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Extraordinary postmortem stability of kappa opioid receptors in guinea-pig brain

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Postmortem delay can be an important variable in biochemical studies on autopsy tissue. We subjected guinea-pig brains to gradual cooling, simulating temperature conditions of human postmortem brains, in order to assess the sensitivity of kappa receptors to postmortem degradation. Kappa receptor specific binding was defined as the (−)-[^{3}H]ethylketocyclazocine bound in the presence of 100 nM D-ala^2-D-leu^5-enkephalin and 30 nM morphine. Postmortem delays of up to 16 h did not alter the affinity or density of kappa binding sites. The remarkable stability of kappa receptors may greatly facilitate the study of this opioid receptor subtype in human brain.

The existence of the kappa opioid receptor subtype, mediating sedation and analgesia, was postulated on the basis of studies with ketocyclazocine in the chronic spinal dog (Martin et al 1976). While radioligand binding assays succeeded in characterizing mu and delta binding sites, the lack of specificity of kappa ligands hampered early attempts to demonstrate binding to this receptor subtype (Hiller & Simon 1979, 1980; Pasternak 1980).

More recently, this problem has been overcome by the choice of selective incubation conditions (Wood et al 1981), incubation with drugs that block the access of the kappa ligands to other binding sites (Kosterlitz et al 1981), and discrimination of multiple binding sites by computerized curve fitting techniques (Pfeiffer & Herz 1982). With the use of these approaches, the pharmacological properties of the kappa receptor subtype have been characterized in different species (Kosterlitz et al 1981; Maurer 1982; Wood & Charleson 1982; Garzon et al 1984), and its anatomical distribution within the central nervous system (CNS) has been delineated (Foote & Maurer 1982). The presence of kappa receptors in the human CNS was also investigated (Bonnet et al 1981; Itzhak et al 1982; Maurer 1982; Pfeiffer et al 1982; Gouarderes et al 1986). As receptors are subject to postmortem changes (Kuhar et al 1973), possible degradation must be considered as an important experimental variable in studies of human autopsy tissues. We, therefore, tested the effects of postmortem delay, using a model simulating human autopsy conditions, on the kappa receptor in the guinea-pig brain, as determined by the binding of (−)-[^{3}H]ethylketocylazocine ((−)-[^{3}H]EKC).

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Materials and methods

The following drugs were purchased: (−)-[^{3}H]EKC (specific activity approximately 30 Ci mmol^{-1}) (New England Nuclear Corp.) and D-leu^2-D-ala^5-enkephalin (DADL) (Peninsula Laboratories, San Carlos, CA). Morphine sulphate and levorphanol tartrate were obtained from the Research Technology Branch, National Institute on Drug Abuse.

Eighteen male, 2-month-old, Hartley guinea-pigs were decapitated after they had lost consciousness by breathing CO_2, and their brains were rapidly removed. Three brains were immediately frozen at −70 °C. The remaining brains were placed with 0·9% NaCl in plastic bags which were then sealed and kept in a water bath in which the temperature was regulated to decrease gradually, simulating human autopsy conditions (Spokes & Koch 1978). The brains, in groups of three, were removed from the bath after 6, 10, 13, and 16 h at temperatures of 25, 13, 6, and 5 °C (groups 2, 3, 4, and 5, respectively), and they were stored frozen at −70 °C until assayed.

Membranes were prepared from the whole brain including the cerebellum, as previously described (Kosterlitz et al 1981). Briefly, each brain was weighed and homogenized in 10 volumes of 50 mM Tris-HCl buffer, pH 7·4 at 0 °C with a Polytron homogenizer at setting 5 for 20 s. The homogenates were centrifuged at 40 000*g* for 10 min, and the resulting pellets were resuspended in 10 volumes of 50 mM Tris-HCl buffer containing 100 mM NaCl and 50 μM GTP. The suspension was incubated at 37 °C for 45 min and centrifuged at 40 000*g* for 10 min; the resultant pellet was finally resuspended in 90 volumes of ice-cold 50 mM Tris-HCl buffer. For the receptor binding assay, 1·8 mL of the freshly prepared membranes from 20 mg of wet brain tissue were added to borosilicate tubes containing solutions of unlabelled drugs or buffer. The reaction was started by the addition of 50 μL of (−)-[^{3}H]EKC at various concentrations as noted below, yielding a final volume of 2 mL. To define non-specific binding, 10 μM levorphanol was used to displace specific binding from opioid receptors.

