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European Journal of Pharmacology 414 (2001) 189–195

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Analysis of [^{3}H] bremazocine binding in single and combinatorial opioid receptor knockout mice

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Received 26 October 2000; received in revised form 29 January 2001; accepted 2 February 2001

Abstract

Despite ample pharmacological evidence for the existence of multiple μ-, δ- and κ-opioid receptor subtypes, only three genes encoding μ-(MOR), δ-(DOR) and κ-(KOR) opioid receptor have been cloned. The KOR gene encodes κ_1-sites, which specifically bind arylacetamide compounds, and the possible existence of κ-opioid receptor subtypes derived from another κ-opioid-receptor gene, yet to be characterized, remains a very contentious issue. κ_2-Opioid receptors are described as binding sites typically labelled by the non-selective benzomorphan ligand [^{3}H]bremazocine in the presence of μ-, δ- and κ_1-opioid receptor blocking ligands. To investigate the genetic origin of κ_2-opioid receptors, we have carried out homogenate binding experiments with [^{3}H]bremazocine in brains of single MOR-, DOR-, KOR- and double MOR/DOR-deficient mice. Scatchard analysis showed that $68 \pm 12\%$ of the binding sites arise from the MOR gene, $27 \pm 1\%$ from the DOR gene and $14.5 \pm 0.2\%$ from the KOR gene, indicating that the three known genes account for total [^{3}H]bremazocine binding. Experiments in the presence of μ-, δ- and κ_1-opioid receptor suppressor ligands further showed that non-κ_1-opioid receptor labelling can be accounted for by binding to both the μ- and δ-opioid receptors. Finally, [^{3}H]bremazocine binding experiments performed on brain membranes from the triple MOR/DOR/KOR-deficient mice revealed a complete absence of binding sites, confirming definitively that no additional gene is required to explain the total population of [^{3}H]bremazocine binding sites. Altogether the data show that the putative κ_2-opioid receptors are in fact a mixed population of KOR, DOR and predominantly MOR gene products.

Keywords: Bremazocine; κ_2-Opioid receptor; Opioid-receptor knock-out mouse; Opioid pharmacology

1. Introduction

Narcotic analgesics produce their biological actions by activating receptors of the nervous system classified as μ-, δ- and κ-opioid receptors. Despite pharmacological evidence for several μ-, δ- and κ-opioid receptor subtypes (for review, see Fowler and Fraser, 1994; Pasternak, 1993; Traynor, 1989; Traynor and Elliot, 1993), only three genes have been cloned (reviewed in Kieffer, 1995). Although these genes clearly encode one μ-(MOR), one δ-(DOR) and one κ-(KOR) opioid receptor protein, the correspondence between the multiple opioid receptor subtypes defined by the pharmacology and the three identified gene products remains an intense matter of debate (see Befort and Kieffer, 1997; Zaki et al., 1996). The precise assignment of opioid receptor subtypes to cloned receptor proteins, or to yet uncloned gene products, is of critical importance in the search for novel useful opioid therapeutic agents freed from adverse side effects.

For over 15 years, it has been suggested that subtypes of the κ-opioid receptor exist because benzomorphan ligands such as bremazocine and ethylketocyclazocine—non-selective opioids—label a greater number of κ-opioid receptors in the brain compared to κ-opioid receptor sites that are specifically labelled by selective κ-opioid receptor ligands such as arylacetamide compounds. In those experiments, [^{3}H]bremazocine binding to μ- and δ-opioid receptors is suppressed by cold competing ligands, and remaining [^{3}H]bremazocine binding sites—which represent

