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Regulation of an opioid-binding protein in NG108-15 cells parallels regulation of δ-opioid receptors

(immunofluorescence/confocal microscopy/antibodies/transfection)

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ABSTRACT **An opioid-binding protein has recently been purified from bovine brain and cloned, and its cDNA sequence has been obtained. Indirect evidence suggests that this protein has a role in opioid-receptor function. However, because direct testing of its function by expression of its cDNA has not yet been possible and because its structure bears no resemblance to G protein-coupled receptors, the role of this protein in opioid-receptor activity is still in question. An antibody raised to a portion of the predicted amino acid sequence of opioid-binding cell-adhesion molecule (OBCAM) specifically labeled the surface of NG108-15 cells, as visualized by immunofluorescence with confocal microscopy. Furthermore, chronic treatment of these cells with opioid agonist, which down-regulates opioid receptors, reduced OBCAM immunoreactivity (ir). Down-regulation of both opioid receptors and OBCAM-ir was greatest after chronic treatment of NG108-15 cells with δ-opioid agonists, as well as with nonselective agonists such as etorphine, whereas other agonists including [D-Ala^2-*N*-MePhe^4-Gly-ol]enkephalin, morphine, levorphanol, dynorphin A-(1–13), and U-50,488H were less effective or ineffective. Chronic treatment of NG108-15 cells with muscarinic agonists had no effect on OBCAM-ir. Furthermore, NG108-15 cells transfected with an antisense construct to OBCAM have a reduced density of opioid-binding sites as well as reduced OBCAM-ir. Taken together, these results strongly suggest that OBCAM has a role in opioid-receptor function in NG108-15 cells.**

Opioid receptors have been extensively characterized pharmacologically and biochemically in the nearly two decades since their identification; yet purification has remained an elusive goal. Although several laboratories have reported the isolation and biochemical characterization of opioid-binding proteins (1–3), definitive proof that any of these proteins is identical to pharmacologically and biochemically relevant opioid receptors is lacking. There have been two reports of cloning of an opioid-binding protein. In the most recent, Xie *et al.* (4) transfected a cDNA library from human placenta into COS-7 cells and identified a clone that expresses opioid-binding activity and curiously has a high homology to the substance K (neurokinin A) receptor.

Several years earlier, we reported the purification of an opioid-binding protein from bovine brain, using a combination of affinity chromatography, lectin chromatography, and gel filtration (3). The cDNA for this protein was subsequently isolated and cloned, revealing a predicted amino acid sequence of 345 amino acids (5). Surprisingly, this sequence displays significant homologies to several members of the immunoglobulin superfamily and particularly to cell-adhesion molecules, such as neural cell adhesion molecule (N-CAM), myelin-associated glycoprotein, and the invertebrate molecules amalgam, fasciclin II, and neuroglian (6). Accordingly, this binding protein was named opioid-binding cell-adhesion molecule (OBCAM).

The most direct test of the function of OBCAM, the successful expression of its cDNA in a cell line, has not yet been done. However, several lines of indirect evidence indicate a role for this protein in opioid function. Monoclonal and polyclonal antibodies against the purified protein inhibit opioid binding to brain membranes as well as to the purified protein (7, 8). In addition to antibodies against the purified protein, polyclonal antibodies were prepared against peptides corresponding to portions of the predicted amino acid sequence of OBCAM. Members of this series of antibodies react positively with NG108-15 cells in an ELISA and adsorb opioid-binding proteins from solubilized NG108-15 cell membranes (Sabita Roy, H.H.L., and N.M.L., unpublished data). More recently, we found that antisense transfection of a 649-base-pair (bp) cDNA corresponding to a portion of OBCAM into NG108-15 neuroblastoma × glioma hybrid cells dramatically decreases opioid binding (9); binding to other cell-surface receptors was not altered.

In the present study, we used immunofluorescence and confocal microscopy to determine the distribution and regulation of OBCAM, as recognized by an antiserum raised against OBCAM-(270–281) (MRIENKGHISTL, hereafter referred to as MN-3). The expression of OBCAM immunoreactivity (OBCAM-ir) has been examined in normal NG108-15 cells, NG108-15 cells transfected with both antisense and sense OBCAM constructs, and in NG108-15 cells chronically incubated with opioid agonists, a treatment known to down-regulate opioid receptors in these cells (10). These results provide further evidence for a role of OBCAM in opioid-receptor function.

