Regulation of Acute and Chronic Opioid Receptor Functions by OBCAM, a Cell Adhesion-Like Molecule

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INTRODUCTION

Opioid receptors were one of the first class of cell surface receptors to be identified using in vitro binding assays with brain tissue, but for many years efforts to purify and clone them were unsuccessful because of their sensitivity to detergents, their heterogeneity, and the lack of a simple biochemical assay for their function (Loh and Smith 1990). Several laboratories, including the authors' own, reported purification of opioid-binding proteins (Cho et al. 1986; Gioannini et al. 1985; Maneckjee et al. 1985; Newman and Barnard 1984), but were unable to demonstrate unequivocally that they possessed all the pharmacological properties of in vivo receptors.

This situation has been dramatically altered by the recent successful cloning of the delta opioid receptor from NG108-15 cells, as reported at the end of 1992 by two independent laboratories (Evans et al. 1992; Kieffer et al. 1992). The two laboratories used a similar approach (expression cloning), preparing a cDNA library from NG108-15 cells, transfecting pools of this cDNA into mammalian cells, and then assaying the cells for binding of a radioactive opioid ligand. The cloned, expressed receptor showed typical binding properties expected of an opioid receptor, including high affinity, stereoselectivity, and preference for a particular class of opioids, namely delta; in addition, Evans and colleagues (1992) demonstrated that binding to the expressed receptor inhibited adenylyl cyclase, as is the case with native delta-opioid receptors on NG108-15 cells.

In NG108-15 cells (Koski and Klee 1981), as well as in at least some areas of the mammalian central nervous system (CNS) (Attali and Vogel 1989; Blume et al. 1979; Childers and Snyder 1978; Law et al. 1981), opioid receptors are coupled to guanosine triphosphate binding proteins (G-proteins), so it was no surprise that the predicted amino acid sequence of the cloned opioid receptor cDNA was homologous to other members of the G-protein-coupled receptor

superfamily (Dohlman et al. 1987); in particular, the sequence displayed seven characteristic hydrophobic regions that are presumed to span the cell membrane. The cloned delta opioid receptor (hereafter referred to as DOR) cDNA possesses several other features in common with other G-protein-coupled receptors, including aspartate residues in characteristic positions in transmembrane regions 2 and 3, which are thought to be involved in ligand binding and coupling to adenylyl cyclase; cysteine residues in extracellular loops 2 and 3, which may form a disulfide bridge between these regions; several putative glycosylation sites in the N-terminal region; and several putative phos-phorylation sites in the C-terminal region, which in other G-protein-coupled receptors are thought to be involved in regulation of the receptor during chronic agonist treatment (e.g., desensitization and downregulation). Among the other members of this superfamily, the somatostatin receptors are most closely related to the delta opioid receptor, exhibiting 35 to 40percent sequence homology, and this is consistent with the report that somatostatin ligands can bind to opioid receptors.

Whenever the cDNA for a receptor becomes available, it becomes of interest to use it as a probe to screen libraries in search of structurally similar sequences. In the case of the delta opioid receptor, this search has taken on particular importance, for it seemed possible that the other types of opioid receptors that have been defined pharmacologically, in parti-cular the mu receptor (the major mediator of opioid antinociception) and the kappa receptor, might have similar nucleotide sequences. This has proved to be the case. Chen and associates (1993) isolated from rat brain a cDNA that expressed receptor selective for mu ligands such as [D-Ala2-MePhe4, Gly-ol5]-enkephalin (DAMGO), and which was negatively coupled to adenylyl cyclase. Yasuda and colleagues (1993) also isolated a clone from rat brain that appears to express a receptor selective for kappa opioids. Both of these receptors exhibit about 60 percent amino acid homology with the delta opioid receptor as well as with each other. The greatest homology, as expected, is in the seven putative transmem-brane regions, while the least is in the N-terminal and C-terminal sequences, and in the second and third extracellular loops formed by amino acids between transmembrane regions 4 and 5 and 6 and 7.