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)00822-6

κ-opioid receptors by substraction of μ- and δ-sites—appear substantially more abundant than κ-sites detected by direct labelling using radiolabelled arylacetamides (for review, see Fowler and Fraser, 1994). Arylacetamide binding sites are referred as to κ_1-sites, while the additional κ-sites labelled by the non-selective benzomorphan ligands have been called κ_2-sites and have been proposed to be distinct from κ_1-sites. κ_2-Sites, which can also be termed "residual" [^{3}H]bremazocine sites labelled in the presence of excess cold selective μ-, δ- and κ_1-opioid receptor ligands, demonstrate an opioid pharmacology (Tiberi and Magnan, 1990). Detailed quantitative autoradiography shows species differences in the relative proportions of these putative κ_1- and κ_2-opioid receptors (Unterwald et al., 1991). More generally, evidence for κ-opioid receptor heterogeneity has been abundant (see Wollemann et al., 1993) and include studies where [^{3}H]bremazocine binding fits two site modelling, showing differential sensitivity to dynorphin A and the arylacetamide *N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspirol[4,5]dec-8-yl]-benzeneacetamide (U69,593; Devlin and Shoemaker, 1990). Others, mostly on the basis of competition binding, have further suggested the existence of multiple classes of κ_2-opioid receptors (Ni et al., 1995) and a κ_3-opioid receptor which is labelled by naloxone benzoylhydrazone has been proposed (Clark et al., 1989; Pan et al., 1996; Tive et al., 1992). κ-Opioid receptors that are distinct from κ_1-opioid receptors have also been proposed to be the previously named ε-opioid receptor which purportedly binds β-endorphin (Nock et al., 1990, 1992). In vivo studies have also been carried out to potentially discriminate κ_1 and κ_2 mediated responses. Ho et al. (1997) have shown that bremazocine and the putative κ_2-opioid receptor agonist methyl4-[(3,4-dichlorophenyl)-acetyl]-3-[(1-pyrrolidinyl)methyl]-1-piperazinecarboxylate (GR89,696) reverse inflammatory hyperalgesia whilst the κ_1-opioid receptor agonist U69,593 is without effect. GR89,696 has also been shown to be effective in reversing hyperalgesia and allodynia in models of peripheral neuritis and neuropathy where U69,593 is only weakly active (Eliav et al., 1999).

At present it is clear that the KOR gene encodes κ_1-opioid receptors, since the recombinant receptor expressed in heterologous host cells bind arylacetamide compounds with high affinity (for a review, see Kieffer, 1995). There is also evidence that the κ_3-opioid receptor subtype may correspond to the recently cloned orphanin FQ/nociceptin receptor (ORL1) since naloxone benzoylhydrazone has been shown to bind to the mouse brain and rat recombinant receptors (Mathis et al., 1997; Noda et al., 1998), and antinociception induced by this compound is abolished in ORL1 knockout mice (Noda et al., 1998). Finally, there are notable differences in the expression pattern of κ-opioid receptor mRNA and [^{3}H]bremazocine-labelled κ-opioid receptor sites which cannot be explained by receptor trafficking and might therefore suggest that [^{3}H]bremazocine is binding to a κ-site derived from another gene (Mansour et al., 1994). The lack of highly selective ligands for the κ_2-opioid receptor subtype and the lack of success in isolating κ-opioid receptor-encoding cDNAs that would differ from the KOR cDNA, has meant the molecular basis for κ_2-subtypes remains unclear. Therefore, the possible existence of κ-opioid receptor subtypes that would be distinct molecular entities from the cloned KOR gene product remains an open issue (Traynor, 1989).

We have recently disrupted the KOR gene by homologous recombination and the phenotype of mutant mice has been published (Simonin et al., 1998). We have shown both by homogenate binding and quantitative receptor autoradiography that there is complete loss of κ-opioid receptors throughout the brain when labelled by the arylacetamide ligand [^{3}H]-((5A-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-4-benzofuranacetamide) (CI-977; Slowe et al., 1999). This finding confirmed the previous binding studies on the cloned κ-opioid receptor, and demonstrated that the KOR gene encodes receptors of the κ_1-type in vivo. To determine whether another population of κ-opioid receptor subtypes remains in the KOR-deficient animals, we now have conducted experiments using [^{3}H]bremazocine binding under conditions where μ- and δ-binding is suppressed. In addition, we have carried out homogenate binding with [^{3}H]bremazocine in single MOR- and DOR-, double MOR/DOR- and the triple MOR/DOR/KOR-deficient mutant mice and collectively our data show that κ_2-opioid receptor sites can be accounted for by a combination of [^{3}H]bremazocine binding to MOR, DOR and KOR gene products.