MATERIALS AND METHODS

Materials. Initial stock cultures of neuroblastoma × glioma NG108-15 hybrid cells were from B. Hamprecht (Hoppe-Seyler Institut fur Physiologie und Chemie, Tubingen, F.R.G.). Ligands used were β-endorphin, dynorphin A-(1–13), [D-Ala^2,D-Leu^5]enkephalin (DADLE), [D-Ala^2,*N*-MePhe^4,Gly-ol]enkephalin (DAMGO), [D-Pen^2,D-Pen^5]enkephalin (DPDPE, where Pen represents penicillamine) (all from Peninsula Laboratories), morphine (Mallinckrodt), U-50,488H (Upjohn), etorphine, naloxone, levorphanol, dextrorphan, and naloxone (all from the National Institute on Drug Abuse, Bethesda, MD). Cross-linking agents were obtained from

Abbreviations: DADLE, [D-Ala^2,D-Leu^5]enkephalin; DAMGO, [D-Ala^2,*N*-MePhe^4,Gly-ol]enkephalin; DPDPE, [D-Pen^2,D-Pen^5]enkephalin, in which Pen is penicillamine; ir, immunoreactivity; MN-3, peptide with sequence MRIENKGHISTL; N-CAM, neural cell adhesion molecule; OBCAM, opioid-binding cell-adhesion molecule; sulfo-MBS, *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester; KLH, keyhole limpet hemocyanin.

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Pierce. Fluorescein isothiocyanate-conjugated goat anti-rabbit and anti-mouse γ globulin were purchased from Antibodies Inc. and Boehringer Mannheim. Anti-N-CAM monoclonal and polyclonal antibodies were purchased from Chemicon. Laminin was purchased from Collaborative Research. All other reagents were purchased from Sigma.

Cell Culture. Stocks of T-75 flasks of NG108-15 cells were maintained by the method of Law *et al.* (11). Only cells between passages 20 and 30 were used. In some experiments cells were cultured for 24 hr before fixation in the presence of opioid and nonopioid agonists (Table 1). NG108-15 cells were transfected with both sense (ST8-4) and antisense (ST7-3) constructs representing a 649-bp segment from the 5′ untranslated region into the open reading frame of OBCAM as reported (9). These cells were grown in the presence of the neomycin analog G418 (600 μg/ml). For immunocytochemical experiments cells were harvested 2 days before immunocytochemistry. Cells were removed from flasks with phosphate-buffered saline (PBS) with EDTA (0.1 M NaCl/0.01 M NaH_2PO_4/1.4 mM EDTA) and spun down for 10 min at 250 × *g*. Freshly harvested cells were seeded at a density of ≈50,000 cells per well on poly(L-lysine)-coated coverslips [12.3 nM poly(L-lysine)] in Volmer's carbonate buffer (7.5 mM Na_2CO_3/17.4 mM $NaHCO_3$/1.5 mM sodium azide, pH 9) in 24-well plates. Alternatively, cells were grown on laminin (2 μg/cm^2); the medium was Dulbecco's modified Eagle's medium supplemented with 15.3 mM glucose, 0.43 M $NaHCO_3$, 10% heat inactivated fetal calf serum, and HAT (10 mM hypoxanthine/10 mM aminopterin/16.1 mM thymidine). This medium was diluted 3:1 with conditioned medium; conditioned medium was made by adding regular medium with 10% heat-inactivated fetal calf serum to flasks of growing cells 4 days after passage. This medium was removed on day 6 and sterilized by filtration through a 0.22-μm filter before use. The medium was changed, and ligands were added 24 hr before fixation.

Preparation of Rat Liver Cells. Fresh liver was obtained from Harlan–Sprague–Dawley rats immediately after decapitation. The liver was chilled to 4°C and washed several times with 25 mM Hepes buffer, pH 7.4. The liver was pressed through a tissue sieve, and the cells were spun down for 5 min at 250 × *g*. The cells were washed once with PBS and spun again as before. Cells resuspended in PBS were plated at 50,000 per well on poly(L-lysine)-coated coverslips. Cells were allowed to settle for 20 min at 4°C before fixation.