Samples were incubated in triplicate for 40 min at 25 °C, filtered through Whatman GF/C filters using a 48-channel cell harvester (Brandel, Gaithersburg, MD), and rinsed three times with 5 mL ice-cold Tris

buffer. Filters were placed in vials with 5 mL Formula 963 liquid scintillation cocktail (New England Nuclear) and counted the next day in a Beckman LS 2800 liquid scintillation counter.

The data were analysed using the BASIC program EBDA (McPherson 1983), which provided estimates of K_d and B_{max}.

Results

(−)-[^{3}H]EKC can be used as a radioligand to assess kappa opioid receptor binding providing that the cross-interference with other binding sites is eliminated (Su 1985; Tam 1985). In preliminary experiments, therefore, the optimal concentrations of unlabelled DADL and morphine necessary to protect the delta and mu binding sites, respectively, from the (−)-[^{3}H]EKC (0·5 nM) were determined.

Inhibition of the binding of (−)-[^{3}H]EKC by the delta opioid ligand DADL was biphasic, showing a plateau of inhibition at concentrations between 50 and 1000 nM. The portion of the binding inhibited by DADL constituted about 40% of the specific (−)-[^{3}H]EKC binding. In addition, DADL at a concentration up to 10 μM could not displace more than 50% of the (−)-[^{3}H]EKC binding (Fig. 1A).

The remaining binding (60% of total) was subjected to further displacement by morphine, in the presence of 100 nM DADL. The portion of (−)-[^{3}H]EKC binding that was resistant to DADL was sensitive to morphine, the displacement being linear and almost complete at a concentration of 5 μM (Fig. 1B).

The binding sites resistant to 100 nM DADL, which could block all the delta and most of the mu binding sites, and 30 nM morphine, which could probably block the remaining mu sites (Su 1985) without any interference at kappa binding sites, were further characterized. The kappa agonists U50488H (*trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzene-acetamide methane sulphonate hydrate) and bremazocine potently and completely inhibited this portion of (−)-[^{3}H]EKC binding. In two assays of (−)-[^{3}H]EKC displacement, K_i estimates for U50488H were 3·19 and 3·75 nM. Values of K_i obtained with bremazocine were 0·12 and 0·16 nM.

Saturation studies of the kappa sites were conducted in the presence of 100 nM DADL and 30 nM morphine. Using a wide range of radioligand concentrations (0·01–80 nM), (−)-[^{3}H]EKC appeared to bind in a saturable manner and with high affinity to a single population of binding sites (data not shown). Hence, only ten concentrations of the radioligand, ranging between 0·01 and 10 nM, were used in all experiments to examine the effects of postmortem delay on kappa receptors. The values of K_d and B_{max} were obtained by Scatchard analysis of the saturation curves (Fig. 2).

The results from experiments performed on membranes from the brains within each treatment group were averaged. No difference in K_d or B_{max} was observed between groups, even with a postmortem delay of 16 h. Values of K_d ranged from 0·23 to 0·30 nM,

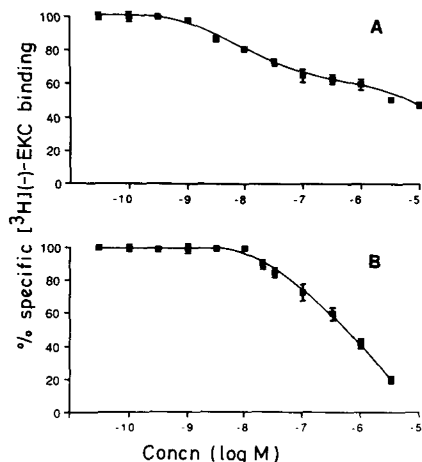

FIG. 1. A. Inhibition of (−)-[^{3}H]EKC (0·5 nM) binding by DADL. B. Inhibition of (−)-[^{3}H]EKC (0·5 nM) binding by morphine in the presence of 100 nM DADL. The results are plotted as the percentage of the maximum (−)-[^{3}H]EKC binding vs log of the molar concentrations of the unlabelled drugs. Each point represents the mean value of three separate experiments performed in triplicate.

