Vol. 133, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1985 Pages 60-66

NMB: A HUMAN NEUROBLASTOMA CELL LINE WITH SPECIFIC OPIATE BINDING SITES

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Received September 13, 1985

The human neuroblastoma cell line designated NMB (Brodeur et al., 1977, Cancer 40: 2256) has been shown to have specific opiate binding sites. These sites are highly stereospecific. Two characteristic delta specific peptides, D-Ala^2-D-Leu^5 enkephalin and D-Thr^2-D-Thr^6 enkephalin, have high affinity for the binding sites. Morphine binds specifically but with a much lower affinity. Dextrorphan and the mu specific peptide morphiceptin (Tyr-Pro-Phe-Pro-CO-NH_2) do not bind to the site. The binding sites are heat and trypsin sensitive. Sodium ions specifically lower agonist binding to the sites. Approximately 14,000 binding sites per cell are found. The binding characteristics of these sites are very similar to those of the delta sites characterized on mouse neuroblastoma cell lines.

Clonal murine neuroblastoma cell lines have proven to be of great value in elucidating the structure and function of opiate receptors. For instance the negative regulation of adenylate cyclase by opiates is most convincingly shown in the neuroblastoma x glioma hybrid line NG 108-15 (1,2). This line has also been instrumental in the biochemical characterization of the opiate receptor (3).

Similar studies on human cells with opiate receptors would be worthwhile because some of the interest in opiates and enkephalins derives from their use in medicine. This motivated us to screen the neuroblastoma cell lines previously developed by one of us (M.N.G.) for the presence of opiate binding sites. In this report we show that one human cell line, NMB, expresses an

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Abbreviations: [^{3}H]DADLE, enkephalin (2-D-alanine-5-D-leucine[Tyrosyl 3,5-^{3}H(n)]); DADLE, (D-Ala^2-D-Leu^5] enkaphalin; DTLET, [D-Thr^2-Leu^5] enkephalin-Thr.

0006-291X/85 $1.50

opiate binding site. The binding properties of the site resemble those of the delta site of murine neuroblastomas.

MATERIALS AND METHODS

NMB cells are from the stock collection of one of us (M.N.G.) in whose laboratory the line was established. NMB cells were grown in Dulbecco's Modified Eagle's Medium with high glucose supplemented with penicillin and streptomycin (100 units/ml each), Gibco non-essential amino acids and 10% fetal bovine serum. The cells were grown to confluency in Falcon T flasks. Cells were harvested by adding 10 ml of chilled .05M Tris HCl, pH 7.5 (Buffer A) to each flask and scraping the cells from the surface. The cells suspended in Buffer A were centrifuged at 17,000xg for 10 min. The supernatant was discarded, the pellet resuspended in Buffer A and homogenized in a glass-Teflon homogenizer. The homogenate was aliquoted and frozen at -70°C until ready for use. Protein concentrations of the homogenates were measured by the Lowry method (4).

Opiate binding studies were performed on membranes by methods similar to those developed in other labs (5,6,7). Homogenates were suspended in Buffer A so that the final concentration was 0.5 mg/ml of protein. [D-Ala^2-D-Leu^5]enkephalin[Tyrosyl 3,5-^{3}H(N)]), 43.6 Ci/mMole, abbreviated [^{3}H]DADLE, was added at the indicated concentration in saturation binding assays and in competition assays. The indicated concentration of non-radioactive opiate was added to the reaction mixtures for competition assays. Assay tubes were incubated at 21-22°C for 1 hour. The homogenates were then filtered over Whatman GF/F glass fiber filters. Filters were washed three times with Buffer A at 4°C. The filters were then placed in 10 ml of 3a70 liquid scintillation fluid (RPI, Inc.) and counted in a liquid scintillation counter.

[D-Ala^2-D-Leu^5] enkephalin was obtained from Sigma Chemical Company, St. Louis. [^{3}H]DADLE was purchased from New England Nuclear Co. [D-Thr^2-Leu^5] enkephalin-Thr (abbreviation DTLET) was purchased from Cambridge Research Biochemicals Limited. Morphiceptin (Tyr-Pro-Phe-Pro-NH_2) was the gift of Dr. G.R. Marshall and Dr. R. Nelson. Naloxone was the gift of the DuPont Corporation. The following drugs were obtained from Dr. T.J. Cicero: morphine, levorphanol, dextrorphan, etorphine and ethylketocyclazocine.

RESULTS

The NMB human neuroblastoma cell line has been karyotyped (8). In order to insure that current stocks have not been accidentally contaminated by other cells, the metaphase chromosomes of NMB cells were analyzed by Dr. Gary Brodeur of the Department of Pediatrics, Washington University School of Medicine. His analysis showed that the karyotype included features which differentiate human from rodent and other non-human species. Furthermore, it showed the two homogeneously staining regions (HSRs) on chromosome 13 which had previously been shown to be characteristic of the NMB cell line.

