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Novel Opioid Binding Sites Associated with Nuclei of NG108-15 Neurohybrid Cells

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Nuclear opioid binding sites have been discovered in NG108-15 neurohybrid cells. Marker enzyme analyses as well as electron and fluorescence microscopy studies attested to the high degree of purity of the nuclear preparations. Immunohistochemical studies on cryostat sections of NG108-15 cells with an antibody to the opioid receptor corroborated a nuclear localization. ^{3}H-[D-Pen^2,D-Pen^5]enkephalin (^{3}H-DPDPE), ^{3}H-[D-Ala^2,D-Leu^5]enkephalin (^{3}H-DADLE), and ^{3}H-diprenorphine binding parameters, K_d and B_{max} values, and heterologous competition binding and stereospecificity data satisfied criteria for the presence of δ-opioid sites in purified nuclear preparations. Neither μ-([D-Ala^2,mephe^4,gly-ol^5] enkephalin), dihydromorphine, nor κ-(U69593) specific binding was detectable in purified nuclear preparations. Rates of association and dissociation of ^{3}H-[D-Ser^2,L-Leu^5]enkephalyl-Thr were comparable to values obtained previously for opioid receptors. Opioid binding was also shown in subnuclear preparations from NG108-15 cell cultures. Agonists, ^{3}H-DADLE and ^{3}H-DPDPE, bind with high affinity to nuclear membranes and with lower affinity to chromatin. In contrast, partial agonist ^{3}H-diprenorphine high-affinity binding sites were predominant in chromatin, while low-affinity binding was found in the nuclear membrane. Accordingly, 5′-guanylylimidodiphosphate sensitivity of ^{3}H-DADLE binding was detected in nuclear membranes but not in chromatin. Both agonist and partial agonist opioid binding to nuclear membrane and chromatin were abolished upon cycloheximide treatment of NG108-15 cells. Taken together, the results suggest that NG108-15 cells contain newly synthesized GTP binding regulatory protein (G-protein)–coupled δ-opioid receptors in nuclear membranes and uncoupled opioid binding sites in chromatin.

[Key words: opioid receptor, nucleus, neurohybrid cells, subcellular fractionation, opioid peptide, immunohistochemistry]

The localization of nuclear binding sites for steroid and thyroid hormones in many target cells has been well documented. In the last several years, evidence has accumulated favoring the hypothesis that certain growth factors and polypeptide hormones may also have nuclear receptors (Burwen and Jones, 1987). It is clear that growth factors interact with their corresponding surface receptors, but the interpretation of the data pertaining to their fate after internalization remains controversial. Both subcellular fractionation and immunohistochemical studies have shown either uptake of growth factors into nuclei of target cells or the presence of specific nuclear binding sites. Epidermal growth factor (EGF) has been reported to accumulate within cell nuclei in cultured rat pituitary, liver, epidermis, and hair follicle cells (Johnson et al., 1980; Green et al., 1987; Marti et al., 1991). Subsequently, specific binding sites for EGF in chromatin isolated from cultured human colorectal carcinoma cells were demonstrated by immunoprecipitation studies (Rakowicz-Szulczynska et al., 1986). A monoclonal antibody generated against the cell-surface EGF receptor bound to chromatin (Rakowicz-Szulczynska et al., 1986). Chromatin binding sites were also reported for angiotensin II (Re et al., 1984) and platelet-derived growth factor (Rakowicz-Szulczynska et al., 1986). However, the data for nuclear binding sites for NGF are contradictory, showing their exclusive localization in chromatin in some reports (Andres et al., 1977; Rakowicz-Szulczynska and Koprowski, 1986) and restriction to nuclear membranes in another (Yankner and Shooter, 1979). A receptor for vasoactive intestinal peptide has also been detected in nuclei of a human colonic adenocarcinoma cell line (Omary and Kagnoff, 1987).

The existence of intracellular opioid receptors has been established by various approaches. It is reasonable to assume that opioid receptors have a life cycle that entails intracellular synthesis and processing followed by migration to the cell surface and then internalization for either cessation of signal transduction, ligand transport, turnover, or translocation activation. Upon the discovery of opioid receptors, subcellular fractionation techniques were adopted to identify their major locus as the plasma membranes (Pert and Snyder, 1973; Terenius, 1973; Pert et al., 1974; Simantov et al., 1976; Smith and Loh, 1976; Leyert and Laduron, 1977; Glasel et al., 1980). Simultaneously, these investigations yielded evidence for the existence of intracellular opioid binding sites. Subsequently, Roth et al. (1981, 1982) initiated an investigation of the subcellular localization of opioid receptors in rat brain with emphasis on the characterization of the various intracellular compartments by marker enzymes, electron microscopy (EM), isopycnic density, and binding assay. Morphological evidence to support the existence of intracellular opioid binding sites came from autoradiographic EM experiments with a μ-selective ^{125}I-labeled opioid peptide analog (Hamel and Beaudet, 1984). In this manner, 13% of the total μ-opioid binding was found over cell bodies of brain neurons with considerable distribution within the cytoplasm. Since some

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opioid binding sites are associated with endoplasmic reticulum (ER) (Roth et al., 1981), it is possible that they may also occur on other intracellular membranes with similar structural composition. Many studies have revealed that the nuclear envelope is functionally a specialized form of ER. Beaudet and coworkers (Hamel and Beaudet, 1987; Beaudet et al., 1989) provided EM-autoradiographic data for the association of intracellular opioid binding sites with the ER and Golgi apparatus, while an additional contingent was associated with the nucleus or nuclear membrane. Nuclear opioid binding sites have also been invoked in a hypothesis on long-term memory (Laduron, 1987). Nuclear localization of opioid receptors is consistent with data from our laboratory as well as that of others indicating a role of opioids in modulating both developing and transformed cell growth (Zagon and McLaughlin, 1987; Zagon and McLaughlin, 1989; Barg et al., 1990).

Recently, we examined opioid receptors from subcellular fractions [heavy membranes (HMs) and light membranes (LMs)] of neurohybrid cell cultures (Coscia et al., 1990; Belcheva et al., 1991). Marker enzymes, ouabain-sensitive Na^+/K^+-ATPase, glucose-6-phosphatase, and UDP-galactose and transferase were used to demonstrate that HMs were enriched in plasma membranes, while LMs contained most of the ER and Golgi complexes, respectively. In the present study, we detected and characterized opioid binding sites in purified nuclear preparations and subnuclear fractions from NG108-15 cells. While satisfying many criteria for authentic opioid receptors, several unique binding properties were found to be associated with the nuclear sites.

Materials and Methods

Chemicals. Unless otherwise indicated, all chemicals were purchased from Sigma, St. Louis, MO. The opioids [D-Ser^2,L-Leu^5]enkephalyl-Thr (DSLET), [D-Ala^2-mephe^4,gly-ol^5]enkephalin (DAMGE), [D-Pen^2, D-Pen^5]enkephalin (DPDPE), [D-Ala^2,D-Leu^5]enkephalin (DADLE), diprenorphine, and (+)- and (−)-naloxone were obtained from Multiple Peptide Systems, San Diego, CA. Naltrindole and dextrorphan were purchased from Research Biochemicals Inc., Natick, MA.

Cell cultures. NG108-15 neuroblastoma × glioma hybrid cells were grown at 37°C in a humidified CO_2 (10%) incubator in Dulbecco's modified Eagle's medium and Ham's Nutrient Mixture F12 containing calf serum (10%). Cells were subcultured in 150 cm^2 flasks, and medium was changed every 3 d. Some cells were treated with cycloheximide (1 μg/ml, 48 hr). This treatment proved to be sufficient to block more than 80% of the protein synthesis in the cultures (Belcheva et al., 1991). Higher concentrations (1.5–3 μg/ml) of cycloheximide caused cell death. Naive and treated cultures were harvested after 48 hr and stored at −70°C. NCB-20 cells were grown and harvested as previously described (Zhu and Chuang, 1988). C_6 glioma cells were grown under the same conditions as NG108-15 cells.

