Pergamon

Biochemical Pharmacology, Vol. 49, No. 7, pp. 883–891, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(94)00512-5

κ-OPIOID RECEPTOR RECOGNITION SITES ARE NOT MODULATED BY LOCAL ANAESTHETICS

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(*Received* 2 *May* 1994; *accepted* 5 *November* 1994)

Abstract—Local anaesthetics and opioid drugs function synergistically to provide analgesia. In the present study, the nature of this synergy has been investigated using *in vitro* radioligand binding to determine whether the local anaesthetics bupivacaine and tetracaine modulate the binding of two κ-opioid receptor ligands, [^{3}H]U-69593 (5-α,7-α,8-β-(-)-*N*-methyl-*N*-[7-(1-pyrrolodinyl)-1-oxaspiro(4,5)dec-8-yl]-benzene acetamide) and [^{3}H](-)-EKC (ethylketocyclazocine). [^{3}H]U-69593 bound with a K_D of 0.88 nM and a B_{max} of 2.39 ± 0.22 fmol/mg wet weight in guinea pig cerebellar membranes. The binding was inhibited by bremazocine and morphine with Hill slopes near unity and pI_{50} values of 9.96 and 6.84–6.86, respectively. [^{3}H]U-69593 binding was inhibited by Gpp(NH)p (5′-guanylyl imidodiphosphate) and NaCl, consistent with an agonist action of the compound. The binding characteristics of the ligand were not changed by bupivacaine or tetracaine. [^{3}H](-)-EKC bound with K_D values of 0.55 and 0.97 nM and B_{max} values of 4.22 and 0.99 fmol/mg wet weight in guinea pig cerebellar membranes and rat spinal cords, respectively. In the rat spinal cord, [^{3}H](-)-EKC appeared to act as an agonist/antagonist, since the presence of Gpp(NH)p and NaCl only produced a small (21%) reduction in binding, but reduced the pI_{50} for the residual binding to inhibition by morphine from 6.33–6.39 to 5.95. As with [^{3}H]U-69593, the binding characteristics of [^{3}H](-)-EKC were not affected by bupivacaine or tetracaine. These studies demonstrate that effects on κ-opioid receptor recognition site conformation are unlikely to explain the clinically observed synergy between local anaesthetics and opioids.

Key words: local anaesthetics; opioid receptors; morphine; U-69593; ethylketocyclazocine; bremazocine; α-neoendorphin; G-protein

Local anaesthetics and opiate analgesics are often administered to treat post-operative and obstetric pain. Clinical and animal studies have shown that concomitant opiate and local anaesthetic therapy improves the magnitude and duration of pain relief when compared to the effects of each drug administered separately [1–3]. These effects of concomitant therapy are consistent with the observation that local anaesthetics potentiate opioid-induced analgesia [4, 5]. Synergistic analgesic responses to these compounds are significant because combination therapy allows for the management of pain with a reduced risk of the patient developing side effects or tolerance to the medication.

The nature of the synergistic interaction between the opioids and local anaesthetics has not yet been determined. It is suggested that local anaesthetics may alter opioid pharmacokinetics [6], attenuate the opioid response via an interaction at the second messenger level [7], or block voltage sensitive Ca^{2+} channels in primary afferent terminals to facilitate the pre-synaptic inhibition of neurotransmitter release by opioids [5].

Alternatively, a recent investigation of opioid binding in the rat spinal cord suggested that bupivacaine altered the properties of the κ-opioid recognition site [8]. Thus, in the presence of the synthetic opioid peptides DAMGO‡ and DADLE (to block non-selective binding of the ligand to μ- and δ-opioid sites, respectively), very low concentrations of bupivacaine potentiated both [^{3}H]-EKC binding and, in particular, the inhibition of [^{3}H]EKC binding by morphine. The increase in potency of morphine found in the presence of low concentrations of bupivacaine was rather dramatic. Thus, at a morphine concentration of 15 nM, the presence of 15–154 nM bupivacaine increased the inhibition of [^{3}H]EKC binding from 27% to 79–83% [8]. Such a large change would mean that in the presence of low concentrations of bupivacaine, morphine acts as a κ- and μ-opioid receptor agonist rather than as a μ-opioid receptor selective agonist. These effects were presented as evidence that local anaesthetics induce a conformational change in κ-opioid receptors.

However, the investigation summarised above [8] used only the combination of bupivacaine and morphine, so whether the observations reflect a general effect for all local anaesthetics and opioids remains unanswered. Also, in view of the tissue- and ligand-dependent pharmacology of κ-opioid receptor recognition sites [see 9], the study did not indicate as to whether such changes in recognition

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‡ Abbreviations: U-69593, 5-α,7-α,8-β-(−)-*N*-methyl-*N*-[7-(1-pyrrolodinyl)-1-oxaspiro(4,5)dec-8-yl]-benzene acetamide; EKC, ethylketocyclazocine; DADLE, [D-Ala^2,D-Leu^5]-enkephalin; DAMGO, [D-Ala^2,*N*-Met-Phe^4, Gly-ol^5]-enkephalin; Gpp(NH)p, 5′-guanylyl imidodiphosphate.

site pharmacology are specific to spinal cord [^{3}H]-EKC binding sites or are found globally.

In consequence, the present investigation considers the interaction of bupivacaine and tetracaine with several opioids at the κ-receptor site defined by the selective ligand [^{3}H]U-69593 [10] in the κ-rich guinea pig cerebellum [11]. Comparisons are made with the [^{3}H]EKC binding sites in rat spinal cord. Some of the present results have been presented elsewhere in abstract form [12] and in a review article [9].

