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EFFECTS OF CYCLOHEXIMIDE AND TUNICAMYCIN ON OPIATE RECEPTOR ACTIVITIES IN NEUROBLASTOMA X GLIOMA NG108-15 HYBRID CELLS

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Abstract—The molecular mechanism of opiate receptor down-regulation and desensitization was investigated by studying the effects of cycloheximide and tunicamycin on opiate receptor activities in neuroblastoma X glioma NG108-15 hybrid cells. Cycloheximide inhibited [^{35}S]methionine and [^{3}H]-glucosamine incorporation by hybrid cells, while tunicamycin inhibited [^{3}H]glucosamine incorporation only. Exposing hybrid cells to these two agents did not alter the viability of the cell. Treatment of NG108-15 cells with cycloheximide or tunicamycin produced a decrease in [^{3}H]diprenorphine binding dependent on both time and concentrations of inhibitors, with no measurable modification in the ability of etorphine to regulate intracellular cyclic AMP production. Cycloheximide attenuated [^{3}H]-diprenorphine binding by decreasing both the number of sites, B_{max}, and the affinity of the receptor, K_d. Tunicamycin treatment produced a decrease in B_{max} with no apparent alteration in K_d values. Cycloheximide and tunicamycin did not potentiate the rate or magnitude of etorphine-induced down-regulation or desensitization of opiate receptor in NG108-15 cells. Furthermore, there was an apparent antagonism in cycloheximide action on receptor down-regulation. The reappearance of opiate binding sites after agonist removal was affected by these two inhibitors. Cycloheximide and tunicamycin eliminated the increase in [^{3}H]diprenorphine binding in the chronic etorphine-treated cells after agonist removal. These two inhibitors did not alter the resensitization of hybrid cells to etorphine. Thus, the site of opiate agonist action to induce receptor down-regulation and desensitization is not at the site of protein synthesis or protein glycosylation. These data substantiate previously reported observations that receptor down-regulation and receptor desensitization are two different cellular adaptation processes.

When neuroblastoma glioma NG108-15 hybrid cells are chronically exposed to opiate agonists, at least three separate cellular adaptation processes occur [1]. There is first a loss of the ability of opiate agonists to regulate adenylate cyclase, or receptor desensitization, with a concomitant increase in adenylate cyclase activity. These two processes precede the disappearance of opiate binding sites, or receptor down-regulation. In earlier studies, Sharma *et al.* [2] suggested that the loss of opiate activity after chronic agonist treatment is due to an increase in adenylate cyclase activity. However, our recent findings indicate that all three of these cellular processes are separated from each other, as substantiated by dose-, time- and opiate ligand-dependent studies [1].

Chronic opiate treatment of NG108-15 hybrid cells does not alter the activities of other receptors in this clonal cell line [1]. Muscarinic, α_2-adrenergic and prostaglandin E_1 (PGE_1) receptor activities remained after complete opiate receptor desensitization and down-regulation. Thus, regulation of opiate receptor turnover may be important in the specific adaptation of the hybrid cell to chronic opiate treatment. To determine whether this is so, we decided to test the effects of two agents that affect turnover: cycloheximide, a general inhibitor of protein synthesis, and tunicamycin, an inhibitor of protein glycosylation. Both agents have been used in studies of the regulation of other receptors. Cycloheximide potentiates down-regulation induced by muscarinic agonists in NG108-15 cells [3], a receptor system that, like opiate, inhibits adenylate cyclase activity. Tunicamycin potentiates insulin-induced down-regulation of insulin receptors in 3T3-L1 adipocytes [4] and acetylcholine binding in embryonic chicken muscle cells [5]. Since evidence has indicated that opiate receptors are glycoproteins [6, 7], tunicamycin might modulate the acute and chronic effects of opiate agonists. Therefore, in this paper, the effects of these two inhibitors on receptor binding, opiate inhibition of adenylate cyclase, opiate receptor desensitization, and opiate receptor down-regulation are reported.