Of particular surprise and interest, however, is the very strong sequence homology—about 90 percent—of the third cytoplasmic loop, between transmembrane regions 5 and 6, in all three of these opioid receptor sequences. In other members of the G-protein-

coupled superfamily, this region is thought to be critical in coupling to G-proteins, and consistent with the wide variety of G-proteins available for coupling to different receptors, the homology in this area is relatively low even between highly related receptors. For example, the somatostatin 1 and 2 receptors have about 40 percent sequence identity in this region, as do the ß1- and ß2-adrenergic receptors. The unexpectedly high homology in the case of opioid receptors suggests that these three receptors may couple to the same G-proteins. This raises the question of how these receptors are regulated so that they mediate distinct physiological responses.

Studies in the authors' laboratory have begun to address the question of opioid receptor regulation. To determine which G-proteins the receptors couple to, a technique involving labeling G-proteins with a stable guanosine triphosphate (GTP) analog has been employed. After stable transfection of mu or delta opioid receptor cDNA into Chinese hamster ovary (CHO) cells, the authors determined the ability of various opioid ligands to induce covalent labeling of the alpha subunit of G-proteins by the radioactive GTP analog [32P]-alpha-azidoanilido GTP. In CHO cells, four different Gas could be separated by SDS/urea gel electrophoresis and identified by appropriate antibodies: Go2a, Gi2a, Gi3a, and another, unidentified Goa. All four of these Gas were labeled by selective opioid ligands (mu or delta) in CHO cells transfected with mu or delta opioid receptor cDNA, though there was some preference demonstrated. In most cases, there was no correlation between the potency of a ligand to label a G-protein and its affinity for the expressed receptor or its potency to inhibit adenylyl cyclase.

The authors have also begun to identify the genetic elements that may be responsible for regulating the synthesis of opioid receptors. The sequence of the mu opioid receptor gene has been determined, and a number of sequences have been found that fit the consensus for certain regulatory elements. These include AP-1 and AP-2, which are regulated by cyclic adenosine monophosphate (AMP), and NF-GMb and NF-IL6, which are involved in the regulation of cytokine receptors. The existence of potential cyclic AMP (cAMP) regulatory elements is of interest, as opioid receptors in many systems inhibit cAMP synthesis. On the other hand, the discovery of potential immune regulatory sites is intriguing in light of accumulating evidence of links between opioids and the immune system.

Another possibility, however, is that opioid receptors may be regulated by some other molecule. For the past several years, the authors'

laboratory has characterized a protein, opioid binding cell adhesion molecule (OBCAM), that appears to play a regulatory role in opioid receptor function. OBCAM has homology to neural cell adhesion molecule, amalgam, and other cell adhesion molecules, possessing three immuno-globulin domains that are presumed to be oriented on the extracellular surface of the cell membrane. Since it has no putative cytoplasmic domain, it is difficult to see how it could behave as a complete signal transducing unit, yet it might still regulate opioid receptor function in some way. In this chapter, some of the evidence that OBCAM plays such a role will be discussed.

ROLE OF OBCAM IN OPIOID RECEPTOR BINDING AND ACUTE ACTION OF OPIOIDS

OBCAM was originally isolated on the basis of its ability to bind opioids, though it required the additional presence of acidic lipids (Hasegawa et al. 1987). As discussed above, both the lack of a putative cytoplasmic domain in OBCAM, as well as the demonstration that G-protein-coupled receptors bind opioids, make it unlikely that OBCAM directly binds opioids. Nevertheless, several studies in the authors' laboratory indicate that OBCAM may regulate opioid binding.

First, antibodies have been raised both to purified OBCAM and to peptides corresponding to portions of its predicted amino acid sequence, and have demonstrated that these antibodies inhibit opioid binding to the purified protein as well as to brain membranes (Roy et al. 1988a, 1988b). In addition, antibodies to OBCAM also block opioid antinociception when injected into the brain. These antibody data do not establish that OBCAM itself binds opioid ligands in situ, but they strongly suggest some kind of close association between OBCAM and the opioid receptor.