MATERIALS AND METHODS

Materials. [^{3}H]U-69593 was purchased from Amersham International plc, U.K. (specific activity 57 Ci/mmol). [^{3}H](-)-EKC was purchased from DuPont NEN, Bad Homburg, Germany (specific activity 28.5 Ci/mmol). Levallorphan tartrate was obtained from Roche, Switzerland. The local anaesthetics used in this study were bupivacaine (Astra Pain Control AB, Södertälje, Sweden) and tetracaine (Astra, MA, U.S.A.). Gpp(NH)p, α-neoendorphin and BSA were obtained from the Sigma Chemical Co., St Louis, MO, U.S.A. Bremazocine HCl, non-radioactive U-69593, DADLE and DAMGO were purchased from Research Biochemicals International, MA, U.S.A. Morphine HCl was obtained from Apoteksbolaget, Göteborg, Sweden. With the exception of α-neoendorphin, which was dissolved in distilled water and stored at −20° in aliquots, all drugs were weighed out fresh daily, dissolved in distilled water and diluted to the final assay concentration in 50 mM Tris buffer, pH 7.4.

Tissue preparation. Radioligand binding assays were conducted on pre-frozen cerebella from male white Dunkin–Hartley guinea pigs aged 30–40 days or pre-frozen (unless otherwise stated) spinal cords from male Sprague–Dawley rats weighing 200–250 g (aged 30–40 days). All animals were supplied by BK Universal AB, Sollentuna, Sweden.

The guinea pigs were killed by decapitation and the cerebella rapidly dissected on ice and preserved between −70° and −85° prior to preparation of homogenates. Cerebella had been stored between 14 and 24 months prior to assay. However, storage has been shown not to affect the B_{max} or K_D values of [^{3}H](-)-EKC binding sites in this tissue [13]. Homogenates were prepared from 0.8–1.0 g wet weight of cerebellum essentially as described by Sharif *et al.* [14]. Tissue was homogenised by Ultra-Turrax in 50 mM Tris buffer (pH 7.4) containing 1 mM Na_2EDTA and centrifuged at 44,000 *g* for 20 min. The pellet was resuspended in the same buffer and incubated for 45 min at 37° in order to degrade endogenous opioids. Homogenates were centrifuged twice more and the final pellet resuspended in 0.32 M sucrose (200 mg wet weight/mL) and stored at −70° to −85° until the day of the assay.

Rats were killed by decapitation and the spinal cord regions rostral to L-5 or L-6 rapidly dissected on ice and, unless otherwise stated, preserved at temperatures between −70° and −85° for 0–12 months prior to preparation of homogenates. Homogenates were prepared from 2.5–3.5 g wet weight of spinal cord in the same manner as for the guinea pig cerebella described above. The final pellet was suspended in 0.32 M sucrose (600 mg wet weight/mL) and stored at −70° to −85° until the day of the assay.

Radioligand binding assay procedures. On the day of the assay, guinea pig cerebella tissue homogenates were thawed and diluted to approximately 15 mg wet weight/mL with 50 mM Tris buffer, pH 7.4, prior to addition to assay vials. Similarly, rat spinal cord homogenates were diluted to approximately 60 mg wet weight/mL with 50 mM Tris buffer, pH 7.4. The spinal cord homogenates were sonicated three times at 5 sec intervals using a Vibra Cell VCX 400 (Sonics and Materials Inc, U.S.A.) to improve the consistency of the homogenate. Binding was measured by the addition of [^{3}H]U-69593 or [^{3}H]-(-)-EKC (the latter in the presence of 100 nM DAMGO and 100 nM DADLE to inhibit binding of this ligand to μ- and δ-opioid receptors) in guinea pig cerebella homogenates and [^{3}H](-)-EKC in rat spinal cord homogenates at the concentrations indicated in the table and figure legends. The assay volume was 500 μL, of which 350 μL was the membrane preparation. Non-specific binding was defined as binding measured in the presence of 1 μM levallorphan.

Samples were incubated for 60–90 min at room temperature (20–25°) upon which binding was rapidly terminated by vacuum filtration using a Brandel Cell Harvester. Separation of bound ligand from free ligand was achieved by glass fibre filters (Whatman GF/B) which had been pre-soaked for 4 hr in 0.1% polyethylenimine to decrease non-specific binding. After separation, filters were washed three times with 5 mL 50 mM Tris buffer, pH 7.4, at room temperature and allowed to dry prior to addition of 4 mL of scintillant (Ultima Gold, Packard) and analysis by liquid scintillation spectroscopy with quench correction. Individual assays were performed in duplicate, triplicate or quadruplicate.

Analysis of data. Radioligand binding data are expressed either as fmol/mg wet weight or as a percentage of control values where appropriate. Control values were assayed concurrently in the absence of test compound. Control samples, test substances and levallorphan were arranged in a consistent order on assay filters to minimise error due to interfilter variations.

Data from the [^{3}H]U-69593 and [^{3}H](-)-EKC saturation curves were analysed by Rosenthal transformation [15] to determine K_D and B_{max} values. Hill slopes and pI_{50} values were calculated from linear regression analyses of individual plots of $\log_{10}$(% inhibition/[100 − % inhibition]) vs $\log_{10}$[drug]. Only % inhibitions in the contiguous range between 10–90% were used since values <10% or >90% will bias regressions. In all cases the regressions were significant ($P < 0.05$). Statistical significance of data was determined using one- and two-way ANOVAs.

RESULTS

Saturation curves of the binding of [^{3}H]U-69593 and [^{3}H](-)-EKC to the different preparations were

