal [^{14}C]ACh release only. On the other hand, the D-1 DA receptor-stimulated cyclic AMP efflux from striatal slices appeared to be inhibited by activation of μ as well as of δ receptors. In this case, the reversible μ antagonist, naloxone (0 1 μM), fully antagonized the inhibitory effect of the μ agonist, DAGO, without changing the effect of the δ agonist DPDPE but was ineffective as an antagonist in slices pretreated with FIT (1 μM). The inhibitory effect of DAGO on the electrically evoked [^{3}H]NA release was antagonized by naloxone whether the receptors were irreversibly blocked by FIT or not. These data not only further support the existence of independent presynaptic μ-, δ- and κ-opioid receptors in rat brain but also evidence strongly that μ and δ receptors mediating the inhibition of DA-sensitive adenylate cyclase could share a common binding site (for naloxone and FIT) and, therefore, may represent constituents of a functional opioid receptor complex.

Opioid receptors; Neurotransmitter release; Adenylate cyclase; Fentanyl isothiocyanate; Brain slices

1. Introduction

Evidence from numerous investigations, both in vivo and in vitro, has led to the generally accepted view that the effects of opioid drugs on the central and peripheral nervous system are mediated via multiple receptors. The existence of μ receptors, for which morphine is the prototype agonist, of δ receptors, which display selectivity for the endogenous opioid peptides methionine- and leucine-enkephalin, and κ receptors, for which dynorphin A and related peptides may be the endogenous agonists, has been well established (for reviews, see Paterson et al., 1983; Martin, 1984; Goldstein, 1987). Although experiments aimed at isolation of these different types of opioid receptors suggest strongly that they represent structurally different

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proteins (Itzhak et al., 1984, Gioannini et al., 1985; Howard et al., 1986, Simon, 1987), it remains to be found whether signal specificity is encoded in these different structures and whether or not these receptor types (always) represent independent entities. It is worth noting that μ and δ receptors in the brain could be coupled to the same effector systems in certain cells. Thus, both of these opioid receptor types appeared to mediate the inhibition of adenylate cyclase activity (Schoffelmeer et al, 1986, 1987) and to enhance potassium conductance (North et al., 1987), probably involving the activation of guanine nucleotide binding proteins (Pfeiffer et al., 1982; Abood et al., 1985; Kazmi and Mishra, 1987). Nonetheless, in view of the differential regional localization of these receptor types in the brain (Tempel and Zukin, 1987), suggesting involvement in different physiological functions, their pharmacological differences could have an important therapeutic impact.

With regard to the heterogeneity of functional opioid receptors in the brain, recent studies indicate that the depolarization-induced release of different neurotransmitters from brain tissue is liable to inhibition by presynaptic μ, δ and κ receptors (Hagan and Hughes, 1983; Mulder et al., 1984, 1987; Jackisch et al., 1986a,b) Thus, results obtained earlier in our laboratory indicated that the release of noradrenaline (NA) from rat brain cortex slices and synaptosomes is reduced by activation of μ-opioid receptors only (Mulder et al., 1984, 1987), whereas the release of acetylcholine (ACh) and dopamine (DA) from striatal slices is inhibited by activation of homogeneous populations of δ and κ receptors, respectively (Mulder et al., 1984). Although these functional studies suggest that there are independent opioid receptor types, evidence is accumulating that the distinct opioid receptors may (also) exist as non-competitively (allosterically) interacting proteins, possibly as constituents of an opioid receptor complex (Bowen et al., 1981; Lee et al., 1982; Smith et al., 1983; Holaday and Tortella, 1984; Long et al., 1984; Rothman et al., 1985; Demoliou-Mason and Barnard, 1986; Bowen et al., 1988). The existence of such a putative receptor complex is particularly intriguing in view of the possibility that its pharmacological nature might depend on (and regulated by) local environmental factors in the cell membranes in which it is embedded. We recenty reported that a μ/δ-opioid receptor complex could indeed have a functional role in rat brain, being coupled in an inhibitory fashion to DA-sensitive adenylate cyclase in the neostriatum (Schoffelmeer et al., 1986, 1987). We now investigated whether the μ and δ receptors involved might share a common ligand binding site. We compared the effects of the δ-opioid receptor-selective, irreversible ligand fentanyl isothiocyanate (FIT, Rice et al., 1983) on presynaptic opioid receptors mediating the inhibition of neurotransmitter release and on the hypothetical opioid receptor complex linked to adenylate cyclase in superfused rat brain slices.