2. Material and methods

2.1. Generation of knock-out mice for opioid receptors

The generation of MOR-, KOR- and DOR-deficient mice was described previously (Filliol et al., 2000; Matthes et al., 1996; Simonin et al., 1998) Double mutant mice lacking DOR/MOR genes and triple mutant mice lacking DOR/MOR/KOR genes were produced by interbreeding of single knock-out mice. The ablation of μ- and δ-opioid receptor sites in the double mutant mice was verified by confirming the absence of [^{3}H]-[D-Ala^{2}MePhe^{4}Gly-ol^5] enkephalin (DAMGO) and [^{3}H]-[D-Penicillamine^2, D-Penicillamine^5] enkephalin (DPDPE) binding on brain membrane preparations (not shown). Similarly, we confirmed the lack of [^{3}H]DAMGO, [^{3}H]DPDPE and [^{3}H]CI-977 binding sites in the triple mutant mice (not shown).

2.2. Chemicals

[^{3}H]bremazocine (984.2 Gbq/mmol), [^{3}H]DAMGO (2146 Gbq/mmol) and [^{3}H]DPDPE (1960 Gbq/mmol)

were purchased from New England Nuclear. [^{3}H]-[3,5-^{3}H-Tyr]-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 (deltorphin I, 1739 Gbq/mmol) was custom synthesised by Zeneca (Cheshire, UK). [^{3}H]CI-977 (1810 Gbq/mmol) was from Amersham. Naloxone, DAMGO and DPDPE were purchased from Sigma. CI-977 was a gift from John Hughes (Parke Davis Neuroscience Research Center, Cambridge, UK). D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) was from Research Biochemical International and naltrindole was from TOCRIS.

2.3. Homogenate binding

Brain membranes were prepared from wild-type and knockout animals as previously described (Ilien et al., 1988) and in parallel experiments to avoid variations between preparations. One or two brains were used for each preparation from each genotype, and the final protein concentration was identical for each brain membrane preparation as estimated by the Bradford assay (Bradford, 1976). Binding assays were carried out as described (Kieffer et al., 1992). Briefly, 100 μg total brain membrane proteins were incubated in 50 mM Tris–HCl pH 7.4, 1 mM EDTA for 1 h at 25°C with [^{3}H]bremazocine. For saturation analysis, [^{3}H]bremazocine concentrations ranging from 0.01 to 2 nM were used. Non-specific binding was determined using naloxone (10 μM). Binding experiments were performed two times in triplicate, using two distinct brain membrane preparations for each genotype. Competing ligands were added when indicated (Fig. 2). DAMGO (100 nM and 1 μM), DPDPE (100 nM and 1 μM) and CI-977 (20 and 200 nM) were used to block μ-, δ- and κ_1-opioid receptor sites, respectively. All three competitors were added to wild-type brain preparations while DAMGO, DPDPE and C-I977, respectively, were omitted when MOR-, DOR- and KOR-deficient brain preparations were used. Analysis of saturation experiments was performed using PRISM (Biosoft). Following a significant effect by one-way analysis of variance (ANOVA), comparison of binding between wild-type and knockout brains was analyzed using Dunnett's test.

3. Results

3.1. [^{3}H]bremazocine binding in wild-type, single and double mutant mice

Brain membranes from wild-type and single MOR, DOR or KOR knockout mice were prepared under strictly identical conditions (see Section 2) in order to minimize experimental variations due to the preparation itself. We first examined specific binding of [^{3}H]bremazocine at a single saturating concentration (2 nM; Fig. 1). In the absence of any other opioid ligand, [^{3}H]bremazocine labels all opioid receptor sites in wild-type mice. We compared

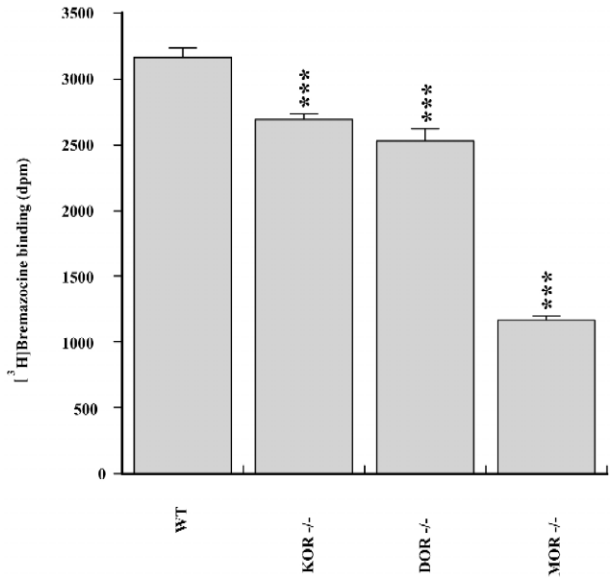

Fig. 1. [^{3}H]Bremazocine binding in whole brain homogenates from wild-type (WT), KOR-, DOR- and MOR-deficient mice. Brain membranes (100 μg per assay) were incubated in the presence of [^{3}H]bremazocine (2 nM). Nonspecific binding was determined in the presence of 10 μM naloxone. Results are expressed as mean ± SEM of three experiments performed in triplicate and conducted on three distinct membrane preparations. $^{***}P < 0.001$, comparisons between wild-type and mutants brain membrane preparations (Dunnett's test).

