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Effects of cations on binding, in membrane suspensions, of various opioids at μ-sites of rabbit cerebellum and κ-sites of guinea-pig cerebellum

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1 At the μ-sites of rabbit cerebellum, NaCl, LiCl, KCl, choline chloride and $MnCl_2$ were tested for potentiation and inhibition of the binding of several opioids. Naloxone, (−)-bremazocine and diprenorphine are μ-antagonists in pharmacological assays and their binding is potentiated by the lower concentrations and inhibited by the higher concentrations of NaCl. The binding of the agonists [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin and [^{3}H]-dihydromorphine is inhibited. $MnCl_2$ potentiates the binding of the agonist [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin but not the binding of the antagonists. The thresholds of inhibition and slopes of the dose-response curves for inhibition by $MnCl_2$ and LiCl vary. This finding may indicate that potentiating effects of $MnCl_2$ and LiCl are masked by simultaneous inhibition.

2 At the κ-sites of guinea-pig cerebellum, NaCl, KCl and $MnCl_2$ inhibit the binding of [^{3}H]-dynorphin A (1–8), [^{3}H]-dynorphin A (1–9), [^{3}H]-(−)-bremazocine, [^{3}H]-tifluadom, and [^{3}H]-diprenorphine. NaCl also causes a small potentiation of the binding of [^{3}H]-diprenorphine, which is a κ-agonist in the guinea-pig myenteric plexus but a κ-antagonist in the rabbit vas deferens. The slopes of the inhibitory dose-response curves and the thresholds of inhibition vary with the different ligands. Therefore some potentiating effects may have been masked.

3 The results support the view that NaCl, and perhaps LiCl, but not KCl and choline chloride, potentiate the binding of μ-antagonists but not the binding of μ-agonists. It is not yet possible to decide whether, at the κ-site, there is a similar differentiation of the binding of agonists and antagonists.

Introduction

Prior to the characterization of the μ-, δ- and κ-opioid binding sites it was suggested that the effects of NaCl and LiCl on the binding of μ-opioid agonists may be different from their effects on μ-opioid antagonists (Pert & Snyder, 1974). Recently, a systematic investigation of the effects of the chloride salts of various monovalent and divalent cations on binding at selectively labelled μ-, δ- and κ-opioid sites in guinea-pig brain has shown that regulation by cations of opioid binding is different at each site and is partly ligand-dependent (Paterson *et al.*, 1986). The aim of this paper was to determine with a wider range of ligands, the extent to which the effects of cations at μ-sites and at κ-sites are ligand-dependent. One of the major difficulties with a detailed investigation is the fact that there are at present relatively few selective ligands. It is best to avoid the usual method (Gillan & Kosterlitz, 1982) of using unlabelled ligands to restrict binding of labelled ligands to a single site since the binding of the unlabelled ligands may be modified by changes in the ionic composition of the assay medium. Thus, in order to restrict the binding of non-selective ligands largely to a single site, the rabbit cerebellum, in which the opioid binding sites are mainly of the μ-type (Meunier *et al.*, 1983), and the guinea-pig cerebellum, in which the opioid sites are mainly of the κ-type (Robson *et al.*, 1984), have been used.

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Methods

Binding assays

With previously described methods (Gillan & Kosterlitz, 1982; Gillan *et al.*, 1985; Paterson *et al.*, 1986) binding was assayed at 25°C in membrane suspensions (10 mg tissue ml^{-1} Tris-HCl; 50 mM, pH 7.4) prepared

from the cerebella of Dunkin Hartley guinea-pigs or Californian rabbits. To determine whether, as reported for the New Zealand White rabbit (Meunier *et al.*, 1983), the cerebellum of the Californian rabbit has a predominance of μ-binding sites, the maximum binding capacities and the equilibrium dissociation constants at μ-, δ- and κ-sites were determined by Hill analysis (Hill, 1910) of saturation curves obtained with the selective labelling techniques of Gillan & Kosterlitz (1982). Details are given in the legend of Table 1.