MATERIALS AND METHODS

Cell culturing. Initial stock cultures of neuroblastoma X glioma NG108-15 hybrid cells were gifts of Dr. B. Hamprecht (Physiologischemishes Institut des Universitat, Wurzburg, FRG). Hybrid cells were cultured in Dulbecco's modified Eagle medium containing HAT (0.1 mM hypoxanthine, 10 μM amino-

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pterin and 17 μM thymidine) plus 10% fetal calf serum in a humidified atmosphere of 10% CO_2 and 90% air. The cells were detached from the growing surface by the addition of Saline D (NaCl, 137 mM; KCl, 5 mM; $Na_2PO_4 \cdot 7H_2O$, 0.17 mM; KH_2PO_4, 0.22 mM; glucose, 6 mM; sucrose, 59 mM; pH 7.0) for propagation or experimentation. Cells in confluency with passage number between 20 and 30 were used in all experimentation.

Opiate receptor binding. Opiate receptor binding to intact cells in suspension using [^{3}H]diprenorphine was carried out as described [8]. Briefly, incubation with [^{3}H]diprenorphine in the presence or absence of 5 μM diprenorphine was carried out at 37° for 20 min with 10^6 cells/1.0 ml of Krebs–Ringer–Hepes buffer (KRHB) [NaCl, 110 mM; KCl, 5 mM; $MgCl_2$, 1 mM; $CaCl_2$, 1.8 mM; glucose, 25 mM; sucrose, 55 mM; and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), 10 mM; at pH 7.4]. Afterwards, incubations were terminated by collecting hybrid cells on Whatman glass fiber GF/B filters which were washed with KRHB at 4° with 3 × 5 ml. Radioactivity retained by filters was determined by liquid scintillation counting in Liquiscint (National Diagnostics, Somerville, NJ). Specific binding was defined as the difference in radioactivity of triplicate samples in the absence and the presence of non-radioactive diprenorphine. Scatchard analysis of binding data was carried out for saturation equilibrium binding of [^{3}H]diprenorphine from 0.2 to 10 nM. K_d and B_{max} values were obtained from a non-linear hyperbolic curve fitting program run on a Hewlett–Packard HP9825 computer. The effects of cycloheximide, tunicamycin or chronic opiate treatment on opiate receptor binding were determined by measuring the amount of [^{3}H]diprenorphine, 4–5 nM, specifically bound to hybrid cells after treatment. In all drug treatments, excess drug was washed away prior to binding assays. Etorphine in chronic treatment was removed by a washing procedure as described [1]. To equalize the number of specific sites exposed to etorphine, the same concentration of etorphine as in chronic treatment was added to control cells for 5 min prior to harvesting.

Measurement of opiate inhibition of intracellular cAMP production. The ability of opiates to inhibit PGE_1-stimulated increase in intracellular [^{3}H]cAMP levels was determined by labeling intracellular ATP pools with [^{3}H]adenine as outline by Schultz and Daly [9] and as described by Law *et al.* [8] for hybrid cells. Opiate inhibition was carried out in cell suspensions, 0.5×10^6 cells/0.5 ml, and in KRHB, unless otherwise specified. [^{3}H]cAMP formed was separated from other radioactive nucleotides by Dowex 50 and alumina column chromatography as described by White and Karr [10].

Incorporation of [^{35}S]methionine and [^{3}H]glucosamine by NG108-15 cells. Effects of cycloheximide and tunicamycin on protein synthesis and protein glycosylation were determined by monitoring [^{35}S]methionine ([^{35}S]Met) or [^{3}H]glucosamine ([^{3}H]Glu) incorporation by hybrid cells in the presence of these inhibitors. For these experiments, hybrid cells were cultured in 17 mm plates for 4 days. The cells were treated with various concentrations of either cycloheximide or tunicamycin for 5 or 18 hr. Cells in four separate plates were used for the concentration of each inhibitor. Then growth medium was removed, and excess fetal calf serum was washed away with DMEM. To each individual plate, identical concentrations of cycloheximide or tunicamycin used in chronic treatment together with 1 μCi/ml of [^{35}S]Met or [^{3}H]Glu in DMEM supplemented with HAT were added. Incubation with radioisotopes was then carried out for 1 hr at 37°. Afterwards, medium was removed, and 0.5 ml of 10% trichloroacetic acid (TCA) was added. Excess radioactivity was removed by washing cells which remained attached to the surface of plates with 3 × 1 ml of 10% TCA. The precipitated cells were then dissolved in 1 ml of 1% sodium dodecyl sulfate (SDS)–1 N NaOH–water (1:1:1). Aliquots were removed for radioactivity determination by liquid scintillation counting and for protein determination by the method of Lowry *et al.* [11].

