

The regulation of δ -opiate receptor density on 108CC15 neuroblastoma \times glioma hybrid cells

Margaret A. Moses & Christopher R. Snell

MCR Neuroendocrinology Unit, Newcastle General Hospital, Westgate Road, Newcastle upon Tyne NE4 6BE

- 1 The effect of exogenous substances on the expression of opiate receptors on 108CC15 neuroblastoma \times glioma hybrid cells has been studied.
- 2 Cell differentiation by culture in the presence of N⁶-O²-dibutyryl adenosine 3',5'-cyclic monophosphate induced a three fold increase in opiate receptor density.
- 3 When the cells were grown in the presence of 10⁻⁵ M morphine hydrochloride for up to 23 days, opiate receptor densities were reduced by only 30% when compared with matched controls.
- 4 Culture in the presence of 10⁻⁷ M D-Ala²-D-Leu⁵-enkephalin produced opiate receptor down regulation of 73% compared to controls after only 4 h of treatment.
- 5 The down regulation process could be inhibited by continued exposure to D-Ala² D-Leu⁵-enkephalin at concentrations greater than 4 nM; below this concentration down regulation was rapid and irreversible.
- 6 A model to explain these observations is described.

Introduction

The 108CC15 neuroblastoma \times glioma hybrid cells are a clonal cell line derived from fusion of a mouse neuroblastoma C1300 and rat glioma C6 (Hamprecht, 1974; Hamprecht, 1977). They possess many of the characteristics of neurones and have been shown to express membrane bound δ -opiate receptors (Klee & Nirenberg, 1974; Chang, Miller & Cuatrecasas, 1978; Chang & Cuatrecasas, 1979) that are functionally linked to adenylate cyclase in an inhibitory manner (Sharma, Nirenberg & Klee, 1975b; Traber, Fischer, Latzin & Hamerecht, 1975; Wahlstrom, Brandt, Moroder, Wunsch, Lindeberg, Ragnarsson, Terenius & Hamprecht, 1977). These cells provide a suitable model for studying the mechanisms involved in the regulation of opiate receptor densities by exogenous substances. In this paper we show that δ -opiate receptor levels can be increased three fold by induction of neurite outgrowth in these cells, and decreased dramatically by culture in the presence of the opioid peptide D-Ala²-D-Leu⁵-enkephalin (DADLE), but not the opiate alkaloid morphine. We show that down regulation of receptor numbers is initiated only when ligand concentration falls below 4 nM and propose a model to explain these observations.

Methods

The hybrid cells were grown at 37°C in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, 0.1 mM hypoxanthine, 1 μ M aminopterin, 16 μ M thymidine, 0.01% (w/v) kanamycin and 0.004% (w/v) gentamycin in a humidified atmosphere of 10% CO₂ plus 90% air. Cells with passage number between 14 and 20 were used to avoid possible variation due to chromosome loss.

Exposure to modulators

Treatment with dibutyryl cyclic AMP After seeding at low density (3.5 \times 10⁵ cells/75 cm² flask) the cells were cultured until 5 to 10% confluent (usually taking 48–72 h), whereupon N⁶-O²-dibutyryl adenosine 3',5'-cyclic monophosphate (dibutyryl cyclic AMP) was added to the culture medium to a final concentration of 0.77 mM. The cells were harvested after a further eight days culture for opiate receptor assay. Untreated cultures of the same passage number and at approximately the same degree of confluency were used as control cells.

Treatment with morphine hydrochloride Cells were cultured in the usual way but the medium was supplemented with 10^{-5} M morphine hydrochloride. Culture was continued through one or more passages, and matched controls were grown in parallel. Cells were harvested after up to 23 days continuous exposure to morphine hydrochloride, and assayed for δ -opiate binding sites.

Treatment with D-Ala²-D-Leu⁵-enkephalin (DADLE) Cells were cultured in the usual way until approximately 80% confluent, when they were supplemented with DADLE to give a final concentration in the medium of 10^{-7} M. After 3 or 4 h exposure, treated cells and matched controls were harvested for opiate receptor assay. In other experiments identically treated cells were removed from exposure to DADLE by changing the medium. After further incubation for 1, 4, 20 or 44 h the cells were harvested with similarly treated matched controls and assayed for δ -opiate binding sites.