A second line of evidence indicating that OBCAM regulates opioid binding has come from studies using antisense cDNA. This technique has gained widespread use in recent years as a relatively easy and highly specific way of testing the role of a particular gene product in some function by selectively inhibiting expression of that gene. The authors have applied this technique to NG108-15 neuroblastoma x glioma hybrid cells, which contain a homogeneous population of delta opioid receptors coupled to adenylyl cyclase (Chang and Cuatrecasas 1979; Sharma et al. 1975a). By transfecting neuroblastoma x glioma NG108-15 cells with antisense cDNA to OBCAM, a stable cell line has been created (ST7-3) in which opioid binding is greatly reduced relative to that of cells transfected with OBCAM

sense (ST8-4), as well as nontransfected cells (Ann et al. 1992). The selectivity of this effect is suggested by the observation that binding of ligands to other cell surface receptors in ST7-3 cells was unaffected. Scatchard analysis of the binding indicated that most of the reduction was due to a decrease in receptor number, not affinity. Moreover, the remaining receptors could be further downregulated by chronic opioid agonist treatment of the cells. Thus, it appears that the OBCAM antisense has greatly reduced the number of opioid receptors on the cell without affecting their intrinsic response to acute or chronic opioid agonist treatment. This result again is consistent with an association between OBCAM and another molecule functioning as the receptor.

The antisense OBCAM cDNA has also been used to create a line of transgenic mice. The antisense was injected into mouse oocytes, which were implanted into pseudopregnant females; upon birth and maturation, the mice were selectively bred to create a stable line of transgenics. Founder as well as first- and second-generation transgenic mice showed a reduced response to the antinociceptive effect of morphine, as determined by the tailflick test, and also reduced sensitivity to acute tolerance to morphine. In the latter test, the animals were pretreated with a fixed dose of morphine, followed by determination of the morphine median analgesic dose (AD50) in the presence of a fixed dose of the antagonist naloxone. Normal mice show an increased AD50 in the presence of naloxone, and the higher the pretreatment dose, the higher the AD50. This increase was greatly reduced in the transgenic mice.

These studies all suggest that OBCAM plays a role in opioid receptor function, but do not really address the question of how it could do so. The authors have recently shown that coupling to G-proteins is altered in ST7-3 (OBCAM antisense-transfected) cells (Gavitrapong et al. 1993). To demonstrate this, the authors made use of the fact that cholera toxin (CTX) induces adenosine diphosphate (ADP)-ribosylation of G-proteins only in the presence of ligand, which promotes coupling between receptor and G-protein. Thus ribosylation can be used as a convenient assay of the degree of coupling induced by ligand for a specific type of receptor. In untransfected cells or cells transfected with OBCAM sense (ST8-4), CTX was shown to induce, in the presence of opioid agonist, ADP-ribosylation of Gi and Go on the basis of the reactivity of SDS gel bands with antibodies for these G-proteins. In the antisense (ST7-3) cells, in contrast, this labeling was greatly inhibited; as shown in table 1, the median effective dose (ED50) for DADLE to induce labeling was increased thirtyfold to fiftyfold.

TABLE 1. DADLE-induced, CTX-catalyzed ADP-ribosylation.

Membrane	ED50 (nM)
NG108-15	20
ST8-4 (sense)	30
ST7-3 (antisense)	-1,000

The reduced coupling between receptor and G-protein in ST7-3 cells was also manifested in reduced ability of opioid ligand to inhibit either basal or forskolin-stimulated adenylyl cyclase. The basal data are shown in figure 1a. While [2-D-Ala-5-D-Leu-] enkephalin (DADLE) at