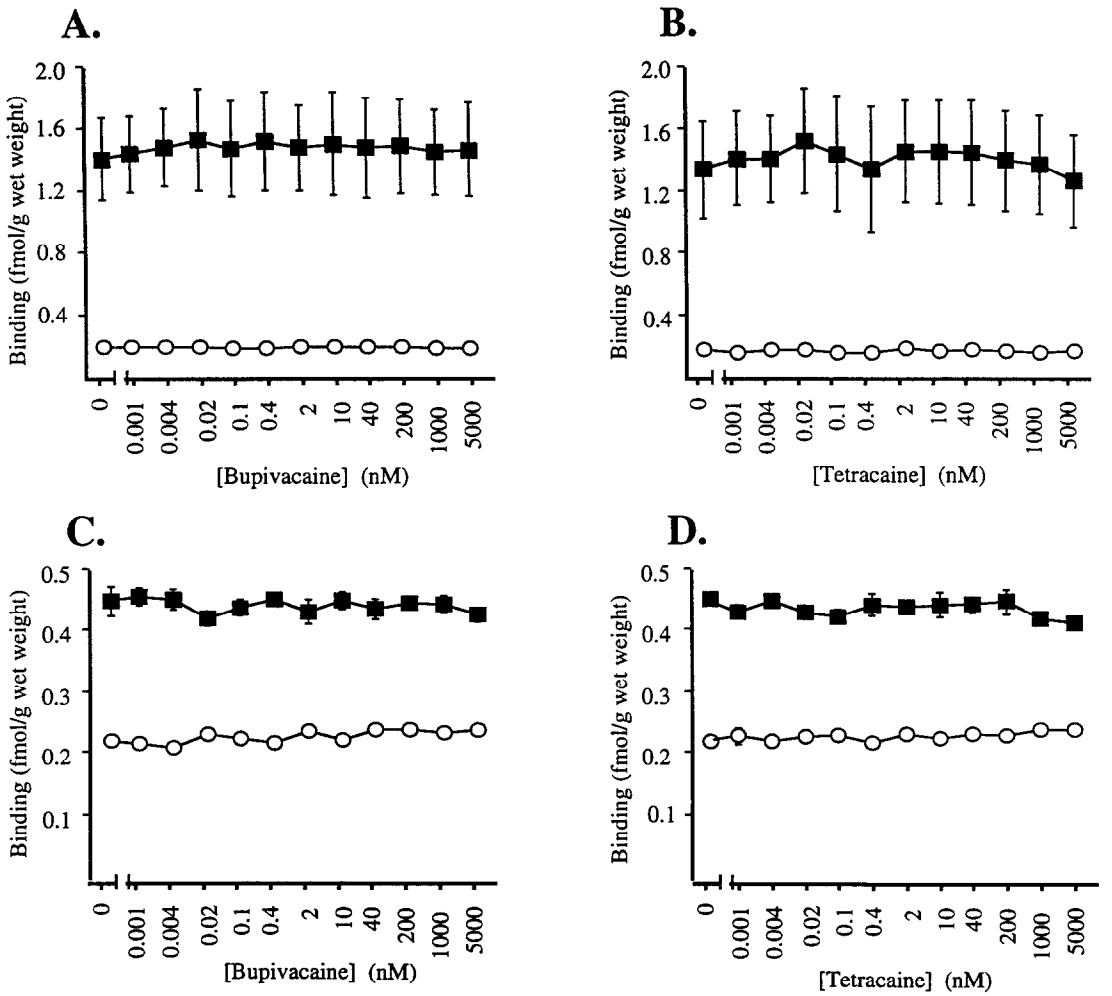

Fig. 1. Effects of local anaesthetics on [^{3}H]U-69593 and [^{3}H](-)-EKC binding in guinea pig cerebellar membranes and rat spinal cord. Data are means ± SEM (when larger than the symbols), N = 3 for specific (filled squares) and non-specific (unfilled circles) binding. (A) and (B) represent [^{3}H]U-69593 binding performed on guinea pig cerebellar membranes at a ligand concentration of 0.85 ± 0.008 nM. (C) and (D) represent [^{3}H](-)-EKC binding performed on rat spinal cords at a ligand concentration of 1.67 ± 0.026 nM. One-way ANOVA in each case indicated no significant effects of the local anaesthetics on specific or non-specific binding ($F_{11,24} < 1.1$, $P > 0.42$).

undertaken. Binding of [^{3}H]U-69593 to the rat spinal cord membranes was not undertaken since it has been established that this ligand shows very low specific binding in this tissue [10]. In each case, the binding was saturable, and Rosenthal analysis of [^{3}H]U-69593 binding to guinea pig cerebellar membranes gave a K_D value of 0.88 ± 0.10 nM and a B_{max} of 2.39 ± 0.22 fmol/mg wet weight (means ± SEM, N = 4). For [^{3}H](-)-EKC binding, K_D values of 0.55 ± 0.09 and 0.97 ± 0.02 nM and B_{max} values of 4.22 ± 0.12 and 0.99 ± 0.07 fmol/mg wet weight (means ± SEM, N = 4 and N = 3) were found for guinea pig cerebellar membranes and rat spinal cords, respectively.

The effects of a wide range of concentrations of bupivacaine and tetracaine on the binding of [^{3}H]U-69593 (guinea pig cerebellum) and [^{3}H](-)-EKC (rat spinal cord) are summarised in Fig. 1. One-way ANOVA analyses indicated that there was no significant effect of either local anaesthetic on the specific binding of either ligand.

The effects of bupivacaine and tetracaine upon the inhibition of radioligand binding by morphine are shown in Figs 2 and 3, and the pI_{50} values and Hill coefficients determined from the data are summarised in Table 1. In addition, the effects of bupivacaine upon the pI_{50} values and Hill coefficients for inhibition of ligand binding by bremazocine and α-neoendorphin are given in Table 1. In all these experiments, irrespective of ligand or tissue, neither bupivacaine nor tetracaine had a measurable effect on the affinities of the various competitor compounds for the ligand binding sites as determined by one-way ANOVA analysis of the pI_{50} values ($F_{3,7-12} < 1.25$, $P > 0.36$). The local anaesthetics also had no measurable effects on the Hill slopes as