To determine the effects of salts on binding, the equilibrium binding of concentrations of tritiated ligands close to their K_D values in guinea-pig brain was measured at 25°C; the incubation times were 30 min for [^{3}H]-dynorphin A (1–8) and [^{3}H]-dynorphin A (1–9) or 40 min for the other tritiated ligands. When [^{3}H]-dynorphin A (1–8) or [^{3}H]-dynorphin A (1–9) was used, the peptidase inhibitors bestatin (30 μM) and captopril (300 μM) were added to the membrane suspensions to reduce degradation of the ligands (Gillan *et al.*, 1985). The effects of concentrations of up to 200 mM of monovalent salts or of up to 16 mM of divalent salt were investigated. Specific binding was defined as the difference between binding in the absence and presence of unlabelled diprenorphine (1.2 μM) or of MR2266 (1.5 μM) when [^{3}H]-diprenorphine was the ligand. The effects of the added salts are expressed as the changes in specific binding as a proportion (%) of control binding in their absence. If binding was reduced below control levels and the regression of the change (%) in binding against the logarithm of salt concentration (mM) was linear, the slope and the IC_{50} value could be obtained. The salt concentration at which the extrapolated dose-response curve intercepted the abscissa (0% change in binding) was determined. This value was the threshold of inhibition except when there was potentiation of binding.

Labelled ligands

The labelled ligands used were: [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (47–56 Ci mmol^{-1}), [^{3}H]-dihydromorphine (66 Ci mmol^{-1}), [^{3}H]-naloxone (65–68 Ci mmol^{-1}), [^{3}H]-[D-Ala^2, D-Leu^5]enkephalin (27–53 Ci mmol^{-1}), [^{3}H]-dynorphin A (1–9) (34 Ci mmol^{-1}), and [^{3}H]-diprenorphine (31 Ci mmol^{-1}) from Amersham International, [^{3}H]-dynorphin A (1–8) (26–32 Ci mmol^{-1}) from Amersham International or New England Nuclear and [^{3}H]-(−)-bremazocine (24–40 Ci mmol^{-1}) from Dr D. Römer (Sandoz) or New England Nuclear. Reverse-phase h.p.l.c. of the tritiated ligands on a μBondapack C_{18} column was used to ensure purities of >95%. The specific activities of the purified ligands were assessed as previously described (Gillan *et al.*, 1985).

Drugs, peptides and salts

The drugs and peptides used were diprenorphine base from Reckitt & Colman, MR 2266 base ((−)-α-5, 9-diethyl-2-(3-furylmethyl) -2′-hydroxy-6, 7-benzomorphan) from Dr H. Merz (C.H. Boehringer Sohn), U-50, 488H base (*trans*-3, 4-dichloro-N-methyl-N-(2-(1-pyrrolldinyl)-cyclohexyl)-benzeneacetamide) from the Upjohn Company, [D-Ala^2, McPhe^4, Gly-ol^5]enkephalin from Dr D. Römer (Sandoz), [D-Ala^2 D-Leu^5]enkephalin from Dr S. Wilkinson (Wellcome Laboratories), [D-Pen^2, D-Pen^5]enkephalin from Peninsula Laboratories Europe Ltd., bestatin from Cambridge Research Biochemicals and captopril from Squibb. Tris(hydroxymethyl) methylamine and the chloride salts of monovalent and divalent cations were from B.D.H. Chemicals (AnalaR grade). Dilutions of drugs and peptides were made in Tris-HCl solution and dilutions of salts, in distilled water.

Results

Cerebellum of guinea-pig and of rabbit as tissues selective for κ-binding sites and μ-binding sites

It has been shown from analysis of saturation curves that under our assay conditions 84% of the opioid binding sites in guinea-pig cerebellum are of the κ-type (Robson *et al.*, 1984). The maximum binding capacities of the μ-, δ- and κ-sites in rabbit cerebellum were obtained from analysis of saturation curves and are shown in Table 1. From the sum of the binding capacities (between 9 and 10 pmol g^{-1} tissue), it is estimated that 75–83% of the opioid sites are μ-sites, 15–17% are κ-sites and less than 10% are δ-sites.

In the guinea-pig cerebellum, at concentrations similar to those used in this investigation, 95.3 ± 4.1% (n = 11) of the binding of [^{3}H]-dynorphin A (1–9) (0.1 nM) and 93.5 ± 2.6% (n = 3) of the binding of [^{3}H]-tifluadom (0.4 nM) were attributed to κ-sites, since these proportions of binding remained when 100 nM each of the unlabelled μ-ligand, [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin and the unlabelled δ-ligand, [D-Ala^2, D-Leu^5]enkephalin, were present in the assay medium. With less selective ligands, the proportions attributed to κ-sites were 86.1 ± 3.1% (n = 8) for [^{3}H]-dynorphin A (1–8) (0.5 nM), 88.2 ± 2.0% (n = 31) for [^{3}H]-(−)-bremazocine (0.1 nM) and 79.4 ± 2.4% (n = 13) for [^{3}H]-diprenorphine (0.1 nM).