³H-methionine enkephalin binding assay Cell monolayers were suspended by mechanical agitation and the cells harvested by centrifugation at 800 g for 4 min. After washing three times with buffer (137 mM NaCl, 5.4 mM KCl, 0.17 mM Na₂HPO₄, 0.22 mM KH₂PO₄, 5.5 mM D-glucose) the cells were counted in a haemocytometer and suspended in assay buffer (0.32 M sucrose, 0.01 M Tris, 15 mM MgCl₂, 0.005% (w/v) bacitracin, pH 8.0) at a concentration of 10^6 cells ml⁻¹. In experiments designed to prevent down regulation 6 nM DADLE was included in all the washing solutions, and the cells suspended in DADLE-free assay buffer immediately before assay. In all cases the binding assay was performed within 1 h of harvest.

The washed cells (5×10^5 /tube) were added to a serial dilution of [³H]-methionine enkephalin (36 Ci mmol⁻¹; Amersham International plc; 0.3–16 nM) in assay buffer, and a parallel series of tubes containing, in addition, 10^{-7} M unlabelled methionine enkephalin. The total volume in each tube was 1 ml. After incubation for 10 min at 37°C the tubes were centrifuged at 14,000 g for 2 min to separate bound from free labelled enkephalin. The supernatant was aspirated off and the pellets superficially washed with ice-cold assay buffer (0.5 ml). The tips of the tubes containing the pellets were cut off and the pellets suspended in water (0.5 ml). Packard scintillant 299 (5 ml) was added and the radioactivity in the pellet measured on a Packard 460CD scintillation counter with on line d.p.m. correction. The assays were performed in triplicate or quadruplicate. Specific binding was taken as the difference between that bound in the absence and presence of excess unlabelled methionine-enkephalin. The data were

analysed by the method of Scatchard (1949). The contributions to biphasic Scatchard plots were separated mathematically by the method described by Burt & Snyder (1975).

Results

The density of [³H]-methionine-enkephalin binding sites was found to increase as the cell monolayer became confluent. Figure 1 shows the variation of receptor density (B_{\max}) with the extent of monolayer cover for cells in serial culture between passages 14 and 18. A linear correlation was obtained between receptor density and cell density, however there was no observable correlation between time in culture or passage number and receptor density.

Treatment with dibutyryl cyclic AMP

After 3 days in culture with dibutyryl cyclic AMP the cell bodies became more rounded and refractile with many short neurite outgrowths. The rate of cell proliferation decreased and after 8 days in culture with dibutyryl cyclic AMP, treated cells were only 40% confluent and had many long neurite processes. Protein assay by the method of Lowry, Rosebrough, Farr & Randall (1951) showed that both treated and untreated cells contained 0.74 mg protein/ 10^6 cells. However, the number of opiate receptors on the dibutyryl cyclic AMP treated cells had increased over three fold compared with untreated controls, whereas the affinity of the receptors remained the same, see Figure 2.

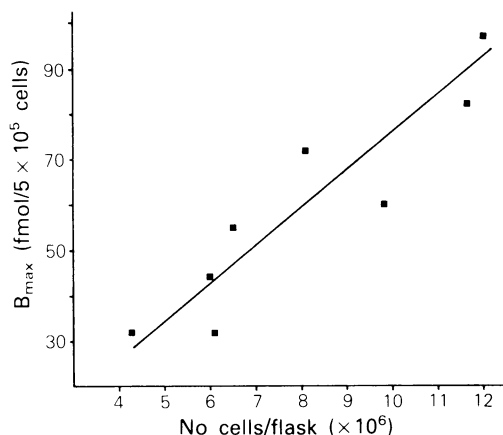


Figure 1 The effect of monolayer cover on B_{\max} for the δ -opiate receptor. B_{\max} was obtained by Scatchard analysis of receptor saturation using [³H]-methionine-enkephalin as labelled ligand as described in the Methods, and is plotted against monolayer cover for cells harvested between passage 14 and 18.