specific [^{3}H]bremazocine binding in wild-type and mutant mice and the difference shows the relative contribution of MOR, DOR and KOR to [^{3}H]bremazocine binding sites. In MOR-deficient mice, [^{3}H]bremazocine binding was 37% (±1) of wild-type, indicating that [^{3}H]bremazocine binding to μ-opioid receptors accounts for 63 % of total [^{3}H]bremazocine binding. Similarly, 80% (±2) and 85% (±2) [^{3}H]bremazocine binding remained in DOR- and KOR-deficient mice, indicating that δ- and κ_1-opioid receptor sites contribute to 20% and 15% of total [^{3}H]bremazocine sites, respectively. Altogether, the total number of [^{3}H]bremazocine binding sites due to MOR, DOR and KOR represents 100% of the binding.

To verify that the decrease of [^{3}H]bremazocine binding was indeed due to a loss of binding sites, we then performed a saturation analysis of [^{3}H]bremazocine binding (Table 1). B_{max} values obtained for wild-type, MOR- and DOR-deficient mice indicate a contribution of MOR and DOR to [^{3}H]bremazocine binding of 68% (±12) and 27% (±1), respectively. Because of the low number of κ sites in mouse brain (Kitchen et al., 1997), we decided to use brain membranes from double MOR/DOR-deficient mice to get a direct measurement of the contribution of KOR to [^{3}H]bremazocine binding. The B_{max} value was estimated to be 56 ± 1 fmol per mg of protein, representing 14.5% (±0.2) from binding at wild-type mice. These results are consistent with data obtained using a single concentration of [^{3}H]bremazocine and confirm that the contribution of MOR, DOR and KOR accounts for the total number of

Table 1
Saturation analysis of [^{3}H]Bremazocine binding on brain membranes of wild-type, MOR, DOR and MOR/DOR mutant mice

	K_d (nM)	B_{max} (fmol/mg protein)
wild-type	0.29 ± 0.03	383 ± 10 (μ + δ + κ)
MOR −/−	0.22 ± 0.04	121 ± 22 (δ + κ)
DOR −/−	0.24 ± 0.04	280 ± 11 (μ + κ)
MOR/DOR double −/−	0.04 ± 0.005	56 ± 1 (κ)

Values are means ± SEM and were obtained from at least four experiments performed in triplicate on two separate membrane preparations.

binding sites. Therefore [^{3}H]bremazocine binding in wild-type brain is due to μ-, δ- and κ_1-opioid receptors and no additional gene seems to be required to explain the total population of [^{3}H]bremazocine binding sites.

In this experiment (Table 1), the determination of K_d values indicated highest affinity of [^{3}H]bremazocine for κ-opioid receptor sites in MOR/DOR −/− mice, consistent with previous binding studies (Corbett et al., 1993). The similar affinity values obtained in single MOR −/− and DOR −/− result from binding at multiple opioid receptor sites (δ- and κ-opioid receptors in MOR −/− mice, μ- and κ-opioid receptors in DOR −/− mice) and mainly reflect the lower binding affinity of [^{3}H]bremazocine toward δ- and μ-opioid receptors (Corbett et al., 1993).