In the rabbit cerebellum, 91.9 ± 1.8% (n = 4) of the binding of [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (1 nM), 91.5 ± 1.9% (n = 4) of the binding of [^{3}H]-dihydromorphine (1.1 nM) and 85.0 ± 3.3% (n = 4) of the binding of [^{3}H]-naloxone (1 nM) were displaced by 30 nM of the unlabelled μ-ligand, [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin, indicating that these tritiated ligands bind mainly to μ-sites. The corresponding

Table 1 Binding characteristics of tritiated opioids at μ-, δ- and κ-sites in suspensions of rabbit cerebellar membranes

Binding site	*Tritiated ligand*	*Dissociation constant*	*Binding capacity* (pmol g^{-1} tissue)	
μ	[D-Ala², MePhe⁴, Gly-ol⁵]enkephalin	1.09 ± 0.32	7.47 ± 1.24	(3)
δ	[D-Ala², D-Leu⁵]enkephalin*	—	< 1 pmol g^{-1}	(3)
κ	(−)-Bremazocine**	0.041 ± 0.004	1.54 ± 0.09	(3)

Values were obtained from analysis of saturation curves and are the means ± s.e.mean. The number of observations is given in parentheses. *Suppression of the μ-binding was obtained by a constant ratio of 10 nM of unlabelled [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin to 1 nM of free [³H]-[D-Ala², D-Leu⁵]enkephalin. The level of κ-sites was too low for determination of satisfactory saturation curves. **Suppression of the μ- and δ-binding was obtained by a constant ratio of 100 nM of unlabelled [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin and 100 nM unlabelled [D-Ala², D-Leu⁵]enkephalin to 0.14 nM of free [³H]-(−)-bremazocine.

value for the non-selective ligand, [³H]-(−)-bremazocine (0.1 nM) was 71.4 ± 2.38% ($n = 6$) and that for another non-selective ligand, [³H]-diprenorphine (0.1 nM) was 73.4 ± 1.13% ($n = 5$). The inference that about 30% of the binding of these ligands is to κ- and δ-sites was confirmed by the finding that 70.2 ± 4.4% ($n = 6$) of the binding of [³H]-(−)-bremazocine and 73.2 ± 6.4% ($n = 5$) of the binding of [³H]-diprenorphine remained when the unlabelled κ-ligand, U-50, 488H (100 nM), and the unlabelled δ-ligand, [D-Pen², D-Pen⁵]enkephalin (45 nM), were added to the assay medium.

Effects of salts on opioid binding in the rabbit cerebellum

NaCl caused dose-dependent inhibition of the binding of the peptide μ-agonist, [³H]-[D-Ala², MePhe⁴, Gly-ol⁵]enkephalin, and of the alkaloid μ-agonist, [³H]-dihydromorpine (Figure 1a, Table 2). The two dose-response curves were similar and no potentiating effects were observed. However, when [³H]-naloxone, [³H]-(−)-bremazocine or [³H]-diprenorphine were the ligands, the effect of NaCl was different. The binding of [³H]-naloxone and, to a smaller extent, that of [³H]-(−)-bremazocine or of [³H]-diprenorphine was enhanced by NaCl (Figure 1a). This enhancement was reduced at concentrations of NaCl above 3 mM when [³H]-naloxone was the ligand, and above 12 mM when [³H]-(−)-bremazocine or [³H]-diprenorphine was the ligand. At higher salt concentrations the binding was reduced below control levels (0% change). The concentrations of NaCl which inhibited the binding of [³H]-naloxone, [³H]-(−)-bremazocine or [³H]-diprenorphine were higher than the concentrations which inhibited the binding of [³H]-[D-Ala², MePhe⁴, Gly-ol⁵]-enkephalin and [³H]-dihydromorphine. Nevertheless, the slope of the dose-response curve for inhibition of [³H]-(−)-bremazocine binding was similar to the slopes obtained with [³H]-[D-Ala², MePhe⁴, Gly-ol⁵]enkephalin and [³H]-dihydromorphine (Table 2).