The characteristics of [^{3}H]DADLE enkephalin binding to NMB homogenates were measured by adding increasing concentrations of the ligand to a fixed

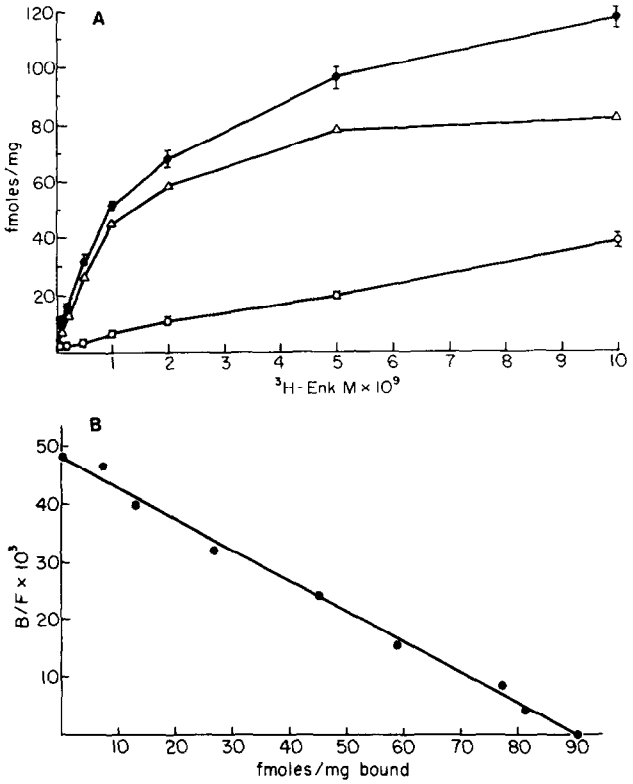

Figure 1. (A) Binding of [^{3}H]DADLE as a function of concentration. [^{3}H]DADLE was added at the concentrations shown on the abscissa either in the absence (filled circles) or presence (open circles) of 2.5 μM levorphanol. Femtomoles bound per mg of protein (average of triplicates with the standard deviation shown) is plotted on the ordinate. Specifically bound drug (Δ) is defined as total binding minus binding in the presence of levorphanol. (B) Scatchard plot of the specific binding in Part A.

quantity of homogenate. Non-specific binding is determined by measuring binding in the presence of 2.5 μM levorphanol. Figure 1 is a typical binding curve and a Scatchard plot of the data. The binding curve shows that specific binding is saturable, and that the ratio of specific to non-specific binding is high. The Scatchard plot is a single straight line which is consistent with binding to a single class of sites. The B_{max} is 89.4 fmoles/mg of homogenate protein. The K_{Diss} is 9.2×10^{-10}M. Table I summarizes the results of this experiment and two other binding experiments. The average value of B_{max} from Table I was used to calculate the number of binding sites per cell. The calculation showed that the NMB cells express 14,000 binding sites per cell.

Table I

Binding of [^{3}H]DADLE to NMB cell homogenates

Experiment	B_{max}	K_{Diss}
1	89.4	9.2×10^{-10}
2	85.2	7.4×10^{-10}
3	83.2	1.0×10^{-9}

The B_{max} is in fmoles/mg protein. The K_{Diss} is in M. The table presents the results from three separate experiments. Experiment 1 is illustrated in Fig. 1; the others were performed in the same way.

Opiate binding sites and receptors have been classified into subtypes on the basis of their interaction with various alkaloids and peptides. We further characterized the binding sites on NMB cells by measuring the ability of a panel of active agents to displace bound [^{3}H]DADLE. The results are shown in Figure 2 and Table II. In each displacement assay [^{3}H]DADLE was added to NMB cell homogenate either alone or with the indicated concentration of unlabelled drug or peptide. Specific binding for each condition was defined as total binding minus the binding in the presence of 2.5 µM levorphanol. Specific binding of [^{3}H]DADLE alone was taken as 100% binding for each inhibition curve. In each assay, points were done in triplicate.

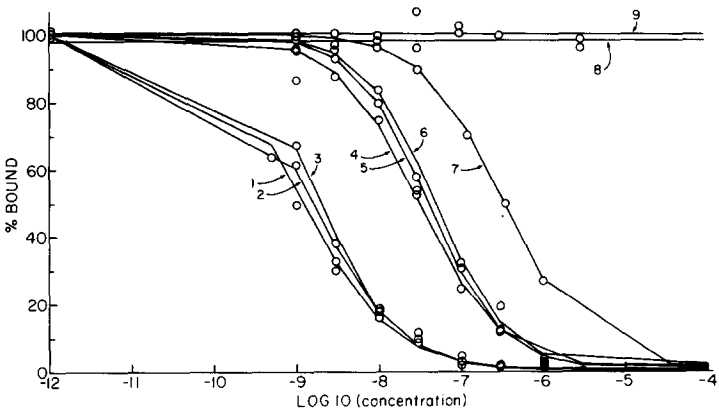

Figure 2. Inhibition of [^{3}H]DADLE binding by opiate alkaloids and peptides. Specific binding is shown on the ordinate with binding in the absence of inhibitors taken as 100%. Concentrations of competing ligands are given on the abscissa. Each curve is obtained by averaging three separate experiments. Competing ligands: 1, DTLET; 2, etorphine; 3, DADLE; 4, levorphanol; 5, ethylketocyclazocine; 6, naloxone; 7, morphone; 8, morphiceptin; 9, dextrorphan.