3.2. Competition of [^{3}H]bremazocine binding with cold suppressor ligands in wild-type and single knockout mice

To further investigate the genetic origin of κ_2-opioid receptors, we performed [^{3}H]bremazocine binding experiments in the presence of cold suppressors for μ- and δ-opioid receptors, as well as for κ_1-opioid receptors (Fig. 2). We first used 100 nM DAMGO, 100 nM DPDPE and 20 nM CI-977, corresponding to classically used conditions (Tiberi and Magnan, 1990) for the study of κ_2-sites (Fig. 2, left panel). In wild-type mice, a substantial binding of [^{3}H]bremazocine was clearly observed, accounting for 23% (±1) of the binding observed without suppressors. These sites that we refer to as "residual" [^{3}H]bremazocine binding represent κ_2-opioid receptor sites. Interestingly, this binding was significantly lower in the brains of all mutant mice: levels were 18% (±1), 19% (±1) and 7% (±0.5) of total [^{3}H]bremazocine binding in KOR-, DOR- and MOR-deficient mice, respectively (Fig. 2, left panel). This indicates that each of the three known genes, participate in forming the subpopulation of κ_2-sites. Further, the ratio of their respective contribution strikingly paralleled that of [^{3}H]bremazocine binding in the absence of blockers: residual binding at KOR-, DOR- and MOR-deficient mouse brains was 20%, 17% and 68% lower, respectively, from that of wild-type mice (Fig. 2, left panel). Therefore, similarly to what we observed for total [^{3}H]bremazocine binding (Fig. 1), 100% of the "residual" [^{3}H]bremazocine binding in wild-type brains could be accounted for by MOR, DOR and KOR. This result further supports the notion that the so-called κ_2-opioid receptor sites indeed arise from a combination of the three known gene products.

We then increased the concentration of blocking ligands for μ-, δ- and κ_1-opioid receptors (Fig. 2, right panel). In the presence of 1 μM DPDPE, 1 μM DAMGO and 200 nM CI-977, the binding of [^{3}H]bremazocine was further reduced. In wild-type mice [^{3}H]bremazocine binding was 4% (±0.3) of the binding in the absence of suppressors and this binding was significant ($P < 0.001$) when compared to the background. The binding of [^{3}H]bremazocine in DOR- and KOR-deleted brains (3.6 ± 0.3% and 4.3 ± 0.3%, respectively) was not significantly different from binding at wild-type brain. In contrast, brains lacking the MOR gene showed significantly less binding (1.2 ± 0.1%) compared to wild-type. This result indicates that in the presence of high concentrations of blockers, very low but

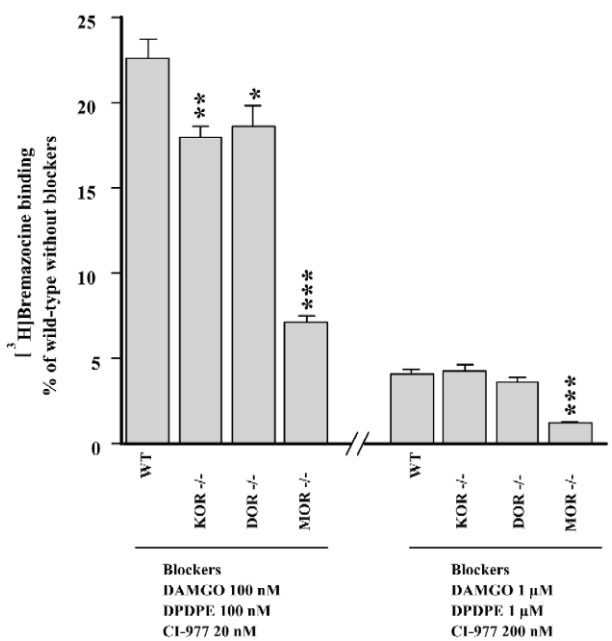

Fig. 2. [^{3}H]Bremazocine binding in the presence of μ-, δ- and κ_1-opioid receptor suppressor ligands using whole brain homogenates from wild-type (WT), KOR-, DOR- and MOR-deficient mice. Brain membranes (100 μg per assay) were incubated in the presence of [^{3}H]bremazocine (2 nM) and in the presence of the cold suppressors DAMGO (100 nM or 1 μM, μ), DPDPE (100 nM or 1 μM, δ) and CI-977 (20 or 200 nM, κ_1). In membranes from KOR-, DOR- and MOR-deficient mice, CI-977, DPDPE and DAMGO, respectively, were omitted. Nonspecific binding was determined in the presence of 10 μM naloxone. Results are expressed as percent of total specific [^{3}H]bremazocine binding in wild-type mice, defined as 100%. Data are mean ± SEM of three experiments performed in triplicate and conducted on three distinct membrane preparations. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$, comparisons between wild-type and mutant brain membrane preparations (Dunnet's test).

significant [^{3}H]bremazocine binding remains detectable in wild-type brains and that these sites are mostly due to binding at MOR.