Materials. [^{3}H]Diprenorphine (9 Ci/mmole) and [^{35}S]methionine (1250 Ci/mmole) were purchased from Amersham (Arlington Heights, IL). New England Nuclear (Boston, MA) and ICN (Irvine, CA) supplied the [^{3}H]adenine (16 Ci/mmole) and [^{3}H]-glucosamine (20 Ci/mmole) respectively. Tunicamycin was from Calbiochem-Behring (San Diego, CA). Cycloheximide and other reagents were obtained from Sigma (St. Louis, MO). ZK62711 was a gift from Schering (Berlin, FRG).

RESULTS

Effects of cycloheximide and tunicamycin on protein synthesis and protein glycosylation. Protein synthesis and protein glycosylation in NG108-15 hybrid cells were determined by monitoring [^{35}S]Met and [^{3}H]Glu incorporation into TCA insoluble pools respectively. Pretreating hybrid cells with different concentrations of cycloheximide for 5 or 18 hr reduced the amount of [^{35}S]Met incorporated in the TCA insoluble pools (Fig. 1a). The magnitude of decrease after 5 or 18 hr of cycloheximide treatment was identical. Cycloheximide treatment also reduced the amount of [^{3}H]Glu incorporated by hybrid cells (Fig. 1a). However, the cycloheximide effect of [^{3}H]Glu incorporation was considerably less in cells treated with cycloheximide for 5 hr than in cells treated for 18 hr (Fig. 1a).

The decreases in both protein synthesis and protein glycosylation after cycloheximide treatment were not due to cytotoxicity of the inhibitor. As summarized in Table 1, when viability of NG108-15 cells was determined by the nigrosin dye exclusion method [12], exposing hybrid cells to 1 μg/ml of cycloheximide for 18 hr or 30 μg/ml cycloheximide for 6 hr did not reduce viability of cells as compared to control. Although there was a significant decrease in total amount of protein in the cycloheximide-treated plates, the number of cells in those plates remained similar to those in control plates (Table 1). There was a greater percent reduction in the total protein content in cells treated with cycloheximide for 18 hr when compared to those treated with the protein synthesis inhibitor for 6 hr.

When hybrid cells were treated with various con-

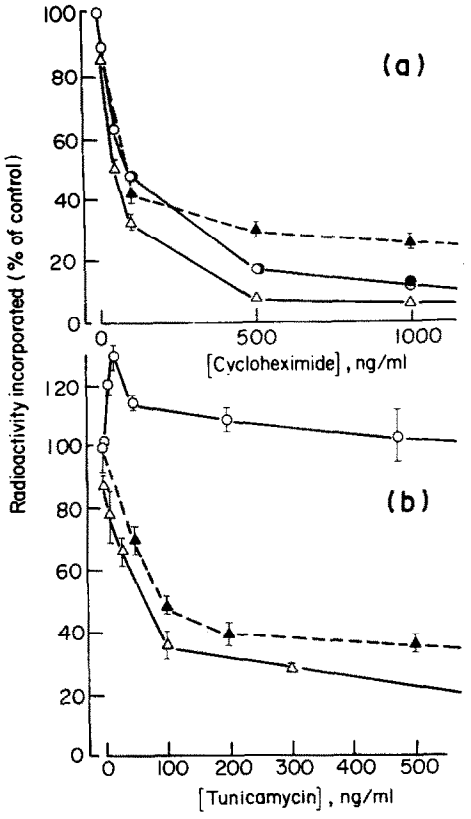

Fig. 1. Effect of (a) cycloheximide and (b) tunicamycin on [^{35}S]methionine and [^{3}H]glucosamine incorporation. NG108-15 cells in 17 mm plates were treated with various concentrations of cycloheximide or tunicamycin for 5 (---) or 18 (——) hr. Then 1 μCi/ml of [^{35}S]methionine or [^{3}H]glucosamine was added and cells were incubated for 1 hr. Afterwards, the amount of radioactivity incorporated into TCA insoluble pools was determined as described in Methods. Key: (●) and (○) represent the relative amount of [^{35}S]methionine incorporated after 5 and 18 hr of treatment respectively; (▲) and (△) represent [^{3}H]glucosamine incorporated after 5 and 18 hr of treatment respectively. Amounts of [^{35}S]methionine and [^{3}H]glucosamine incorporated in control cells were $1.92 \pm 0.07 \times 10^4$ cpm/mg protein and 1.37×10^3 cpm/mg protein respectively.

centrations of tunicamycin for 5 or 18 hr, decreases in [^{3}H]glycosamine incorporated by the cells were observed (Fig. 1b). Similar to the cycloheximide effect, treating NG108-15 cells with tunicamycin for 18 hr produced a larger decrease in [^{3}H]Glu incorporation than treating cells for 5 hr. On the other hand, tunicamycin concentrations used in these experiments did not attenuate [^{35}S]Met incorporation (Fig. 1b). Again, these tunicamycin effects were not due to cytotoxicity. As summarized in Table 1, at the highest concentration of tunicamycin used, 5.0 μg/ml, this inhibitor did not alter the viability of hybrid cells.