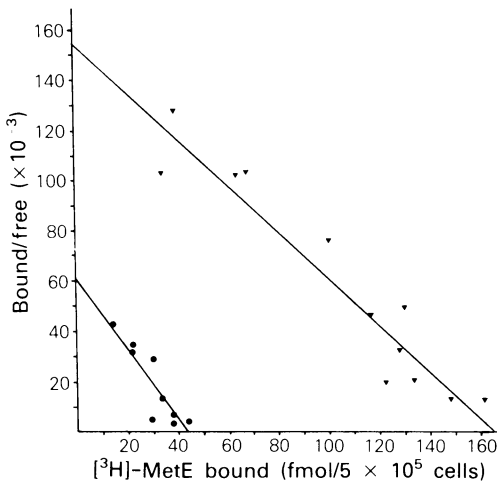


Figure 2 Scatchard plot of [3 H]-methionine-enkephalin ([3 H]-Met E) binding to control and dibutyl cyclic AMP treated cells. The treated cells were cultured in the presence of 0.77 mM dibutyl cyclic AMP for seven days prior to harvest and assayed as described in the text. (▼) Dibutyl cyclic AMP treated cells, K_d 1.1 nM; B_{max} 153 fmol/ 5×10^5 cells, ($r = 0.953$). (●) Control cells, K_d 0.8 nM; B_{max} 60 fmol/ 5×10^5 cells, ($r = 0.919$).

Treatment with morphine hydrochloride

Morphine-treated and control cells showed no apparent differences in morphology or growth rate. Figure 3 shows a typical experiment where treated cells were

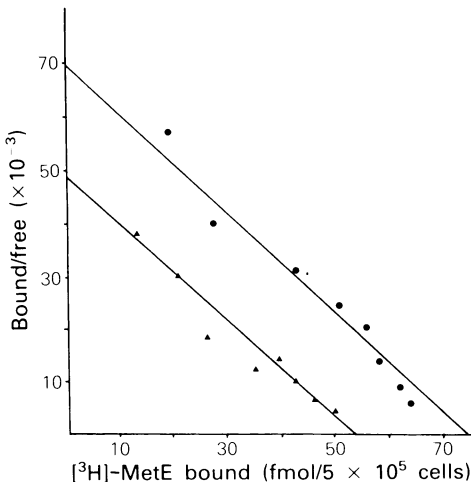


Figure 3 Scatchard plot of [3 H]-methionine-enkephalin binding to control and morphine-treated cells. The treated cells were cultured in the presence of 10^{-5} M morphine and assayed as described in Methods. (▲) Morphine-treated cells, B_{max} 54 fmol/ 5×10^5 cells. (●) Control cells, B_{max} 74.5 fmol/ 5×10^5 cells.

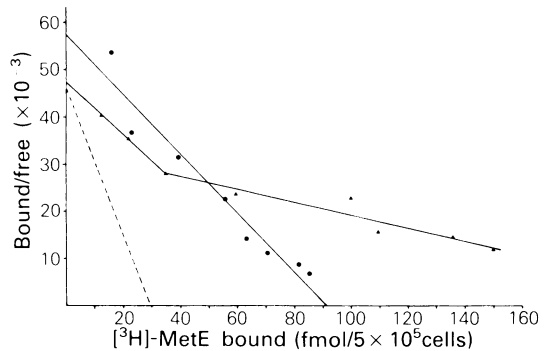


Figure 4 Scatchard plot of [3 H]-methionine-enkephalin binding to control and morphine-treated cells where the treated cells show two binding sites. Cells were cultured as described in Methods. (●) Control cells show a single binding site, K_d 1.9 nM; B_{max} 91 fmol/ 5×10^5 cells. (▲) Morphine-treated cells, K_{d1} 0.6 nM; B_{max1} 29.1 fmol/ 5×10^5 cells; K_{d2} 7.5 nM; B_{max2} 235 fmol/ 5×10^5 cells. The dashed line represents the high affinity component for treated cells after mathematical separation by the method of Burt & Snyder (1976).