These data also indicate that the concentrations of competitor ligands classically used to block μ-, δ- and κ_1-sites are not high enough to compete with [^{3}H]bremazocine binding at those sites. Since all the suppressor ligands that were used are agonist compounds and may poorly bind to low-affinity sites, we tested whether specific μ- or δ-opioid receptor antagonists would be more efficient at displacing [^{3}H]bremazocine binding in wild-type and opioid receptor deficient mice. When naltrindole (10 and 100 nM) or CTOP (100 nM and 1 μM) were used instead of DPDPE and DAMGO to block δ- and μ-opioid receptor sites, a virtually identical pattern of [^{3}H]bremazocine binding was observed (data not shown). This suggests that the tiny residual binding of [^{3}H]bremazocine that we observe does not correspond to low affinity DAMGO- and DPDPE-binding sites.

3.3. [^{3}H]bremazocine binding to the triple mutant mice

Finally, we performed [^{3}H]bremazocine binding experiments in animals deficient in the three receptor genes (MOR/DOR/KOR-deficient mice). Using [^{3}H]bremazocine at 0.5 and 5 nM concentrations, we could not detect any specific [^{3}H]bremazocine binding in brain membrane homogenates from those animals (Fig. 3). This result definitely excludes the possible existence of an additional opioid receptor gene encoding κ_2-opioid receptor.

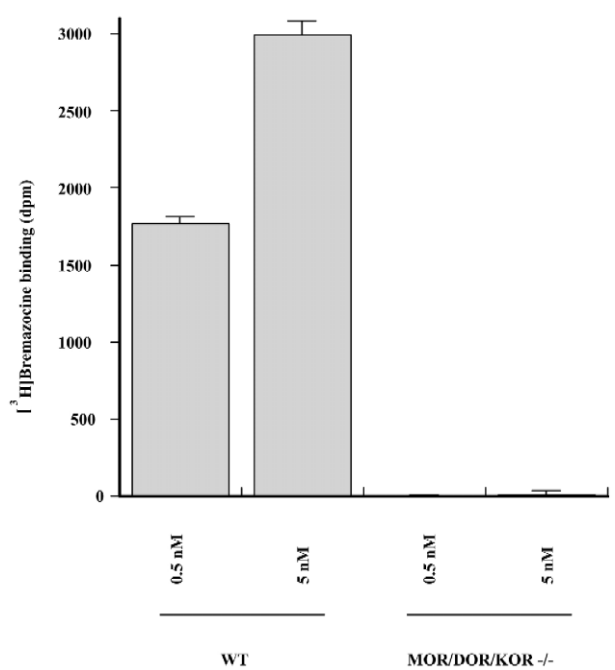

Fig. 3. [^{3}H]Bremazocine binding in whole brain homogenates from wild-type (WT) and MOR/DOR/KOR-deficient mice. Brain membranes (100 μg per assay) were incubated in the presence of [^{3}H]bremazocine (0.5 and 5 nM). Nonspecific binding was determined in the presence of 10 μM naloxone. Results are expressed as mean ± SEM of three experiments performed in triplicate and conducted on two distinct membrane preparations.

4. Discussion

What is evident from our homogenate binding data is that virtually all [^{3}H]bremazocine binding sites can be accounted for by labelling of mostly μ-opioid, but also some δ- and κ_1-opioid receptors. This is supported by three observations. First, our parallel study of MOR-, DOR- and KOR-deficient mice shows that the number of sites arising from MOR, DOR and KOR gene products represent 100% of [^{3}H]bremazocine binding, both in the absence and presence of classical (low) concentrations of cold competing ligands. The second observation is that high concentrations of blocking ligands eliminate most [^{3}H]bremazocine binding in wild-type and opioid receptor knockout mice. The third observation is that no [^{3}H]bremazocine binding is detectable in the triple MOR/DOR/KOR-deficient mice. We conclude that we have strong evidence to rule out the possibility that the κ_2-opioid receptor subtype is encoded by a gene that is different from MOR, DOR and KOR genes.