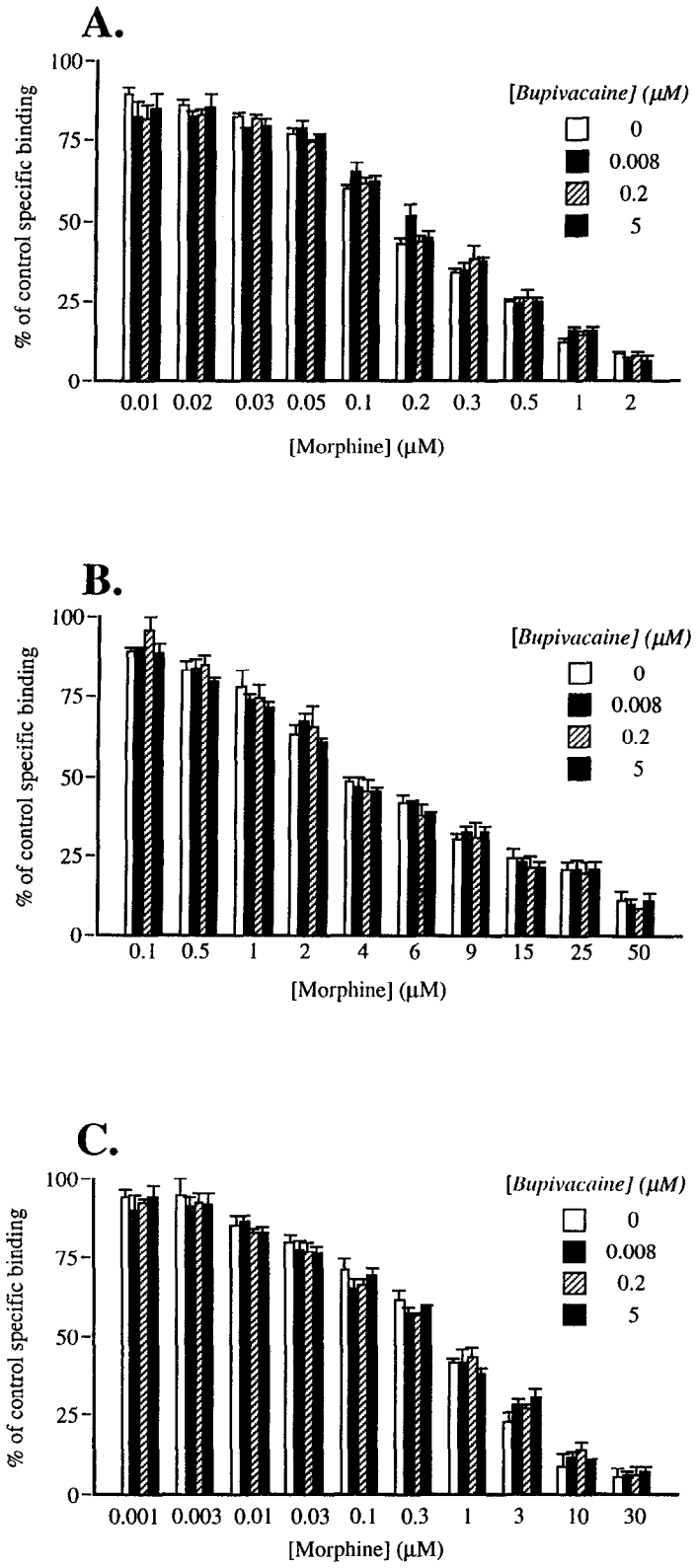

Fig. 2. Inhibition by morphine of 0.84 ± 0.025 nM [^{3}H]U-69593 binding in guinea pig cerebellar membranes (panel A), 1.49 ± 0.17 nM [^{3}H](-)-EKC binding in guinea pig cerebellar membranes (panel B) and 1.62 ± 0.08 nM [^{3}H](-)-EKC binding in rat spinal cord membranes (panel C). The bupivacaine concentrations present are indicated in the figure. Data are means ± SEM, N = 3–4.

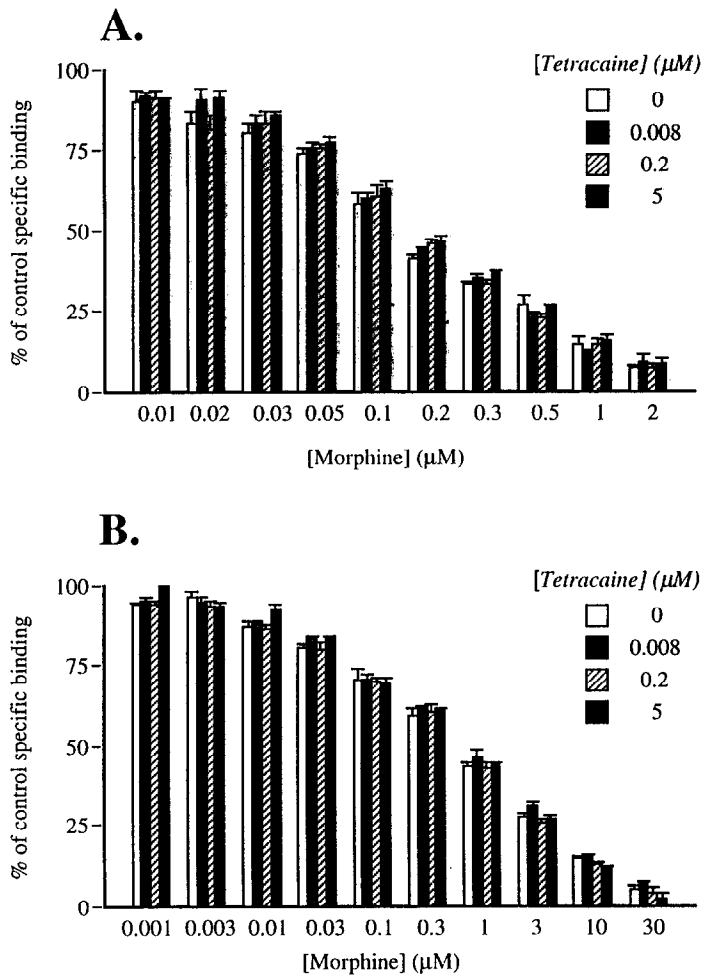

Fig. 3. Inhibition by morphine of 0.83 ± 0.012 nM [^{3}H]U-69593 binding in guinea pig cerebellar membranes (panel A) and 1.48 ± 0.10 nM [^{3}H](-)-EKC binding in rat spinal cord membranes (panel B). The tetracaine concentrations present are indicated in the figure. Data are means ± SEM, N = 3.

determined by one-way ANOVA analysis ($F_{3,7-12} < 2.5$, $P > 0.13$) with two exceptions, the bupivacaine effect on morphine inhibition of [^{3}H]U-69593 in guinea pig cerebellar membranes ($F_{3,12} = 4.4$, $P < 0.05$) and the tetracaine effect on morphine inhibition of [^{3}H](-)-EKC in rat spinal cord ($F_{3,8} = 6.7$, $P < 0.05$). With respect to these cases, examination of the untransformed data from which these values were derived (Figs 2A and 3B) indicate that the local anaesthetics had at best very small effects upon the inhibition of ligand binding by morphine and the significance is thus of no physiological relevance.