exposed to morphine for 17 days. There was no difference in receptor affinity after treatment, but the B_{max} was reduced by 28%. In all cases where morphine exposure was continued for more than 4 days and the Scatchard analysis showed a single binding site there was a reduction in [3 H]-methionine-enkephalin binding sites of $30.8 \pm 2.2\%$ ($n = 7$) when compared to matched controls. In 6 out of 27 experiments, however, the Scatchard plot of treated cells was clearly biphasic, see Figure 4 (control cells K_d 1.9 nM, B_{max} 91 fmol/ 10^6 cells; treated cells K_{d1} 0.62 nM, B_{max1} 29.1 fmol/ 10^6 cells, K_{d2} 7.4 nM, B_{max2} 235 fmol/ 10^6 cells). The second lower affinity binding site had not been observed in control cultures or cultures treated in any other way. The biphasic data could be preceded or followed by assays at adjacent passage levels with monophasic Scatchard plots.

Treatment with D-Ala²-D-Leu⁵-enkephalin (DADLE)

Cells treated with 10^{-7} M DADLE showed no visible difference from control cells. In all cases exposure of the cells to DADLE for 3 to 4 h produced a profound down regulation in receptor density, as measured by [3 H]-methionine-enkephalin binding assay of $73.3 \pm 4.6\%$ ($n = 10$). When DADLE was removed from the culture a further 44 h incubation was needed to restore receptor densities to that of control cells.

When exposure to DADLE was maintained by including 6 nM DADLE in all the washing buffers the receptor down regulation could be inhibited. During

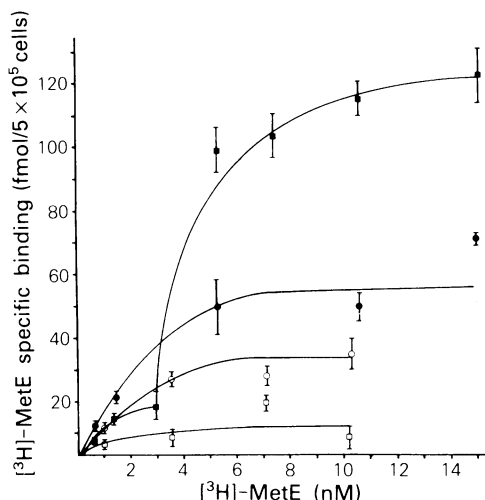


Figure 5 Saturation of [^3H]-methionine-enkephalin binding to hybrid cells after 4 h exposure to DADLE with and without cold DADLE (6 nM) in the washing buffers. The cells were exposed to DADLE for 4 h, harvested with and without washing buffer containing cold DADLE (6 nM), and assayed with matched controls as described in the Methods. (○) Controls and (□) treated cells without added cold DADLE. (●) Controls and (■) treated cells with added cold DADLE.

the subsequent receptor binding assay, ligand concentrations below 4 nM produced a rapid down regulation and higher concentrations continued to prevent this down regulation, see Figure 5. The presence of unlabelled or labelled ligand maintained δ -opiate receptor densities at the level of control cells. On some occasions δ -opiate receptor densities in these cells up to 100% higher than in control cells were observed. [^3H]-methionine-enkephalin binding assays yielded the same receptor densities for lysed and unlysed cells showing that the uptake of labelled enkephalin observed in N4TG1 neuroblastoma cells did not contribute to the observed specific binding (Blanchard, Chang & Cuatrecasas, 1983).