Subcellular fractionation. The fractionation protocol to obtain LMs, HMs, and the pellet (P fraction) followed the procedure given by Sweat and Klee (1985) with some minor modifications (Belcheva et al., 1991). NG108-15 and NCB-20 cells were pretreated with Concanavalin A (ConA) to increase the density difference between plasma and intracellular membranes. Cells were then homogenized by polytron, and the entire homogenate was layered onto a sorbitol step gradient (20%, 32%, and 54%) followed by a centrifugation at 100,000 × *g* for 2 hr. The 20–32% sorbitol interface (LMs) and the P fraction were recovered by centrifugation in 50 mM Tris-HCl, pH 7.4, at 150,000 × *g* for 2 hr. HMs were collected from the 32–54% interface in the same way except that ConA was displaced with 0.5 M α-methylmannoside prior to the final washing (Belcheva et al., 1991).

Isolation of purified nuclei, nuclear membrane, and chromatin. Purified nuclei, nuclear membranes, and chromatin were prepared using a modification of the procedure by Rakowicz-Szulczynska et al. (1986). Although routinely used by the authors in the original protocol, the detergent Triton X-100 was avoided in our procedures for two reasons. Recent studies indicate detergents can strip proteins from nuclear membranes (Burwen and Jones, 1987). Moreover, opioid receptor binding can be diminished by many detergents. After harvesting, NG108-15 cells were washed twice with 10 mM phosphate buffer, pH 7.75, and frozen. Cells were suspended in 0.35 M sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.6), 12 mM 2-mercaptoethanol; disrupted with a "cell cracker" (Balch and Rothman, 1985) by forcing the cell suspension through a 36.83 μm precision bore opening in a stainless steel block; and centrifuged at 600 × *g* for 10 min. In prior experiments, we established that this clearance affords homogenization of NG108-15 cell cultures that is optimal for the isolation of intact nuclei as determined by EM. The supernatant from the 600 × *g* spin was centrifuged further to obtain a 20,000 × *g* membrane preparation (P_{20}), which was washed with 50 mM Tris-HCl (pH 7.4) before use. The 600 × *g* pellet was washed twice in 0.2 M sucrose, 3 mM $CaCl_2$, 50 mM Tris-HCl (pH 7.6) by centrifugation at 700 × *g* for 10 min. Washing of the pellet was then repeated twice with 50 mM Tris-HCl, pH 7.4, and this preparation (referred to as the purified nuclear preparation in this article) was used immediately for binding assays. In some experiments, purified nuclear preparations were spun through 2.1 M buffered sucrose at 25,000 × *g* for 30 min before washing with 50 mM Tris-HCl, pH 7.4. To obtain subnuclear fractions, the 700 × *g* pellet was suspended in 5 ml of 1 mM Tris-HCl (pH 7.9), 1 mM phenylmethylsulfonyl fluoride, and kept for 1 hr on ice. After Douncing, the suspension was layered onto 1.7 M sucrose, 10 mM Tris-HCl, pH 7.9, and was centrifuged at 150,000 × *g* for 2 hr. Chromatin sedimented to the bottom of the tubes, while nuclear membranes were collected at the interface and a fraction of nucleoplasmic proteins, too small to subject to binding assay, remained on the top of the gradient. For opioid binding and marker enzyme assays, nuclear membranes and chromatin were washed with 50 mM Tris-HCl, pH 7.4, and used immediately.

Marker enzymes. The purity of subcellular fractions was assessed with ouabain-sensitive Na^+/K^+-ATPase, glucose-6-phosphatase, and UDP-galactose transferase, marker enzymes of plasma membranes, ER, and Golgi complexes, respectively (Swanson, 1950; Fleisher, 1974; Ariano and Appelman, 1979).

Immunohistochemical localization of opioid binding sites. NG108-15 cells were harvested at confluence, culture medium was removed by centrifugation, and cells were resuspended in 0.2% solution of agarose (agarose type VIII) in PBS, pH 7.4. Cells were then centrifuged at maximum speed (about 8000 × *g*) on a microfuge B (Beckman, Palo Alto, CA) for 2 min, and pellets were frozen on dry ice (−70°C) and stored at −20°C. Pellets were cut into 10-μm-thick sections at −20°C with a cryostat (Miles Cryo-Tek), and were thaw mounted onto gelatin-covered microscopic slides.

Cryostat sections were used for the immunohistochemical demonstration of nuclear binding of the monoclonal anti-idiotypic anti-opioid receptor antibody (Ab2-AOR). Ab2-AOR was generated against an anti-morphine antibody but, like enkephalins, displayed affinity for both μ- and δ-opioid receptors (Coscia et al., 1991). Unfixed cryostat sections of NG108-15 cells were incubated with mouse ascites containing Ab2-AOR (the first antibody) and subsequently with anti-mouse IgG peroxidase conjugate (as a second antibody). The peroxidase bound to the cells was visualized with 3,3′-diaminobenzidine/H_2O_2. In some experiments, an IgG fraction purified by protein A chromatography from ascites containing Ab2-AOR was used as first antibody. The staining procedure was adopted from Polak and Van Noorden (1986) with the following modifications. Prewashing of cryostat sections, preincubations, and dilution of the first antibody were performed with 50 mM Tris/maleate buffer, pH 5.0 or pH 7.4. PBS, pH 7.4, was used for washing the cryostat sections after incubation with the first antibody, for dilution and washing of the second antibody.

Several control experiments were performed to verify the specificity of the immunohistochemical reaction: (1) the first antibody was replaced with the dilution buffer; (2) the second antibody was omitted to control for endogenous peroxidase; (3) the first antibody was replaced by the nonspecific mouse ascites (control mouse ascites fluid, clone NS-1); (4) cryostat sections of C_6 glioma cells, which lack opioid receptors (Tocque et al., 1984), were subjected to the same immunohistochemical procedure; (5) 0.01–1 mM concentrations of naltrindole or DSLET were preincubated with cryostat sections and included with the first antibody solution to control for opioid binding specificity.

Opioid receptor binding assays. Membrane fractions, purified nuclei, nuclear membranes, and chromatin from neurohybrid cells were assayed for opioid binding activity as described (Belcheva et al., 1991). Purified nuclear and membrane preparations (200–600 μg protein) were incu-

bated in duplicate with 1 nM of each of ^{3}H-DADLE ([30 Ci/mmol; New England Nuclear (NEN)–DuPont, Boston, MA), ^{3}H-U69593 (45.7 Ci/mmol; Amersham, Arlington Heights, IL), ^{3}H-DHM (88.6 Ci/mmol; NEN), and ^{3}H-DAMGE, (48 Ci/mmol; NEN) at 25°C for 1 hr; 1 nM ^{3}H-diprenorphine (47 Ci/mmol; Amersham) at 37°C for 20 min; or 2 nM ^{3}H-DPDPE (37.1 Ci/mmol; NEN–DuPont, Boston, MA) at 25°C for 3 hr. B_{max} and K_d values for the above opioids were estimated from homologous competition binding assays performed in the presence of 10–12 different concentrations of the corresponding unlabeled ligand. In heterologous competition binding assays with ^{3}H-DSLET (35 Ci/mmol; Multiple Peptide Systems, San Diego, CA), the following drugs were used: DADLE, DPDPE, naltrindole, diprenorphine, DAMGE, and dextrorphan. ^{3}H-DSLET dissociation studies were performed by preincubation with the radioligand for 60 min at 25°C, adding etorphine (1 μM) or naltrindole (10 nM) and determining membrane-bound label remaining at different time intervals. In several experiments, a range (1–1000 μM) of concentrations of either 5′-guanylimidodiphosphate (Gpp(NH)p; Boehringer Mannheim, Indianapolis, IN) or (−)- or (+)-naloxone (NIDA; Drug Supply, Research Triangle Park, NC) were added to the incubation mixture with 1 nM ^{3}H-DADLE. Nonspecific binding was measured in the presence of 10 μM etorphine and was <6%. Incubations were terminated by addition of ice-cold 50 mM Tris-HCl, (pH 7.4), and samples were collected on Whatman GF/B filters after 3 × 5 ml washing with buffer using a Brandel cell harvester (Gaithersberg, MD). In the ^{3}H-DPDPE and ^{3}H-diprenorphine experiments filters were presoaked in the same buffer containing 0.02% polyethylene glycol. Filters were dried and counted in a Packard Tri-carb 1500 liquid scintillation analyzer. Protein concentrations were determined by the method of Lowry (1951) with bovine serum albumin as a standard. DNA content was assayed by the method of Giles and Myers (1965). Statistical analyses of data were performed using the Student's t test. Binding affinities and densities (K_d and B_{max}) were estimated by the LIGAND program (Munson and Rodbard, 1980). Heterologous competition curves were generated with the SIGMAPLOT program (JANDEL Scientific, Corte Madera, CA) using an equation from the ALLFIT program (DeLean et al., 1988). IC_{50} values were calculated by an iterative procedure using the same mathematical function.