Attenuation of opiate receptor binding by cycloheximide and tunicamycin. Treatment of NG108-15 cells with either cycloheximide or tunicamycin resulted in a decrease in [^{3}H]diprenorphine binding, which was dependent on the concentrations of the inhibitors. For cycloheximide, a concentration of 30 μg/ml produced maximal inhibition of opiate receptor binding after 6 hr of treatment. The onset of the cycloheximide effect was not immediate, for there was a 2-hr lag period in the effect of the inhibitor (Fig. 2a). Since, in [^{35}S]Met incorporation studies, isotope incorporation was inhibited by cycloheximide immediately, the lag time was not due to inability of cycloheximide to inhibit protein synthesis during the first 2 hr.

When saturation binding of [^{3}H]diprenorphine was carried out with hybrid cells treated with 30 μg/ml cycloheximide for 5 hr, computer analysis of Scatchard plots revealed reduction in both the affinity and B_{max} value of [^{3}H]diprenorphine binding in treated cells as compared to that in control cells (Fig. 3a). [^{3}H]Diprenorphine binding to control cells from the separate passage numbers was determined to have a K_d value of 2.77 ± 0.22 nM and a B_{max} value of 0.468 ± 0.044 nM (or 659.2 ± 62 fmoles/mg protein). After cycloheximide treatment, the K_d value of [^{3}H]diprenorphine increased to 4.97 ± 0.41 nM and the B_{max} value decreased to 0.364 ± 0.015 nM (or 689.4 ± 28.4 fmoles/mg protein). Note that when the B_{max} values are expressed in relation to the amount of protein present in the assay, cycloheximide treatment did not

Table 1. Effects of tunicamycin and cycloheximide on viability and growth of neuroblastoma X glioma NG108-15 hybrid cells*

	No. of cells	% Viability	Total protein (mg)
Control	$2.23 \pm 0.34 \times 10^6$	92.7 ± 0.6	3.43 ± 0.11
+ Tunicamycin			
0.5 μg/ml, 18 hr	$1.82 \pm 0.14 \times 10^6$	91.7 ± 3.0	3.13 ± 0.07
5.0 μg/ml, 18 hr	$2.35 \pm 0.09 \times 10^6$	94.4 ± 3.4	3.57 ± 0.01
+ Cycloheximide			
1 μg/ml, 18 hr	$2.32 \pm 0.14 \times 10^6$	89.6 ± 0.8	2.31 ± 0.07†
30 μg/ml, 6 hr	$2.15 \pm 0.08 \times 10^6$	94.1 ± 0.1	3.00 ± 0.07‡

* NG108-15 hybrid cells, 1×10^5, were seeded in 35 mm culture plates and were cultured for 3 additional days as described in Methods. On day 3, hybrid cells were treated with the respective drug for the time period as stated. Afterwards, cells were detached from the surface with Saline D, and viability was determined by the nigrosin dye exclusion method. Values represent the average determinations from three separate culture plates.

† $P < 0.005$ when compared to control.

‡ $P \leq 0.05$ when compared to control.

