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SYMPATHETIC GANGLION CELL X NEUROBLASTOMA HYBRIDS WITH OPIATE RECEPTORS

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Abstract—A number of cultured cells of nerve or smooth muscle origin have been surveyed for stereospecific binding of either [^{3}H]naloxone or [^{3}H]dihydromorphine. Of the cell lines tested, three somatic cell hybrids, TCX17, NX1 and Z5, exhibited substantial specific binding of both naloxone and dihydromorphine. In addition, specific binding of [^{3}H]naloxone by the hybrid clone 108cc15 was confirmed. The neuroblastoma, smooth muscle, glial, and other hybrid cell lines demonstrated little or no specific binding. Specific binding of [^{3}H]dihydromorphine is dose dependent. Dissociation constants for the three hybrid clones range from 15 to 90 nM and maximal binding values range from 75 to 225 fmoles/mg of protein. The order of effectiveness of other opiates in displacing bound [^{3}H]dihydromorphine in TCX17 cells agrees well with that found in brain. Sodium ions selectively inhibit agonist binding (dihydromorphine) but not antagonist binding (naloxone), while potassium and magnesium salts have little effect on either.

Since the demonstration of stereospecific binding of opiates to brain homogenates [1–3], the properties of central nervous system (CNS) opiate receptors have been extensively studied [4–13]. Elucidation of receptor function, however, has been complicated by both the organizational complexity of the CNS and the heterogeneity of cell type. Cultured cells provide several advantages for such studies. They can be grown as homogeneous populations which can be derived from either selected tissues or selected cell types within a tissue. In addition, a number of continuous cell lines have been shown to exhibit morphologic, electrical and biochemical properties expressed by neural cells [14, 15]. The present study was undertaken to identify continuous cell lines which possess opiate receptors and to describe the binding properties of these receptors. Recently, a cell hybrid derived from neuro-blastoma and glioma parental lines was reported to have opiate receptors [16]. We describe here properties of opiate receptors found in somatic cell hybrids that resulted from the fusion of sympathetic ganglion cells with neuroblastoma clones.

MATERIALS AND METHODS

The following chemicals were used: [^{3}H]naloxone (23.6 Ci/m-mole) and [^{3}H]dihydromorphine (41 Ci/m-mole) (New England Nuclear, Boston, Mass.), morphine sulfate (Mallinckrodt Chemical Works, St. Louis, Mo.), naloxone hydrochloride (Endo Laboratories), and levorphanol and dextrorphan tartrate (Hoffmann-La Roche, Nutley, N.J.). All other chemicals were purchased from Sigma, St. Louis, Mo.

Cell lines and growth conditions. The NX hybrid cell lines were obtained by fusion of 13-day embryonic mouse sympathetic ganglion cells and the mouse neuroblastoma clone N18TG2 [17]. The Z3 and Z5 hybrid clones were formed by a similar procedure, utilizing the mouse neuroblastoma clone N4TG1.* The 108cc05 and 108cc15 cell hybrids clones are the result of a cross between the neuroblastoma clone N18TG2 and the rat glioma line BuC6 which is resistant to 5-bromodeoxyuridine [18].

Cells were grown in Falcon dishes at 37° in a water-saturated atmosphere of 5% CO_2–95% air. All cell lines were grown in modified F12 media [19] containing 5% fetal calf serum (GIBCO). Additional modifications to this media were made for hybrid cell lines as follows: (1) media for all hybrid cell types (except TCX cells) was supplemented with additional hypoxanthine (30 μM increased to 100 μM), aminopterin and thymidine (3 μM increased to 16 μM) (HAT) to maintain a selective pressure for retention of at least a portion of the normal neural genome [20]; (2) cell lines designated THX were grown in HAT media in the absence of tyrosine to select for cells with high tyrosine hydroxylase activity [21]; (3) TCX clones were grown in a tyrosine-deficient HAT media which was supplemented with carbachol (100 μM) to select for clones with high tyrosine hydroxylase activity and low sensitivity to acetylcholine;† and (4) TX clones were grown in modified F12 media in the absence of tyrosine.