All binding studies were conducted on pre-frozen tissues. However, similar results for the inhibition of rat spinal cord [^{3}H](-)-EKC binding by morphine (mean pI_{50} 6.16, mean Hill slope 0.50, no effects of 0.008, 0.2 or 5 μM bupivacaine upon these parameters) were found in two preparations where fresh rather than pre-frozen spinal cords were used.

The specific binding of [^{3}H]U-69593 to guinea pig cerebellar membranes was inhibited in a concentration-dependent manner by the non-hydrolysable GTP analogue, Gpp(NH)p (Fig. 4A). The residual binding found in the presence of a high (100 μM) concentration of Gpp(NH)p was sensitive to inhibition by NaCl, so that the combination of 100 μM Gpp(NH)p + 100 mM NaCl totally inhibited the specific binding (Fig. 4A). On the other hand, the specific binding of [^{3}H](-)-EKC to rat spinal cord membranes was only reduced by 21 ± 3% (means ± SEM, N = 4) in the presence of 100 μM Gpp(NH)p + 100 mM NaCl (data not shown). The specific binding remaining, however, was less sensitive to inhibition by morphine and the Hill slope for inhibition was higher than when Gpp(NH)p and NaCl were absent (Fig. 4B, Table 1).

The effect of bupivacaine upon the regulation of ligand binding by Gpp(NH)p and NaCl was also investigated in these experiments. In the case of [^{3}H]U-69593 binding to the guinea pig cerebellar membranes, the reduction of specific binding found in the presence of 100 mM NaCl was similar in the absence or presence of bupivacaine (Fig. 4A).

Table 1. Effect of local anaesthetics upon the inhibition by opiates of [^{3}H]U-69593 and [^{3}H](-)-EKC binding

Compounds	[^{3}H]U-69593, guinea pig cerebellum			[^{3}H](-)-EKC, guinea pig cerebellum			[^{3}H](-)-EKC, rat spinal cord		
	pI_{50}	Hill slope	N	pI_{50}	Hill slope	N	pI_{50}	Hill slope	N
Morphine	6.84 ± 0.025	0.88 ± 0.023	4	5.43 ± 0.094	0.74 ± 0.063	3	6.39 ± 0.027	0.52 ± 0.074	4
+ 0.008 μM Bupi	6.82 ± 0.038	0.77 ± 0.022	4	5.42 ± 0.051	0.80 ± 0.055	3	6.43 ± 0.084	0.47 ± 0.052	4
+ 0.2 μM Bupi	6.85 ± 0.033	0.77 ± 0.025	4	5.46 ± 0.12	0.80 ± 0.035	3	6.54 ± 0.052	0.46 ± 0.017	4
+ 5 μM Bupi	6.84 ± 0.040	0.79 ± 0.027	4	5.51 ± 0.021	0.68 ± 0.062	3	6.46 ± 0.085	0.48 ± 0.032	4
Morphine	6.86 ± 0.049	0.86 ± 0.032	3				6.33 ± 0.060	0.52 ± 0.013	3
+ 0.008 μM Tetra	6.82 ± 0.021	0.96 ± 0.063	3				6.25 ± 0.043	0.53 ± 0.005	3
+ 0.2 μM Tetra	6.84 ± 0.056	0.91 ± 0.061	3				6.37 ± 0.038	0.54 ± 0.014	3
+ 5 μM Tetra	6.76 ± 0.031	0.97 ± 0.029	3				6.32 ± 0.030	0.60 ± 0.017	3
Bremazocine	9.96 ± 0.045	1.24 ± 0.18	3				8.84 ± 0.019	0.64 ± 0.028	3
+ 0.008 μM Bupi	9.99 ± 0.11	0.93 ± 0.23	3				8.80 ± 0.040	0.78 ± 0.051	3
+ 0.2 μM Bupi	10.06 ± 0.043	0.87 ± 0.007	3				8.83 ± 0.022	0.79 ± 0.069	3
+ 5 μM Bupi	9.98 ± 0.054	0.96 ± 0.017	3				8.82 ± 0.028	0.83 ± 0.049	3
α-Neoendorphin	8.68 ± 0.22	0.53 ± 0.011	3				5.80 ± 0.241	0.37 ± 0.016	3
+ 0.008 μM Bupi	8.69 ± 0.18	0.52 ± 0.024	3				5.68 ± 0.238	0.36 ± 0.021	3
+ 0.2 μM Bupi	8.67 ± 0.17	0.51 ± 0.030	3				5.70 ± 0.315	0.35 ± 0.017	3
+ 5 μM Bupi	8.71 ± 0.22	0.50 ± 0.026	3				5.86 ± 0.360	0.34 ± 0.038	3
U-69593	8.67 ± 0.089	0.90 ± 0.067	4	7.56 ± 0.014	0.74 ± 0.017	3	5.77 ± 0.058	0.73 ± 0.062	3
+ 0.008 μM Bupi				7.59 ± 0.033	0.72 ± 0.029	3			
+ 0.2 μM Bupi				7.54 ± 0.068	0.72 ± 0.025	3			
+ 5 μM Bupi				7.52 ± 0.055	0.76 ± 0.012	3			
+Gpp(NH)p +NaCl									
Morphine							5.95 ± 0.111	0.69 ± 0.056	4
+ 0.008 μM Bupi							5.83 ± 0.042	0.71 ± 0.024	4
+ 0.2 μM Bupi							5.84 ± 0.038	0.72 ± 0.019	4
+ 5 μM Bupi							5.88 ± 0.055	0.71 ± 0.055	4

Data are means ± SEM for the number of experiments shown. The mean ligand concentration range used was 0.78–1.18 nM ([^{3}H]U-69593) and 1.3–1.8 nM ([^{3}H](-)-EKC). For the experiments with Gpp(NH)p and NaCl, the concentration of these agents was 100 μM and 100 mM, respectively.

