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Stereoselectivity of κ-opiate receptor ligands in inhibiting the binding of [^{3}H][3-MeHis^2]thyrotrophin releasing hormone to brain membranes

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Abstract—The effect of (±)-, (−)- and (+)-isomers of several ligands for κ-opiate receptors on the binding of [^{3}H][3-MeHis^2]-thyrotrophin releasing hormone ([^{3}H]MeTRH) to rat brain membranes has been determined. [^{3}H]MeTRH bound to rat brain membranes at a single high affinity site with maximal binding capacity (B_{max}) of 48 ± 2 fmol(mg protein)$^{-1}$, and an apparent dissociation constant, K_d of 4·6 ± 0·2 nM. At a concentration of 2 nM, the specific binding of [^{3}H]MeTRH was 12·3 ± 0·6 fmol(mg protein)$^{-1}$. The isomers of ketocyclazocine, tifluadom (1-methyl-2-(3-thienylcarbonyl) aminomethyl-5-(2-fluorophenyl) *H*-2,3-dihydro-1,4-benzodiazepine), and α-5,9-diethyl-2′-hydroxy-2-(3-furylmethyl)-6,7-benzomorphan [MR 2266 (−), MR 2267 (+)] were used for interaction studies. The (−)-isomer of each of the above drugs was more potent than the (+)-form in inhibiting the binding of [^{3}H]MeTRH to brain membranes, whereas the (±)-forms had activity intermediate between (−)- and (+)-forms. The order of activity of κ-ligands was tifluadom > MR 2266 > ketocyclazocine. It is concluded that κ-opiate drugs inhibit the binding of [^{3}H]MeTRH to brain membranes in a stereoselective manner with tifluadom being the most potent drug.

Besides releasing thyrotrophin and prolactin from the anterior pituitary (Bowers et al 1971), thyrotrophin releasing hormone (TRH) has been shown to exert direct actions on the central nervous system (CNS), since TRH can produce its actions even in hypophysectomized and thyroidectomized rodents. TRH enhances the stimulant action of L-dopa in pargyline-treated rodents (Nemeroff et al 1979) and antagonizes the pharmacological effects of a variety of CNS depressants including barbiturates and alcohol (Nemeroff et al 1979), Δ^9-tetrahydrocannabinol (Bhargava 1980a; Bhargava & Matwyshyn 1980) and ketamine (Bhargava 1981a). The studies on the antagonism by TRH and its analogues of the pharmacological effects of endogenous and exogenous opiates were reviewed by Bhargava et al (1983). In summary, TRH antagonizes the hypothermic, depressant, cataleptic actions of morphine and β-endorphin but it does not affect opiate-induced analgesia. Furthermore, TRH has been shown to inhibit the development of tolerance to (Bhargava 1981b) and dependence on morphine (Bhargava 1980b; Bhargava & Matwyshyn 1985), and inhibit some symptoms of morphine abstinence (Bhargava 1981c). Recent studies from this laboratory indicate that TRH can inhibit gastrointestinal transit in the mouse (Pillai & Bhargava 1984), an effect which is mediated by stereospecific opiate receptors, since such an action was antagonized by MR 2266 [(−)α-5,9-diethyl-2-hydroxy-2-(3-furylmethyl)-6,7-benzomorphan], a benzomorphan opiate antagonist, but not by its (−)-isomer, MR 2267 (Bhargava & Pillai 1985).

Although the mechanism by which TRH interacts with opiates is not well delineated, evidence suggests that it does not interact with μ-opiate receptors, since TRH does not affect the binding of [^{3}H]dihydromorphine or [^{3}H]naloxone (Martin et al 1977; Tache et al 1977). Studies from this laboratory suggest that ligands for μ-opiate receptors do not affect the binding of [^{3}H][3-MeHis^2]TRH ([^{3}H]MeTRH) to brain membranes, but ligands for δ (ICI 154, 129) and κ-(ethylketocyclazocine) opiates inhibit the binding of [^{3}H]MeTRH (Bhargava & Das 1986). Further studies revealed that the binding of [^{3}H]MeTRH to brain membranes was inhibited by tifluadom, a κ-opiate in a non-competitive manner and did not involve GABA or benzodiazepine receptors. Other κ-opiates U-50,488 H (*trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide-methane sulphonate hydrate) and dynorphin (1-13) also inhibited the binding of [^{3}H]MeTRH (Das & Bhargava 1987). Since the inhibitory effect of TRH on gastrointestinal transit was antagonized by MR 2266, a κ-opiate drug, but not by its (+)-isomer, MR 2267 (Bhargava & Pillai 1985), it was of interest to determine whether κ-agents exhibit stereoselectivity or stereospecificity at the brain TRH receptors.