2. Materials and methods

2.1. Determination of neurotransmitter release

Male Wistar rats (180-220 g body weight) were decapitated and the cortex or neostriatum was rapidly dissected from the brain. Slices ($0.3 \times 0.3 \times 2$ mm) were prepared with a McIlwain tissue chopper, then incubated and superfused essentially as described previously (Mulder et al., 1984). In short, the slices were washed twice with 5 ml Krebs-Ringer bicarbonate medium containing 121 mM NaCl, 1.87 mM KCl, 1.17 mM KH_2PO_4, 1.17 mM $MgSO_4$, 1.22 mM $CaCl_2$, 25 mM $NaHCO_3$ and 10 mM D(+)glucose. The slices were incubated for 60 min in 2.5 ml Krebs-Ringer bicarbonate medium (under an atmosphere of 95% O_2-5% CO_2) in either the absence or presence of fentanyl isothiocyanate (FIT, 0.1-1 μM). A 45-min incubation was followed by incubation for an additional period of 15 min in the presence of 0.05 μM [^{3}H]NA (cortex slices) or 0.1 μM [^{3}H]DA and 1 μM [^{14}C]choline (neostriatal slices), again in the absence or presence of FIT. After labelling, the slices were transferred to each of the 24 chambers of a superfusion apparatus (about 4 mg tissue per chamber; 0.2 ml volume) and were subsequently superfused (0.25 ml/min) with medium (gassed with 95% O_2-5% CO_2) without FIT at 37°C. The

superfusate was collected as 10-min samples after 40 min of superfusion (t = 40). Calcium-dependent neurotransmitter release was induced during superfusion by exposing the slices to electrical biphasic block pulses (cortex slices: 1 Hz, 15 mA, 4 ms pulses; striatal slices: 3 Hz, 24 mA, 4 ms pulses) for 10 min at t = 50 min (electrical field stimulation). Drugs were added to the medium 20 min prior to stimulation. The radioactivity remaining at the end of the experiment was extracted from the tissue with 0.1 N HCl. The radioactivity in superfusion fractions and tissue extracts was determined by liquid scintillation counting.

The efflux of radioactivity during each collection period was expressed as a percentage of the amount of radioactivity in the slices at the beginning of the respective collection period. The electrically evoked neurotransmitter release was calculated by subtracting the spontaneous efflux of radioactivity from the total overflow of radioactivity during stimulation and the following 10 min. A linear decline from the 10-min interval before, to the interval 20-30 min after the start of stimulation was assumed for calculation of the spontaneous efflux of radioactivity. The release evoked was expressed as percent of the ^{3}H and ^{14}C content of the slices at the start of the stimulation period.

2.2. *Determination of adenylate cyclase activity*

The receptor-mediated modulation of adenylate cyclase activity in rat striatal slices was investigated, with the efflux of cyclic AMP from superfused slices used as an accurate and highly sensitive parameter to reflect intracellular cyclic AMP formation (Stoof and Kebabian, 1981; Lazareno et al., 1985). Striatal slices were prepared as described above and were washed twice with 10 ml of Krebs-Ringer bicarbonate medium. The medium contained 1 mM 3-isobutyl-1-methylxanthine in order to prevent phosphodiesterase breakdown of cyclic AMP. The slices were then preincubated for 1 h in 5 ml of this medium in the presence or absence of FIT. Subsequently, the slices were transferred to each of the 24 chambers of a superfusion apparatus (0.2 ml volume; 20 mg of tissue per chamber) and were superfused (0.1 ml/min) with medium (containing 3-isobutyl-1-methylxanthine but no FIT) gassed with 95% O_2-5% CO_2, at 37 °C. A 20-min basal efflux fraction was collected after 60 min of superfusion (t = 60 min). Drugs were added at t = 80 min and a second 20-min fraction was collected from t = 100-120 min.

The cyclic AMP content of triplicate 100 µl aliquots of the first and the second fraction was estimated with a radioimmunoassay (Brooker et al., 1979). The limit of detection of this assay was 5.0 fmol/100 µl. Preliminary experiments had shown that the cyclic AMP content determined in the second fraction did not differ (96 ± 4%, n = 12) from that found in the first fraction (when the slices were superfused with medium without additional drugs) and that the latter varied between experiments (from 12 ± 0.2 to 31 ± 0.5 fmol/min). The receptor-stimulated cyclic AMP efflux was therefore expressed as percent increase in cyclic AMP content of the second fraction relative to the content of the first fraction.