Assay for specific opiate binding. Growth media for log phase cells was replaced with fresh F12 media containing 5% fetal calf serum 24 hr prior to the binding assay. Cells were harvested by scraping cells in 0.05 M Tris–HCl (pH 7.4), and were sonicated for 5 min. The resulting suspension was briefly homogenized by hand in a glass–Teflon homogenizer and then assayed for the presence of specific opiate binding activity using a modification of the assay described by Pert and Snyder [1]. Incubations were performed at 30° or 37° in an assay mixture containing either 200 nM dextrorphan or 200 nM levorphanol, 0.05 M Tris–HCl (pH 7.4), 150–300 μg protein, and either [^{3}H]naloxone or [^{3}H]dihydromorphine in a final volume of 0.25 ml. Preliminary studies indicated that specific binding was essentially complete in 5–7 min. However, to insure that equilibrium was

* L. A. Greene and W. Shain, personal communication.
† P. R. Myers and W. Shain, personal communication.

established, cell homogenates were preincubated with either dextrorphan or levorphanol for 5 min followed by a 15-min incubation with radioactive drug. At the end of this period, the samples were diluted with 2.5 ml of 0.05 M Tris–HCl (4°), immediately poured onto Whatman GF/C glass filters (2.4 cm dia.) under negative pressure, and washed with five 2.5-ml aliquots of buffer. Radioactivity retained on the filters was determined by means of liquid scintillation counting (efficiency of approximately 48 per cent). The mixture contained Triton X-100 (New England Nuclear), toluene and Liquiflor (New England Nuclear) (100:184:16).

The difference between radioactivity retained in the presence of dextrorphan from that in the presence of levorphanol is defined as specific binding and is designated as femtomoles bound per mg of protein. Cell lines were routinely surveyed for specific binding using at least two concentrations of naloxone (6 and 12 nM) and in some cases additionally with [^{3}H]dihydromorphine (10 nM). [^{3}H]naloxone, a specific narcotic antagonist, is known to bind to the opiate receptor in the brain [1, 2]. All assays were performed in triplicate or quadruplicate.

To better determine the properties of opiate binding to cell lines which showed significant binding in the initial survey, cell homogenates were centrifuged at 20,000g for 10 min and the pellet was resuspended in assay buffer. Aliquots containing 200–250 μg protein were assayed for stereospecific binding of labeled drug. Studies indicated that opiate specific binding was linear from 50 to 350 μg protein under standard assay conditions. In typical assays in which homogenates of the TCX17 clone were incubated with 12 nM [^{3}H]naloxone or [^{3}H]dihydromorphine, radioactivity retained in the presence of dextrorphan and levorphanol was 343 ± 20 cpm and 244 ± 5 cpm, respectively, using [^{3}H]naloxone (190 μg protein) and 833 ± 30 and 614 ± 10, respectively, using [^{3}H]dihydromorphine (275 μg protein). Because sonication did not significantly alter the relative amounts of opiate binding under these conditions, sonication was omitted in these and subsequent studies.

Inhibition of dihydromorphine binding by other narcotics or other agents was assessed by preincubating aliquots of the resuspended 20,000g pellet with the appropriate substances for 5 min followed by a 15-min incubation with [^{3}H]dihydromorphine.

Statistics. Results are reported ± standard error of the mean. The apparent dissociation constant (K_d) and maximum binding (R_0) for either [^{3}H]naloxone or [^{3}H]dihydromorphine to cell preparations was calculated using the weighted regression values for a double reciprocal plot from Cleland's FORTRAN program for statistical evaluation [21].