Discussion

The 108CC15 neuroblastoma \times glioma hybrid cells express a variety of membrane bound receptors that can induce transmembrane events or receptor occupancy. Among these the δ -opiate receptor has been identified on these cells (Klee & Nirenberg, 1974; Chang *et al.*, 1978; Chang & Cuatrecasas, 1979) and the ability of opiate ligands to inhibit adenylate cyclase has been demonstrated (Sharma *et al.*, 1975b; Traber *et al.*, 1975; Wahlstrom *et al.*, 1976). In addition, prolonged exposure to opiate ligands leads to an increase in activity of adenylate cyclase such

that on removal of the ligand considerable over production of cyclic AMP ensues. This has been proposed as a model for the induction of tolerance to opiate alkaloids. A corresponding 'withdrawal syndrome' can be induced by subsequent removal from exposure to opiates or by challenge with naloxone (Brandt, Fischer, Moroder, Wunsch & Hamprecht, 1976; Lampert, Nirenberg & Klee, 1976; Sharma, Klee & Nirenberg, 1975a). Down regulation of receptors by exposure to elevated concentrations of ligand has been demonstrated for the insulin (Schlesinger, Schechter, Willingham & Pastan, 1978), epidermal growth factor (Carpenter & Cohen, 1976), nerve growth factor (Johnston, Andrews & Bradshaw, 1978) and catecholamine (Mukherjee, Caron & Lefkowitz, 1975; Wessels, Mullikin & Lefkowitz, 1978; Su, Harden & Perkins, 1979; 1980) receptors. The initial aim of the work described here was to determine, under conditions that induce tolerance, whether opiate ligands could induce opiate receptor down regulation in this cell line. Growth of the hybrid cells in the presence of morphine hydrochloride at 10^{-5} M reproducibly caused down regulation of only 30% of the opiate receptors when compared with matched control cells, even after prolonged exposure. On some occasions a second binding site of lower affinity was observed, that had a total density higher than that of control levels, which must be due to expression of previously inaccessible receptors. When treated with morphine under the same conditions, these cells have been shown to develop tolerance to the alkaloid induced inhibition of adenosine and prostaglandin E_1 stimulated cyclic AMP production (Sharma *et al.*, 1975a; Lampert *et al.*, 1976). Receptor loss due to partial down regulation during induction of morphine tolerance could contribute, but not account completely, for the observed decrease in inhibition of cyclic AMP production.

A dramatic contrast was seen when the cells were cultured in the presence of DADLE. All cultures treated with this opioid peptide showed a profound and irreversible down regulation of [^3H]-methionine-enkephalin binding sites after exposure times of only 3 or 4 h. After down regulation further incubation in the absence of DADLE for up to 44 h was required for receptor densities to recover to that of control levels. The receptors that are lost are clearly not rapidly reincorporated into the membrane after removal of ligand as the recovery times are more consistent with *de novo* biosynthesis of new receptors.

Morphine and DADLE have very different effects on receptor down regulation as only the peptide is capable of initiating rapid receptor loss. In other respects, morphine and opioid peptides have qualitatively identical effects on these cells, both being able to bind to the δ -opiate receptor, inhibit cyclic AMP

production and also induce a tolerant state, yet the peptide, but not the alkaloid, must contain within its structure the additional molecular determinants required to cause receptor internalization, either directly or indirectly.

On some occasions a transient intermediate receptor population could be detected after treatment of the cells with both morphine and DADLE. Morphine treatment could give rise to a reversible biphasic Scatchard plot for [3 H]-methionine-enkephalin binding. A lower affinity binding site being expressed partly at the expense of the higher affinity site (see Figure 4) but also with the appearance of previously undetected lower affinity binding sites. DADLE treatment always gave monophasic Scatchard plots but on occasions receptor levels greater than control were observed. Hazum, Chang & Cuatrecasas (1979) have already shown using a fluorescent-labelled enkephalin analogue that the presence of opioid peptides induces aggregation of opiate receptors on these cell membranes. In other systems that have been studied in more detail, receptor aggregation seems to be a prerequisite for subsequent internalization (Schlessinger *et al.*, 1975; Haigler, Ash, Singer & Cohen, 1978; Schlessinger, Vanobberghen & Kahn, 1980; Hazum, Cuatrecasas, Marian & Conn, 1980; Amsterdam, Berkowitz, Nimrod & Kohen, 1980). It is possible that these intermediates correspond to a transient aggregated state; morphine and DADLE producing receptor aggregates with different affinities for [3 H]-methionine-enkephalin, however only the DADLE treated aggregates progress to the internalized state.