2.3. *Statistics*

The statistical significance of differences was determined by one-way analysis of variance (ANOVA) and subsequently by the two-tailed Student's t-test.

2.4. *Radiochemicals and drugs*

[^{3}H]Noradrenaline (39 Ci/mmol), [^{3}H]dopamine (40 Ci/mmol) and [^{14}C]choline (15 mCi/mmol) were purchased from the Radiochemical Centre (Amersham). The following drugs were obtained commercially: 3-isobutyl-1-methylxanthine (IBMX), dopamine hydrochloride, naloxone hydrochloride (Sigma), [D-Ala^{2},D-Leu^{2}]enkephalin (DADLE), [D-Ala^{2},MePhe^{4},Gly-ol^{5}]enkephalin (DAGO), [D-Pen^{2},D-Pen^{5}]enkephalin (DPDPE) (Bachem). (−)Sulpiride was a gift from DeLagrange and bremazocine from Sandoz. 6,7-Dihydroxy-N,N-dimethyl-2-aminotetralin (TL-99) was kindly donated by Dr A.S. Horn (University of Groningen, Groningen, The Netherlands) and the cyclic AMP antibody by Dr G. Brooker

(Georgetown University, Washington, DC, USA). N-Phenyl-N-[1-(2-(p-isothiocyano)phenylethyl)-4-piperidinyl]propanamide (fentanyl isothiocyanate, FIT) was synthesized in the laboratory of Dr K.C. Rice (NIADDK, Bethesda, USA).

3. Results

3.1. Effects of FIT on presynaptic μ, δ and κ receptors

The electrically evoked release (in excess of spontaneous outflow) of [^{3}H]NA (cortex slices), [^{14}C]ACh (striatal slices) and [^{3}H]DA (striatal slices) amounted to about 5, 6.5 and 2.5% of the total tissue radioactivity, respectively. [^{3}H]NA release was reduced dose dependently (fig. 1) by the δ/μ agonist DADLE and the selective μ agonist DAGO (0.01-1 μM) but not by the selective δ agonist DPDPE (0.01-1 μM) nor by the high-affinity κ agonist bremazocine (0 001-0 3 μM). In contrast, [^{3}H]DA release was inhibited by bremazocine only whereas the electrically evoked release of [^{14}C]ACh was inhibited by both DADLE and DPDPE but not by bremazocine nor by DAGO.

The inhibitory effect of a maximally effective concentration of DADLE (1 μM) on [^{14}C]ACh release was antagonized dose dependently (table 1) on preincubation of the slices with the δ-opioid receptor-selective irreversible ligand FIT (0.1-1 μM) for 60 min. In contrast, the inhibitory effect of the 2-aminotetralin TL-99 (0.1 μM), which displays a high affinity for D-2 DA receptors (Horn et al., 1982), on [^{14}C]ACh release was not altered by preincubation of the slices with FIT. Moreover, whereas preincubation of the slices with 1 μM FIT completely prevented the inhibitory effect of both DADLE (1 μM) and DPDPE (1 μM) on the electrically evoked release of [^{14}C]ACh, the inhibi-