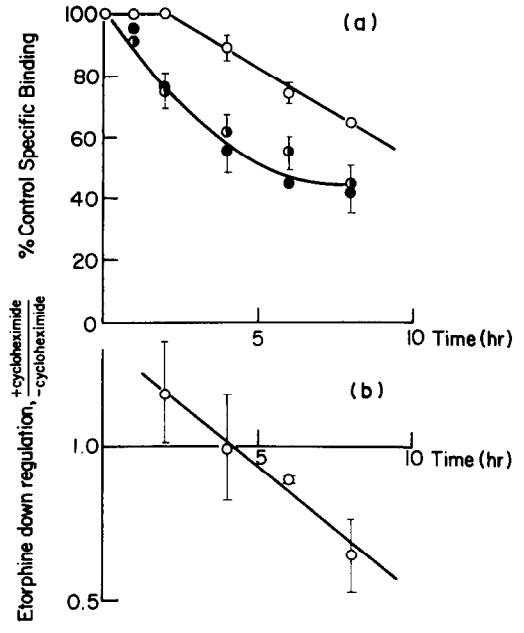

Fig. 2. Time-dependent decrease in [^{3}H]diprenorphine binding in the presence of cycloheximide. NG108-15 cells from three separate passage numbers, cultured in 100 mm plates, were treated with 30 μg/ml cycloheximide (-○-○-), 10 nM etorphine (-●-●-) or etorphine and cycloheximide (-◑-◑-) for various periods of time. Afterwards, cells were washed free of drugs and 5 nM [^{3}H]diprenorphine binding to intact cells was determined as described in Methods. Panel (b) represents the degree of etorphine-induced decrease in [^{3}H]diprenorphine binding in the presence and absence of 30 μg/ml cycloheximide.

alter the number of opiate binding sites in hybrid cells. However, it did reduce the number of binding sites per cell, for the amount of protein per cell was decreased significantly (Table 1). Thus, by equalizing the number of control or antibiotic-treated cells used in a binding assay, it can be observed that cycloheximide reduced the number of binding sites per cell.

Reduction in [^{3}H]diprenorphine binding was observed also after tunicamycin treatment. As shown in Fig. 4a, addition of 0.5 μg/ml or 5 μg/ml tunicamycin to the medium produced a time-dependent decrease in [^{3}H]diprenorphine specifically bound to hybrid cells. Analogous to cycloheximide studies, the tunicamycin effect exhibited a lag period of 1 hr. At the higher concentration of tunicamycin used, 5 μg/ml, the antibiotic effect could be due to inhibition of protein synthesis. However, at 0.5 μg/ml, the effects were caused by inhibition of protein glycosylation since this concentration of tunicamycin did not alter [^{35}S]Met incorporation (Fig. 1b). The reduction in [^{3}H]diprenorphine binding after tunicamycin treatment was due to a decrease in the number of binding sites (Fig. 3b). Computer analysis of Scatchard plots obtained with hybrid cells from three separate passage numbers treated with 0.1 μg/ml of tunicamycin for 18 hr revealed a K_d value of 2.7 ± 0.16 nM and a B_{max} value of 0.213 ± 0.01 nM. The number of binding sites in hybrid cells thus treated with tunicamycin was reduced by 55%.

Cycloheximide and tunicamycin action on etorphine-induced receptor down-regulation. When NG108-15 hybrid cells were treated with 10 nM etorphine, a time-dependent decrease in receptor number or down-regulation can be observed. Addition of cycloheximide, up to 50 μg/ml, did not alter the rate of magnitude of etorphine-induced opiate receptor down-regulation. Etorphine-induced opiate receptor down-regulation was not additive to the attenuation of [^{3}H]diprenorphine binding elicited by 30 μg/ml cycloheximide treatment (Fig. 2).

The effect of tunicamycin on etorphine-induced down-regulation was biphasic. In both time course and concentration-dependent studies, tunicamycin potentiated and attenuated the magnitude of etorphine-induced receptor down-regulation (Figs. 4 and 5). At a tunicamycin concentration less than 30 ng/ml, incubation of hybrid cells with the antibiotic for 18 hr concurrently with 5 nM etorphine attenuated etorphine-induced receptor down-regulation. At higher concentrations of tunicamycin, there was an apparent increase in the magnitude of etorphine-induced receptor down-regulation (Fig. 5). However, the increase was not statistically significant. Unlike the cycloheximide effect, in which the decrease in [^{3}H]diprenorphine binding induced by chronic etorphine treatment was the same in the presence or absence of added cycloheximide, tunicamycin could further decrease the amount of [^{3}H]-