LiCl and KCl, as found with NaCl, inhibited the binding of [³H]-D-Ala², MePhe⁴, Gly-ol⁵]enkephalin without any potentiating effect (Table 2). Since the slopes of the dose-response curves for the three salts did not differ significantly, comparison of the values for threshold of inhibition gives an estimate of their relative potencies. Thus, for inhibition of [³H]-[D-Ala², MePhe⁴, Gly-ol⁵]enkephalin binding, NaCl was about 2 fold and KCl 6 fold less potent than LiCl. LiCl and KCl, in contrast to NaCl, inhibited but did not potentiate the binding of [³H]-naloxone or [³H]-(−)-bremazocine. The threshold values for inhibition by LiCl of the binding of [³H]-naloxone and [³H]-(−)-bremazocine were 15 to 21 times higher than for inhibition of [³H]-[D-Ala², MePhe⁴, Gly-ol⁵] enkephalin binding. However, the corresponding threshold values obtained for inhibition by KCl were only up to 2.2 fold higher than those for [³H]-[D-Ala², MePhe⁴, Gly-ol⁵] enkephalin.

Choline chloride also inhibited binding (Table 2). The characteristics of the dose-response curves were similar for inhibition of the binding of [³H]-[D-Ala², MePhe⁴, Gly-ol⁵]enkephalin, [³H]-naloxone, [³H]-(−)-bremazocine and [³H]-diprenorphine. Furthermore, the dose-response curves for inhibition of [³H]-[D-Ala², MePhe⁴, Gly-ol⁵]enkephalin, [³H]-naloxone and [³H]-(−)-bremazocine binding were indistinguishable from those observed with KCl.

The profile of activity of $MnCl_2$ was different from that of the monovalent salts (Figure 1b, Table 2). Its effects on the binding of [³H]-naloxone, [³H]-(−)-bremazocine and [³H]-diprenorphine were solely inhibitory whereas at concentrations below 0.5 mM the binding of [³H]-[D-Ala², MePhe⁴, Gly-ol⁵]enkephalin was potentiated by up to about 20%. This potentiation was reduced by concentrations of $MnCl_2$, above 0.5 mM, a concentration which is of the same order as the threshold values for inhibition of the binding of [³H]-naloxone and [³H]-(−)-bremazocine. The binding of [³H]-diprenorphine was more sensitive to inhibition by $MnCl_2$, with a threshold of 0.043 mM; further-

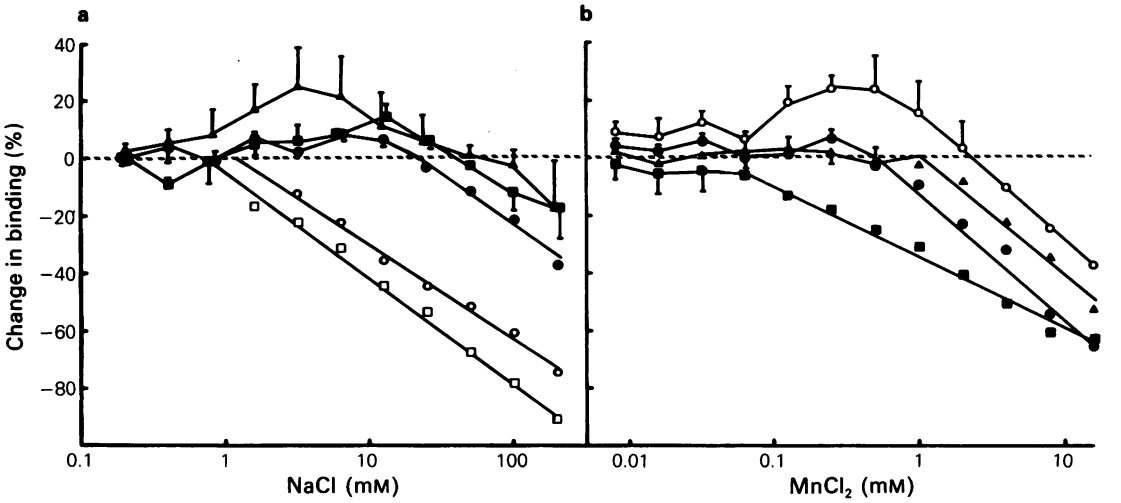

Figure 1 Effects of NaCl (a) and $MnCl_2$ (b) on the binding of tritiated opioids in suspensions of rabbit cerebellar membranes at 25°C: (○) [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]-enkephalin (1 nM); (□) [^{3}H]-dihydromorphine (1.3 nM); (▲) [^{3}H]-naloxone (1 nM); (●) [^{3}H]-(−)-bremazocine (0.1 nM) and (■) [^{3}H]-diprenorphine (0.15 nM). Values are the means of 3 to 6 observations. The correlation coefficients of the regression lines for inhibition of binding varied between 0.987 and 0.999.