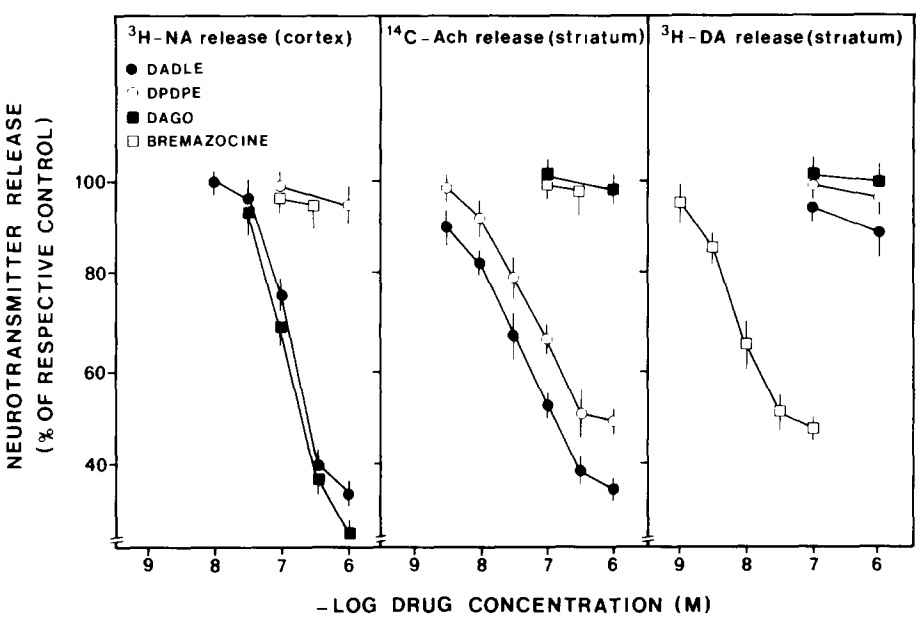

Fig 1. Effect of opioid receptor agonists on the electrically evoked release of neurotransmitters from rat brain slices. After labelling with either [^{3}H]NA (cortex slices) or [^{14}C]choline and [^{3}H]DA (striatal slices), the slices were superfused and stimulated electrically at t = 50 for 10 min Drugs were added to the superfusion medium 20 min prior to depolarization Control [^{3}H]NA, [^{14}C]ACh and [^{3}H]DA release in excess of spontaneous outflow, i e. in the absence of drugs, amounted to 4 9±0.1, 6.6±0.2 and 2.5±0 1% and spontaneous outflow of radioactivity amounted to 2.3±0.1, 1 9±0 1 and 2.9±0.2% of total tissue radioactivity, respectively. The data, expressed as percent of respective control release, represent means±S E M of 12-16 observations Observations were made in quadruplicate

TABLE 1

Effect of FIT on the inhibitory effects of DADLE and TL-99 on the electrically evoked [^{14}C]ACh release from striatal slices Slices were preincubated for 45 min in the absence or in the presence of different concentrations of FIT and were subsequently incubated for an additional 15 min with the media containing [^{14}C]choline. The slices were superfused after labelling and were stimulated electrically for 10 min at t = 50 min Drugs were added to the superfusion medium 20 min prior to stimulation The control [^{14}C]ACh release in the absence of DADLE and TL-99 amounted to 6 5±0.3, 6 2±0 1, 6 4±0 4 and 6 0±0 8% of total tissue ^{14}C following preincubation of the slices with 0, 0 1, 0.3 and 1 0 μM FIT, respectively The data, expressed as percentage of the respective control release, are means ± S.E.M of 8 observations. Observations were made in quadruplicate

Preincubation	[^{14}C]ACh release (% of respective control) in the presence of	
	1.0 μM DADLE	0 1 μM TL-99
–	34±2	44±3
0 1 μM FIT	48±3 [a]	N.D.
0.3 μM FIT	82±4 [a]	N.D.
1 0 μM FIT	102±4 [a]	47±5

[a] Significantly higher than following preincubation in the absence of FIT N.D., not determined.

tory effects of opioids on [^{3}H]NA and [^{3}H]DA release appeared to be independent of whether the slices were preincubated with 1 μM FIT or not (table 2).

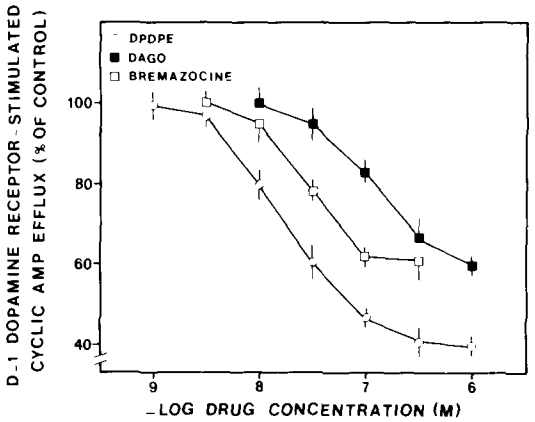

Fig. 2. Effect of opioid receptor agonists on D-1 DA receptor-stimulated cyclic AMP efflux from striatal slices. Striatal slices were superfused and a basal efflux fraction was collected from t = 60-80 min Drugs were added at t = 80 min and a stimulated 20-min fraction was collected at t = 100 min. The stimulated control cyclic AMP efflux induced by 40 μM DA + 10 μM (−)sulpiride amounted to 374±8% of basal efflux. Basal cyclic AMP efflux (in the absence of drugs) averaged 21±0.6 fmol/min. The data, expressed as percent of the stimulated control cyclic AMP efflux induced by DA + (−)sulpiride alone, are means ± S E M. of 12-16 observations. Observations were made in quadruplicate