Table 1. Down-regulation of OBCAM-ir in NG108-15 cells by various opioid ligands

Ligand	Concentration	Percent of control
Etorphine	125 nM	25.9 ± 3.4*
DADLE	125 nM	29.5 ± 3.5*
β-Endorphin	500 nM	44.0 ± 4.6*
β-Endorphin	125 nM	71.3 ± 3.7*
DPDPE	125 nM	54.4 ± 5.6*
Dynorphin A-(1–13)	500 nM	63.7 ± 4.8*
Dynorphin A-(1–13)	125 nM	81.0 ± 2.2*
U-50,488H	125 nM	75.4 ± 5.5*
Morphine	125 nM	95.6 ± 3.3
DAMGO	2 μM	91.3 ± 5.3
DAMGO	500 nM	98.4 ± 5.6
DAMGO	125 nM	87.6 ± 4.5
Levorphanol	125 nM	85.6 ± 6.7
Dextrorphan	125 nM	82.7 ± 5.8
Naloxone	10 μM	96.6 ± 3.9
DPDPE + naloxone	125 nM, 10 μM	85.0 ± 5.5
Etorphine + naloxone	125 nM, 10 μM	86.6 ± 4.7
DADLE + naloxone	125 nM, 10 μM	100.0 ± 4.3
Carbachol	125 nM	100.0 ± 7.8
Atropine	125 nM	100.7 ± 5.4

NG108-15 cells were incubated for 24 hr with the indicated concentration of ligand. Cells were washed once with PBS, fixed, and then processed for immunostaining, as described.
*$P < 0.05$.

Preparation of Antiserum Against MN-3. The peptide MN-3 was synthesized by adding an amino-terminal cysteine for coupling (Peninsula Laboratories). The peptide was coupled to 5 mg of keyhole limpet hemocyanin (KLH) by first mixing the latter with a 40-fold molar excess of *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (sulfo-MBS). Sulfo-MBS that did not react was removed by gel filtration; then the KLH linked to sulfo-MBS was treated with 5 mg of peptide in PBS. The volume was adjusted to 1 ml with PBS. This peptide–KLH complex was emulsified in a 1:1 ratio with Freund's complete adjuvant (Calbiochem), and rabbits were immunized (7). The resulting antiserum was characterized in immunoblotting and binding experiments. Briefly, antiserum was affinity purified and used to construct an immunoaffinity column. The solubilized P2 fraction of NG108-15 membranes was passed over this column, and the retained fraction was further analyzed by immunoblotting and binding. The retained fraction was subjected to SDS/PAGE, transferred to nitrocellulose, and probed with the affinity-purified antibody; proteins were then detected with the immunoblot ECL kit (Amersham). Two major bands (≈55 and 72 kDa) were detected. With [^{3}H]diprenorphine as a ligand, the retained fraction from the immunoaffinity column also demonstrated significant binding compared with controls (data not shown).

Immunocytochemistry. To maintain the antigenicity of the epitope recognized by MN-3 antiserum several different methods of fixation were used, including formaldehyde, cold ethanol, and several cross-linking agents. Based upon intensity of immunostaining, sulfo-MBS was selected. All incubations and washes were done at 4°C, and all dilutions were made in PBS. Cells were fixed by adding 0.5 ml of 0.7 mM sulfo-MBS to each well and incubated for 2.5 hr (12). Sulfo-MBS that did not react was removed by a 2-hr wash with three changes of PBS. Primary antiserum (0.5 ml, diluted 1:200) or, in some cases, antibody purified over the MN-3 immunoabsorbant (0.5 ml, ≈0.1 μg/ml) was then added to cells and incubated for 2 hr. Unbound antibody was removed with two 20-min PBS washes. Fluorescein isothiocyanate-conjugated goat anti-rabbit γ globulin was then added (0.5 ml per well in a 1:50 dilution), and cells were incubated for 1 hr. Cells were then washed three times over a 30-min period with PBS. Coverslips containing the treated cells were then placed on a drop of anti-fading agent, paraphenylenediamine (13), on microscope slides. Immunofluorescent staining with antisera to MN-3 is referred to as OBCAM-ir.

For anti-N-CAM, a control used in some of these studies, the primary antibody was incubated in 1% bovine serum albumin/PBS at a 1:100 dilution. Fluorescein isothiocyanate-conjugated goat anti-mouse globulin was used as a secondary antibody.