Salt	*Tritiated ligand*	*Threshold** (mM)	*Slope****	IC_{50} (mM)	
NaCl	[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin	1.34 ± 0.40	− 33 ± 2	44.2 ± 4.1	(4)
	Dihydromorphine	0.82 ± 0.24	− 37 ± 4	17.3 ± 2.2	(3)
	(−)-Bremazocine	**	− 44 ± 12	> 200	(4)
LiCl	[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin	0.60 ± 0.13	− 32 ± 2	20.5 ± 1.7	(3)
	(−)-Bremazocine	9.39 ± 3.13	− 35 ± 4	> 200	(3)
	Naloxone	12.69 ± 5.61	− 40 ± 8	> 200	(3)
KCl	[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin	3.72 ± 0.51	− 33 ± 3	145 ± 61	(3)
	(−)-Bremazocine	7.70 ± 2.2	− 32 ± 4	> 200	(3)
	Naloxone	8.02 ± 1.18	− 43 ± 4	128 ± 25	(3)
Choline chloride	[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin	3.19 ± 0.77	− 37 ± 4	94.7 ± 36.9	(3)
	(−)-Bremazocine	3.20 ± 1.09	− 32 ± 4	194 ± 82	(5)
	Diprenorphine	6.03 ± 2.2	− 32 ± 5	> 200	(3)
	Naloxone	7.36 ± 3.68	− 40 ± 3	117 ± 33	(3)
$MnCl_2$	[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin	**	− 47 ± 11	> 16	(3)
	(−)-Bremazocine	0.65 ± 0.12	− 47 ± 2	7.76 ± 1.29	(6)
	Diprenorphine	0.043 ± 0.029	− 24 ± 4	4.90 ± 0.61	(3)
	Naloxone	1.24 ± 0.45	− 45 ± 6	15.3 ± 0.8	(3)

Values are the means ± s.e.mean; the number of observations is given in parentheses. The concentrations of the tritiated ligands were 1 nM for [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin and [^{3}H]-naloxone, 1.3 nM for [^{3}H]-dihydromorphine, 0.1 nM for [^{3}H]-(−)-bremazocine and 0.15 nM for [^{3}H]-diprenorphine. In the absence of added salts, the binding of [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin was 3.84 ± 0.29 (n = 16) pmol g^{-1} issue, that of [^{3}H]-dihydromorphine was 3.70 ± 0.73 (n = 3) pmol g^{-1} tissue, that of [^{3}H]-(−)-bremazocine was 3.04 ± 0.25 (n = 21) pmol g^{-1} tissue, that of [^{3}H]-diprenorphine was 5.41 ± 0.71 (n = 6) pmol g^{-1} tissue and that of [^{3}H]-naloxone was 4.59 ± 0.29 (n = 15) pmol g^{-1} tissue. Correlation coefficients varied between 0.96 and 0.99. *Salt concentration at which extrapolated dose-response curve intercepts with the abscissa (0% change in binding) **Since potentiation of binding preceded inhibition the intercept was not equal to the threshold of inhibition. ***% change in binding versus logarithm salt concentration (mM).

more, the slope of the dose-response curve (− 24% change in binding versus log mM $MnCl_2$) was lower than the slopes for the other three ligands (− 45 to − 47% change in binding versus log mM $MnCl_2$).

Effects of salts on opioid binding in the guinea-pig cerebellum

NaCl inhibited the κ-binding of [^{3}H]-dynorphin A (1–9), [^{3}H]-dynorphine A (1–8), [^{3}H]-tifluadom and [^{3}H]-(−)-bremazocine; no potentiating effects were observed (Figure 2a, Table 3). The threshold value for inhibition of [^{3}H]-tifluadom or of [^{3}H]-(−)-bremazocine binding was 1.5 times or 6 times higher than the values found for the two dynorphin fragments. Furthermore, the slopes of the dose-response curves varied from − 32% change in binding versus log mM NaCl for [^{3}H]-tifluadom to − 50% change in binding versus log mM NaCl for [^{3}H]-dynorphin A (1–9). In contrast, when [^{3}H]-diprenorphine was the ligand, the binding was potentiated by about 10% with 10 mM NaCl and was subsequently reduced by higher salt concentrations (Figure 2a).