3.2. Effect of FIT on opioid receptors mediating inhibition of DA-sensitive adenylate cyclase

When striatal slices were exposed to 40 μM DA in the presence of 10 μM of the selective D-2 DA receptor antagonist (−)sulpiride, the resulting

TABLE 2

Effect of 1 μM FIT on the inhibitory effects of opioids on the electrically evoked release of various neurotransmitters from brain slices The slices were preincubated for 45 min in the absence or presence of FIT (1 μM) and were subsequently labelled (cortex slices [^{3}H]NA; striatal slices. [^{14}C]choline and [^{3}H]DA) in the media for 15 min. After labelling, the slices were superfused and stimulated electrically at t = 50 min for 10 min. Drugs were added to the superfusion medium 20 min prior to depolarization. The control [^{3}H]NA, [^{14}C]ACh and [^{3}H]DA release amounted to 5.2±0 2, 6 8±0.4 and 2 7±0 1% of total tissue radioactivity, respectively, upon preincubation in the absence of FIT and to 5.1±0 3, 6.3±0.2 and 2.3±0.2% in the case of FIT-pretreated slices. The data, expressed as percent of respective control release, are means ± S.E M. of 8-12 observations Observations were made in quadruplicate.

Opioids	Neurotransmitter release (% of respective control)					
	[^{3}H]NA		[^{14}C]ACh		[^{3}H]DA	
	– [a]	FIT	–	FIT	–	FIT
1 μM DADLE	41±3	43±2	34±2	102±4 [b]	93±4	101±3
1 μM DPDPE	98±4	N.D.	48±2	97±2 [b]	96±2	93±4
1 μM DAGO	34±2	30±2	99±1	N D	92±4	N.D
0 1 μM bremazocine	98±3	N D.	94±4	96±2	47±3	45±4

[a] Preincubation. [b] Significantly higher than upon preincubation in the absence of FIT ($P < 0.001$) N.D., not determined

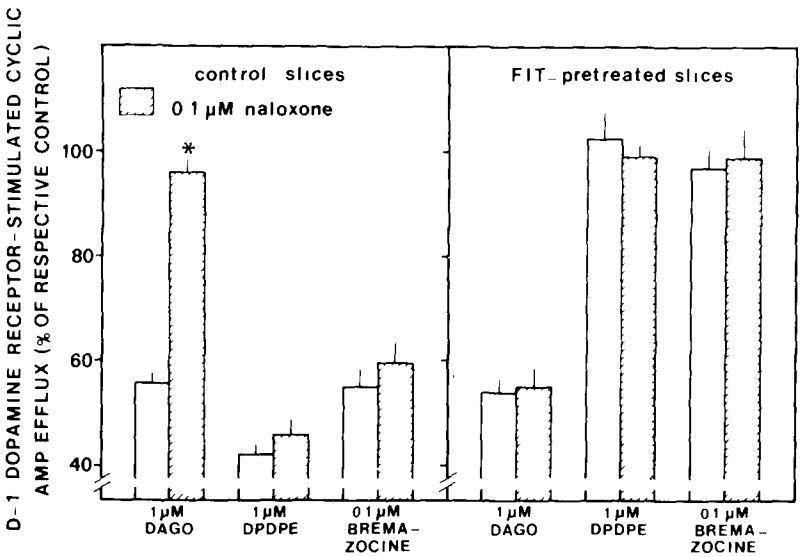

Fig 3. Antagonism of opioid-mediated inhibition of D-1 DA receptor-stimulated cyclic AMP efflux from striatal slices by naloxone and the effect of FIT thereon. Slices were preincubated for 60 min in the absence or presence of 1 μM FIT. The slices were then superfused and a basal efflux fraction was collected from t = 60-80 min Drugs were added at t = 80 min and a stimulated 20-min cyclic AMP efflux fraction was collected at t = 100 min The stimulated cyclic AMP efflux induced by 40 μM DA + 10 μM (−)sulpiride amounted to 353 ± 6% of the basal efflux from control slices and to 317 ± 8% from slices pretreated with FIT The basal cyclic AMP efflux from control and FIT-pretreated slices averaged 15 6 ± 0 4 fmol/min and 16 2 ± 0 3 fmol/min, respectively Naloxone itself did not significantly affect the cyclic AMP efflux. The data, expressed as percent of the stimulated control cyclic AMP efflux induced by DA + (−)sulpiride alone, are means ± S.E M of 16-20 observations Observations were made in quadruplicate. * Significantly higher than in the absence of naloxone ($P < 0.001$)