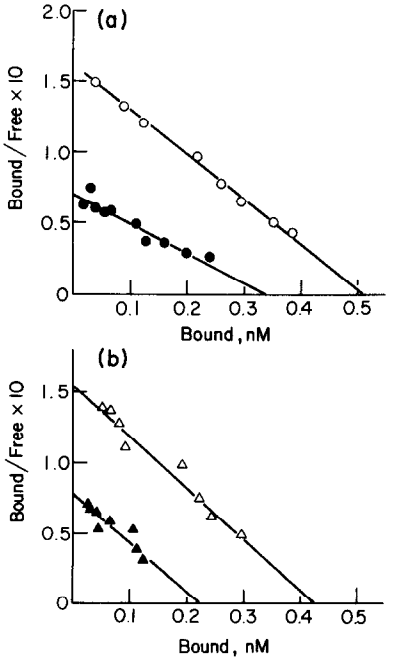

Fig. 3. Scatchard analysis of [^{3}H]diprenorphine binding in (a) control (-○-○-) and cycloheximide-treated cells (-●-●-) and in (b) control (-△-△-) and tunicamycin-treated cells (-▲-▲-). Hybrid cells were treated with 30 μg/ml cycloheximide for 6 hr or with 0.1 μg/ml tunicamycin for 18 hr. The values represent the average from three separate passage numbers of cells.

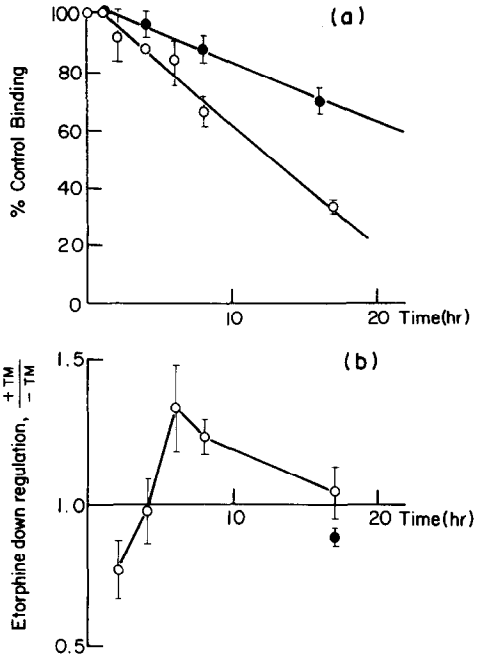

Fig. 4. Time-dependent decrease in [^{3}H]diprenorphine binding in the presence of tunicamycin. Cells from three separate passage numbers were treated with 5 μg/ml (-○-○-) or 0.5 μg/ml (-●-●-) of tunicamycin for various periods of time. Afterwards, cells were washed free of drugs and [^{3}H]diprenorphine binding to intact cells was determined. The amount of [^{3}H]diprenorphine specifically bound to control cells was 625.1 ± 9.0 fmoles/mg protein. Panel (b) represents the degree of opiate receptor down-regulated by 10 nM etorphine in the presence of 5 μg/ml (●) or 0.5 μg/ml (○) of tunicamycin.

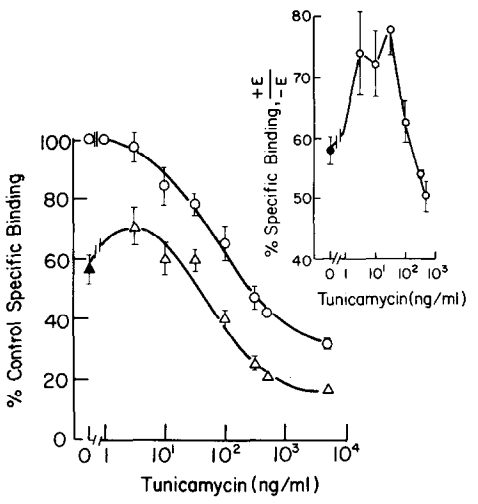

Fig. 5. Tunicamycin concentration-dependent inhibition of [^{3}H]diprenorphine binding in control (-○-○-) and etorphine (-△-△-) -treated cells. NG108-15 cells cultured in 100 mm plates were treated with various concentrations of tunicamycin for 6 hr in the presence or absence of 5 nM etorphine. The closed triangle (▲) represents the amount of [^{3}H]diprenorphine specifically bound after 16 hr of 5 nM etorphine treatment. Specific [^{3}H]diprenorphine binding in control cells was 533.9 ± 47.6 fmoles/mg protein (N = 3 separate passage numbers). Insert: percent of control specific binding after 16 hr of 5 nM etorphine treatment in the presence of various concentrations of tunicamycin.

diprenorphine bound in the hybrid cells in which opiate receptor was down-regulated by etorphine (Fig. 5).