RESULTS

Specific binding by [^{3}H]naloxone. Of the cell lines surveyed, four hybrid cell lines (NX1, TCX17, Z5 and

Table 1. Specific binding of [^{3}H]naloxone and [^{3}H]dihydromorphine to homogenates of cultured cells of nerve or smooth muscle origin*

Cell line	Cell origin	[3H]naloxone (fmoles bound/mg protein)	[^{3}H]dihydromorphine (fmoles bound/mg protein)
Neuroblastoma			
C1300	Mouse	0–2	
N18TG2	Subclone of C1300	0–2	
IMR	Human	0–4	
Hybrids			
NX1	Neuroblastoma (N18TG2) X sympathetic ganglion cell	29 ± 4	22 ± 3
NX 31, 58, 89, 93	Neuroblastoma (N18TG2) X sympathetic ganglion cell	0–2	
TX 23, 27, 30, 32, 42, 53	Subclone of NX 31	0–2	
TX 22, 60, 71	Subclone of NX 31	2.5–7	
THX 03, 05, 13, 20	Subclone of NX 31	0–2	
TCX17	Subclone of NX 31	18 ± 3	19 ± 4
TCX11	Subclone of NX 31	4–13	4–8.5
Z5	Neuroblastoma (N4TG1) X sympathetic ganglion cell		34 ± 2
Z3	Neuroblastoma (N4TG1) X sympathetic ganglion cell		0
108cc15	Neuroblastoma (N18TG2) X glial tumor cell (BuC6)	29 ± 3	19 ± 2
108cc15	Neuroblastoma (N18TG2) X glial tumor cell (BuC6)	0	
Glial tumor			
B6	Rat	0	
BuC6	Rat	0	
Smooth muscle			
U3	Human umbilical cord		0–2

* Homogenates were incubated in the presence of either 12 nM [^{3}H]naloxone (23.6 Ci/m-mole) or 10 nM [^{3}H]dihydromorphine (41 Ci/m-mole). Specific binding is the difference between radioactivity retained in the presence o 200 nM dextrorphan and 200 nM levorphanol. For cell lines which have substantial binding activity, values are expressed ± S. E. In the case of the cell lines which exhibited low binding activity, the range of values has been presented. Cell lines which did not have significant binding activity (i.e. less than 2 fmoles/mg of protein) are designated as 0 or 0–2.

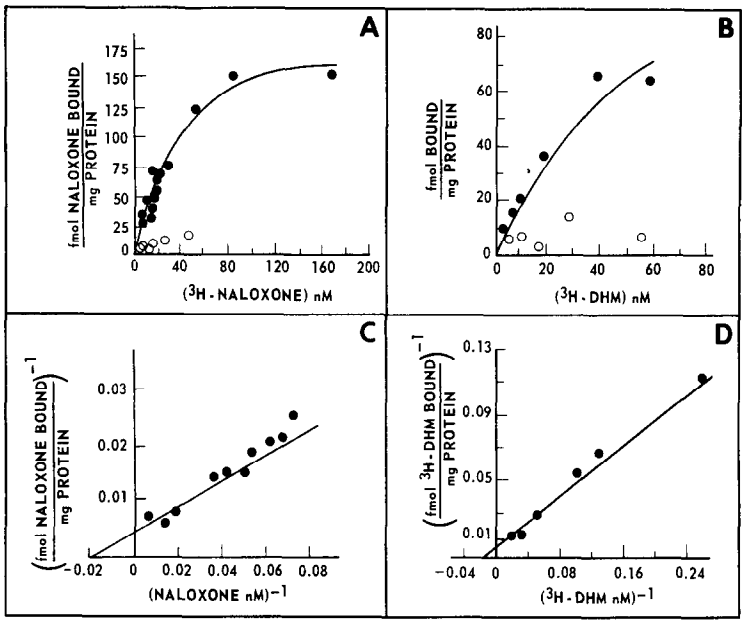

Fig. 1. Binding of [^{3}H]naloxone (panels A and C) and [^{3}H]dihydromorphine ([^{3}H]DHM) (panels B and D) to TCX17 (●) and TCX11 (○).