selective activation of D-1 DA receptors (see Stoof and Kebabian, 1981; Schoffelmeer et al., 1986; 1987) caused a more than 3.5-fold increase of cyclic AMP efflux. This DA-stimulated cyclic AMP efflux was strongly reduced in a dose-dependent fashion by DPDPE (0.001-1 μM), DAGO (0.01-1 μM) and bremazocine (0.003-0.3 μM) (fig. 2. The preferential μ-opioid receptor antagonist, naloxone, did not alter (fig. 3) the inhibitory effects of DPDPE (1 μM) and bremazocine (0.1 μM) in a concentration (0.1 μM) which fully antagonized the effect of 1 μM DAGO. In contrast, preincubation of striatal slices with 1 μM FIT for 60 min prevented the inhibitory effect of DPDPE and bremazocine on DA-stimulated cyclic AMP efflux without altering the effect of DAGO.

3.3. Effect of FIT on the blockade of μ receptors by naloxone

A very intriguing difference was observed when the interaction of FIT with functional μ receptors

TABLE 3

Antagonism by naloxone of the inhibitory effects of opioids on the electrically evoked release of [^{3}H]NA from cortex slices and the effect of FIT thereon The slices were preincubated for 45 min in the absence or presence of FIT (1 μM). The slices were subsequently labelled with [^{3}H]NA in the media for 15 min, superfused and stimulated electrically for 10 min at t = 50 min Drugs were added to the superfusion medium 20 min prior to depolarization The control [^{3}H]NA release amounted to 5 4 ± 0 2% of total tissue tritium and to 5 1 ± 0 1% upon preincubation of the slices with FIT. Naloxone itself did not affect [^{3}H]NA release The data, expressed as percent of the respective control release, are means ± S E M of 12-16 observations Observations were made in quadruplicate

Opioids	[^{3}H]NA release (% of respective control)	
	− [a]	FIT
1 μM DAGO	19 ± 2	23 ± 2
+ 0 1 μM naloxone	83 ± 4 [b]	86 ± 3 [b]
1 μM DADLE	30 ± 1	33 ± 2
+ 0 1 μM naloxone	91 ± 3 [b]	96 ± 2 [b]

[a] Preincubation. [b] Significantly higher than in the absence of naloxone ($P < 0.001$)

was studied further in the two functional set-ups described above. Thus, while preincubation of striatal slices with 1 μM FIT for 60 min did not at all affect the inhibitory effect of DAGO on DA-stimulated cyclic AMP efflux, it completely prevented the antagonism of this inhibitory effect by a maximally effective concentration (0.1 μM) of naloxone (fig. 3). In contrast, the inhibitory effect of DAGO as well as that of DADLE on the electrically evoked release of [^{3}H]NA from cortex slices was fully antagonized by naloxone, irrespective of whether the slices were pretreated with 1 μM FIT or not (table 3).

4. Discussion

Most studies on the molecular basis of opioid receptor heterogeneity in the brain indicate that the pharmacologically distinct μ, δ and κ receptors may represent independent and structurally different entities. Receptor autoradiographic investigations have revealed that these three opioid receptor types each have a characteristic regional distribution in the brain (Tempel and Zukin, 1987) while ligand-receptor binding studies have indicated that the μ, δ and κ receptor binding sites do not appear at the same time during ontogenetic development of the brain (Spain et al., 1985; McDowell and Kitchen, 1987; Kornblum et al., 1987). Studies aimed at isolating μ, δ and κ receptors have provided strong evidence for the existence of structurally different opioid receptor recognition sites. However, it remains to be established whether the reported differences in molecular weight of the polypeptides involved reside in the amino acid composition of the opioid receptors or in post-translational modifications, i.e. whether separate genes code for the μ, δ and κ receptors (Itzhak et al., 1984; Gioannini et al., 1985; Howard et al., 1986; Simon, 1987).