The inhibitory effects of KCl on the binding of [^{3}H]-dynorphin A (1–8) and [^{3}H]-dynorphin A (1–9) were similar; in a paired experiment it was confirmed that the effects of KCl on the binding of these two ligands are indistinguishable. The slopes of the dose-response curves were higher than those found with [^{3}H]-(−)-bremazocine (Table 3).

With $MnCl_2$, dose-dependent inhibition of binding was observed for [^{3}H]-dynorphin A (1–9), [^{3}H]-dynorphin A (1–8), [^{3}H]-(−)-bremazocine and [^{3}H]-diprenorphine; there were no potentiating effects (Figure 2b, Table 3). The thresholds of inhibition varied between 0.05 mM for [^{3}H]-diprenorphine and 0.25 mM for [^{3}H]-dynorphin A (1–9); as with NaCl-induced or KCl-induced inhibition, the slopes of the inhibitory dose-response curves were higher for the two gragments of dnyorphin A than for the non-peptide ligands. When [^{3}H]-tifluadom was the ligand, the inhibitory effect of $MnCl_2$ was much less than for the other four ligands although the thresholds of inhibition were of the same order for all five ligands (Figure 2b).

Discussion

The results of this investigation indicate that at μ- and possibly κ-binding sites NaCl may potentiate or inhibit binding. When potentiation is caused at low concentrations of NaCl it is subsequently reversed by an inhibitory effect at higher concentrations, leading to inhibition. In this context it has to be considered that potentiation may be masked by simultaneous inhibition and, conversely, that one effect of potentia-

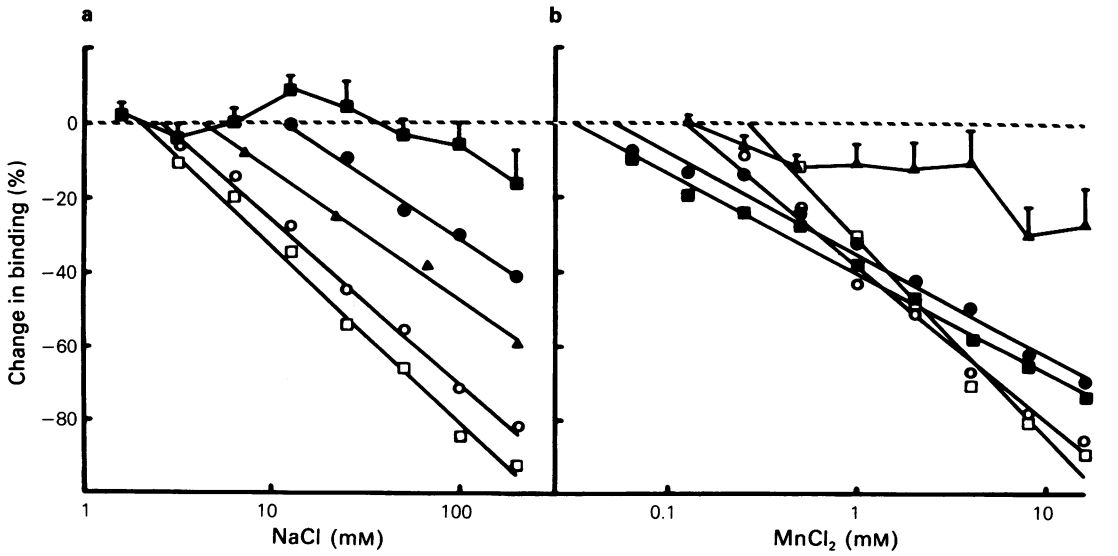

Figure 2 Effects of NaCl (a) and $MnCl_2$ (b) on the binding of tritiated opioids in suspensions of guinea-pig cerebellar membranes at 25°C: (○) [^{3}H]-dynorphin A (1–8) (0.45 nM); (□) [^{3}H]-dynorphin A (1–9) 0.1 nM); (●) [^{3}H]-(−)-bremazocine (0.1 nM); (■) [^{3}H]-diprenorphine (0.15 nM) and (▲) [^{3}H]-tifluadom (0.35 nM). Values are the means of 3 to 7 observations. The correlation coefficients of the regression lines for inhibition of binding varied between 0.987 and 0.996.