Electrophoretic analysis of subcellular fractions. SDS-PAGE was performed as described by Laemmli (1970). Gels were silver stained using a modification of the procedure described by Adams and Sammons (1981). Briefly, the gel (10 × 10 cm) was treated with 100 ml of 50% methanol, 10% acetic acid for about 16 hr. It was then incubated in 10% methanol, 10% acetic acid for 30 min with gentle shaking. This was followed by two 15 min washes in H_2O with gentle shaking. The gel was then exposed to 100 ml of 0.1% silver nitrate with gentle shaking for 30 min, washed for 30 sec in H_2O, and developed in 100 ml of 0.3 M sodium carbonate, 0.037% formaldehyde for 5–10 min. When the development was judged satisfactory, 10 ml of 2 M citric acid was added to stop the reaction. All of the above steps were performed at room temperature.

Results

Characteristics of the P fraction. The original subcellular fractionation protocol adopted in these studies entailed layering ConA-pretreated, polytron-generated homogenates onto a sorbitol step gradient (Belcheva et al., 1991). Upon centrifugation, LMS, enriched in ER and Golgi complexes, were resolved from HMs containing the bulk of the plasma membranes from NG108-15 and NCB-20 cells. An analysis of binding parameters of the fractions resolved by gradient centrifugation revealed that the pellet (P fraction) also contained a substantial amount of opioid binding sites in both cell lines. B_{max} values ranged from 29 ± 4 to 110 ± 6 for ^{3}H-DADLE and from 33 ± 6 to 77 ± 12 fmol/mg protein for ^{3}H-diprenorphine. K_d values of ^{3}H-DADLE and ^{3}H-diprenorphine (0.8–2.5 nM) binding to the P fraction are similar to those for LM (1.1–1.9 nM) and HM (1.0–2.2 nM) opioid binding in these cell lines (Belcheva et al., 1991).

EM examination of the P fraction from NG108-15 cells revealed an absence of intact cells and the prevalence of nuclear remnants including nucleoli (not shown). Moreover, the pellet contained more than 90% of the total cellular DNA content and a DNA : protein ratio that is 4 orders of magnitude higher than that of LMs and HMs. Marker enzyme analyses were consistent with a nuclear origin of these membranes (data not shown).

Isolation and characterization of purified nuclear preparations. Our approach to the isolation of nuclei, nuclear membrane, and chromatin has been to adopt a gentle method of homogenization and to avoid detergents. To this end, NG108-15 cell homogenization was performed with a "cell cracker," which causes minimal destruction of cell organelles (Balch and Rothman, 1985). Upon subcellular fractionation of the homogenates, EM inspection of the purified nuclear preparation revealed the prevalence of intact nuclei (Fig. 1*A*). Furthermore, observation by fluorescence microscopy of the 4′,6′-diamidino-2-phenylindole stained 2.1 M sucrose pelleted nuclear preparation (Fig. 1*B*) showed a uniform distribution of DNA. The absence of appreciable fluorescence in P_{20} membranes (Fig. 1*C*) for this DNA stain served as a negative control.

SDS-PAGE of the subcellular fractions revealed that the purified nuclear preparation and the 2.1 M sucrose pellet were enriched in low-molecular-weight proteins, presumably histones, in the region of the gel corresponding to 14–20 kDa (Fig. 2). Moreover, the nuclear membrane fraction was essentially free of chromatin as indicated by the absence of histone bands. Likewise, certain major proteins in the nuclear membrane fraction were absent in the chromatin fraction.

Although accounting for 9–10% of the total opioid binding (see below), the purified nuclear preparation contained a 15-fold-lower specific activity and 5% of the total ouabain-sensitive Na^+/K^+-ATPase activity found in the 20,000 × g pellet (P_{20}) (Table 1). This plasma membrane marker enzyme activity was not detected in the chromatin fraction. Furthermore, the specific activity of glucose-6-phosphatase (ER marker enzyme) recovered in nuclear membranes was 58% of the LM-associated enzyme activity (19.8 ± 1.1 nmol/min/mg protein). This is consistent with previous data for liver nuclear membranes, which were found to have about half the specific activity of ER-enriched microsomes (Kasper, 1974).

Opioid binding to purified nuclear preparations and subnuclear fractions. ^{3}H-DPDPE, ^{3}H-DADLE, and ^{3}H-diprenorphine binding parameters for the purified nuclear preparation and its subfractions are given in Table 2. K_d and B_{max} values for nuclear opioid binding are similar to those found for the P fraction. However, a major difference was the two-site binding of ^{3}H-DPDPE, and ^{3}H-diprenorphine (Fig. 3, Table 2). Agonists ^{3}H-DPDPE and ^{3}H-DADLE bind with high affinity to opioid sites in nuclear membranes, while a five- to eightfold higher K_d value was found for binding to chromatin. In contrast, the partial agonist ^{3}H-diprenorphine shows high affinity to chromatin binding sites and significantly lower-affinity binding to nuclear membranes. These data imply at least two different populations of nuclear opioid binding sites are present, one that may occur in the nuclear envelope and a second in chromatin. B_{max} data for the high-affinity sites are consistent with this notion and suggest an enrichment of agonist binding in the nuclear membranes and diprenorphine binding in chromatin.

^{3}H-DSLET heterologous competition binding studies with several opioid agonists and antagonists were conducted using purified nuclear preparations (Fig. 4). Naltrindole, which is a highly δ-specific antagonist, expresses the highest selectivity for nuclear binding sites, and its competition curve suggests the existence of as many as three sites compared to one, or at most two, in the P_{20} fraction (Fig. 4*A*). Very high-affinity binding was