108cc15) exhibited substantial binding of [^{3}H]naloxone (Table 1). The neuroblastoma, glial and smooth muscle cells and many of the somatic cell hybrids had no apparent affinity for naloxone. However, several hybrid clones demonstrated low specific binding activity, which, although statistically significant, was difficult to measure in a reproducible, quantitative fashion. An example of low binding activity is illustrated in Fig. 1 (open circles) by the TCX11 subclone. Although both [^{3}H]naloxone and [^{3}H]dihydromorphine binding is evident, it is characteristically low and of questionable linearity when plotted as a function of ligand concentration. In contrast, specific binding to a related subclone, TCX17, shows a dose-response relationship to both naloxone and dihydromorphine (Fig. 1, panels A and B, filled circles). From a double reciprocal analysis (Fig. 1, panel C and D), the maximum ligand bound was estimated to be 210 and 140 fmoles/mg of protein for naloxone and dihydromorphine, respectively, with an apparent dissociation constant of approximately 50 nM for both drugs. Specific binding of [^{3}H]dihydromorphine to NX1 (closed circles) and Z5 (open circles) cells also showed a concentration dependence (Fig. 2).

The apparent dissociation constants and number of binding sites (Table 2) varied as a function of the cell line. For example, the Z5 clone has both the lowest dissociation constant and the fewest binding sites, while NX1 cells have the lowest affinity for dihydromorphine but a relatively larger number of binding sites. The maximum specific binding varies from 75 to 225 fmoles/mg of protein, for TCX17, NX1 and Z5 (Table 2) which is somewhat less than that reported for the 108cc15 hybrid cells, but similar to values found in brain (Table 2). However, the dissociation constants for all the hybrid cells are at least an order of magnitude higher than those measured in rat brain preparations (Table 2).

Competition studies. The stereospecificity of the opiate binding activity is clearly demonstrated by studies in which the quantity of dextrorphan of levorphanol required to displace bound [^{3}H]dihydromorphine is measured. As seen in Fig. 3, levorphanol is nearly 10,000 times more potent than its enantiomer in inhibiting [^{3}H]dihydromorphine binding. Morphine and naloxone are intermediate in potency, requiring a concentration an order of magnitude higher than levorphanol to produce 50 per cent inhibition. A number of non-narcotic drugs including prostaglandin E_1, norepinephrine, dopamine, acetylcholine, histamine and serotonin were ineffective at 10^{-5} M in blocking [^{3}H]dihydromorphine binding.

Effect of salts. Sodium salts enhance antagonist binding and inhibit agonist binding to opiate recep-

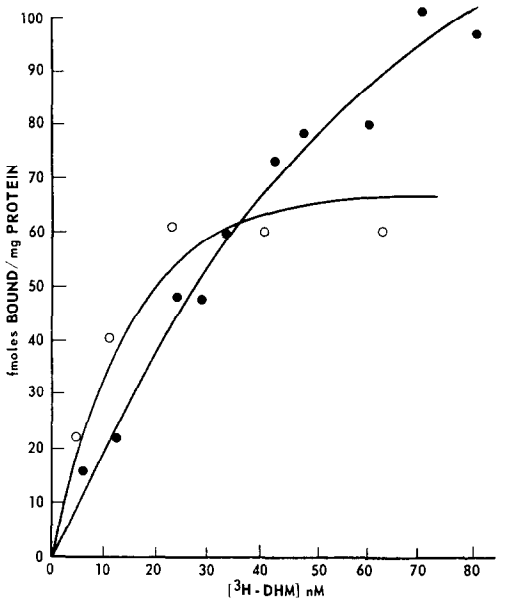

Fig. 2. Binding of [^{3}H]dihydromorphine to NX1 (●) and Z5 (○) cells.