Confocal Microscopy. Confocal micrographs and quantitative data were obtained using the Bio-Rad MRC 600 confocal microscope and the method of White *et al.* (14), as applied by Brelje *et al.* (15). For pictorial presentation, eight planes of images taken at levels spanning the lower to upper surfaces of cells were obtained and pseudocolored so as to depict fluorescence-staining intensity (see Fig. 2). For quantitative studies, cells were selected on the basis of morphologic integrity. A single focal plane through each cell was scanned 10 times by using the Kalman averaging algorithm. The grey level (representing fluorescence intensity) of 10 points on each cell was determined with SOM software (Bio-Rad). These 10 points were averaged, and each data point reported represents the mean of 30 cells taken at 10 cells per coverslip. Statistical analysis was done with a two-tailed, paired Student *t* test (Statview, Abacus Concepts, Berkeley, CA).

RESULTS

Incubation of NG108-15 cells with MN-3 antiserum resulted in distinct, but somewhat uneven, labeling of the plasma membrane (Fig. 1), with highest intensity observed near processes of cells (Fig. 2*a*). Similar results were found by using antibody obtained after affinity purification over MN-3–agarose. In contrast, when cells were incubated with either preimmune serum or with MN-3 antiserum pretreated with the MN-3 peptide, the intensity of the fluorescent signal was ≈10% that of cells labeled with MN-3 antiserum alone (Fig. 2*b*). However, the intensity of OBCAM-ir did not change when MN-3 antiserum was preincubated with KLH. In addition, there was no significant labeling by the MN-3 antiserum of rat liver cells, which do not contain opioid receptors (data not shown).

As one indication of whether the protein labeled by MN-3 antiserum has a role in opioid function, the effects of chronic treatment of NG108-15 cells with various opioid ligands on OBCAM-ir were examined. Incubation of these cells with 125 nM DADLE, a δ-opioid agonist, for 24 hr has been shown to cause the loss of as much as 80% of opioid-receptor binding (10). Fig. 2*c* shows that such treatment dramatically decreased OBCAM-ir. Furthermore, the reduction in OBCAM-ir was dose-dependent (Fig. 3) and blocked by naloxone (Fig. 2*d*; Fig. 3; Table 1).

Chronic treatment of NG108-15 cells with other opioid ligands, including β-endorphin, DPDPE, dynorphin A-(1–13), and U-50,488H also significantly decreased OBCAM-ir (Table 1). Etorphine proved the most potent ligand tested, with a 125 nM concentration sufficient to eliminate >74% of OBCAM-ir (i.e., somewhat more potent than DADLE; Fig. 3, Table 1); again, the effect was blocked by naloxone. In contrast, no significant decrease in OBCAM-ir followed chronic incubation of cells with the μ-agonists DAMGO and levorphanol, the analgetically inactive enantiomer dextrorphan, or the antagonist naloxone.

Several types of control experiments demonstrated the specificity of opioid agonist down-regulation of OBCAM-ir. Incubation of the cells with 125 nM carbachol or atropine for 24 hr, which is known to down-regulate muscarinic receptors present on these cells (16), had no effect on OBCAM-ir (Table 1). In addition, treatment of NG108-15 cells with 100 nM DADLE for 24 hr had no effect on N-CAM immunoreactivity.

Transfected cell lines ST7-3 (antisense) and ST8-4 (sense) demonstrated altered OBCAM-ir. OBCAM-ir was decreased to 65 ± 7.3% (n = 30) in ST7-3 cells compared with the parental NG108-15 cells ($P < 0.001$). In contrast OBCAM-ir was increased to 132 ± 4.7% (n = 30) in ST8-4 cells compared with NG108-15 cells ($P < 0.0025$). N-CAM-ir was not significantly different from NG108-15 in either cell line.

DISCUSSION

An antibody raised to a portion of a protein cloned in a strategy to structurally characterize opioid receptors labeled the plasma membrane of NG108-15 cells. These cells have been widely studied for their expression and regulation of the δ subtype of opioid receptors (1, 9–11, 17–19). The specificity of the labeling we obtained was demonstrated by the virtual absence of labeling by preimmune sera or by MN-3 antisera pretreated with its cognate peptide (Fig. 2) and by the decrease in immunostaining in cells transfected with an antisense construct related to OBCAM. This result indicates that membranes of NG108-15 cells contain either OBCAM or a homologous protein containing all or most of the MN-3 sequence. However, a search of the Swiss-Prot (release 21), GenBank (release 71.0), and GenPept (release 71.0) data bases found no other sequence with significant homology to MN-3. In addition, the pattern of immunostaining we obtained resembles localizations of enkephalin-binding sites reported on NG108-15 cells (20), as well as opioid receptors on NG108-15 cells as determined with anti-idiotype antibodies (19, 21).