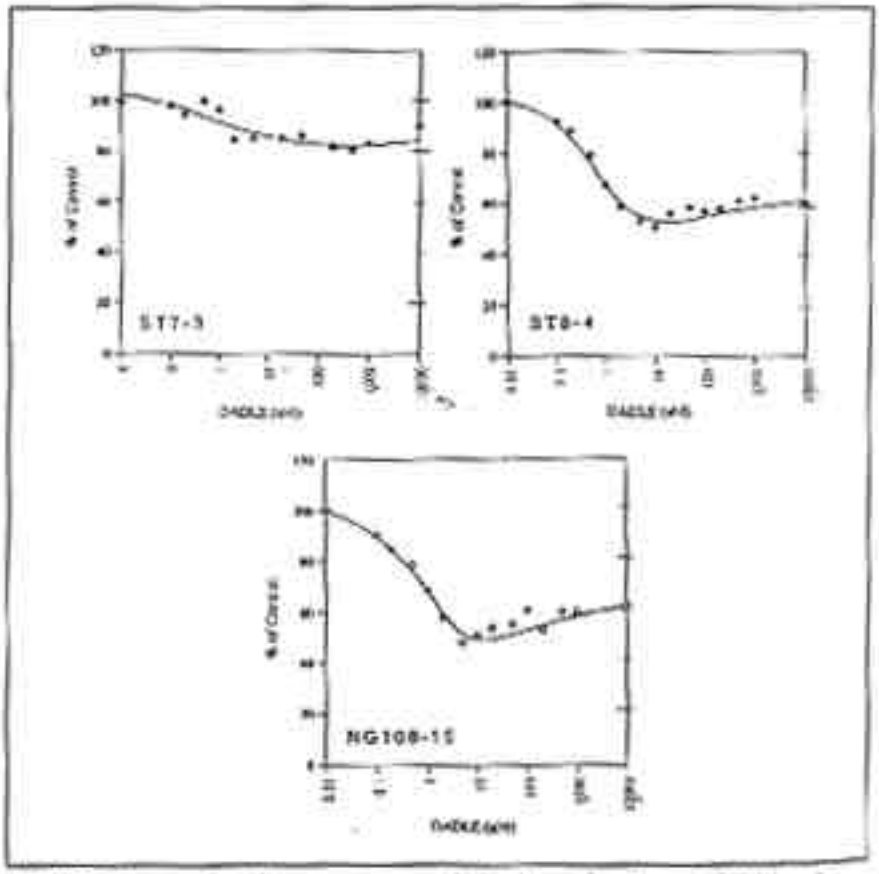

FIGURE 1a. *Opioid receptor coupled adenylyl cyclase inhibition in intact cells.*

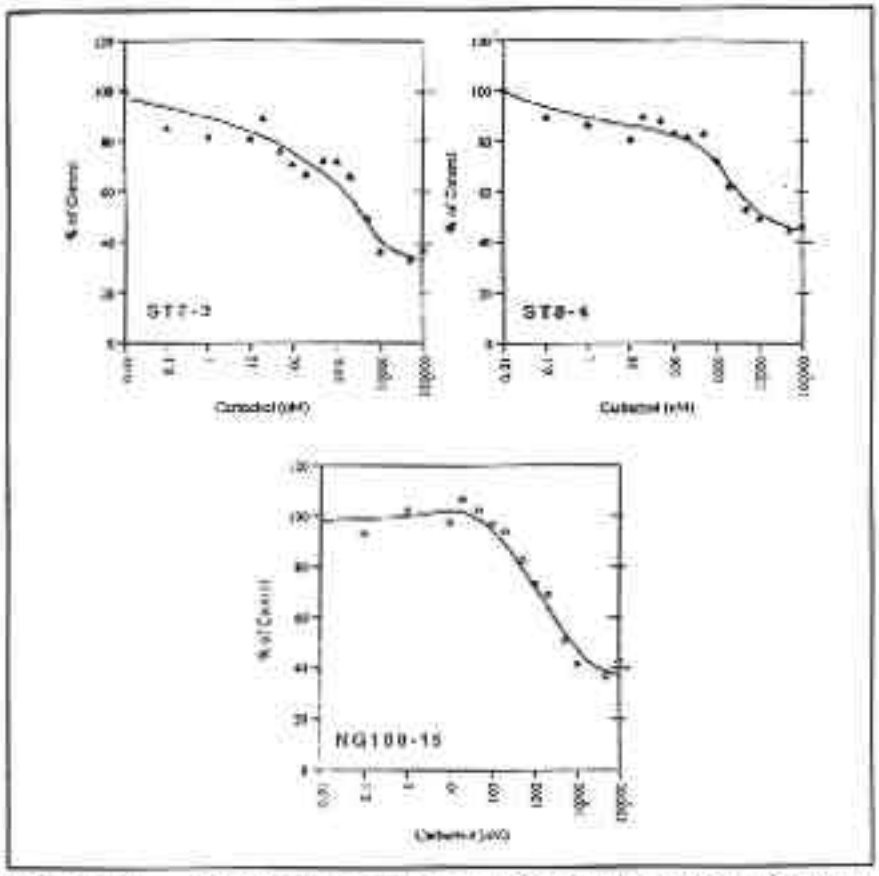

FIGURE 1b. *Muscarinic receptor coupled adenylyl cyclase inhibition in intact cells.*