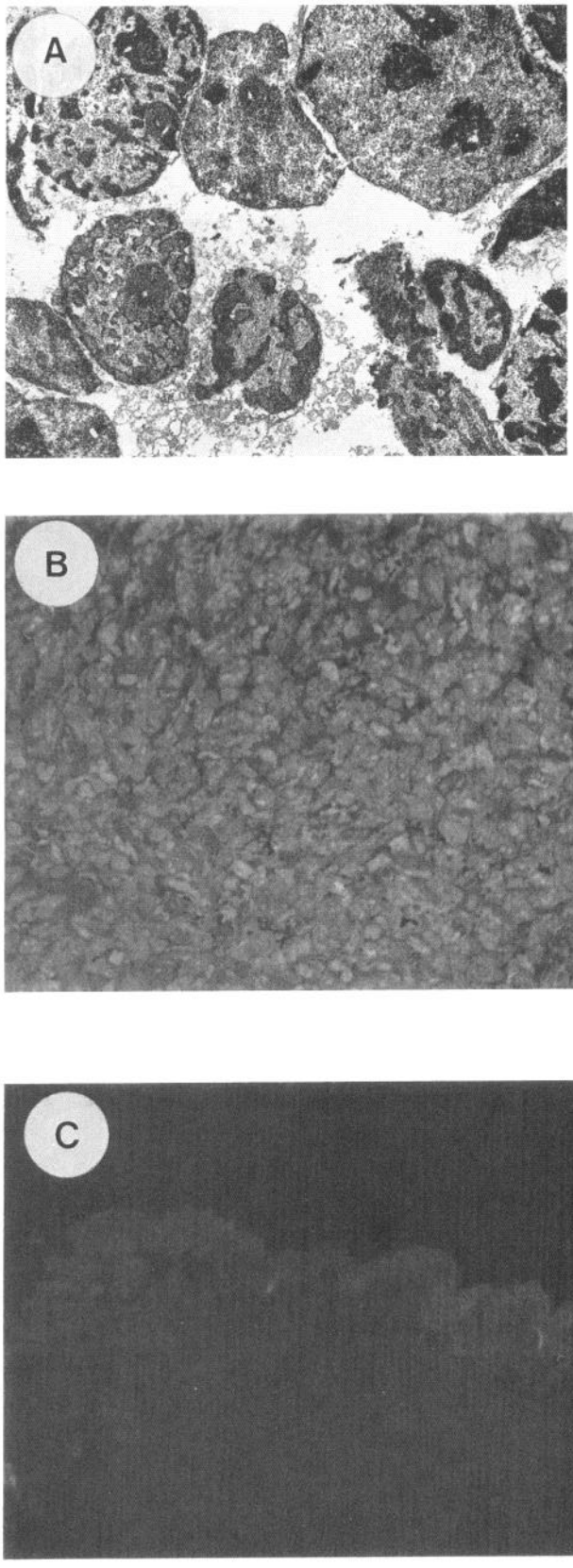

Figure 1. Electron and fluorescence microscopy of subcellular fractions from NG108-15 cells. *A*, Electron micrograph of a representative field of a purified nuclear preparation from NG108-15 cells that was isolated as described in Materials and Methods. Note the homogeneity and prevalence of intact nuclear envelope. Magnification, 3,800×. *B* and *C*, Fluorescence micrographs of 4′,6′-diamidino-2-phenylindole staining for DNA in the 2.1 M sucrose pelleted nuclear preparation (*B*) and in P_{20} membranes (*C*). Magnification, 125×.

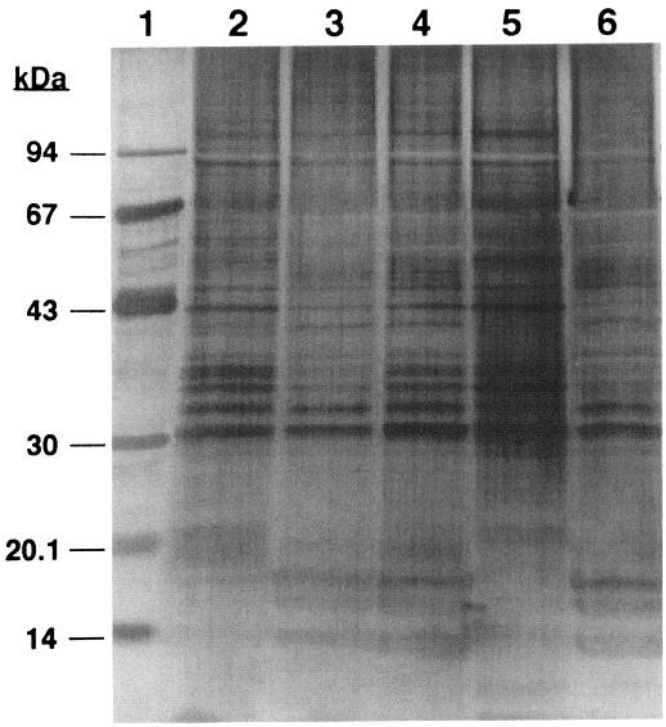

Figure 2. SDS-PAGE analysis of subcellular fractions from NG108-15 cells. One microgram of total protein from each subcellular fraction was applied to a 9% SDS-polyacrylamide gel, resolved, and silver stained as described in Materials and Methods. *Lanes: 1*, molecular weight standards; *2*, P_{20}; *3*, purified nuclear preparation; *4*, 2.1 M sucrose pellet; *5*, nuclear membrane; *6*, chromatin.

seen in nuclear preparations (IC_{50} = 0.54 ± 0.07 fM, 129 ± 22 fM, and 0.19 ± 0.03 nM). P_{20} membranes also displayed high-affinity naltrindole binding with IC_{50} values at 0.026 ± 0.003 nM and possibly 18 ± 2.1 fM. The less selective agonist DADLE displayed monophasic competition (IC_{50} = 3.3 ± 1.2 nM) for the ^{3}H-DSLET binding sites (Fig. 4*C*). Binding of the δ-selective agonist DPDPE and partial agonist diprenorphine to ^{3}H-DSLET sites in the same preparation was biphasic, consistent with the results from homologous competition binding assays. In both cases, the higher-affinity site was in the nanomolar range (0.14 ± 0.03 and 2.4 ± 0.9 nM, respectively) as seen with most δ-selective ligands, while the lower IC_{50} was 0.013 ± 0.002 and 0.13 ± 0.02 μM, respectively. In contrast, diprenorphine and DPDPE competition with ^{3}H-DSLET for P_{20} sites gave monophasic isotherms (IC_{50} = 0.77 ± 0.07 and 8.8 ± 0.7 nM, respectively; curves not shown). The μ-specific enkephalin analog DAMGE displayed low affinity to both nuclear preparations and P_{20} (IC_{50} = 325 ± 34 and 365 ± 49 nM, respectively) (Fig. 4*B*). Dextrorphan, the inactive (+)-isomer, failed to affect ^{3}H-DSLET binding to purified nuclear preparations (Fig. 4*C*). These results were consistent with affinity data on (+)- and (−)-naloxone for P fraction sites that also manifested specificity for levorotatory opiate alkaloids (data not shown).

As shown in Table 2 chromatin contains high-affinity diprenorphine binding but only lower-affinity DPDPE and DA-

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Table 1. Ouabain-sensitive Na^+/K^+-ATPase and glucose-6-phosphatase specific and total activities in subcellular fractions from NG108-15 cells

Preparation	Na^+/K^+-ATPase		Glucose-6-phosphatase	
	Specific (nmol/min/mg protein)	Total (nmol/min)	Specific (nmol/min/mg protein)	Total (nmol/min)
P_{20}	79 ± 4	1755 ± 147	9.6 ± 0.6	167 ± 21
Purified nuclei	5.2 ± 0.8	101 ± 17	5.7 ± 0.6	83 ± 2.4
Nuclear membranes	4.1 ± 0.4	26 ± 4.7	11.5 ± 0.9	47 ± 5.2
Chromatin	ND	ND	2.6 ± 0.03	30 ± 3.1

Subcellular fractions from NG108-15 cells were prepared as described in Materials and Methods.
ND, Not detected. $N = 3$.

DLE binding. This binding behavior has not been seen for HMs (Belcheva et al., 1991). Since diprenorphine also binds to μ- and κ-opioid receptors, it was of interest to test purified nuclear preparations with ligands more specific for these classes. In homologous competition binding assays with tritiated μ- (DAMGE and dihydromorphine) and κ- (U69593) selective ligands, specific binding was not detected (data not shown). These and the above (Fig. 4*B*) results indicate the absence of μ- and κ-binding sites in nuclear preparations from NG108-15 cells. In summary, nuclear preparations may contain as many as two very high-affinity δ-opioid binding sites, detected with naltrindole, and a third site with a nanomolar IC_{50} comparable to that of plasma membrane opioid receptors.

Purified nuclear preparations were also centrifuged through 2.1 M sucrose and resultant pellets were extracted with 0.42 M NaCl according to the procedure of Dignam et al. (1983) (Table 3). Interestingly, ^{3}H-diprenorphine binding to the pellet obtained prior to NaCl treatment revealed a single high-affinity binding site, in comparison with two binding sites found with purified nuclei (Table 2). This suggests that some of the nuclear membrane proteins might have been removed during the spin through the 2.1 M sucrose. The amount of protein recovered in the NaCl-insoluble fraction represented about 50% of the protein in nuclear preparation, and the total binding was 61% of the opioid binding in 2.1 M sucrose pellet. The NaCl-insoluble fraction accounted for 94% of the binding recovered. Very little opioid binding was detected in 0.42 M NaCl soluble fraction. This finding suggests that nuclear opioid binding sites belong to the proteins that are tightly bound to DNA and contamination with loosely bound plasma or intracellular membrane proteins may be excluded.