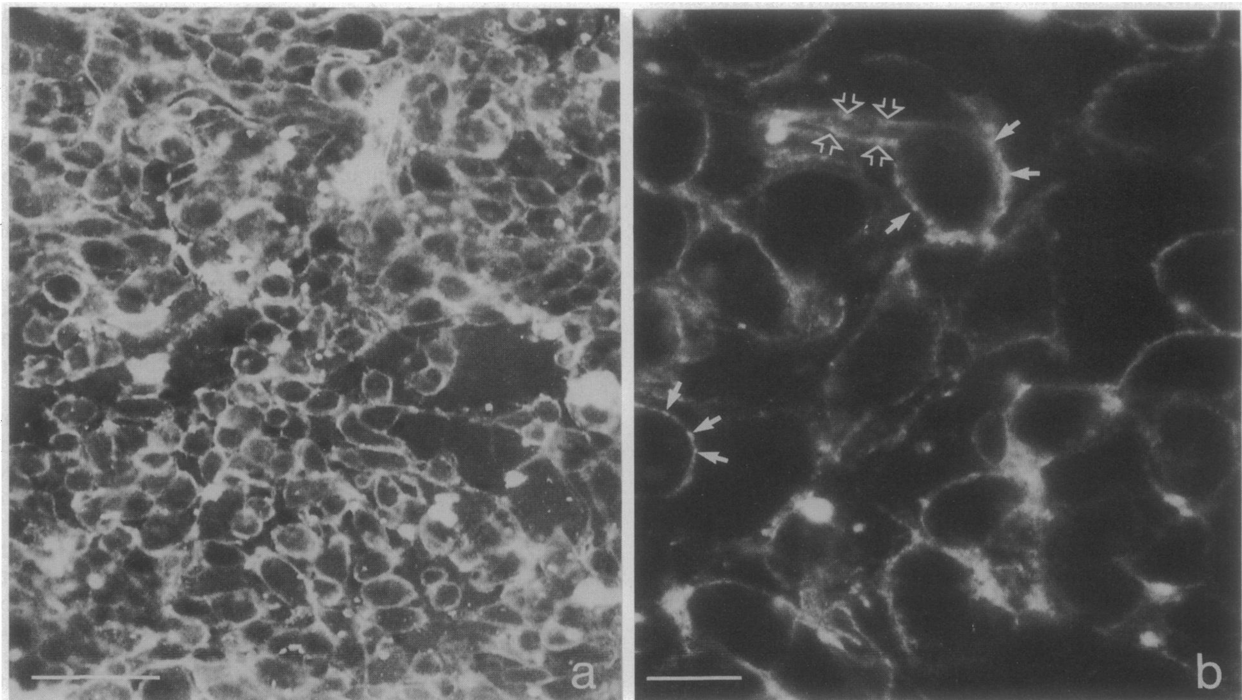

FIG. 1. Confocal immunofluorescence micrographs of NG108-15 cells after incubation with antiserum to MN-3. (*a*) Most cells in confluent cultures display OBCAM-ir associated with the plasma membrane. (*b*) At higher magnification, OBCAM-ir is concentrated in spots along membranes (arrows) and along the membranes of cellular processes (open arrows). Staining within the cytoplasm is not apparent. [Bar = 100 μm (*a*) and 25 μm (*b*).]