concentrations of 1 to 10 nanomalors (nM) inhibited cyclase approximately 50percent in untransfected or sense-transfected cells, maximal inhibition was less than 20 percent in antisense-transfected cells, and even then required much higher DADLE concentrations. Furthermore, the antisense effect appeared to be specific to opioid receptors, as inhibition of adenylyl cyclase by adrenergic and muscarinic ligands was the same in ST7-3 cells as in ST8-4 or untransfected cells (figures 1b and 1c). Finally, the ability of DADLE to stimulate GTPase, a normal concomitant of coupling, was also inhibited in ST7-3 cells.

In summary, the presence of antisense OBCAM cDNA in NG108-15 cells has somehow interfered with coupling of opioid receptors to G-proteins, reducing the ability of the receptors to inhibit adenylyl

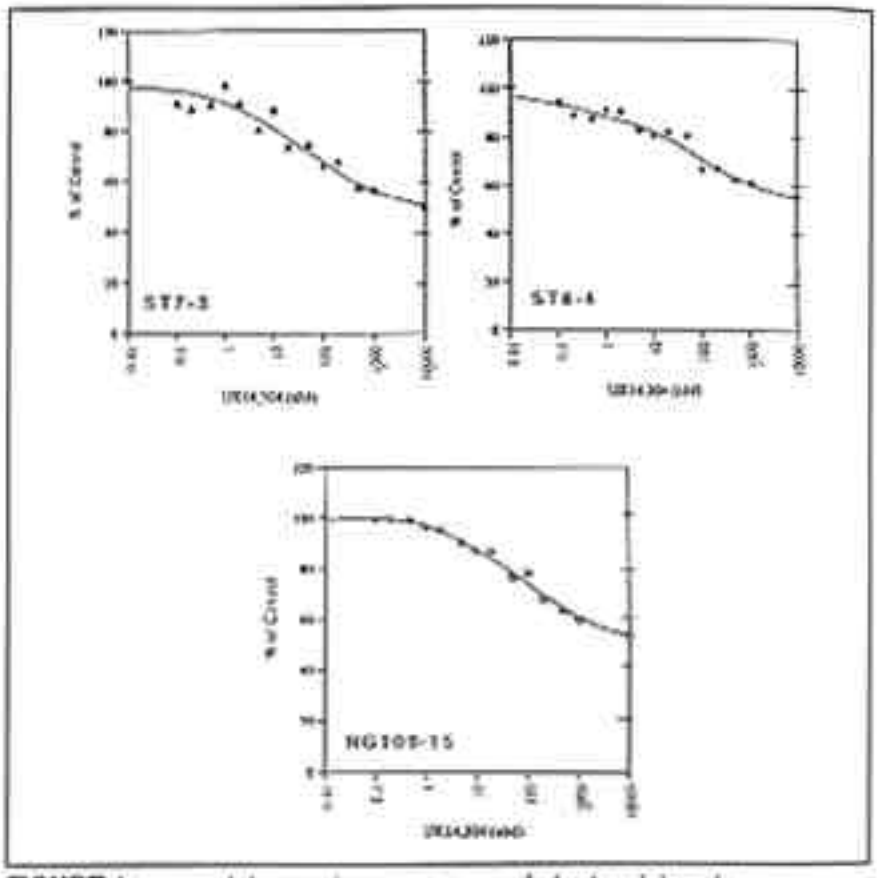

FIGURE 1c. *α_2-Adrenergic receptor coupled adenylyl cyclase inhibition in intact cells.*

cyclase. It is not clear how OBCAM, an extracellular molecule, could couple a seven transmembrane receptor to G-proteins, which are located on the cytoplasmic face of the cell membrane. One possibility is that OBCAM interaction with the receptor alters its conformation, so that those portions of the receptor that are located in the cell, and couple directly to G-proteins, are altered. Alternatively, the presence of OBCAM may be necessary to promote a lipid environment in which receptor and G-protein may couple.