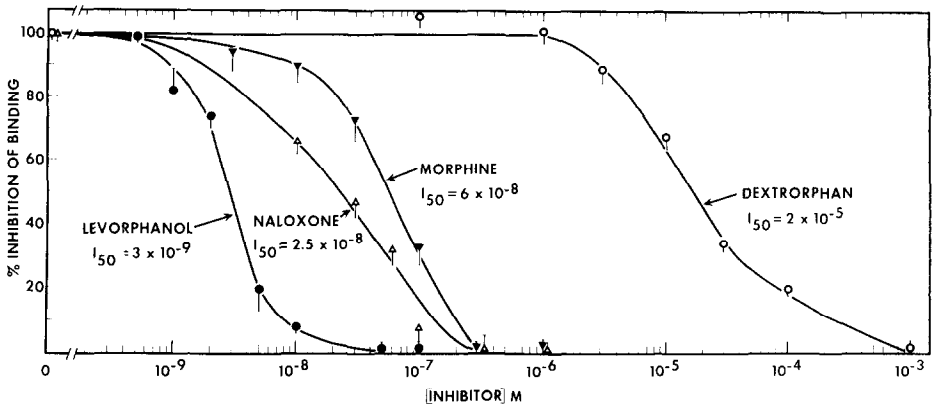

Fig. 3. Inhibition of [^{3}H]dihydromorphine binding to TCX17 by opiates and opiate analogues. Approximately 45 fmoles/mg of protein of [^{3}H]dihydromorphine (20 nM) is specifically bound in the absence of inhibitor.

tors in the central nervous system [6, 11]. Increasing concentrations of NaCl added to homogenates of TCX17 cells resulted in a selective inhibition of specific binding by the agonist, dihydromorphine, while binding of the antagonist, naloxone, remained unchanged (Fig. 4). Potassium and magnesium showed little or no inhibitory effect on the binding of either drug (Table 3), suggesting that the inhibition by cations is specific for sodium.

DISCUSSION

The opiate receptors found in the somatic hybrid TCX17 possess a number of properties which are similar to those described for brain receptors: (1) the binding is highly stereospecific; (2) sodium ion selectively inhibits agonist but not antagonist binding; and (3) narcotic analogues are capable of displacing specifically bound dihydromorphine while other neurohumoral agents have no effect. The rank order effectiveness of the narcotic analogues tested for displacement of dihydromorphine is in good agreement with that found for the 108cc15 hybrid cells by Klee and Nirenberg [16] and is consistent with the relative binding affinities in brain [2, 8, 9].

These opiate receptors differ from those found in brain in that the apparent dissociation constants measured for dihydromorphine binding to the hybrid cells are greater than those found in homogenates of an amygdala–pyriform cortex region or than has been determined in crude synaptosomal fractions from rat brain (Table 2). In addition, specific binding of agonists and antagonists is less sensitive to inhibition by 100 mM potassium and 5 mM magnesium. Pert and Snyder [4] demonstrated that both K^+ and Mg^{2+} reduce [^{3}H]naloxone binding by over 50 per cent and Simon *et al.* [11] reported 15 and 35 per cent inhibition of [^{3}H]naloxone and [^{3}H]levorphanol binding, respectively, by 100 mM potassium. We have observed no appreciable inhibition utilizing similar concentrations of these ions.

The sympathetic ganglion X neuroblastoma hybrids express fewer receptors than reported in the 108cc15 hybrids [16]. This comparison can best be made by estimating the number of receptors per cell from the maximum binding values (assuming one ligand bound/receptor) in Table 2, and the number of cells per mg of protein. Thus, for TCX17 there are approximately 3.2×10^6 cells/mg of protein (protein determined in the 20,000 *g* pellet), and therefore 3×10^4 receptors/cell. Similar calculations give 8×10^4 receptors/cell for NX1 and 3×10^4 receptors/cell for Z5 (1.6×10^6 cells/mg of protein for both cell lines). This is in contrast with the findings of Klee and Nirenberg [16] who reported 3×10^5 receptors/cell in 108cc15.