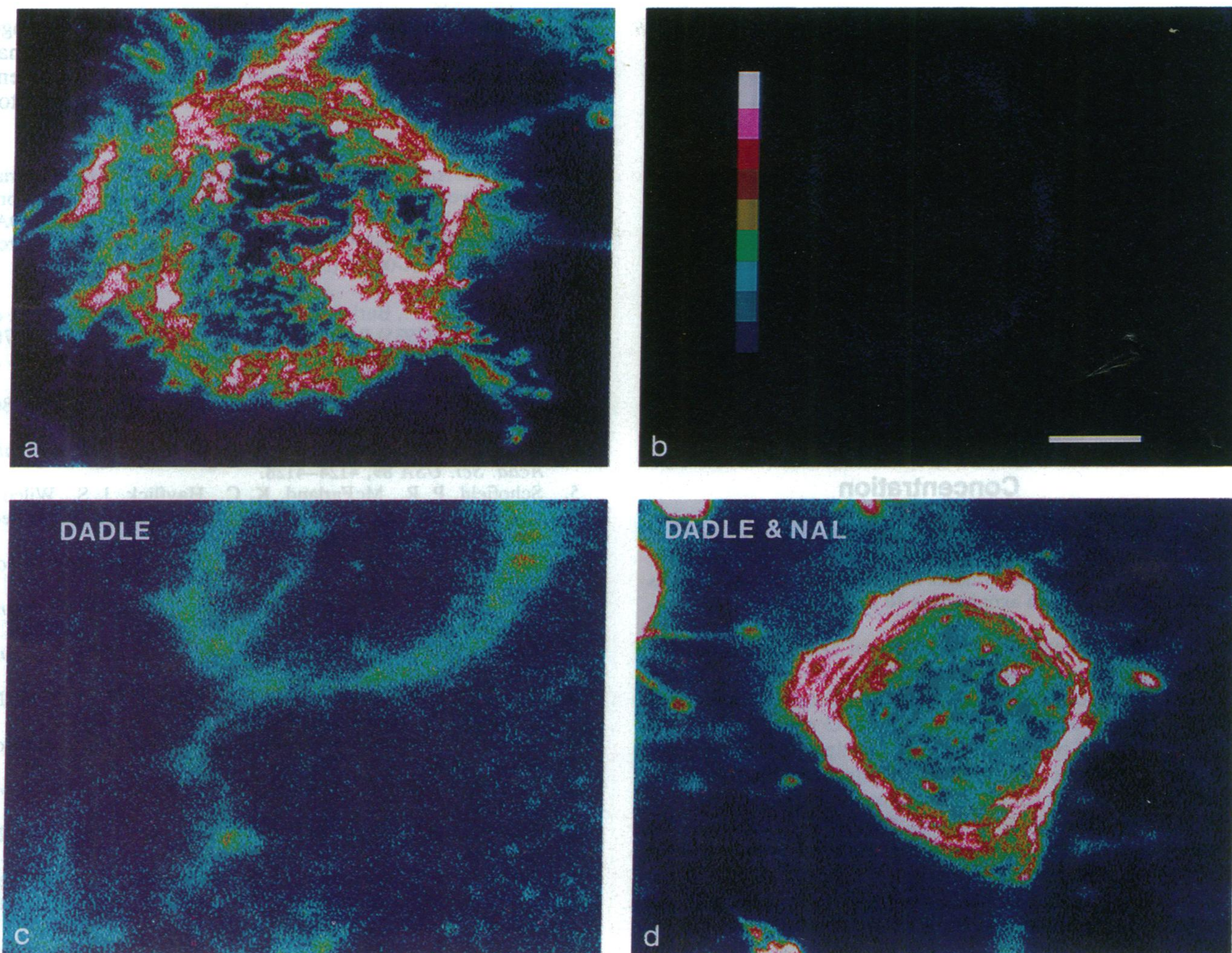

FIG. 2. Pseudocolor immunofluorescence confocal micrographs of NG108-15 cells after incubation with antiserum to MN-3 (*a*, *c*, and *d*) and antiserum to MN-3 pretreated with the peptide MN-3 (*b*). Each micrograph represents the projection of eight optical sections taken at 2-μm intervals. (*a*) NG108-15 cells grown under normal conditions exhibit patches of intense OBCAM-ir (white areas; see intensity scale in *b*), especially near the origin of cellular processes. (*b*) Pretreatment of the antiserum with the MN-3 peptide before incubation with cells blocks all staining of NG108-15 cells grown under normal conditions. (*c*) OBCAM-ir is decreased in NG108-15 cells grown in the presence of 125 nM DADLE. (*d*) OBCAM-ir is nearly normal (compare with *a*) in NG108-15 cells grown in the presence of 125 nM DADLE plus 10 μM naloxone (NAL). Color scaling (*b*) is identical (*a–d*), where white represents the highest intensity, and dark blue represents the lowest intensity. [Bar = 10 μm (*b*; all figures are of equal magnification).]