ROLE OF OBCAM IN CHRONIC OPIOID EFFECTS

One of the signal features of opioid drugs is the ability to induce tolerance and dependence upon chronic administration to humans or animals. Tolerance may be defined as a state in which the dose of drug required to achieve a given effect is larger than normal. Dependence is a state in which regular doses of drug are required to prevent withdrawal symptoms.

The molecular basis of opioid tolerance and dependence is unknown, but a useful model system is provided by NG108-15 cells. When treated chronically with an appropriate opioid agonist such as DADLE, these cells become less responsive to opioids in a tolerant-like manner (Sharma et al. 1975b). Studies of the molecular basis of these effects have shown that several processes underlie this reduced sensitivity, including uncoupling of the opioid receptors from adenylyl cyclase, an effector for opioids in this system, and downregulation of the receptors, a process by which they are lost from the cell surface (Law et al. 1984).

Studies in the authors' laboratory have suggested that OBCAM plays a role in these processes also. The fate of OBCAM during chronic opioid treatment of NG108-15 cells has been studied by the use of fluorescent antibody labeling in conjunction with confocal microscopy. The antibody used was raised against a 12-amino-acid peptide (MN-3) corresponding to a portion of OBCAM in the third immunoglobulin domain. Initial studies demonstrated heavy fluorescence on the surface of NG108-15 cells treated with this antibody, which was blocked by pretreatment of the antibody with the peptide. In addition, cells transfected with OBCAM antisense cDNA (see above) showed much less immunofluorescence.

When NG108-15 cells were chronically treated with a delta opioid agonist such as DADLE, there was a significant reduction in the amount of antibody staining. The dose dependence and timecourse of this reduction closely paralleled the downregulation of opioid receptors; furthermore, the ability of a number of different agonists to induce downregulation of OBCAM-like immunoreactivity also paralleled their ability to downregulate opioid receptors. Thus delta agonists were the most effective, while mu and kappa opioids were much less so; the addition of an antagonist such as naloxone blocked the downregulation of OBCAM-like immunofluorescence. The specificity of this effect for opioid receptors was demonstrated by control experiments in which OBCAM-like immunoreactivity was

unchanged following chronic treatment of the cells with ligands for other types of receptors, such as muscarinic and adrenergic. Furthermore, chronic treatment with DADLE had no effect on the surface levels of neuronal cell adhesion molecule (N-CAM).

The recent cloning of the delta opioid receptor has made it possible to study the relationship of this receptor with OBCAM more directly, by transfection of cDNA into mammalian cells. With the collaboration of Dr.Ping Law, the authors have obtained CHO cells stably transfected with DOR cDNA. OBCAM expression, as measured by the appearance of M2 antibody fluorescence, is greatly increased in these cells, though interes-tingly, there is no correlation with increased opioid binding; that is, the level of immunofluorescence is about the same in the transfected CHO cells as in untransfected NG108-15 cells, though the level of opioid recep-tor binding is several times higher in the former (Ko and Loh, unpublished data). In addition, the authors have stably transfected neuro 2A cells with DOR, and OBCAM immunofluorescence does not correlate with opioid binding levels in different stable transfectants. Nevertheless, this work, together with the downregulation studies, indicates that manipu-lations that raise or lower the level of opioid receptors on the surface of NG108-15 cells result in a corresponding increase or decrease in OBCAM levels.

CONCLUSION

The cloning of mu, delta, and kappa opioid receptors has ended a two- decade search in pharmacology, but raised new questions concerning the mechanisms by which these receptors are regulated. The authors' studies with OBCAM suggest that this cell adhesion-like molecule may play a role in both the acute and chronic actions of opioids. Thus OBCAM seems to be necessary for normal coupling between opioid receptors and G-proteins, as well as undergoing downregulation in parallel to that of opioid receptors upon chronic opioid treatment. Further studies should clarify the role of OBCAM in opioid receptor function.

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Click here to go to page 154