Regarding the functional aspects of opioid receptor heterogeneity in the brain, we recently presented evidence that activation of μ, δ and κ receptors causes differential inhibition of the potassium-induced release of various neurotransmitter from rat brain slices (Mulder et al., 1984). This hypothesis is supported by our present data indicating that the μ receptor agonist DAGO, the δ agonist DPDPE and the high affinity κ ligand bremazocine (Goldstein, 1987) display very high selectivity towards the opioid receptors mediating inhibition of the electrically evoked release of [^{3}H]NA (cortex slices), [^{14}C]ACh (striatal slices) and [^{3}H]DA (striatal slices), respectively. Indeed, the δ receptor-preferring agonist DADLE (Paterson et al., 1983) showed an almost 10-fold higher affinity for opioid receptors mediating inhibition of [^{14}C]ACh release than for the receptors causing a reduction of [^{3}H]NA release, and was inactive when the release of [^{3}H]DA was studied. Furthermore, whereas naloxone was previously shown to be a selective antagonist for opioid receptors mediating the inhibitory effect of Leu-enkephalin on [^{3}H]NA release, we now showed that the δ receptor-selective, irreversible ligand FIT (Rice et al., 1983) prevented the inhibitory effect of DPDPE and DADLE on [^{14}C]ACh release only. Together, these data leave little doubt that noradrenergic nerve terminals, cholinergic interneurons and dopaminergic nerve terminals in these brain regions are equipped with homogeneous populations of functional μ-, δ- and κ-opioid receptors, respectively.

Other opioid receptor models in addition to independent opioid receptor types have recently been proposed, which have in common that the different opioid binding sites could interact allosterically as physically associated proteins in cell membranes. In this case, binding of an (ant)agonist to a given opioid receptor type could interfere with the interaction of ligands with the other (associated) receptor type(s). In addition, the pharmacological characteristics of such an opioid receptor complex might be regulated by local factors in its immediate environment. These factors could vary between different species and cells and may change, e.g. during brain development. Thus Rothman et al. (1985) suggested, on the basis of ligand-receptor binding studies, the existence of a receptor complex with non-competitively interacting μ and δ binding sites in rat brain. Also receptor autoradiographic data reported by Bowen et al. (1981) indicated the occurrence of such a receptor complex in patches of rat neostriatum, which could interconvert between a μ and a δ

conformation. Holaday and coworkers presented physiological evidence for mutually interacting opioid receptor types involved in the anticonvulsant properties of opioids (Holaday and Tortella, 1984) and in endotoxic shock hypotension (Long et al., 1984). Finally, Smith et al. (1983) integrated physical and pharmacological data and proposed a protein-lipid model of the opioid receptor termed the 'multiple site β-endorphin receptor'. We have recently obtained functional evidence for the existence of such closely associated μ and δ receptors in rat neostriatum (Schoffelmeer et al., 1986; 1987). We showed in these experiments that μ- as well as δ-opioid receptors mediate the inhibitory effects of opioids on D-1 DA receptor-stimulated adenylate cyclase, and that selective activation of δ receptors blocked μ receptor efficacy. It was proposed that these functional receptors both reside on the same cells in the neostriatum, possibly sharing inhibitory guanine nucleotide binding proteins. Interestingly, we found then that simultaneous activation of D-2 DA receptors appeared to prevent the inhibitory effect of δ but not of μ receptor activation on DA-sensitive adenylate cyclase activity, indicating a permissive role of D-2 receptors in the local environment of the purported opioid receptor complex.

Our present data showing that preincubation of striatal slices with 1 μM FIT completely prevented the inhibitory effect of DPDPE on D-1 DA receptor-stimulated cyclic AMP efflux from the slices without changing the effect of DAGO further support the view that both μ and δ receptors are coupled to DA-sensitive adenylate cyclase. Moreover, since the inhibitory effect of bremazocine on D-1 DA receptor-stimulated cyclic AMP efflux was also blocked by preincubation of striatal slices with FIT, the κ-opioid receptors do not seem to be coupled to DA-sensitive adenylate cyclase. Interestingly, bremazocine somehow seems to act as a potent δ agonist in this functional set-up as suggested previously (Schoffelmeer et al., 1987). In this respect it is worth noting that bremazocine has also been shown to be a potent antagonist for certain μ- and δ-opioid receptors in the brain (Dunwiddie et al., 1987), including the presynaptic receptors described above (Mulder et al., in preparation). These observations, added to the selective κ-opioid receptor-mediated inhibitory effect on [^{3}H]DA release in the neostriatum (Mulder et al., 1984; this study), suggest that bremazocine might represent a highly selective tool to diminish dopaminergic neurotransmission in the brain.