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Materials and methods

Animals. Male Sprague-Dawley rats, 225–250 g, from King Animals, Oregon, WI were acclimatized to the laboratory environment for at least four days before being used. They were housed three to a cage in rooms with controlled temperature (23 ± 1 °C), humidity (50 ± 10%) and artificial lighting (0600–1800h). Food and water were freely available.

Drugs. The drugs used were (−)- and (+)-α-5,9-diethyl-2-hydroxy-2-(3-furylmethyl)-6,7-benzomorphan, respectively, MR 2266 and MR 2267 (Boehringer Ingelheim, W. Germany, Dr H. Merz), ketocyclazocine isomers (Sterling-Winthrop Research Institute, Rensselaer, New York, Mr A. E. Soria), (±)-tifluadom (Sandoz Ltd, Basel, Switzerland, Dr D. Römer), and (−)- and (+)-isomers of tifluadom (Kali-Chemie Pharma, Hannover, W. Germany, Drs Zeugner and Benson). [^{3}H]MeTRH (specific activity 70·4 Ci mmol^{-1}) was purchased from Du-Pont-New England Nuclear Corporation, Boston, MA. Nonradioactive TRH was a gift from the Hoechst-Roussel Pharmaceuticals Inc., Somerville, New Jersey through the courtesy of Mr Val R. Wagner.

Determination of binding of [^{3}H]MeTRH to brain membranes. Whole brain without the cerebellum, obtained from male Sprague-Dawley rats, was homogenized in 20 mL of 0·32 M sucrose with a Brinkman Polytron homogenizer (PT 10 setting No. 5 for 8 s). The homogenate was centrifuged for 10 min at 1500*g*. The pellet (crude nuclear fraction, (P_1) was discarded and the supernatant suspension was centrifuged at 17 500*g* for 30 min in a Sorvall RC-5B refrigerated centrifuge. The second pellet (P_2 fraction) was suspended in 20 mL of 20 mM sodium phosphate buffer (pH 7·4) using the Polytron homogenizer (setting No. 5 for 45 s) and was used for the binding of [^{3}H]MeTRH. The binding assay was essentially based on the method of Simasko & Horita (1982) using 0·2 mL of the homogenate in a total volume of 0·5 mL and containing 0·2 mL of sodium phosphate buffer (pH 7·4) and 0·1 mL of

[^{3}H]MeTRH. [^{3}H]MeTRH was dissolved in the buffer containing 0·1% bovine serum albumin to limit its loss to the glassware. Incubation was in triplicate in a shaking ice bath for 5 h. The incubation was terminated by the addition of 4 mL of ice-cold saline to the incubation tubes. The contents of the tube were rapidly filtered under reduced pressure through a glass fibre filter (GF/F) using a Millipore filtration manifold. The filters were washed twice with 4 mL of ice-cold physiological saline. The filters were transferred to liquid scintillation vials containing 10 mL of 3a 70 cocktail (Research Products International, Elk Grove Village, IL). After an overnight equilibration, the radioactivity in the samples was determined in a Packard liquid scintillation spectrometer (Model 4640) with a counting efficiency of 54%. The specific binding of [^{3}H]MeTRH was defined as the difference in binding obtained in the absence and presence of 100 μM TRH. The specific binding of [^{3}H]MeTRH accounted for 60–70% of the total binding. The concentration of protein in the samples was determined by the method of Lowry et al (1951). The amount of [^{3}H]MeTRH specifically bound was expressed as fmol of ligand bound per mg protein. For the determination of B_{max} and K_d values of [^{3}H]MeTRH the concentration of the ligand used was 1–10 nM. All assays were in triplicate. The resulting data were subjected to Scatchard analysis. The binding constants were determined after subjecting the data to linear regression analysis.

For drug interaction studies, the concentration of [^{3}H]MeTRH used was 2 nM. The concentration of tifluadom ranged from 10^{-8} to 10^{-3} M, whereas that of ketocyclazocine and MR compounds ranged from 10^{-7} to 10^{-2} M. When an interaction was found, the concentration of the drug inhibiting the binding of [^{3}H]MeTRH to 50% of that obtained in the absence of the drug (IC50 values) was calculated. The data are expressed as means ± s.e.m. The differences in the values were analysed by the analysis of variance. A value of $P < 0.05$ was considered significant.