Abbreviations: Bupi, bupivacaine; Tetra, tetracaine.

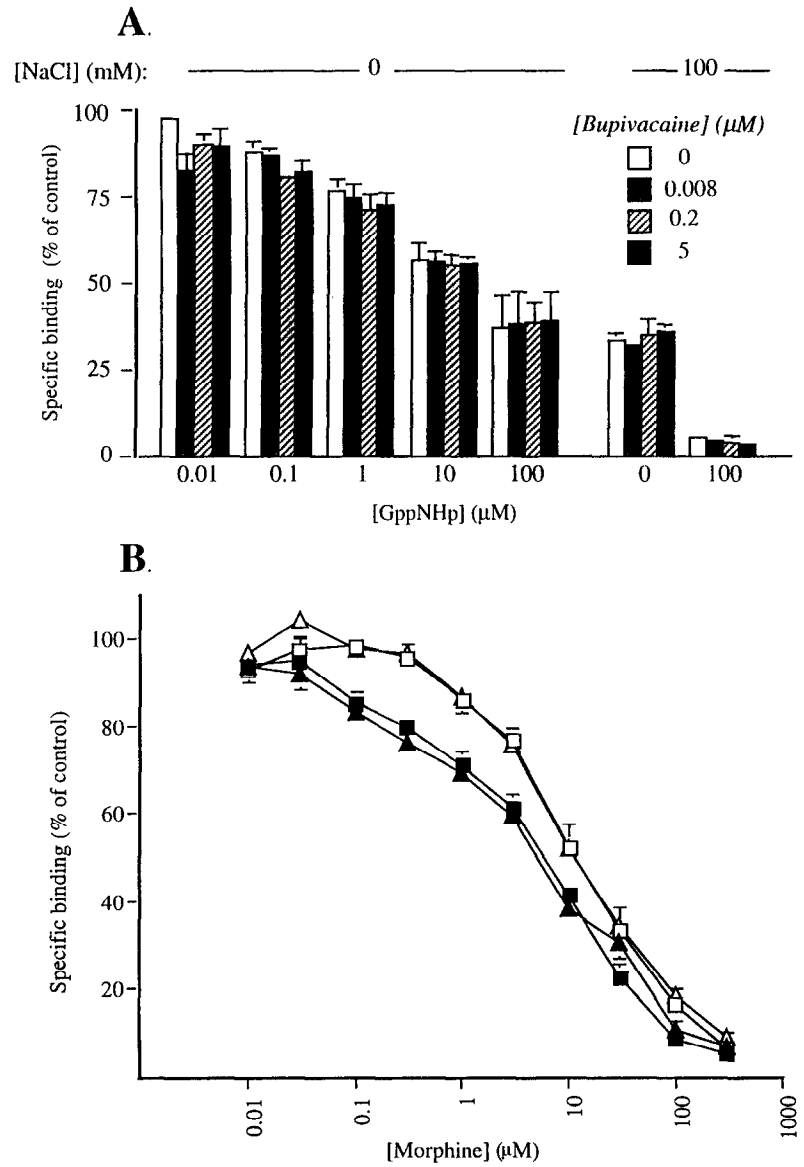

Fig. 4. Panel A. Effect of bupivacaine on the sensitivity of [^{3}H]-U69593 (0.82 ± 0.03 nM) binding to Gpp(NH)p in guinea pig cerebellar membranes. Data are means ± SEM, N = 3. Two-way ANOVA was performed on the untransformed data (i.e. as fmol binding/wet weight). Gpp(NH)p had a significant effect on ligand binding ($F_{5,48} = 92.4$, $P < 0.0001$). Similarly, NaCl had a significant effect on the binding ($F_{1,16} = 560$, $P < 0.001$). No significant effects for bupivacaine ($F_{3,48} = 1.50$, $P = 0.23$), for the interaction bupivacaine × Gpp(NH)p ($F_{15,48} = 0.38$, $P > 0.97$), or for the interaction bupivacaine × NaCl ($F_{3,16} = 0.37$, $P > 0.77$) were found. Panel B. Effect of bupivacaine on the inhibition by morphine of [^{3}H](-)-EKC binding to rat spinal cord homogenates. Data are means ± SEM (shown when their size exceeded the symbol), N = 4 for assays in the absence (filled symbols) or presence (unfilled symbols) of 100 μM Gpp(NH)p and 100 mM NaCl. Shown are the curves for no bupivacaine (□–□) or 5 μM bupivacaine (△–△). The ligand concentration was 0.91 ± 0.04 nM. One-way ANOVA indicated that there was no significant effect of bupivacaine on the response to morphine/Gpp(NH)p and NaCl for either the pI_{50} values ($F_{3,12} = 0.68$, $P > 0.58$) or the Hill slopes ($F_{3,12} = 0.08$, $P > 0.97$).

Bupivacaine did not significantly affect the inhibition of [^{3}H]U69593 binding by morphine under these conditions (Fig. 5). The inhibitory effect of Gpp(NH)p was also not modulated by bupivacaine (Fig. 4A).