Binding kinetics of purified nuclear preparations. A time course study of the binding of 1 nM ^{3}H-DSLET to purified nuclear preparations from NG108-15 cells was performed (Figs. 5, 6). DSLET association reached steady state equilibrium in 60 min at 25°C with a $t_{1/2}$ of about 25 min. A derivative plot (Fig. 5, left panel) proved to be biphasic. The apparent on-rates (k_{+1} (observed) were estimated to be 2.9×10^{-4} and 1.0×10^{-3} sec^{-1}. Dissociation of ^{3}H-DSLET from the nuclear preparation was also determined by introducing either the agonist etorphine (1 μM) or the antagonist naltrindole (10 nM) after an association of 60 min (Fig. 6). Both curves were biphasic with off-rates (k_{-1}) of $1.0–1.9 \times 10^{-3}$ sec^{-1} (a $t_{1/2}$ of about 15 min) calculated for this rapid phase of dissociation and $6.7–10 \times 10^{-5}$ sec^{-1} for the slow phase (Fig. 6). The pseudofirst-order rate constants (k_{+1}) could be calculated using the equation $k_{+1} = (k_{+1}(\text{observed}) - k_{-1})/[L]$, where $[L]$ is the free ligand concentration and k_{-1} is the first-order rate constant for dissociation. Using the above two k_{+1} (observed) values and the four k_{-1} determinations, eight k_{+1} values may be calculated. From the ratio $K_d = k_{-1}/k_{+1}$, it is possible to calculate 32 kinetically derived equilibrium dissociation constants. More than eight K_d values were in the nanomolar range whereas at least two were of higher affinity, consistent with the data from steady state binding data. The biphasic on- and off-rates also support the notion of the existence of at least two δ-opioid binding sites in purified nuclear preparations. These data are in a good agreement with previous reports on opioid binding kinetics with other subcellular fractions (Pryhuber et al., 1982; Scheibe et al., 1984).

Immunohistochemical localization of opioid binding sites. Immunostaining of cryostat sections of NG108-15 cells using the antibody to the opioid receptor AB2-AOR resulted in the de-

Table 2. Opioid binding parameters of subcellular fractions from NG108-15 cells

Preparation	^{3}H-DPDPE		^{3}H-DADLE		^{3}H-diprenorphine	
	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}
P_{20}	2.1 ± 0.2	579 ± 48	1.6 ± 0.2	843 ± 73	1.4 ± 0.2	733 ± 69
Purified nuclei						
First site	1.2 ± 0.1	68 ± 11	2.1 ± 0.2	115 ± 11	1.8 ± 0.1	98 ± 13
Second site	91 ± 10*	371 ± 52	—	—	76 ± 26*	848 ± 265
Nuclear membranes	2.2 ± 0.2	126 ± 15**	2.5 ± 0.3	160 ± 19	24 ± 7.7*	413 ± 89
Chromatin	17 ± 3.9*	445 ± 16	12 ± 3.3*	85 ± 15	3.8 ± 0.3	175 ± 27**

Subcellular fractions were prepared as described in Materials and Methods. LIGAND program analyses indicated a two-site model fit better than a one-site paradigm for ^{3}H-DPDPE and ^{3}H-diprenorphine binding to purified nuclei. K_d is expressed in nM and B_{max} in fmol/mg protein. $N = 3–9$.

* P_{20} versus subnuclear fractions, $p < 0.05$.

** High-affinity site of purified nuclei versus those of subnuclear fractions, $p < 0.05$.

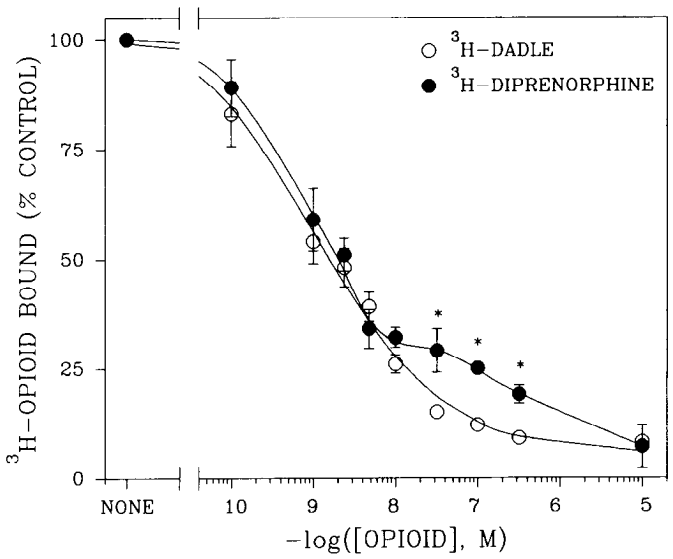

Figure 3. Homologous competition binding of ^{3}H-DADLE and ^{3}H-diprenorphine to purified nuclear preparations. Purified nuclear preparations from NG108-15 cells were obtained as described in Materials and Methods. ^{3}H-DADLE- and ^{3}H-diprenorphine-specific binding ranged from 1230 to 3450 dpm/tube. Analysis with the LIGAND program (Munson and Rodbard, 1980) demonstrated that a two-site model for ^{3}H-diprenorphine binding fits better than a one-site paradigm. The curves shown are derived from means of data obtained from three experiments ± SEM. *, Significantly different from values obtained with the same concentrations of DADLE, $p < 0.05$.

tection of histochemical reaction product over some regions of cell nuclei as well as over the cytoplasm (Fig. 7*A*). The reaction product over the cytoplasm was diffuse and of moderate intensity. Immunostaining of nuclei, however, revealed discrete, more intense spots against the negative background of the remainder of the nuclei. There were one to five of the positive spots of variable shape in each of the nuclear profiles. The diameter of these structures varied from 1 to 3 μm.

Omission of Ab2-AOR–containing ascites fluid from the immunohistochemical procedure resulted in completely negative staining of cells (both cytoplasm and nuclei; Fig. 7*B*), indicating that there was no nonspecific binding of the second antibody conjugated with peroxidase. In addition, negative results were obtained when the second antibody conjugated with peroxidase was omitted (not shown), demonstrating the absence of detectable endogenous peroxidase.

Another control, substitution of nonspecific antibody containing ascites fluid for Ab2-AOR–containing ascites, gave mod-

