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Differences in the characteristics of opioid receptor binding in the rat and marmoset

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Abstract—The density of μ and δ opioid binding sites in homogenates of rat and marmoset brain was determined using radiolabelled peptides with high receptor selectivity. In addition the affinity of μ and δ receptor binding of five drugs of the fentanyl series was measured. The ratio of $\mu:\delta$ sites was similar within each species but the total receptor binding capacity in the rat was double that of the marmoset. The binding affinities for the δ site were dissimilar between the species and supports the hypothesis that the structure of this receptor shows phylogenetic differences. In contrast, μ site binding affinity was identical in rat and marmoset.

In the nervous system of many animals there exist at least three different types of binding site for opioid compounds and species differences have been shown in the proportions, distributions and densities of these sites (Gillan & Kosterlitz 1982; Moskowitz & Goodman 1984; Robson et al 1985). However, some of the radioligands employed in the characterization of these binding sites lacked high selectivity for one class of site, so that for example, 'suppression' (Spain et al 1985) of the μ binding of the poorly selective ligand [D-Ala^2, D-Leu^5] enkephalin is required in order to estimate δ binding. Radiolabelled opioid peptides with high selectivity for μ (Kosterlitz & Paterson 1981) and δ (Mosberg et al 1983) receptors have recently become available and now allow accurate determinations of μ and δ binding sites. There is only limited information on opioid binding in primates and we report here binding studies with highly selective ligands for the μ and δ receptors in the brains of marmosets and rats which indicate differences in the receptors between the two species.

Methods

Preparation of tissue homogenates and radioligands. Adult male Wistar albino rats (University of Surrey strain, 200–300g) were decapitated and the brains rapidly removed and placed into ice-cold buffer (Tris HCl 50mM, pH 7·4). Adult (350–400g) marmosets (*Callithrix jacchus*) of either sex were anaesthetized with halothane (5% in O_2) then placed in a chamber flushed with CO_2. When no ventilatory movements were observed over a 2 min period, the animals were decapitated, and the brains removed as above. The procedure for preparation of membranes for use in binding studies was identical to that of Gillan & Kosterlitz (1982).

Radiolabelled peptides were purified by reverse phase HLPC as previously described (Bailey & Kitchen 1984) and stored as a frozen solution at −20°C.

Ligand binding experiments. Saturation binding experiments were based on the methods of Gillan & Kosterlitz (1982) and Cotton et al (1985), and incubation conditions were identical except for the range of ligand concentrations used and the duration of incubation. Non-specific and total binding of tritiated [D-Ala^2-MePhe^4-Glyol^5]enkephalin ([^{3}H] DAGO, 0·03–9·0nM, 12 concentrations) and tritiated [D-Pen^2, D-Pen^5] enkephalin ([^{3}H]DPDPE, 0·20–25nM, 10 concentrations) were determined in the presence and absence, respectively, of 100-fold excess of unlabelled diprenorphine or naloxone. Incubations in 50mM Tris HCl buffer, pH 7·4, were of 60 min duration at 25°C.

For each radioligand and tissue homogenate, the time required for equilibrium binding to be reached was determined. In addition, experiments were performed to ensure that the amount of tissue used was on the linear portion of the relationship between tissue content and the amount of specific binding, and that the concentrations of naloxone and diprenorphine were sufficient to displace all the specifically bound radioligand. The protein content of membranes from the brain was measured according to the method described by Lowry et al (1951).

For inhibition studies, the concentration of tritiated ligand was held constant at K_D ([^{3}H]DAGO in rat and marmoset brain homogenates and [^{3}H]DPDPE in marmoset brain homogenates) or 0·5 K_D ([^{3}H]DPDPE in rat brain homogenates) whilst that of the unlabelled fentanyl-class of drug was increased (6–8 concentrations). In addition, the incubation times were increased by 30–40%, since the presence of unlabelled inhibiting drug increases the time required for equilibrium binding to be reached according to Weiland & Molinoff (1981).

Data from saturation experiments were analysed by Scatchard plots and those from inhibition assays by Hill plots. Slopes and intercepts were calculated from the values of at least four independent experiments, derived from linear regression of the data. The K_I values were calculated from the IC50 values obtained from Hill plots according to the method of Cheng & Prusoff (1973).