Results

[^{3}H]MeTRH bound specifically to rat brain membranes at a single high affinity binding site with a B_{max} value of 48 ± 2 fmol (mg protein)$^{-1}$, and an apparent dissociation constant, K_d, value of 4·6 ± 0·2 nM. A typical saturation curve and the Scatchard plot for the binding of [^{3}H]MeTRH to membranes prepared from one rat brain is shown in Fig. 1. At 2 nM concentration of [^{3}H]MeTRH which was used for the interaction studies, the specific binding was found to be 12·3 ± 0·6 fmol(mg protein)$^{-1}$.

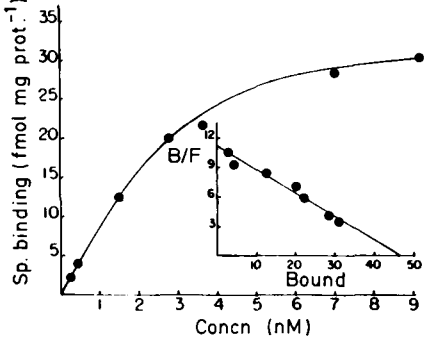

FIG. 1. Saturation curve and Scatchard plot (insert) of the binding of [^{3}H]MeTRH to membranes prepared from one rat brain. The Scatchard plot was drawn after subjecting the data to linear regression analysis.

The effect of the isomers of various drugs acting at κ-opiate receptors is given in Table 1. MR 2266, the (−)-isomer, was active in displacing the binding of [^{3}H]MeTRH with an IC50 value of 1·08 × 10^{-4} M. The (+)-isomer, MR 2267, was 50 times less active than MR 2266 with an IC50 value of 5·25 × 10^{-3} (F 1/7 = 220·7; $P < 0.0005$). (−)-Ketocyclazocine had an IC50 value of 1·66 × 10^{-4} M, whereas the (±)-isomer had an IC50 value of 2·13 × 10^{-4} (F 1/7 = 6·10; $P < 0.04$). The (±)-form of ketocyclazocine exhibited intermediate activity. (−)-Tifluadom inhibited the binding of [^{3}H]MeTRH to brain membranes with an IC50 value of 2·30 × 10^{-6} M. (−)-Tifluadom was five times more potent than the (+)-isomer. The ANOVA analysis indicated a significant difference in the potency of the two isomers (F 1/6 = 372·6; $P < 0.0005$).

Table 1. Effects of racemic and optical isomers of κ-opiate drugs on the binding of [^{3}H]MeTRH to brain membranes.

Test drug	Optical rotation	Concn to inhibit the binding of [^{3}H]MeTRH to brain membranes[a] IC50 value (M) Mean ± s.e.m. (n = 4)
MR 2266	(−)	1·08 ± 1·93 × 10^{-4}[c]
MR 2267	(+)	5.25 ± 0·32 × 10^{-3}
Ketocyclazocine	(−)	1·66 ± 0·10 × 10^{-4}[b]
Ketocyclazocine	(±)	3·12 ± 0·45 × 10^{-4}
Ketocyclazocine	(+)	2·13 ± 0·18 × 10^{-4}
Tifluadom	(−)	2·30 ± 0·10 × 10^{-6}[c]
Tifluadom	(±)	2·40 ± 0·10 × 10^{-6}
Tifluadom	(+)	1·20 ± 0·30 × 10^{-5}

[a] The binding of [^{3}H]MeTRH to rat brain membranes was carried out at 2 nM concentration. The concentration of tifluadom used was in the range of 10^{-8} to 10^{-3} M whereas MR compounds and ketocyclazocine isomers were used in the concentration range of 10^{-7} to 10^{-2} M.

[b] $P < 0.05$; [c] $P < 0.0005$ vs (+)-isomer.