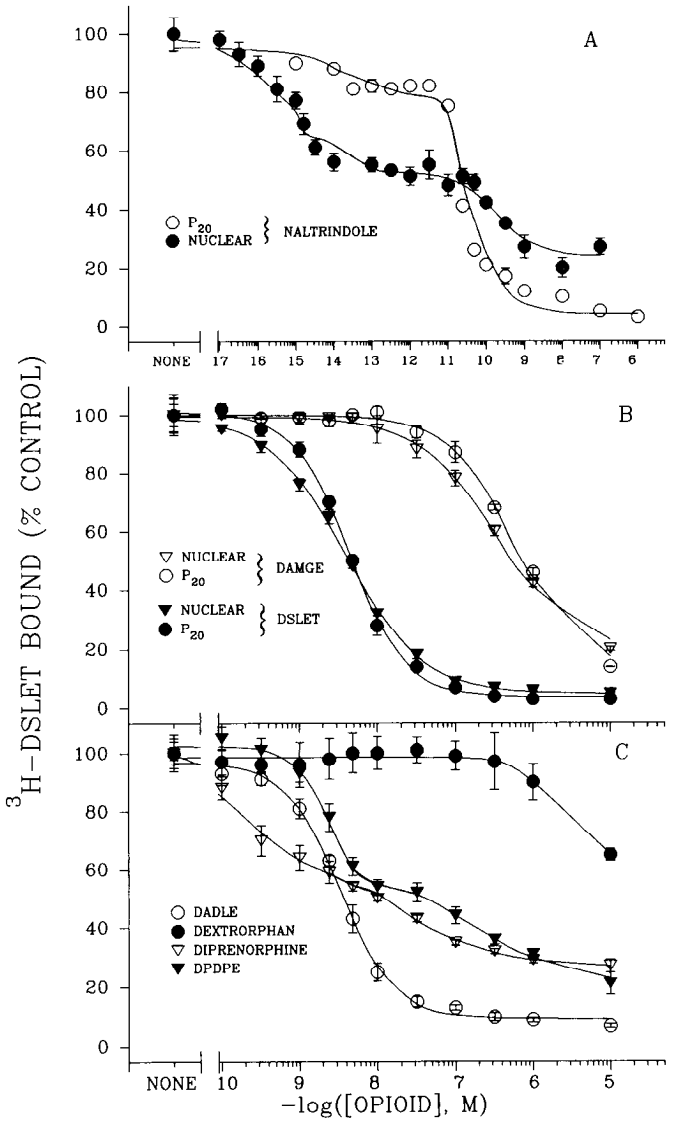

Figure 4. Heterologous competition binding of ^{3}H-DSLET with selected opioid ligands. Assays were performed with 1 nM ^{3}H-DSLET and 12–18 concentrations of opioid ligands with the P_{20} membrane fraction or purified nuclear preparations from NG108-15 cells. Only nuclear preparations were used for the competition curves shown in *C*. Control binding (2460–3890 dpm/tube) represents the specific binding of the radioligand determined as the difference between binding in the presence of 10 μM etorphine (nonspecific) and binding in the absence of any inhibitor (total). Curves shown are derived from means of data obtained from three to six experiments ± SEM.

Table 3. ^{3}H-diprenorphine binding to nuclei centrifuged through 2.1 M sucrose and treated with 0.42 M NaCl

Preparation	K_d (nM)	B_{max} (fmol/mg protein)	Protein (mg)	Total binding (fmol/fraction)
P_{20}	1.2 ± 0.1	556 ± 56	18 ± 2.4	10,133 ± 1679
2.1 M sucrose pellet	3.8 ± 1.0	178 ± 43	19 ± 2.0	3374 ± 649
NaCl -insoluble	2.8 ± 0.2	222 ± 27	9.5 ± 0.9	2067 ± 57
NaCl -soluble	2.4 ± 0.4	35 ± 10	4.2 ± 0.4	136 ± 28

Purified nuclei were prepared from NG108-15 cells as described in Materials and Methods and centrifuged through 2.1 M sucrose for 30 min at 25,000 × *g*. The pellet from this spin was treated with 0.42 M NaCl for 30 min at 4°C and then dialyzed (Dignam et al., 1983). The subcellular fractions were washed in 50 mM Tris buffer, pH 7.4, and then assayed for opioid binding with ^{3}H-diprenorphine. $N = 3$.

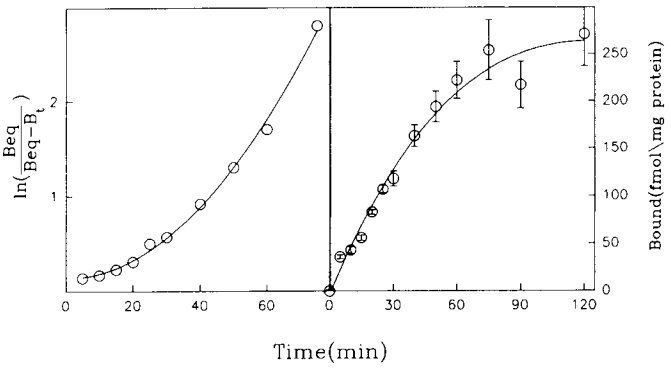

Figure 5. Time dependence of ^{3}H-DSLET specific binding to purified nuclear preparations from NG108-15 cells. Association plots (*right*) were obtained with 1 nM ^{3}H-DSLET at 25°C. Nonspecific binding was determined in the presence of 10 μM etorphine. The rate of association [k_{+1}(observed)] of the radioligand to purified nuclear preparation was calculated from the slope of the line, where ln $\beta_{eq}/(\beta_{eq} - \beta_t)$ was plotted versus time (*left*). β_{eq} = the amount of ^{3}H-DSLET bound at equilibrium (60 min), and β_t = the amount of radioligand bound at each time interval. The data are the mean of three experiments ± SEM.

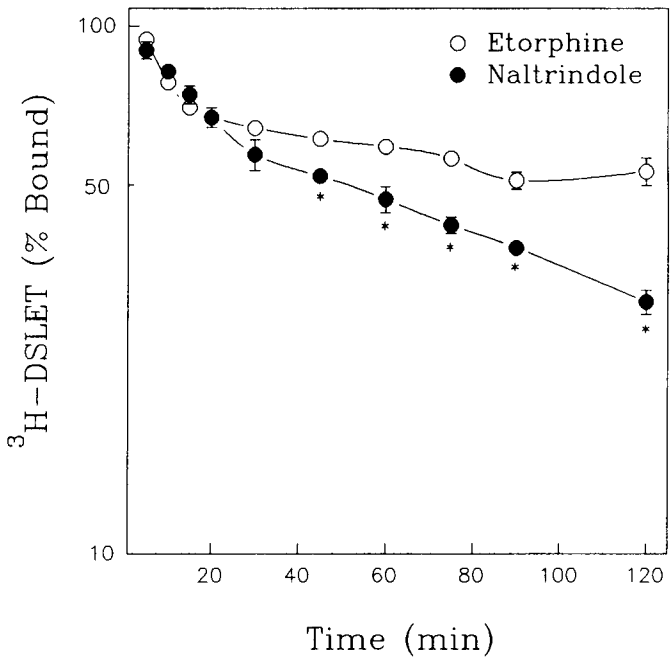

Figure 6. Dissociation of ^{3}H-DSLET from nuclear opioid binding sites in the presence of an agonist or antagonist. Aliquots of purified nuclear preparations from NG108-15 cells were incubated with 1 nM ^{3}H-DSLET for 60 min at 25°C, whereupon etorphine (1 μM) or naltrindole (10 nM) was added and the dissociation of radioligand was monitored at different time intervals. Values are expressed as percentage bound of the amount obtained after 60 min of association (100%). Control specific binding ranged from 1570 to 2340 dpm/tube. *, Significantly different from the values obtained at the same time intervals in the presence of etorphine, $p < 0.05$. The data are the mean of three experiments ± SEM.

erately intense cytoplasmic reactions but no trace of staining in nuclei. When C_6 glioma cells were used, Ab2-AOR–containing ascites fluid again gave slightly positive cytoplasm and negative nuclei (Fig. 7*D*). Since C_6 cells do not normally express opioid receptors, this result provides an additional negative control supporting opioid receptor specificity of Ab2-AOR for nuclear sites. To demonstrate opioid binding specificity of the nuclear sites bound by AB2-AOR, we preincubated cryostat sections with 1 mM naltrindole or 1 mM DSLET. The same concentration of ligands was also included in the Ab2-AOR–containing ascites solution used for incubation. This control gave completely negative results for nuclear structures and almost complete inhibition of cytoplasmic staining, when the procedure was carried out at pH 5.0 and 7.4 (Fig. 7*C*). Lower concentrations of naltrindole or DSLET (10–100 μM) were not inhibitory at either pH 5.0 or pH 7.4. This is consistent with previous immunohistochemical studies with anti-id-14, an anti-idiotypic antibody to the opioid receptor generated from an anti-β-endorphin antibody wherein millimolar concentrations of opioid were required to displace the antibody (Hassan et al., 1989).

Opioid binding of subnuclear preparations in the presence of Gpp(NH)p. Since the partial agonist diprenorphine binds to chromatin with high affinity while the agonists DADLE and DPDPE display higher K_d values (Table 2), it is possible that this subnuclear fraction contains G-protein–uncoupled opioid receptors. One means to estimate the extent of G-protein coupling is to conduct agonist binding in the presence of GTP analogs that are inhibitory (Belcheva et al., 1991). As seen in Figure 8, ^{3}H-DADLE binding to chromatin sites was insensitive to Gpp(NH)p whereas that to nuclear membranes was even more sensitive than HMs and LMs (Belcheva et al., 1991). These results are consistent with the notion that chromatin contains a unique population of opioid binding sites that are uncoupled from G-proteins. The fact that diprenorphine binding to chromatin retains its high affinity is in agreement with previous data that indicate an insensitivity of the binding of this partial agonist to GTP analogs (Costa and Herz, 1989) and to pertussis toxin–mediated uncoupling from G-protein (Hsia et al., 1984).