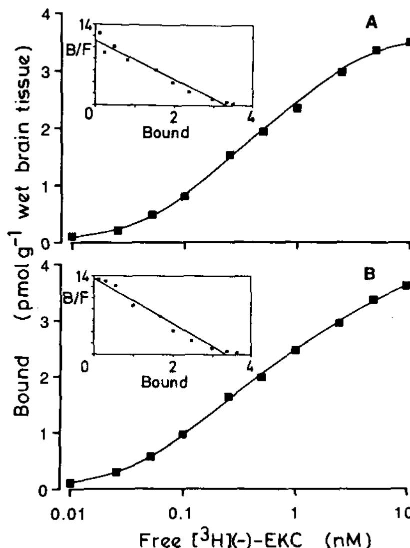

FIG. 2. Saturation curves and Scatchard plots (inserts) of specific (−)-[^{3}H]EKC binding in presence of 100 nM DADL plus 30 nM morphine to guinea-pig brain membranes from groups 1 and 5 (A and B, respectively. See Table 1 for definition of groups). Each value is the mean of three experiments in triplicate. Best-fit lines were determined by linear regression analysis.

Table 1. Effect of postmortem delay on binding characteristics of (−)-[^{3}H]EKC in the presence of 100 nM DADL and 30 nM morphine.

Group	Time after death (h)	K_d (nM)	B_{max} (pmol g^{-1} wet brain tissue)
1	0	0·30 ± 0·04	3·25 ± 0·33
2	6	0·23 ± 0·04	3·68 ± 0·36
3	10	0·24 ± 0·01	3·65 ± 1·23
4	13	0·22 ± 0·01	3·53 ± 0·53
5	16	0·23 ± 0·01	3·25 ± 0·31

The values are the mean ± s.e.m. of three experiments, each performed in triplicate.

and B_{max} varied from 3·25 to 3·68 pmol g^{-1} wet brain tissue (Table 1).

To test the possibility that storage could influence (−)-[^{3}H]EKC binding, the brains were removed and immediately used to prepare the membranes, which were promptly used in binding assays. Again, no significant change in (−)-[^{3}H]EKC binding was observed. The three experiments gave a mean K_d of 0·23 ± 0·08 nM and a B_{max} of 3·89 ± 0·41 pmol g^{-1} wet weight (mean ± s.e.m.).

Discussion

Radiolabelled (±)- and (−)-EKC have been used to characterize the kappa opioid receptor subtypes. Although more specific than (±)-[^{3}H]EKC, which can interact with sigma binding sites, (−)-[^{3}H]EKC is somewhat non-selective, interacting at mu and delta sites. Furthermore, there is no agreement about the relative potencies of the stereoisomers of EKC at kappa binding sites. Kosterlitz et al (1981) observed that the K_d values of (±)-[^{3}H]EKC and (−)-[^{3}H]EKC at the kappa receptor were surprisingly similar, while Su (1985) pointed out the extreme potency of (−)-EKC, in comparison with (±)-EKC, in displacing 10 nM (±)-[^{3}H]EKC binding. Tam (1985) defined kappa binding sites as those to which (−)-[^{3}H]EKC bound in the presence of 500 nM DADL and 20 nM sufentanil. However, the rationale for using these concentrations of DADL and sufentanil to block delta and mu receptors, respectively, was not presented. Therefore, we performed displacement studies with DADL and morphine to determine optimum concentrations of these competing ligands. The data obtained confirmed that 100–500 nM DADL would fully block (−)-[^{3}H]EKC binding to delta receptors without significant interactions at kappa sites. Furthermore, data obtained with morphine were consistent with the reported K_i of morphine at kappa receptors (Tam 1985). Finally, the remaining portion of the binding was characterized as easily displaceable by the kappa-selective ligands, bremazocine and U50488H.

In the saturation experiments, a single class of binding sites with high affinity was observed with B_{max} ranging between 3·25 and 3·68 pmol g^{-1} wet brain tissue, very close to the value reported by Kosterlitz et al (1981). We also confirmed that a high proportion of (−)-[^{3}H]EKC binding in the guinea-pig brain was to kappa receptors. Several studies demonstrated a high concentration of kappa binding sites in the human brain (Itzhak et al 1982; Maurer 1982; Pfeiffer et al 1982). However, the reports made no mention of possible receptor degradation by postmortem delay, which might have differentially affected receptor subtypes. Indeed, delta receptors seemed to be more susceptible to degradation than mu receptors (Syapin et al 1987). A decrease in [^{3}H]dihydromorphine binding, measuring binding to mu receptors, has been associated with prolonged postmortem delay (Kuhar et al 1973).