Drugs. Tyrosyl-3,5-[^{3}H]DAGO (57·5Ci mmol^{-1}) and tyrosyl-3,5-[^{3}H]DPDPE (28·0Ci mmol^{-1}) were purchased from Amersham International (UK). Alfentanil hydrochloride hydrate, and carfentanil, fentanyl and sufentanil as citrate salts were gifts from Janssen Pharmaceuticals (Belgium). The following were also gifts: naloxone hydrochloride (Dupont, UK); diprenorphine hydrochloride (Reckitt & Colman, UK); lofentanil cis (−) oxalate (Professor P. B. Bradley, Birmingham). All unlabelled drugs were dissolved in 50mM Tris HCl buffer (pH 7·4 at 4°C) and stored at 4°C for periods of no longer than 4 weeks.

Results

Binding of tritiated analogues of enkephalin. The specific binding of both [^{3}H]DAGO and [^{3}H]DPDPE to membranes of brains of marmosets (Figs 1, 2) and rat demonstrated a rectangular hyperbolic relationship to free ligand concentration and Scatchard plots were linear. In brain homogenates from rat and marmoset, [^{3}H]DAGO bound with identical affinity (Table 1). However, the binding affinity of [^{3}H]DPDPE for marmoset δ sites was significantly greater than for rat δ sites ($P < 0·001$). The binding capacities of homogenates of marmoset brain for both the radiolabelled peptides were half those of rat brain although the number of μ and δ sites were similar within each species

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Table 1. Binding affinity and capacity for [³H]DAGO and [³H]DPDPE of brain homogenates from rat and marmoset.

		Marmoset	Rat
[³H]DAGO	K_D	1·1±0·2	1·1± 0·2
	B_{max}	75·0±2·0	167·0±11·0
[³H]DPDPE	K_D	1·6±0·3	8·3± 0·6
	B_{max}	74·0±2·0	149·0± 9·0

Values are mean ± s.e. mean of 4–5 independent determinations of K_D (nM) or B_{max} (fmol (mg protein)$^{-1}$).

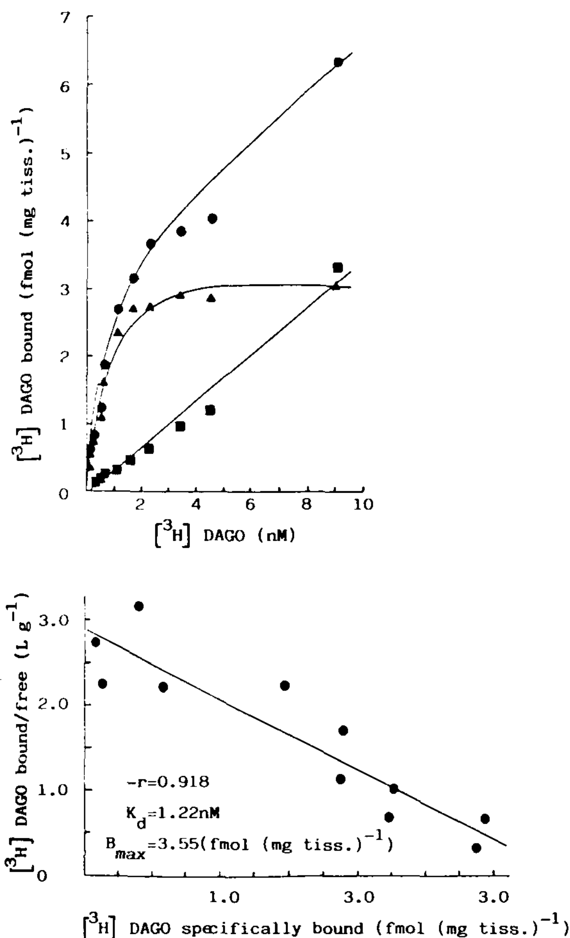

FIG. 1. Saturation binding characteristics of [³H]DAGO in marmoset whole brain. The upper graph shows the concentration dependency of total (circles), non-specific (squares) and specific (triangles) binding of DAGO to marmoset brain membranes. The lower graph is the Scatchard plot derived from the specific binding data. The plots are typical of four independent determinations. The protein concentration was 47mg g^{-1} wet weight tissue, giving B_{max} = 75·6 fmol (mg protein)$^{-1}$.

(Table 1). Non-specific binding of [³H]DAGO was the same (8%) in both species, whereas that of [³H]DPDPE was greater in rat (31%) than in marmoset (16%). The time required for half-maximal specific binding of the peptides to be attained was greater in marmoset than in rat for both [³H]DAGO (14 vs 7·5 min) and [³H]DPDPE (17 vs 10·5 min).