Table 2. [^{3}H]dihydromorphine binding to cultured cells and brain homogenates

	K_d (nM)	R_0 (fmoles/mg protein)
TCX17	50	140
NX1	90	225
Z5	15	75
108cc15*	20–30	600
Amygdala–pyriform Cortex homogenate†	3.5	100
Brain (P2)‡	1.0	200–250

* See Ref. 16. †J. Blosser and J. Abbott, unpublished results. ‡ See Ref. 9.

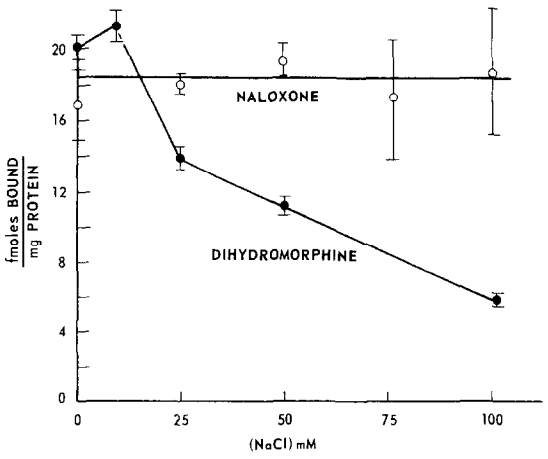

Fig. 4. Effect of NaCl on the binding of [^{3}H]dihydromorphine (10 nM) and [^{3}H]naloxone (8 nM) to TCX17.

Table 3. Effect of cations on [3H]dihydromorphine and [^{3}H]naloxone binding to TCX17*

Salt	[^{3}H]dihydro-morphine (% of control)	[^{3}H]naloxone
NaCl (100 mM)	29 ± 2	103 ± 11
KCl (100 mM)	94 ± 7	85 ± 14
$MgCl_2$ (5 mM)	87 ± 10	85 ± 11

* Cell membrane suspensions (from 20,000 *g* pellet) were incubated as described in Methods with 10 nM of either [^{3}H]dihydromorphine of [^{3}H]-naloxone. Specific binding of dihydromorphine and naloxone was 20 and 18 fmoles/mg of protein, respectively, in the absence of added salts.

Cells with opiate receptors appear to vary phenotypically on the basis of their affinity for [^{3}H]hydromorphine (K_d = 15–90 nM). suggesting that there may be alterations in receptor structure or the membrane environment. It does not appear possible to identify the parental origin of the opiate receptors in any of the somatic cell hybrids since no significant specific binding has been observed in the neuroblastoma parent, N18TG2. Specific opiate binding has not been measured in embryonic mouse sympathetic ganglion tissue nor in the neuroblastoma clone N4TG1. The possibility remains that receptors may originate from either, or both, parental lines and are only expressed in hybrid clones or under certain growth conditions. This may well occur in the case of the TCX17 subclone derived from the NX31 clone which has no apparent specific binding activity.

There is growing evidence that opiate receptors of somatic cell hybrids are functionally related to other membrane phenomena. Morphine has recently been shown to inhibit prostaglandin E_1 (PGE_1) stimulation of adenylate cyclase [22, 23] and increase the levels of cGMP [24] in 108cc15 hybrids. The former interaction has also been reported to occur in homogenates of rat brain [25]. Myers and Livengood [26] have described a dopamine-induced depolarization response in 108cc15 cells, which can be blocked by morphine [27]. Subsequent administration of naloxone in the presence of morphine restores the dopamine response [27]. The TCX17 somatic hybrid also has a depolarization response to dopamine, which can be inhibited by morphine.* Further studies into the nature of these morphine effects should lead to a clearer understanding of the function of opiate receptors and their relationship to other receptors in cell hybrids as well as provide insight into the role of opiate receptors in the CNS.

* P. R. Myers, personal communication.

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