In the case of [^{3}H](-)-EKC binding to the rat spinal cord, the reduction of binding produced by 100 μM Gpp(NH)p + 100 mM NaCl was similar at each concentration of bupivacaine tested, values (means ± SEM) of 21 ± 3, 26 ± 4, 26 ± 4 and

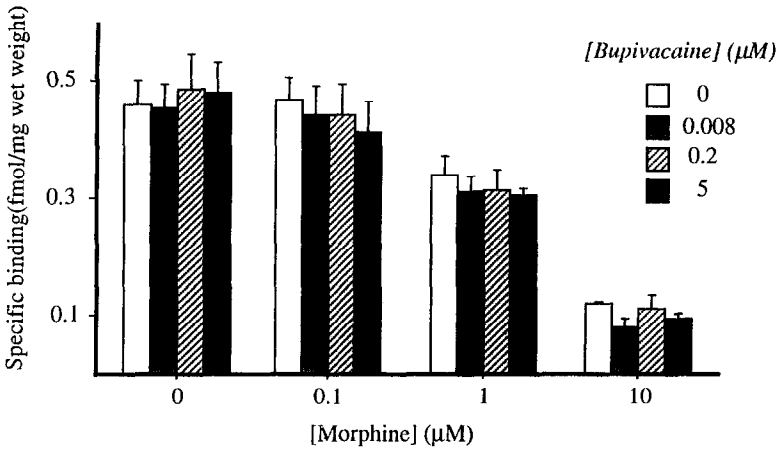

Fig. 5. Effect of bupivacaine upon inhibition of [^{3}H]U-69593 binding to guinea pig cerebellar membranes at an assay NaCl concentration of 100 mM. Data are means ± SEM, N = 3. The ligand concentration was 0.82 ± 0.03 nM. Two-way ANOVA performed on the untransformed data (i.e. as fmol binding/wet weight) indicated a significant effect of morphine ($F_{3,32} = 78.4$, $P < 0.0001$) but not of bupivacaine ($F_{3,32} = 0.46$, $P > 0.71$) or of the interaction bupivacaine × morphine ($F_{9,32} = 0.16$, $P > 0.9$).

29 ± 6% being found at 0, 0.008, 0.2 and 5 μM bupivacaine, respectively. Bupivacaine did not significantly affect either the pI_{50} values or the Hill slopes for the inhibition by morphine of [^{3}H](-)-EKC binding to rat spinal cords assayed under these conditions (Table 1, Fig. 4B).

DISCUSSION

In the present study, the pharmacological properties of opioid binding sites labelled by [^{3}H]U-69593 have been compared with those labelled by [^{3}H](-)-EKC (after blocking binding to μ- and δ-opioid recognition sites). The data with [^{3}H]U-69593 are in line with the literature for guinea pig cerebellar membranes both with respect to B_{max} and K_D values and with respect to the pI_{50} values obtained for the opioid compounds tested [10, 16, 17]. These binding sites correspond to the κ_1-opioid receptor as defined by Zukin *et al.* [18]. The low Hill coefficient for the inhibition of [^{3}H]U-69593 binding by α-neoendorphin is also consistent with the literature [19].

[^{3}H](-)-EKC is a non-selective opioid ligand that has been often used to label κ-opioid receptors in combination with selective compounds to block labelling to μ- and δ-opioid receptor sites. Under these conditions, the B_{max} and K_D values determined agree very well with reported values for [^{3}H]EKC binding in guinea pig cerebellar membranes [13, 16]. Comparison of pI_{50} values for non-radioactive U-69593 competition versus the ligand are also consistent with the literature [16, 18].

[^{3}H](-)-EKC binds to a larger population of sites in guinea pig cerebellum than [^{3}H]U-69593, and in the rat spinal cord the binding of [^{3}H](-)-EKC is inhibited with Hill slopes considerably lower than unity, unlike the situation for [^{3}H]U-69593 binding to guinea pig cerebellar membranes. These observations are consistent with findings from other studies conducted in a variety of tissues [18, 20, 21], although data for [^{3}H](-)-EKC in the rat spinal cord had previously only been obtained in the absence of DAMGO and DADLE (or corresponding compounds) [see 9], with the exception of the study by Tejwani *et al.* [8], who reported 27 and 33% inhibition of specific binding of [^{3}H]EKC by 15 and 30 nM morphine (in the absence of bupivacaine, see below).

These differences in binding characteristics for the two ligands have been interpreted by a number of investigators as evidence for κ-receptor heterogeneity, whereby [^{3}H]EKC binds to the defined κ_1-opioid receptor as well as a novel U-69593-insensitive "κ_2" binding site [13, 16, 22], although the identity of this latter site has been questioned [21, 23]. Alternatively, Richardson *et al.* [24] suggested that apparent κ-receptor heterogeneity might be a consequence of differential labelling of G-protein coupled and uncoupled forms of the receptor secondary to differences in the intrinsic activities of the ligands used. This would provide a simple explanation for the differences in recognition site densities found for the two ligands in guinea pig cerebellum. Our own data on the different Gpp(NH)p sensitivities of the binding sites are consistent with this explanation.

The present study has focused upon the modulation of the [^{3}H]U-69593 and [^{3}H](-)-EKC binding sites by local anaesthetics, in response to the recent suggestion [8] that bupivacaine at low (15–154 nM) concentrations greatly increases the sensitivity of rat spinal cord [^{3}H]EKC binding to inhibition by morphine. These authors suggested that local anaesthetics may induce conformational changes in the recognition sites and that such effects may have relevance with respect to the well-established synergistic effects of local anaesthetics and opioids in man and in experimental animals [1–5].

The present study demonstrated, however, that the local anaesthetics, bupivacaine and tetracaine,

when tested over a wide range of concentrations, did not have any effect on the binding of [^{3}H]U-69593 to guinea pig cerebellar membranes or the binding of [^{3}H](-)-EKC (after blockade of μ- and δ-opioid receptors) to rat spinal cord homogenates. Further, these local anaesthetics did not affect, to any obvious extent, the inhibition of these ligands by a variety of opioid compounds in competitive binding studies. In addition, modulation of the recognition site binding profiles by NaCl and/or Gpp(NH)p was unchanged. These data would suggest that local anaesthetic-induced changes in the properties of the κ-opioid receptor recognition sites cannot explain the observed synergistic properties between local anaesthetics and opiates seen in the clinic. This does not rule out, however, the possibility that there is a functional synergy *in vitro*, such as a change in the signal transduction response to opioid receptor stimulation. Such a possibility is under investigation.