Inhibition studies. Inhibition experiments produced Hill plots with slopes and coefficients of linear regression near unity (Table 2). The rank order of affinities of the 5 drugs tested at the μ and δ binding sites were identical in rats, but showed minor dissimilarities between the two sites and with the order in marmosets. Compounds with the highest affinities (lowest K_I

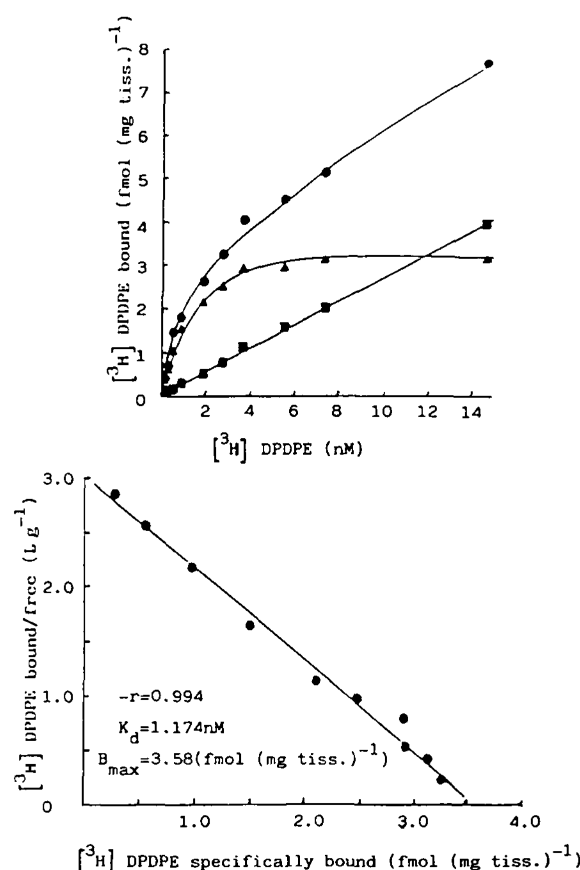

FIG. 2. Saturation binding characteristics of [³H]DPDPE in marmoset whole brain. The upper graph shows the concentration dependency of total (circles), non-specific (squares) and specific (triangles) binding of DPDPE to marmoset brain membranes. The lower graph is the Scatchard plot derived from the specific binding data. The plots are typical of four independent determinations. The protein concentration was 47mg g^{-1} wet weight tissue, giving B_{max} = 76·2 fmol (mg protein)$^{-1}$.

values) possessed the lowest site selectivity, and correlation between log μ-site K_I and log selectivity for the μ site was strong in both species (Fig. 3). For all the drugs of the fentanyl series, μ selectivity was greater in marmoset than in rat, and this increase was particularly notable for carfentanil. In rat brain, carfentanil displayed no site preference, having equal affinity for μ and δ sites. In marmoset brain, however, this compound exhibited a preference for μ over δ sites of 20.

Discussion

Species differences in the density of μ and δ opioid binding sites have been reported (Robson et al 1985; Meunier et al 1983; Gillan & Kosterlitz 1982). In most studies [³H]DAGO has been used to investigate the μ site, and K_D values are consistently close to 1nM in guinea-pigs (Kosterlitz & Paterson 1981), rabbit (Robson et al 1985) and rat (Gillan & Kosterlitz 1982). The affinity of [³H]DPDPE for the δ site, however, shows considerable variation from species to species and even with strains. For example, K_D values for [³H]DPDPE binding have been reported which range from 2·7nM (James & Goldstein 1984) and 4·2nM (Corbett et al 1984) in two strains of guinea-pig to 5·3nM (Cotton et al 1985) and 11·1nM in rat (Mansour et al 1986). Because these ligands are opioid agonists, it is important to note that all the values quoted were obtained in 50mM Tris HCl buffer at pH 7·4. It is therefore unlikely that the apparent species differences on [³H]DPDPE binding affinity are a reflection of differences in

Table 2. Displacement of [^{3}H]DAGO and [^{3}H]DPDPE binding by fentanyl derivatives