This conclusion implies that the non-selective opioid ligand bremazocine and the selective ligands compete for binding to the three known MOR, DOR or KOR gene products. It raises the question of why residual [^{3}H]bremazocine binding remains, even in the presence of high concentrations of DAMGO, DPDPE or CI-977. Up to now, it has been common practice to use [^{3}H]bremazocine or [^{3}H]ethylketocyclazocine binding in order to label the total κ-opioid receptor population in brain homogenates and autoradiographic experiments, under conditions where 100–200 nM DAMGO and DPDPE, or similar δ-opioid receptor ligands, are added to suppress binding at μ- and δ-opioid receptors, respectively (Benyhe et al., 1990; Gillan and Kosterlitz, 1982; Kitchen et al., 1997; Mansour et al., 1994, 1987; Robson et al., 1985; Unterwald et al., 1987; Zukin et al., 1988). It is now clear from the present study that these concentrations are too low to completely suppress [^{3}H]bremazocine binding at DOR and MOR. Indeed, even at tenfold higher concentrations of DAMGO and DPDPE, there is still measurable binding in wild-type mice, mainly contributed for by the MOR gene (Fig. 2, right panel). This contrasts with the claim that suppression with 1 μM DAMGO and 1 μM [D-Ala^2, D-Leu^3] enkephalin (DADLE) in autoradiography reveals only κ-opioid receptors (Morris and Herz, 1986). The need for high concentrations of suppressors accords with the work of Devlin and Shoemaker who used morphiceptin and [D-Thr^2, D-thr^6] Leu-enkephalin (DTLET) which are ligands with similar affinities to DAMGO and DPDPE, re-

spectively, at concentrations of 5 μM (Devlin and Shoemaker, 1990). Bremazocine is a non-selective opioid and is also very different in structure from the opioid peptides DAMGO, DPDPE and CTOP, or from the morphinan naltrindole. It is therefore reasonable to suggest that [^{3}H]bremazocine and the various competitors under study interact with MOR, DOR and KOR at overlapping but distinct sites, and that this prevents full competition to occur. This is supported by our previous opioid receptor mutagenesis studies, which revealed an unexpectedly wide diversity of ligand-receptor interaction modes (Befort et al., 1996). Also, a similar hypothesis was recently proposed by Coward et al. (1998), who suggested the existence of separate binding sites for small κ-opioid receptor agonists and endogenous opioid peptides from the study of mutant κ-opioid receptors.

A recent work of Jordan and Devi (1999) could provide an alternative explanation for residual [^{3}H]bremazocine binding in the presence of high concentrations of selective opioids. The authors presented biochemical and pharmacological evidence for the heterodimerization of κ- and δ-opioid receptors and suggested that these heterodimers could form the molecular basis for κ_2-opioid receptor subtype. Indeed, their data show that, in transfected cells, the δ–κ dimer displays a significant loss of affinity for either κ or δ selective agonists while [^{3}H]bremazocine could still bind with high affinity. In our data, the binding of [^{3}H]bremazocine, observed in presence of blocking ligands, is mostly due to μ-opioid receptors. Consequently, we would propose that κ_2-opioid receptors could also be formed by μ–κ and/or μ–δ heterodimers, in vivo, a hypothesis which remains to be demonstrated.

The correspondence between pharmacological subtypes and opioid receptor gene products is essential in the search of novel therapeutic agents to treat pain and addiction. Analgesic development programs in pharmaceutical industry for κ_1-opioid receptor selective ligands produced highly potent alternative analgesics to morphine in the late 1980s (Millan, 1990) but they failed in the clinic due to psychotomimetic side effects. The potential value of compounds with activity at other κ-opioid receptor subtypes appeared therefore extremely high but search for such compounds has proven difficult because of the lack of selective compounds. Our studies in knockout mice represent an important step in the search for alternative κ-opioid receptor compounds because they highlight the nature of κ_2-sites. These findings in mice should apply to other mammalian species. Indeed, although the relative aboundancy of μ-, δ- and κ-opioid receptors is slightly variable among rodents, κ_2-sites in this study, are observed under experimental conditions highly similar to those described in rat and guinea pig. In conclusion, our data suggest that κ_2-opioid receptors are not encoded by a gene, yet to be characterized, but indeed arise from a mixed population of receptor proteins produced by the three known opioid receptor genes.

Acknowledgements

This work was funded by the Centre National de la Recherche Scientifique and Association pour la Recherche sur le Cancer. S.J. Slowe received a Pfizer-funded studentship. I.K and B.K received support from the Biomed 2 programme of the European Community; EC BMH4-CT96-0510. We thank J.F. Poirier and N. Schallon for animal care. We are grateful to P. Chambon and J.C. Stoclet for constant support.

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