We recently investigated the effects of the highly selective κ-opioid receptor agonist, U 50,488, on the functional opioid receptors in the brain regions now described. The results obtained with this drug fully support the above interpretation of our present data. Thus, in concentrations below 1 μM, U 50,488 did not affect the release of [^{3}H]NA and [^{14}C]ACh whereas the κ agonist strongly reduced [^{3}H]DA release. Moreover, unlike bremazocine, U 50,488 did not inhibit dopamine-sensitive adenylate cyclase (Heijna et al., in preparation).

Most importantly, whereas the preferential μ-opioid receptor antagonist, naloxone (0.1 μM), fully antagonized the inhibitory effect of DAGO on D-1 DA receptor-stimulated cyclic AMP efflux, leaving the effect of DPDPE and bremazocine unchanged, FIT (1 μM) did not alter the effect of DAGO but prevented its antagonism by naloxone. Obviously, this could have been due to an irreversible interaction of FIT with a site on μ-opioid receptors distal from the agonist recognition site, somehow preventing the binding of naloxone. However, if structural homogeneity is assumed for μ-opioid receptors throughout the brain this explanation seems very unlikely in view of the fact that naloxone completely antagonized the μ receptor-mediated effect of DAGO on the electrically evoked release of [^{3}H]NA from cortex slices (see above) irrespective of whether or not the slices were pretreated with FIT. Therefore, it seems much more likely that, although naloxone and FIT (in the concentrations used) interact selectively with μ and δ receptors, these drugs interact with a binding site shared by μ- and δ-opioid receptors coupled to DA-sensitive adenylate cyclase. We have presented this interaction between FIT and naloxone schematically in fig. 4.

In conclusion, our present data indicate that presynaptic μ-, δ- and κ-opioid receptors may exist as independent functional entities in rat brain. However, the μ and δ receptors appear to be

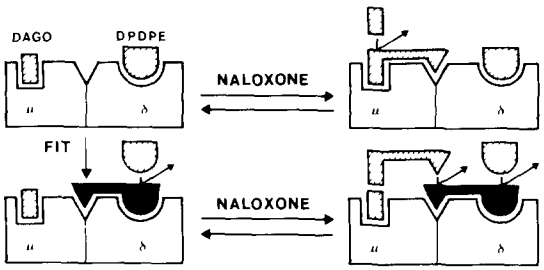

Fig. 4 Schematic representation of the opioid receptor complex presumed to mediate the effects of opioids on DA-sensitive adenylate cyclase in rat neostriatum. This hypothetical opioid receptor complex can be activated by agonists such as DAGO and DPDPE, selectively interacting with the distinct μ and δ recognition sites, respectively Naloxone and FIT block these μ and δ binding sites selectively, respectively, and it is proposed that they interact with an additional common binding site shared by the associated μ- and δ-opioid receptors Thus, irreversible binding of FIT to the δ-binding site impairs the ability of naloxone to interact with the μ binding site.

physically associated at the level of adenylate cyclase stimulated by activation of postsynaptic D-1 DA receptors in the neostriatum. Since we have now shown that these latter μ and δ receptors can be selectively activated by highly selective agonists such as DAGO and DPDPE and have reported earlier that selective δ receptor activation blocks μ receptor efficacy (Schoffelmeer et al., 1987), these functional opioid receptors seem to have separate but allosterically interacting agonist recognition sites mediating the inhibitory effect of opioids on DA-sensitive adenylate cyclase. Moreover, since the interation between the δ-opioid receptor-selective, irreversible ligand, FIT, and the μ-opioid receptor antagonist, naloxone, indicates that these μ and δ receptors may share a common ligand binding domain, we suggest tentatively that they exist as constituents of a functional opioid receptor complex. A recent ligand-receptor binding study with membrane preparations (whole brain minus cerebellum) reported by Bowen et al. (1988) led the authors to suggest that although δ-opioid receptors are coupled inhibitorily to adenylate cyclase by way of guanine nucleotide binding proteins, this may not hold true for the physically associated μ and δ binding sites in rat brain. Obviously, our present functional data argue strongly against this proposal although it cannot be excluded that opioid receptor binding sites distinct from those mediating inhibition of DA-sensitive adenylate cyclase (also measured in binding assays) and in brain regions other than the neostriatum function via other mechanisms.

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