Since nuclear membrane opioid sites are candidates for newly synthesized receptors enroute to the cell surface, it was of interest to determine whether inhibition of protein synthesis would affect their expression. Upon cycloheximide (1 μg/ml, 48 hr) treatment of NG108-15 cells, specific opioid binding measured with ^{3}H-DSLET, ^{3}H-DADLE, or ^{3}H-diprenorphine was completely abolished in nuclear membrane and chromatin fractions (data not shown). Isolation of chromatin from cycloheximide-treated cells proved to be more difficult than from control cells, suggesting that this fraction had undergone structural changes. Both DADLE- and diprenorphine-specific binding to P_{20} membranes were reduced by 63% upon cycloheximide treatment.

Discussion

The presence of authentic nuclear opioid sites in NG108-15 neurohybrid cells has been critically addressed using two different approaches, subcellular fractionation, and immunohistochemistry. The criteria of marker enzyme analysis, electron and fluorescence microscopy, competition binding assays, and binding kinetics were also applied. The procedure for the isolation of nuclei and its subfractions yielded preparations that had little or no appreciable contamination with plasma membrane on the basis of their low-ouabain-sensitive Na^+/K^+-ATPase activity (Table 1). In fact, the extent of contamination by this enzyme (5% of the total activity detected) was less than the relative amount of opioid binding found in purified nuclear fractions (9–10%; Table 2). Moreover, a high specific activity for glucose-6-phosphatase was found in the nuclear membranes. This enzyme, which is generally considered a marker for ER, is present in the nuclear envelope at 58% the level for LMs. The data support the hypothesis that glucose-6-phosphatase is an

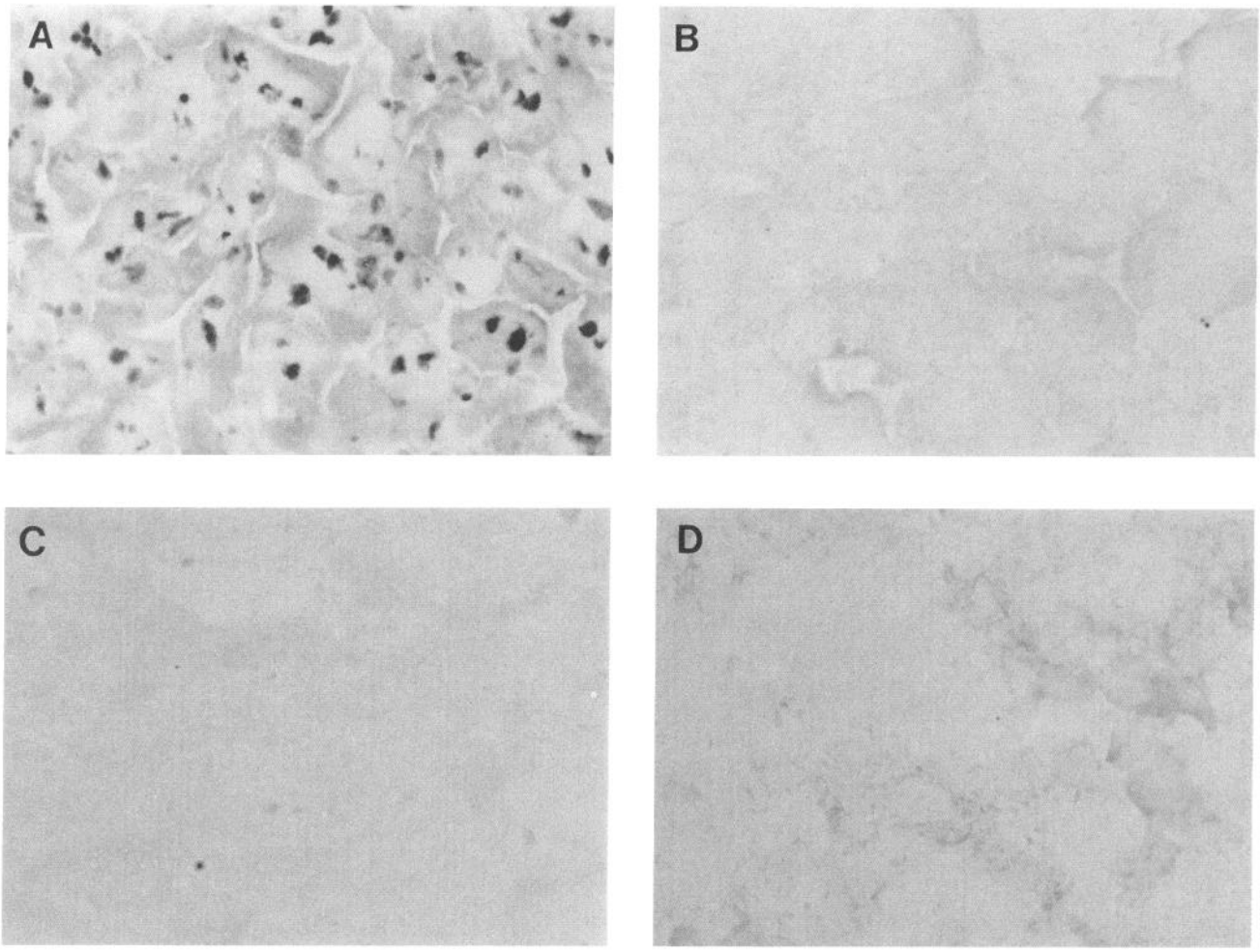

Figure 7. Immunohistochemical staining of NG108-15 cells or C_6 glioma cells: cryostat sections of NG108-15 cells immunostained with Ab2-AOR and anti-mouse-IgG peroxidase conjugate (*A*) or with the second antibody only (control staining) (*B*), at pH 7.4. Note the positive reaction present in the cytoplasm and in certain areas of nuclei. In control staining, only background reaction is seen. Suppression of both cytoplasmic and nuclear immunostaining was achieved by including 1 mM naltrindole in the preincubation and incubation steps of the procedure at pH 5 (*C*). *D* shows background staining of C_6 glioma cells, lacking opioid receptors, when subjected to complete immunohistochemical procedure at pH 7.4. No counterstaining. Magnification, 1000×.

intrinsic enzyme for both the nuclear envelope and ER (Kasper, 1974). In addition, previous histochemical data demonstrated the localization of glucose-6-phosphatase activity on the periphery of the nucleus. Moreover, electron micrographs of purified nuclear preparations indicated the presence of primarily intact nuclei with only traces of cytoplasmic structures such as Golgi, cytoskeletal, and other non-nuclear elements (Fig. 1*A*). Fluorescence microscopy of 4′,6′-diamidino-2-phenylindole staining revealed a uniform distribution of DNA in the 2.1 M sucrose pelleted nuclear preparation (Fig. 1*B*). Extraction of loosely bound proteins from the 2.1 M sucrose nuclear pellet with 0.42 M NaCl excludes the possibility for plasma membrane contamination, since most binding sites were recovered in the NaCl-insoluble material (Table 3). Binding characteristics of these nuclear sites, for example, nanomolar and femtomolar δ-opioid affinity, stereospecificity, saturability, Gpp(NH)p sensitivity, kinetic parameters, time dependency, and reversibility, are consistent with the presence of opioid receptors.

The results of immunostaining of NG108-15 cell cryostat sections, taken together with the control experiments, indicate that the nuclei of these cells contain sites that specifically bind Ab2-AOR (Fig. 7). Since a nuclear localization of the opioid receptors is relatively novel, the specificity of our immunohistochemical procedure was considered critically. Ab2-AOR possesses characteristics of μ- and δ-opioid receptor ligands (Coscia et al., 1991). Here we report that it is possible to block specifically AB2-AOR nuclear immunostaining by the δ-opioid receptor ligands, naltrindole, and DSLET (Fig. 7*C*). Localization of the opioid receptors in intact NG108 cells using monoclonal anti-idiotypic anti-opioid receptor antibody anti-id-14 has been reported previously by Hassan et al. (1989). Specific immunostaining of cell surface in NG108-15 cells was demonstrated. Since cells were not cut or permeabilized, information on the presence/absence of nuclear binding sites was not obtained. The same authors demonstrated the cytoplasmic/cell membrane, but not nuclear, localization of the opioid receptors using anti-id-14 antibody for immunostaining of neurons in cryostat sections of rat brain (Hassan et al., 1989).