To show that the OBCAM-ir in NG108-15 cells plays a role in opioid-receptor function, its concentration after chronic incubation of these cells with opioid agonists, a treatment known to down-regulate opioid receptors, was examined. OBCAM-ir was markedly decreased by incubation of the cells for 24 hr with several opioid agonists (Table 1), and this decrease was concentration dependent and naloxone sensitive. Moreover, the ability of different opioid agonists to down-regulate OBCAM-ir correlated well with their ability to down-regulate opioid receptors. The opioid receptors present on the NG108-15 cells are exclusively of the δ type (17), and δ agonists are the most effective in down-regulating these receptors (10). In these studies, the δ-agonists DADLE and DPDPE markedly decreased OBCAM-ir, as did the nonselective agonists β-endorphin and etorphine, which also potently down-regulate opioid receptors. Agonists such as dynorphin A and U-50,488H decreased OBCAM-ir weakly. In contrast, agonists morphine, levorphanol, and DAMGO, which do not down-regulate NG108-15 receptors, were ineffective in decreasing OBCAM-ir (Table 1).

Receptor down-regulation is a widespread phenomenon observed with chronic treatment of many different agonists, and to some extent these mechanisms probably make use of common cellular processes. That the down-regulation of the protein with OBCAM-ir is specifically related to opioid function, however, is suggested by the absence of any decrease in labeling after the incubation of NG108-15 cells with carbachol, which down-regulates the muscarinic receptors on these cells. Also, the failure of DADLE to down-regulate N-CAM, a molecule in the same superfamily (immunoglobulin) as OBCAM, refutes the notion that the concentration of any cell-surface molecule may be altered by opioid agonist or that the MN-3 antiserum reacts with many different homologous molecules.

In a recent report (9) we have demonstrated that the transfection of NG108-15 cells with an antisense construct to OBCAM reduced opioid binding. Interestingly, in the present study we found that immunostaining for OBCAM with the antisera against the MN-3 peptide was correspondingly decreased. This finding provides additional evidence that the immunostaining seen in the present study was selective for OBCAM. Secondly, the coordinate decrease in opioid binding and OBCAM-ir as a consequence of antisense transfection further strengthens the suggestion that OBCAM is a component of opioid receptors in NG108-15 cells.

The close relationship between OBCAM and the δ receptor in NG108-15 cells is somewhat surprising because OBCAM

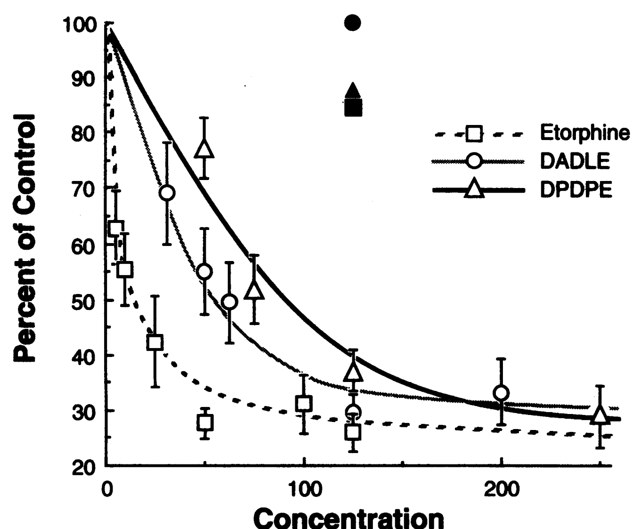

FIG. 3. Intensity of OBCAM-ir as a function of growth for 24 hr of NG108-15 cells in various concentrations (nM) of etorphine, DADLE, or DPDPE. Solid symbols represent values from cultures grown in 125 nM of each agonist plus 10 μM naloxone.

does not have the expected structure of an opioid receptor. At least some opioid receptors, including the δ type found in NG108-15 neuroblastoma × glioma hybrid cells, are thought to be coupled to second messengers via G proteins (17). All other cell-surface receptors reported to date that are associated with G proteins share extensive structural homologies, particularly the presence of seven hydrophobic regions thought to span the membrane (22). OBCAM, in contrast, has no putative membrane-spanning regions but has only a short hydrophobic sequence at its carboxyl terminus that is probably inserted into the cell membrane. The immunoglobulin domains that compose the bulk of its structure are almost certainly extracellular, as assumed for almost all other members of this superfamily. Thus that OBCAM alone could directly associate with a G protein or by itself function in signal transduction is unlikely.