	Lofentanil	Carfentanil	Sufentanil	Fentanyl	Alfentanil
Marmoset					
K_I at μ site (nM)	0·055±0·006	0·22±0·08	0·24±0·05	1·32±0·35	14·4±4·2
	(0·93±0·10)	(1·08±0·18)	(1·33±0·24)	(0·79±0·07)	(0·96±0·19)
K_I at δ site (nM)	0·42±0·05	4·41±0·40	12·9±4·5	127±17	15030±8700
	(1·02±0·07)	(1·07±0·05)	(1·01±0·16)	(0·87±0·07)	(1·01±0·16)
Selectivity for μ site	8·0±0·6	20·0±7·1	65±14	96±13	1330·0±400
Rat					
K_I at μ site (nM)	0·13±0·02	0·42±0·04	0·77±0·10	3·3±0·45	38·9±3·7
	*(1·34±0·11)	(1·00±0·07)	*(0·78±0·05)	(1·01±0·06)	*(0·81±0·05)
K_I at δ site (nM)	0·72±0·09	0·42±0·02	20·2±1·52	246±41	21200±3500
	(1·40±0·15)	(0·99±0·03)	(0·97±0·07)	(0·83±0·09)	(0·79±0·08)
Selectivity for μ site	6·7±0·6	1·1±0·06	25·0±1·9	83·0±8·4	600±50

Each value is the mean±s.e. mean of at least 4 independent determinations.
The slopes of Hill plots are given in parentheses.
* Significantly different from unity, $P<0·05$.

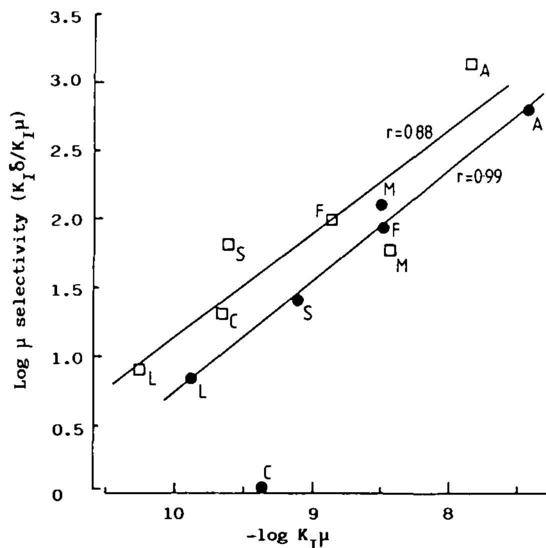

FIG. 3. Correlation between affinity and selectivity for the μ binding site in rat and marmoset brain homogenates. $K_I\mu$ and $K_I\delta$ are the μ and δ binding site affinities (nM), respectively, calculated from the IC50 values obtained in inhibition experiments. Closed circles indicate values in rat and open squares the values in marmoset. The identity of the points for alfentanil, carfentanil, fentanyl, lofentanil and sufentanil are indicated by initial letters.

experimental design. The similarities of the K_D values reported here in rat and marmoset for [^{3}H]DAGO are in accord with the suggestion that μ site structure and/or membrane environment has been well conserved across phylogenetic lines. The marked differences in site binding affinity of the highly selective ligand [^{3}H]DPDPE between species are in such clear contrast with the findings at the μ site that they point to dissimilarities in the structure or environment of the δ site in different species. The dissimilarities in the binding characteristics of some of the fentanyls are also supportive of greater species differences in respect of δ than μ binding. Carfentanil, for example, binds to the marmoset δ site with 10-fold lower affinity than to the equivalent site in rat ($P<0·001$) although the comparative μ affinities are not significantly different ($P>0·05$). The differences in μ site affinity for the fentanyl series between rat and marmoset follow a consistent pattern, being slightly greater in the latter species, and may reflect small differences in the receptor or in its environment within the membrane. The finding that the tritiated peptides reached equilibrium with their binding sites somewhat later in marmoset brain homogenates than was the case with rat may be taken to support the latter contention. Additional evidence is provided by the markedly greater degree of non-specific binding of the δ ligand in rat, a feature which renders determination of δ binding difficult in regions of rat brain relatively deficient in these sites (Yeadon & Kitchen 1988). In contrast, the non-specific binding of the μ ligand is identical in the two species.

In conclusion, there are differences in the binding characteristics of the ligands investigated in brain homogenates of rat and marmoset; these dissimilarities are most marked at the δ opioid binding site.

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Increased vasodilator response to acetylcholine of renal blood vessels from diabetic rats

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Abstract—The vasodilator effect of acetylcholine (ACh) and nitroprusside and the vasoconstrictor effect of noradrenaline was assessed in the perfused kidney of streptozocin diabetic rats. Compared with control animals injected with acidified saline, the renal vasoconstrictor effect of noradrenaline was increased in diabetic rats both in terms of the dose required to produce 50% of the maximal effect (EC50) and in the maximal response achieved. The renal vasodilator effect of ACh (but not nitroprusside) was similarly enhanced in diabetic animals. The effect of ACh (but not nitroprusside) in the perfused kidney of both control and diabetic rats was reduced or abolished by mepacrine (10 μM), metyrapone (10 μM) or methylene blue (100 μM) suggesting that ACh exhibits vasodilator activity in the rat kidney by virtue of releasing endothelium derived relaxing factor (EDRF). These results are in contrast to previous published reports demonstrating reduced biosynthesis of EDRF in the aorta of diabetic rats. The mechanism which underlies the increased renal vascular response to ACh is not known. However, increased endothelial cell turnover or cholinoceptor number, elevated activity of enzyme(s) which synthesise EDRF or hyperresponsiveness of vascular smooth muscle to released EDRF should all be considered.