It was possible to inhibit the DADLE induced down regulation by including the peptide ligand in all the washing buffers at a concentration of 6 nM, as described in Methods. In this way the transient aggregated state could be studied. Receptor densities as high and sometimes up to 100% greater than controls could be detected by saturation analysis with [3 H]-methionine-enkephalin. However, when less than 4 nM [3 H]-methionine-enkephalin was present during the assay, rapid and irreversible down regulation of δ -opiate receptors was observed. Hence the unusual saturation curve in Figure 5. It can be concluded that the internalization process is initiated only after receptor occupancy falls below a critical level, i.e. when approximately 50% of the receptors are occupied.

On the basis of these observations we propose the following model to describe the down regulation process occurring under the influence of DADLE, and the lack of dramatic down regulation with morphine. The peptide ligand when occupying the receptors initiates aggregation of the opiate receptors, as described by Hazum *et al.* (1979), with possible expression of previously cryptic receptors to explain

the occasional elevated receptor densities when compared to controls. The down regulation process is not initiated until receptor occupancy falls below 50% whereupon loss of receptors takes place rapidly, in less than 15 min at 37°C. From these experiments it is clear that Hazum *et al.*, (1979) did not observe loss of receptors during incubation with fluorescent-labelled enkephalin because all their experiments involved continuous exposure of cells to the peptide ligand at concentrations sufficient to inhibit the down regulation process. The internalized receptors are not rapidly incorporated again into the membrane but are apparently replenished by biosynthesis of new receptors. Morphine, on the other hand, produces only limited down regulation over a much longer time period. We suggest that morphine can induce an aggregated state of δ -opiate receptors, with occasionally observed increased receptor numbers. However, the transition state is of lower affinity for [3 H]-methionine-enkephalin and after removal from morphine exposure the aggregated receptors rapidly relax back, principally to the non internalized state.

In the withdrawal syndrome adenylate cyclase activity is elevated after prolonged treatment with both morphine and opioid peptides (Sharma *et al.*, 1975a; Brandt *et al.*, 1976; Lampert *et al.*, 1976). As this effect is produced by both the alkaloid and enkephalin under conditions where only the peptide will have induced opiate receptor down regulation, the withdrawal syndrome cannot be a consequence of receptor loss and must be a direct effect on the adenylate cyclase system.

In addition to down regulation, we have been able to stimulate biosynthesis of δ -opiate receptors on the 108CC15 hybrid cells by inducing neurite formation by exposure to growth medium containing 0.77 mM dibutyryl cyclic AMP (Hamprecht, 1977). When compared with untreated controls at the same degree of confluency, levels of δ -opiate receptors were increased three fold. These increases were not just a consequence of greater membrane protein synthesis due to neurite outgrowth, as after correction for protein concentration the increase in receptor density was unaffected. It would seem that differentiation of 108CC15 neuroblastoma \times glioma hybrid cells by exposure to 0.77 mM dibutyryl cyclic AMP leads to elevated levels of δ -opiate receptors presumably incorporated into the extending neurites. A similar elevation of opiate receptor levels during development in the rat has been observed by Clendeninn, Petraitis & Simon (1976). These changes in receptor levels in the hybrid cells could reflect similar differentiation processes *in vivo*.

In conclusion, we have shown that δ -opiate receptor densities on 108CC15 neuroblastoma \times glioma hybrid cells can be increased by induction of neurite outgrowth after exposure to dibutyryl cyclic AMP,

and down regulated rapidly by exposure to D-Ala²-D-Leu⁵-enkephalin, but not by morphine. In addition, we demonstrated that the down regulation can be inhibited by exposure to opiate peptides at con-

centrations greater than 4 nM. The 108CC15 hybrid cells should be valuable for further elucidation of the molecular mechanism of receptor regulation.

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