Table II

IC_{50} of opiates and peptides as displacers of [^{3}H]DADLE

Compound	$IC_{50} \times 10^{-9}$
DTLET	1.22
Etorphine	1.60
DADLE	2.02
Levorphanol	30.97
Ethylketocyclazocine	39.85
Naloxone	48.84
Morphine	330.62
Morphiceptin	---
Dextrorphan	---

The IC_{50} was taken from the curves shown in Fig. 2.

Each inhibition curve was repeated three times. The inhibition curves were determined using the SIGMOID computer program (9) which also calculated the IC_{50} values. The curves in Figure 2 and the values in Table II are the averaged values from the three independent inhibition curves performed by the SIGMOID program. The results show that the delta specific agonist DTLET (10) is the most potent inhibitor of [^{3}H]DADLE binding, being slightly more potent than DADLE itself. Of the alkaloids tested, only etorphine has a potency matching that of DTLET and DADLE. The alkaloids levorphanol, ethylketocyclazocine and naloxone showed an intermediate potency. Morphine was several hundred fold less potent than DTLET and DADLE. Dextrorphan is inactive on the NMB binding site as it is on most opiate binding sites. The mu specific peptide morphiceptin (7) is also inactive on the NMB site.

Opiate binding is extremely sensitive to ionic conditions. Sodium ions decrease agonist binding (11,12). Sodium ions lower the binding of the agonist peptide [^{3}H]DADLE to NMB binding sites. Potassium ions are far less effective indicating the specificity of the sodium effect (data not shown). In the rat brain, opiate binding sites are inactivated by trypsin (11). Table III shows that the NMB binding site is sensitive to trypsin and to heating. These characteristics are consistent with the binding site being a protein or associated with a protein.

Table III

Effect of trypsin, heating on binding activity

Condition	Specific Counts Bound	% Control
1. Control	459±54	100
2. Trypsin experiments		
a. Trypsin alone	35±21	8
b. Trypsin inhibitor	372±54	81
c. Premixed trypsin + trypsin inhibitor	392±65	85
3. Heating	-8±19	-2

Control binding was measured under standard conditions in Buffer A. For the trypsin experiments homogenates were incubated in final concentrations of trypsin alone, .025%; trypsin inhibitor, .125%; premixed trypsin (.025%) + trypsin inhibitor (.125%). Incubation with homogenate was carried out at 37°C for 30 minutes prior to addition of [^{3}H]DADLE. Heating was at 65°C for 5 minutes followed by standard assay.

DISCUSSION

The value of continuous cell lines expressing opiate receptors to the field of enkephalin research is well-established. All of the cell lines in common use have been derived from the mouse, or in one case a mouse x rat hybrid cell line. A human cell line expressing opiate receptors would be of interest because of the widespread use of opiates in medicine and as drugs of abuse. This report documents opiate binding sites expressed by a human neuroblastoma cell line, NMB, which was established by one of us (M.N.G.) from cells metastatic to bone marrow in a 2-year-old child.

Our studies are restricted to binding measurements and do not characterize functional aspects of the binding site. Therefore, we refer to binding sites rather than receptors throughout the text. The present results show that NMB cell membranes have saturable binding sites for the enkephalin analog DADLE. The methods used to define the binding site and the dissociation constant which we obtained are similar to those for the delta subtype of opiate receptor defined in the rat brain (6). Additional points of similarity emerge from a series of competition studies which measure the ability of cold ligands to displace bound [^{3}H]DADLE. The highly delta specific ligand DTLET is a potent displacer having a slightly lower IC_{50} than

DADLE itself. A number of opiate alkaloids including etorphine, levorphanol, ethylketocyclazocine and naloxone are potent displacers of [^{3}H]DADLE binding. Interestingly, morphine itself is a relatively weak displacer and the mu specific peptide morphiceptin is completely inactive. Therefore, the binding site of the NMB cells corresponds closely to the delta subtype of opiate receptor found in rat brain and on murine cell lines. The NMB binding site is also heat and trypsin labile and shows the "sodium effect" towards agonists first described for rat brain receptors (11,12). Thus, NMB cells should provide a convenient source for the analysis of human delta-like binding sites.

ACKNOWLEDGEMENTS

We wish to thank Dr. Raoul Nelson and Dr. Garland Marshall for advice and supplies of morphiceptin. Dr. Gary Brodeur, Department of Pediatrics, Washington University School of Medicine, kindly evaluated the karyotype of the NMB cells. Dr. T. Cicero generously supplied opiate alkaloids. Supported by a grant from the Monsanto Company.

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