Table 3 The inhibitory effects of the chloride salts of cations on binding of tritiated ligands at the κ-sites in suspensions of guinea-pig cerebellar membranes

Salt	*Tritiated ligand*	*Threshold** (mM)	*Slope***	IC_{50} (mM)	
NaCl	Dynorphin A (1–9)****	2.57 ± 0.86	− 50 ± 4	24.1 ± 5.3	(3)
	Dynorphin A (1–8)	2.64 ± 0.22	− 44 ± 1	35.6 ± 0.99	(3)
	Tifluadom	3.88 ± 1.58	− 32 ± 5	129 ± 14	(3)
	(−)-Bremazocine****	16.2 ± 4.4	− 38 ± 5	> 200	(6)
KCl***	Dynorphin A (1–9)****	5.7 ± 1.2	− 54 ± 4	47.6 ± 6.4	(7)
	Dynorphin A (1–8)	11.2 ± 2.4	− 53 ± 1	97.7 ± 17.9	(4)
	(−)-Bremazocine****	16.9 ± 3.7	− 39 ± 4	> 200	(3)
$MnCl_2$	Dynorphin A (1–9)****	0.25 ± 0.07	− 51 ± 4	2.27 ± 0.38	(3)
	Dynorphin A (1–8)	0.12 ± 0.01	− 42 ± 1	1.93 ± 0.13	(3)
	Tifluadom	> 0.125	—	—	(4)
	(−)-Bremazocine****	0.08 ± 0.02	− 28 ± 1	3.89 ± 0.64	(6)
	Diprenorphine	0.05 ± 0.02	− 30 ± 3	2.68 ± 0.21	(3)

Values are the means ± s.e.mean; the number of observations is given in parentheses. The concentrations of the tritiated ligands were 0.1 nM for [^{3}H]-dyporphin A (1–9) and [^{3}H]-(−)-bremazocine, 0.15 nM for [^{3}H]-diprenorphine, 0.35 nM for [^{3}H]-tifluadom and 0.45 nM for [^{3}H]-dynorphin A (1–8). In the absence of added salts, the binding of [^{3}H]-dynorphin A (1–9) was 2.19 ± 0.19 ($n = 13$) pmol g^{-1} tissue, that of [^{3}H]-dynorphin A (1–8) was 3.26 ± 0.37 ($n = 10$) pmol g^{-1} tissue, that of [^{3}H]-tifluadom was 1.99 ± 0.27 ($n = 7$) pmol g^{-1} tissue, that of [^{3}H]-(−)-bremazocine was 2.45 ± 0.15 ($n = 15$) pmol g^{-1} tissue and that of [^{3}H]-diprenorphine was 1.34 ± 0.14 ($n = 6$) pmol g^{-1} tissue. Correlation coefficients varied between 0.93 and 0.99. *Salt concentration at which extrapolated dose-response curve intercepts with the abscissa (0% change in biding). **% change in binding versus logarithm salt concentration (mM). ***In a paired experiment with [^{3}H]-dynorphin A (1–9) and [^{3}H]-dynorphin A (1–8) the threshold values were 8.2 and 7.0 mM, the slopes were − 56 and − 51% and the IC_{50} values were 65 and 66 mM. ****Values from Paterson *et al.* (1986).

tion may be to increase the threshold of inhibition.

It is of particular importance that, at the κ-sites of the guinea-pig cerebellum, the effects of NaCl, KCl and $MnCl_2$ are inhibitory except that NaCl causes a slight potentiation of the κ-binding of [^{3}H]-diprenorphine. Since the thresholds of inhibition and slopes of the dose-response curves vary markedly for the interaction of the salts with different ligands the reasons for these differences are probably complex. The effects of NaCl, KCl and $MnCl_2$ on the binding of the tritiated endogenous ligand, dynorphin A (1–8), are sufficiently similar to their effects on the binding of [^{3}H]-dynorphin A (1–9) to suggest that the κ-binding of these two peptide ligands is comparable. The enzymatic degradation of these two labile peptide ligands may be affected by changes in the ionic composition of the assay medium. Therefore to decide whether there is a fundamental difference between the binding of peptide and non-peptide ligands at the κ-sites it will be necessary to test peptidase-resistant peptides. These are not yet available.