However, because OBCAM-ir was found to be coordinately regulated with opioid-receptor function in the present study, OBCAM must play a role in opioid-receptor function, at least in NG108-15 cells. This conclusion is consistent with several other lines of evidence from our laboratory. We have shown that monoclonal and polyclonal antibodies against the purified protein inhibit opioid binding to brain membranes as well as to the purified protein (7, 8). Moreover, polyclonal antibodies directed against portions of the predicted amino acid sequence of OBCAM react positively with NG108-15 cells and tissue from several brain regions in ELISA (Sabita Roy, H.H.L., and N.M.L., unpublished data).

Together these findings show that OBCAM levels change in response to agonist treatment in a manner parallel with that of pharmacologically identifiable opioid receptors, consistent with an important role for this molecule in opioid-receptor function.

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1. Simonds, W. F., Burke, T. J., Rice, K. C., Jacobson, A. E. & Klee, W. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4974–4978.
2. Gioannini, T. L., Howard, A. D., Hiller, J. M. & Simon, E. J. (1985) *J. Biol. Chem.* **260,** 15117–15121.
3. Cho, T. M., Hasegawa, J., Ge, B. L. & Loh, H. H. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 4138–4142.
4. Xie, G. X., Miyajima, A. & Goldstein, A. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4124–4128.
5. Schofield, P. R., McFarland, K. C., Hayflick, J. S., Wilcox, J. N., Cho, T. M., Roy, S., Lee, N. M., Loh, H. H. & Seeburg, P. H. (1989) *EMBO J.* **8,** 489–495.
6. Loh, H. H. & Smith, A. P. (1990) *Annu. Rev. Pharmacol. Toxicol.* **30,** 123–147.
7. Roy, S., Zhu, Y. X., Lee, N. M. & Loh, H. H. (1988) *Biochem. Biophys. Res. Commun.* **150,** 237–244.
8. Roy, S., Zhu, Y. X., Loh, H. H. & Lee, N. M. (1988) *Biochem. Biophys. Res. Commun.* **154,** 688–693.
9. Ann, D. K., Hasegawa, J., Ko, J.-L., Chen, S.-T., Lee, M. N. & Loh, H. H. (1992) *J. Biol. Chem.* **267,** 7921–7926.
10. Law, P. Y., Hom, D. S. & Loh, H. H. (1983) *Mol. Pharmacol.* **24,** 413–424.
11. Law, P. Y., Hom, D. S. & Loh, H. H. (1982) *Mol. Pharmacol.* **22,** 1–4.
12. Hassell, J. & Hand, A. R. (1974) *J. Histochem. Cytochem.* **22,** 223–229.
13. Johnson, G. D., Davidson, R. S., McNamee, K. S., Russell, B., Goodwin, D. & Holborow, E. J. (1982) *J. Immunol. Methods* **55,** 231–242.
14. White, J. G., Amos, W. B. & Fordham, M. (1987) *J. Cell Biol.* **105,** 41–48.
15. Brelje, T. C., Scharp, D. W. & Sorenson, R. L. (1989) *Diabetes* **38,** 808–814.
16. Klein, W. L., Nathanson, N. & Nirenberg, M. (1979) *Biochem. Biophys. Res. Commun.* **90,** 506–512.
17. Chang, K. J. & Cuatrecasas, P. (1979) *J. Biol. Chem.* **254,** 2610–2618.
18. Law, P. Y., Hom, D. S. & Loh, H. H. (1983) *Mol. Pharmacol.* **23,** 26–35.
19. Ornatowska, M. & Glasel, J. A. (1992) *J. Chem. Neuroanat.* **5,** 95–106.
20. Hazum, M., Chang, K. J. & Cuatrecasas, P. (1979) *Science* **206,** 1077–1079.
21. Hassan, A. H., Almeida, O. F., Gramsch, C. & Herz, A. (1989) *Neuroscience* **32,** 269–278.
22. Dohlman, H. G., Caron, M. G. & Lefkowitz, R. J. (1987) *Biochemistry* **26,** 2657–2664.