Discussion

Considerable evidence suggests that multiple opiate receptors exists in various mammalian tissues; however, the physiological functions assigned to them are still not clear. Evidence also suggests that opiates interact with TRH. Pharmacological evidence for such an effect has been in existence for some time (Bhargava et al 1983) but the biochemical evidence is only recently emerging. Studies from our laboratory suggest that μ-opiate drugs do not interact with brain TRH receptors labelled with [^{3}H]MeTRH; however, ethylketocyclazocine (EKC, κ-drug) and ICI 154, 129 (δ-drug) appear to inhibit the binding of [^{3}H]MeTRH in a non-competitive manner (Bhargava & Das 1986). However, the IC50 values of opiates in inhibiting the binding of [^{3}H]MeTRH to brain membranes are in the micromolar range or higher.Similarly, tifluadom inhibits the binding of [^{3}H]MeTRH to brain membranes with an IC50 value of 2 μM (Das & Bhargava 1987).

To explore this interaction further, it was of interest to determine whether κ-opiate drugs exhibit stereoselectivity or stereospecificity in inhibiting the binding of [^{3}H]MeTRH. The present studies showthat κ-opiates inhibit the binding of [^{3}H]MeTRH to brain receptors and confirm our finding with EKC (Bhargava & Das 1986), and further show that other κ-agents exhibit differential potency at the brain TRH receptors. Of the three agents tested, tifluadom was the most potent drug which was followed in decreasing order by MR 2266 and ketocyclazocine. In each case, the (−)-isomer was

more potent (2 to 50 times) than the (+)-isomer or the racemate. The greatest separation in the activity of the isomers was observed with MR 2266 in inhibiting the binding of [^{3}H]MeTRH to brain TRH receptors. The stereoselectivity of the action of tifluadom at TRH receptors also compares favourably at the opiate receptor in the absence of sodium (Kley et al 1983).

In summary, the present studies indicate that κ-opioid drugs inhibit the binding of [^{3}H]MeTRH to brain receptors in a stereoselective manner with tifluadom being the most potent agent. However, absolute stereospecificity is not exhibited by κ-opiate drugs in the above effect. These results help in further elucidating the biochemical mechanism for the in-vivo interaction between TRH and opiates.

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Structure-activity relationship of hylambatin and its fragments as studied in the guinea-pig ileum

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Abstract—Hylambatin (Hyl), a dodecapeptide isolated from the skin of the African frog, *Hylambates maculatus*, belongs to the family of tachykinin or physalaemin-like peptides. Hylambatin and its 12 fragments were tested in the guinea-pig ileum preparation for contractile activities. All fragments except 3 had contractile activities. The *C*-terminal fragment as short as the octapeptide sequence was at least as active as the parent molecules. The heptapeptide fragment (Hyl_{6-12}) and the hexapeptide fragment (Hyl_{7-12}) were less active and the *C*-terminal pentapeptide fragment (Hyl_{8-12}) and the *N*-terminal hexapeptide fragment (Hyl_{1-6}) were much less active. The *N*-terminal pentapeptide fragment (Hyl_{1-5}) and the *N*-terminal fragment from which the *N*-terminal Asp or Asp-Pro residues were removed (Hyl_{2-6}, Hyl_{3-6}), were inactive at doses used.

Yasuhara et al (1981) elucidated the structure of a new tachykinin peptide, named hylambatin, isolated from the skin of the South African rhacopharid frog (*Hylambates maculatus*). It is a physalaemin-like dodecapeptide and contains the *C*-terminal amino acid sequence common to all other tachykinins replacing the Leu residue at position 2 from the *C*-terminal by a Met residue (Fig. 1). In parallel bioassay on smooth muscle preparations, blood pressure and salivary secretion, hylambatin and physalaemin were nearly indistinguishable from each other (Falconi Erspamer et al 1984). Hylambatin injected intravenously significantly increased both plasma glucose and plasma insulin in rats (Güllner et al 1984). In guinea-pig ileum, hylambatin was 0·42 times less active than kassinin (Okamoto et al 1984). In the present work, to elucidate the structure-activity relationship, the contractile activities of hylambatin and its fragments were examined in the guinea-pig ileum.

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	1	2	3	4	5	6	7	8	9	10	11	12
A.	Asp	Pro	Pro	Asp	Pro	Asp	Arg	Phe	Tyr	Gly	Met	Met-NH_2
B.		Pyr	Ala	Asp	Pro	Asn	Lys	Phe	Tyr	Gly	Leu	Met-NH_2

FIG. 1. Structures of (A) hylambatin and (B) physaraemin.

Materials and methods

The guinea-pig ileum was mounted in a 20 mL organ bath containing Tyrode solution thermostated at 32 °C and bubbled