The five ligands used in the rabbit cerebellum can be divided into two groups on the basis of the effect of NaCl on their μ-binding. The effects of NaCl on the binding of the peptide agonist [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin and the non-peptide agonist [^{3}H]-dihydromorphine are the same and are solely inhibitory. In contrast the effects of NaCl on the binding of [^{3}H]-naloxone, [^{3}H]-(−)-bremazocine and [^{3}H]-diprenorphine combine potentiation and, at higher concentrations, inhibition. There are quantitative differences in the parameters of these three dose-response curves, possibly because at the ligand concentrations used in these experiments about 30% of the binding of [^{3}H]-(−)-bremazocine or of [^{3}H]-diprenorphine and about 15% of the binding of [^{3}H]-naloxone were not at μ-sites.

There may be species differences in the effects of salts on binding. For instance in rabbit cerebellum (Figure 1, Table 2) and in guinea-pig whole brain (Paterson *et al.*, 1986) low concentrations of NaCl potentiate the μ-binding of [^{3}H]-naloxone but inhibition occurs at higher concentrations. In rat whole brain, however, the potentiating effect of NaCl is maintained when the concentration is as high as 200 mM and this effect is mimicked by LiCl (Pert & Snyder, 1974). Although in rabbit cerebellum a potentiating effect of LiCl cannot be observed, it is important that LiCl discriminates between the μ-binding of [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin for which it has a threshold of 0.6 mM, and that of [^{3}H]-naloxone and [^{3}H]-(−)-bremazocine, for which it has thresholds of 9.4 and 12.7 mM (Table 2). A possible potentiating effect on the binding of the latter two compounds may have been masked. Finally, the effects of KCl and choline chloride are only inhibitory and are of the same order for all ligands tested, a finding that indicates that the potentiating effect is not a property

characteristic of all monovalent cations. The inhibitory effect of monovalent cations appears to be non-specific for μ-opioid ligands since it has been induced by many inorganic and organic cations (Pert & Snyder, 1974; Paterson *et al.*, 1986; Kosterlitz & Paterson, unpublished observations).

In *in vitro* pharmacological assays on the guinea-pig myenteric plexus and the rat vas deferens, diprenorphine and bremazocine are, like naloxone, antagonists at μ-receptors (Gillan *et al.*, 1981; Corbett & Kosterlitz, 1986; Traynor *et al.*, 1987). Our finding that at the μ-sites of the rabbit cerebellum a potentiating effect of NaCl on binding occurs with [^{3}H]-naloxone, [^{3}H]-(−)-bremazocine and [^{3}H]-diprenorphine, but not with [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin and [^{3}H]-dihydromorphine indicates that the potentiating effect of sodium ions at the μ-site occurs with antagonists but not with agonists. This supports, as far as the binding of [^{3}H]-naloxone and [^{3}H]-dihydromorphine are concerned, the results obtained in rat brain (Pert & Snyder, 1974). In this context it is of interest that diprenorphine is an antagonist at the κ-receptor in the rabbit vas deferens but an agonist at the κ-receptor in the guinea-pig myenteric plexus (Traynor *et al.*, 1987). Therefore, it cannot be decided whether, in analogy with the finding at the μ-sites, the small potentiation by NaCl of the κ-binding of [^{3}H]-diprenorphine in guinea-pig cerebellum reflects κ-antagonist activity.

In contrast to the findings with NaCl, the effects of $MnCl_2$ on the binding of the μ-agonist [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin in the rabbit cerebellum comprise both potentiating and inhibitory components; its effects on the binding of the μ-antagonists [^{3}H]-naloxone, [^{3}H]-(−)-bremazocine and [^{3}H]-diprenorphine cause inhibition, apparently without potentiation at lower concentrations. Since the threshold values and slopes of the dose-response curves are of the same order for inhibition of the binding of the agonist [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin, and the antagonists [^{3}H]-naloxone and [^{3}H]-(−)-bremazocine but much lower for a third antagonist [^{3}H]-diprenorphine, the ligand-dependent differences in the effects of $MnCl_2$ at the μ-site cannot at present be explained by differences in agonist or antagonist activities. It will be necessary to investigate the effects of the salts after separately blocking potentiation or inhibition.

The findings obtained in this investigation emphasize our view that, in order to determine the properties of opioid binding sites, it is important that ligands interact with only one type of site. While these ligands should be enzyme-resistant, it will be important to develop experimental methods that will prove whether identical results are obtained with their endogenous, enzyme labile counterparts. Furthermore, to arrive at reliable correlations, the binding and pharmacological properties of each ligand should be assessed in the same tissue.

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