What is the origin of nuclear opioid binding sites? Are they newly synthesized and enroute to the cell surface, distinct binding sites present in the nucleus in the absence of ligand, or are they plasma membrane receptors that translocate to the nucleus as a complex with ligand? There is precedence from studies with other receptors that favors these possibilities (Burwen and Jones,

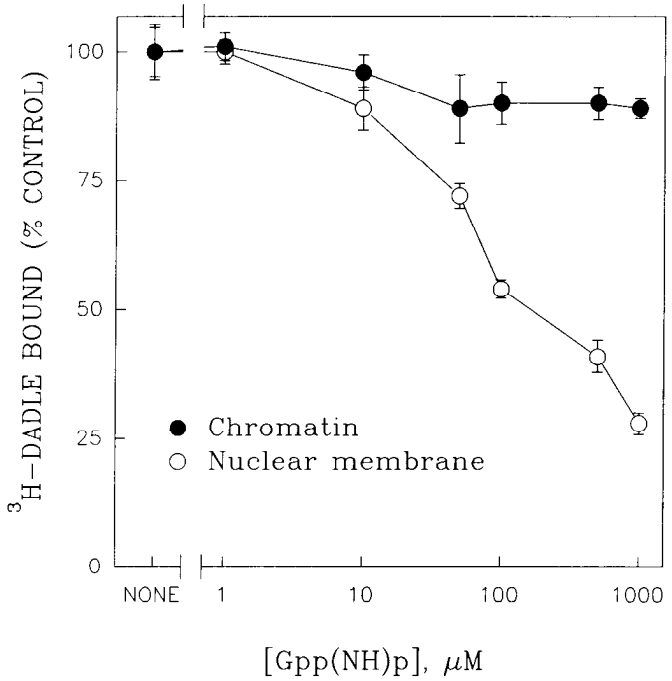

Figure 8. Concentration-dependent Gpp(NH)p inhibition of ^{3}H-DADLE-specific binding to nuclear membranes and chromatin. Nuclear membranes and chromatin were obtained from NG108-15 cells as described in Materials and Methods. Nonspecific opioid binding was determined in the presence of 10 μM etorphine. Specific binding was >1780 dpm/tube. The data are the mean of three experiments ± SEM.

1987, and references cited therein) as well as criticism of these interpretations (Evans and Bergeron, 1988). Recently, more definitive evidence on internalization has appeared. Photoaffinity-labeled insulin receptors on hepatocyte surfaces are transported into the nucleus (Podlecki et al., 1987). Moreover, reconstitution studies have been performed on cells incapable of expressing the EGF receptor (Jiang and Schindler, 1990). When functional EGF receptors are introduced into these cells at the plasma membrane, they migrate to the nucleus and affect transnuclear transport.

Is there a ligand dependency for opioid receptor internalization? Binding studies with purified nuclear preparations, nuclear membranes, and chromatin described here as well as growth factor data (Burwen and Jones, 1987; Marti et al., 1991) indicate the presence of nuclear binding sites without preincubation of cells with ligand. However, we cannot rule out a role of exogenous growth factors in serum added to culture medium or endogenous opioid peptides, which are known to be secreted by NG108-15 cells (Braas et al., 1983).

The results reported here suggest the existence of at least two and possibly three populations of nuclear opioid sites. This is bolstered by the following: (1) ^{3}H-DPDPE and ^{3}H-diprenorphine binding parameters fit a two-site model better than a one-site binding isotherm, with K_d values in the range of 1.2–1.8 and 76–91 nM (Table 2, Fig. 3); (2) analysis of binding experiments in which naltrindole, diprenorphine, and DPDPE compete with ^{3}H-DSLET showed at least biphasic displacement curves; (3) K_d values differ for δ-opioid agonists and partial agonist diprenorphine binding to nuclear membranes as well as chromatin; (4) association and dissociation plots are biphasic; and (5) opioid sites in nuclear membranes exhibit high Gpp(NH)p sensitivity, while those in chromatin are insensitive.

Nuclear membrane opioid binding sites in NG108-15 cells include a pool of G-protein–coupled receptors as shown by Gpp(NH)p sensitivity studies (Fig. 8). Since they display a higher affinity for agonists than the partial agonist diprenorphine, they may represent newly synthesized sites on the outer nuclear membrane, which is contiguous with the cell rough ER. Studies on nuclear envelope structure have led to the postulation that the outer nuclear membrane could be a site of membrane-bound synthesis of glycoproteins (Franke et al., 1981; Puddington et al., 1985), a class to which opioid receptors may belong (Gioannini et al., 1984; Liu-Chen and Phillips, 1987). Moreover, Puddington et al. (1985) have proposed that in some cells with large nuclei and little rough ER, the nuclear membrane is the major site for synthesis of membrane glycoproteins. As shown in this study, cycloheximide, which blocks >80% of new protein synthesis, eliminates all nuclear opioid binding. In contrast, residual plasma membrane opioid binding is retained under the same conditions of cycloheximide treatment. Intracellular opioid receptors that are inhibited by GTP analogs have been postulated to be newly synthesized and en route to the cell surface rather than internalized (Belcheva et al., 1991). These data are consistent with the notion that nuclear membranes contain newly synthesized, G-protein–coupled, opioid sites.

A second population of opioid binding sites may consist of G-protein–uncoupled receptors that are localized in chromatin of the nucleus. Diprenorphine has a higher affinity for chromatin-associated than nuclear membrane sites and as a partial agonist would be capable of binding to G-protein–uncoupled receptors. Agonists bind with lower affinity to chromatin sites, and their binding is insensitive to Gpp(NH)p. As seen here (Table 2), agonist K_d values would be expected to increase with uncoupling, while that of a partial agonist should be unaffected. There is a growing body of evidence supporting the idea that plasma membrane opioid receptors undergo internalization without G-proteins and exist in an uncoupled form (Roth et al., 1981; Law et al., 1985; Sweat and Klee, 1985; Coscia et al., 1990; Belcheva et al., 1991). These receptors may be localized in chromatin. By the same line of reasoning, cycloheximide abolishes chromatin binding by blocking protein synthesis, possibly preventing internalization into the nucleus and causing recycling of residual chromatin-associated sites to the cell surface to compensate for the absence of new synthesis.

Internalized nuclear receptors could be involved in translocation activation. It is possible that receptor-bound opioid or the opioid alone can act as a third messenger to impart specificity to nuclear events. The existence of such a mechanism could shed light on the question of neurotransmitter specificity recently intensified by the discovery of stimulus–transcription coupling of the immediate-early genes c-*fos* and c-*jun* (Morgan and Curran, 1989). At the present time, it is thought that second messengers communicate with the nucleus via protein phosphorylation to alter gene expression and protein synthesis (Cantley et al., 1991). There is a report on translocation of activated protein kinase C from the plasma membrane to the nucleus via a growth factor receptor complex. This translocation is followed by modifications in the nuclear structure and chromatin organization (Fields et al., 1988). However, multiple receptor systems in the same cell appear to use common signal transduction systems. If this theory is correct, then an additional messenger is needed to interact in the nucleus to furnish the necessary specificity. Nuclear receptors may be candidates for this function.

There are opioid functions that may require gene transcription. Opioid tolerance and dependence are delayed processes, demonstrated after 12–24 hr of opioid action. The fact that inhibitors of RNA and protein synthesis diminished the development of tolerance and dependence to morphine and other opioids without affecting their analgesic effects supports the notion of a mechanism leading to a change in gene expression (Laduron, 1987). Taken together, the abundant data brought forth in recent studies afford a compelling argument for nuclear binding sites for not only hormones and growth factors but neurotransmitters such as opioids as well.

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