The vasodilator effect of acetylcholine (ACh) in preconstricted rings or bands of isolated arteries and on resistance blood vessels of perfused organs is mediated by a chemically unstable substance called endothelium derived relaxing factor (EDRF) (Furchgott 1983). The synthesis and release of EDRF from vascular endothelial cells, by regulating the calibre of resistance blood vessels, may play a part in controlling vascular perfusion within individual organs (Vanhoutte 1987). Conversely, impaired production of EDRF may contribute to the vasospasm of coronary arteries associated with angina pectoris (Ganz & Alexander 1985) and with the increased peripheral resistance in hypertension (Konishi & Su 1983; Lockette et al 1986). Recently, Oyama et al (1986) provided evidence that aortic EDRF biosynthesis was similarly reduced in rats made diabetic by streptozocin injection. This finding raises the possibility that a deficiency of vascular EDRF may contribute to the cardiovascular pathology of this condition. Since diabetes in man is associated with profound changes in the structure and function of the renal vasculature we have examined the response to an endothelium dependent (ACh) and an endothelium independent (nitroprusside) vasodilator drug of the rat perfused kidney taken from streptozocin diabetic rats.

Methods

Induction of experimental diabetes in rats. Male rats (Wistar, 250–300 g) were injected intraperitoneally with streptozocin (80 mg kg^{-1}) dissolved in saline (0·9% NaCl) acidified to pH 4·2 with HCl (0·1M). Control animals were injected with an equal volume of acidified saline. Blood samples (0·5 mL) were collected by cardiac puncture of ether anaesthetized animals, anticoagulated with heparin (2 u mL^{-1}) and centrifuged (1,000 g, 20 min, room temperature) to prepare plasma. Animals were killed either before (T=0) or 12 days after streptozocin or acidified saline injection. Plasma glucose was assayed spectrophotometrically at a wavelength of 520 nm using a commercially available diagnostic kit (Sigma).

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Perfusion of rat kidney. Kidney perfusion was performed essentially as described by Armstrong et al (1976). Briefly, the left rat kidney cannulated via the descending abdominal aorta was removed to a heated, water-jacketed chamber. Preparations were perfused (8 mL min^{-1}) with warmed (37°C), oxygenated (95% O_2 : 5% CO_2) Krebs solution (composition, mM; NaCl 118, KCl 4·75, $MgSO_4$ 1·19, $NaHCO_3$ 25, K_2HPO_3 1·19, $CaCl_2$ 1·9, glucose 11·1, pH 7·2) containing indomethacin (8 μM) to prevent release of vasodilator prostanoids such as prostacyclin (PGI_2). Perfusion pressure was monitored continuously by means of a Bell & Howell pressure transducer connected to a Devices pen recorder. Drugs were injected in volumes of less than 20 μL to prevent vascular effects due to an injection artifact. After an equilibration period of 30 min, noradrenaline was injected using a dose cycle time of 3 min. Vasodilator responses to ACh and nitroprusside were assessed in so-called "high tone" preparations which were partially vasoconstricted by inclusion in the perfusing Krebs solution of a concentration of noradrenaline producing approximately 60–80% of the maximal response. A dose cycle time of 5 min was employed for both vasodilator drugs. Inhibitor drugs were added to the perfusing Krebs solution and allowed to perfuse the kidney for 20 min before injection of vasodilator drugs. Preparations were weighed before and after each experiment which typically had a duration of 3–4 h.

Statistics. Results show mean ± s.e. mean with the number of observations shown in parentheses. Statistically significant differences between groups were determined using Student's unpaired *t* test. A probability (*P*) value of 0·05 or less was taken to indicate statistical significance.

Materials. Acetylcholine chloride, histamine dihydrochloride, indomethacin, mepacrine dihydrochloride, methylene blue, metyrapone, sodium nitroprusside, streptozocin and (−)-noradrenaline bitartrate were obtained from Sigma Ltd. Indomethacin was dissolved in Na_2CO_3 (0·5% w/v). All other drugs were dissolved in NaCl (0·9% w/v).