Gouarderes et al (1986), investigating kappa receptor binding in human lumbo-sacral spinal cord, attempted to simulate human postmortem conditions by keeping rats at 4 °C for 24 h after death, and then removing the spinal cords for study. However, it is not appropriate to equate postmorten cooling of the brain in a small animal to the condition of the brain in the human body. To address this point, Spokes & Koch (1978) subjected rat brains to temperatures simulating the conditions of human brains in-situ during the period following death. By using the experimental design of Spokes & Koch (1978), we demonstrate the remarkable postmortem stability of kappa opioid receptors in guinea-pig brain. This stability may reflect the resistance of kappa receptors to the action of proteolytic enzymes (Pasternak 1980).

Postmortem biochemical changes are a very important issue when dealing with studies involving human autopsy tissues. The differential sensitivities of opioid receptor subtypes to postmortem delay might influence the evaluation of the relative amounts of the subtypes in the tissue. Therefore, the low density of delta receptors detected in human brain (Maurer 1982; Pfeiffer et al 1982) may be explained by their sensitivity to postmortem degradation. On the other hand, the remarkable stability of kappa opioid receptors may account for their reported high concentration in autopsy samples. The biochemical stability of kappa opioid receptors can facilitate the advancement of our understanding of this receptor subtype in the human brain.

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The effects of the calcium channel agonist, Bay K-8644, on guinea-pig ileum and rat uterine horn

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Bay K-8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate) concentration-dependently caused contractions of the partly depolarized ileum, but at higher concentrations (10^{-6} to 10^{-5} M) produced relaxation. 10^{-8} to 4×10^{-7} M nifedipine antagonized while 10^{-9} and 4×10^{-9} M potentiated Bay K-8644. On the partly depolarized uterus, Bay K-8644 (10^{-9} to 10^{-6} M) had only a spasmogenic effect whereas nifedipine (4×10^{-9} to 10^{-7} M) was spasmolytic. Verapamil and diltiazem (each at 10^{-4} M) both reduced the maximal response to Bay K-8644, while other spasmolytics were ineffective. Thus, Bay K-8644 activates ileum and uterus by opening voltage-operated Ca^{2+} channels, but its relaxant action at high concentration and its potentiation by nifedipine is not seen in both organs. Such differences probably depend on the concentration of the compounds used and the polarization state of the cell membranes.

The dihydropyridine, Bay K-8644, is a Ca^{2+} channel activating drug which promotes transmembrane Ca^{2+} influx through voltage-operated channels (Schramm et al 1983). Its effects are competitively antagonized by nifedipine, while non-dihydropyridine Ca^{2+} antagonists, such as verapamil and diltiazem, exert only functional antagonism (Schramm et al 1983; Ishii et al 1985). Although voltage-operated Ca^{2+} channels play an important role in the potential-dependent contractile responses of all smooth muscles (Hurwitz 1986), only a few studies (Allen et al 1985; Spedding 1985) have been made of Bay K-8644's effects on non-vascular smooth muscles. The purpose of the present study was to evaluate the effects of Bay K-8644 on ileum and uterus.

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Materials and methods

The first 5 cm of the ileum nearest to the ileocaecal valve were dissected from male guinea-pigs (300–350 g) and vertically mounted in a 20 mL organ bath under a tension of 0·5 g. Mechanical activity was recorded isotonically on smoked drums. During the experiments different bathing solutions were used. The normal medium had the following composition (mM): NaCl 119, KCl 4·7, $MgSO_4 \cdot 7H_2O$ 1·5, $CaCl_2$ 2·5, KH_2PO_4 1·18, $NaHCO_3$ 25, glucose 11. An 8 mM KCl-enriched medium was prepared by equimolar substitution of NaCl in the normal medium, whereas an 8 mM K^+-rich, Ca^{2+}-free medium was obtained from the K^+-enriched medium by omitting $CaCl_2$ and adding the equimolar amount of Cl^- as NaCl and 0·1 mM of EGTA. The bathing fluids were gassed with a mixture of 5% CO_2 and 95% O_2 to give a pH of 7·3–7·4, and were maintained at 37 °C.

After 60 min equilibration in normal medium, the preparations were repeatedly exposed to 50 mM KCl until the contractile responses were reproducible, and these were defined as 100% control responses. Afterwards the effect of Bay K-8644 was tested on different groups of tissues in the following experimental conditions: (i) in preparations perfused in normal medium; (ii) in preparations partly depolarized by perfusion with 8 mM K^+-enriched medium (subthreshold concentration for contraction) for 30 min; (iii) in preparations perfused with 8 mM K^+-rich, Ca^{2+}-free medium for 30 min. Bay K-8644 was added to the muscle bath in a