Acknowledgements—The present work was undertaken as part of an exchange programme between McMaster University, Canada and Astra. The authors would like to thank Drs Ray Fynes, P. K. Rangachari and Dennis Crankshaw for making such an exchange possible. The authors would also like to thank Liisi Alari, Inga-Britt Hellberg, Anne Petrén and Mats Sandberg for their help in providing some of the frozen tissue used in the study.

REFERENCES

1. Rawal N, Indications for the use of intraspinal opioids. In: *Spinal Narcotics* (Eds. Rawal N and Coombs DW), pp. 43–61. Kluwer Academic Publishers, Dordrecht, 1990.
2. DeCastro J, Meynadier J and Zenz M, *Regional Opioid Analgesia: Physiopharmacological Basis, Drugs, Equipment and Clinical Application*. Kluwer Academic Publishers, Dordrecht, 1991.
3. Meert TF and Melis W, Interactions between epidurally and intrathecally administered sufentanil and bupivacaine in hydroxypropyl-β-cyclodextrin in the rat. *Acta Anaesthesiol Belg* **43**: 79–89,1992.
4. Åkerman B, Arweström E and Post C, Local anesthetics potentiate spinal morphine antinociception. *Anesth Analg* **67**: 943–948, 1988.
5. Penning JP and Yaksh TL, Interaction of intrathecal morphine with bupivacaine and lidocaine in the rat. *Anesthesiology* **77**: 1186–1200, 1992.
6. Maves TJ and Gebhart GF, Antinociceptive synergy between intrathecal morphine and lidocaine during visceral somatic nociception in the rat. *Anesthesiology* **76**: 91–99, 1992.
7. Lambert DG, Signal transduction: G proteins and second messengers. *Br J Anaesth* **71**: 86–95, 1993.
8. Tejwani GA, Rattan AK and McDonald JS, Role of spinal opioid receptors in the antinociceptive interactions between intrathecal morphine and bupivacaine. *Anesth Analg* **74**: 726–734, 1992.
9. Fowler CJ and Fraser GL, μ-, δ-, κ-Opioid receptors and their subtypes. A critical review with emphasis on radioligand binding experiments. *Neurochem Int* **24**: 401–426, 1994.
10. Lahti RA, Mickelson MM, McCall JM and Von Voigtlander PF, [^{3}H]U-69593 a highly selective ligand for the opioid κ-receptor. *Eur J Pharmacol* **109**: 281–284, 1985.
11. Kosterlitz HW, Paterson SJ and Robson LE, Characterization of the κ-subtype of the opiate receptor in the guinea-pig brain. *Br J Pharmacol* **73**: 939–949, 1981.
12. Fraser G and Fowler CJ, Do local anaesthetics affect kappa-opioid receptor binding? *Pharmacol Toxicol* **72** (Suppl. II): 67, 1993.
13. Ori C, Su T-P, Weissman AD and London ED, Extraordinary postmortem stability of kappa opioid receptors in guinea-pig brain. *J Pharm Pharmacol* **39**: 951–954, 1987.
14. Sharif NA, Durie E, Michel AD and Whiting RL, Dog cerebral cortex contains μ-, δ- and κ-opioid receptors at different densities: apparent lack of evidence for subtypes of the κ-receptor using selective radioligands. *Brain Res* **510**: 108–114, 1990.
15. Rosenthal HE, Graphical method for the determination and presentation of binding parameters in a complex system. *Anal Biochem* **20**: 525–532, 1967.
16. Kinouchi K and Pasternak GW, Evidence for κ_1 opioid receptor multiplicity in the guinea pig cerebellum. *Eur J Pharmacol (Mol Pharmacol Sect)* **207**: 135–141, 1991.
17. Chen J-C, Smith ER, Cahill M, Cohen R and Fishman JB, The opioid receptor binding of dezocine, morphine, fentanyl, butorphanol and nalbuphine. *Life Sci* **52**: 389–396, 1993.
18. Zukin RS, Eghbali M, Olive D, Unterwald EM and Tempel A, Characterization and visualization of rat and guinea pig brain κ opioid receptors: evidence for κ_1 and κ_2 opioid receptors. *Proc Natl Acad Sci USA* **85**: 4061–4065, 1988.
19. Clark JA, Liu L, Price M, Hersh B, Edelson M and Pasternak GW, Kappa opiate receptor multiplicity: evidence for two U50,488-sensitive κ_1 subtypes and a novel κ_3 subtype. *J Pharmacol Exp Ther* **251**: 461–468, 1989.
20. Unterwald EM, Knapp C and Zukin RS, Neuroanatomical localization of κ_1 and κ_2 opioid receptors in rat and guinea pig brain. *Brain Res* **562**: 57–65, 1991.
21. Nock B, Giordano AL, Moore BW and Cicero TJ, Properties of the putative epsilon opioid receptor: identification in rat, guinea pig, cow, pig and chicken brain. *J Pharmacol Exp Ther* **264**: 349–359, 1993.
22. Nock B, Rajpara A, O'Connor LH and Cicero TJ, [^{3}H]U-69593 labels a subtype of kappa opiate receptor with characteristics different from that labeled by [^{3}H]ethylketocyclazocine. *Life Sci* **42**: 2403–2412, 1988.
23. Nock B, Giordano AL, Cicero TJ and O'Connor LH, Affinity of drugs and peptides for U-69593-sensitive and -insensitive *kappa* opiate binding sites: the U-69593-insensitive site appears to be the *beta* endorphin-specific *epsilon* receptor. *J Pharmacol Exp Ther* **254**: 412–419, 1990.
24. Richardson A, Demoliou-Mason C and Barnard EA, Guanine nucleotide-binding protein-coupled and -uncoupled states of opioid receptors and their relevance to the determination of subtypes. *Proc Natl Acad Sci USA* **89**: 10198–10202, 1992.