Effects of cycloheximide and tunicamycin on etorphine inhibition of adenylate cyclase activity and opiate receptor desensitization. The ability of protein synthesis and protein glycosylation inhibitors to modulate opiate regulation of adenylate cyclase was measured. After exposing hybrid cells to cycloheximide, 30 μg/ml for 6 hr, or to tunicamycin, 0.1 μg/ml for 16 hr, etorphine inhibition of PGE_1-stimulated increase in adenylate [^{3}H]cAMP production was measured in cell suspension. Treating hybrid cells with these two inhibitors did not alter the amount of [^{3}H]cAMP produced in the presence or absence of 10 μM PGE_1 (Table 2). There was no measurable difference in the potency or maximal inhibitory level exhibited by etorphine in hybrid cells treated with either cycloheximide or tunicamycin (Table 2). Under the same treatment conditions, there was a 25 and 35% decrease in [^{3}H]diprenorphine binding in cells treated with cycloheximide and tunicamycin respectively. These data are not surprising because the coupling efficiency of etorphine is greater than 1, suggesting the presence of "spare" receptor [8].

Opiate receptor desensitization after chronic etorphine treatment was not modified by the concurrent addition of cycloheximide or tunicamycin to cells with etorphine (Fig. 6). Treatment of hybrid cells with 30 nM etorphine for 6 hr abolished the ability of etorphine to inhibit PGE_1-stimulated increase in the intracellular [^{3}H]cAMP levels (Fig. 6). Addition

Table 2. Etorphine inhibition of cAMP production in NG108-15 hybrid cells after prolonged tunicamycin and cycloheximide treatment*

	IC_{50} (nM)	Maximal inhibition (%)	n_H
Control	1.78 ± 0.46	44.7 ± 2.7	1.24 ± 0.12
+ Cycloheximide	1.44 ± 0.48	44.4 ± 4.5	1.15 ± 0.05
Control	1.91 ± 0.26	48.0 ± 0.1	1.34 ± 0.08
+ Tunicamycin	2.20 ± 0.28	47.6 ± 2.9	1.09 ± 0.06

* The ability of etorphine to inhibit PGE_1-stimulated increase in intracellular cAMP production in NG108-15 cells suspension was determined with hybrid cells treated with cycloheximide (30 μg/ml, 6 hr) or with tunicamycin (0.1 μg/ml, 16 hr). The control cells in the cycloheximide studies were treated with saline and the control cells in the tunicamycin studies were treated with dimethylformamide–ethanol (3:1). The IC_{50} values of etorphine were obtained from regression analysis of Log–Logit plots of concentration-dependent curves of twelve different concentrations of etorphine. The amounts of [^{3}H]cAMP produced in the absence and in the presence of 10 μM PGE_1 and in the absence of any added etorphine were determined to be 3.23 ± 0.09 × 10^3 and 1.94 ± 0.15 × 10^5 cmp/mg/20 min respectively. The values represented average ± S.E.M. determinations from three consecutive passages of hybrid cells.

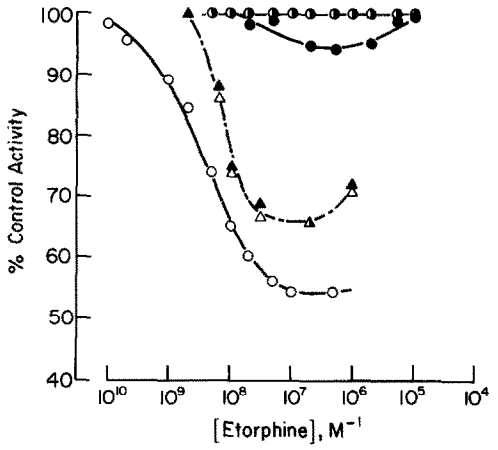

Fig. 6. Etorphine inhibition of cAMP production in control and hybrid cells treated with 2 nM etorphine for 18 hr. The ability of etorphine concentration-dependent inhibition of PGE_1 (10 μM) -stimulated increase in cAMP production was measured in control (○) and chronic etorphine-treated cells. Key: (-▲-▲-) hybrid cells treated with 2 nM etorphine for 18 hr and (-●-●-) cells treated with 30 nM etorphine for 6 hr. The ability of etorphine to regulate cAMP production in cells treated with 0.1 μg/ml tunicamycin and 2 nM etorphine for 18 hr (-△-△-) and in cells treated with 10 μg/ml cycloheximide and 30 nM etorphine for 6 hr (◑—◑) was measured. The values represent the average of three incubations.

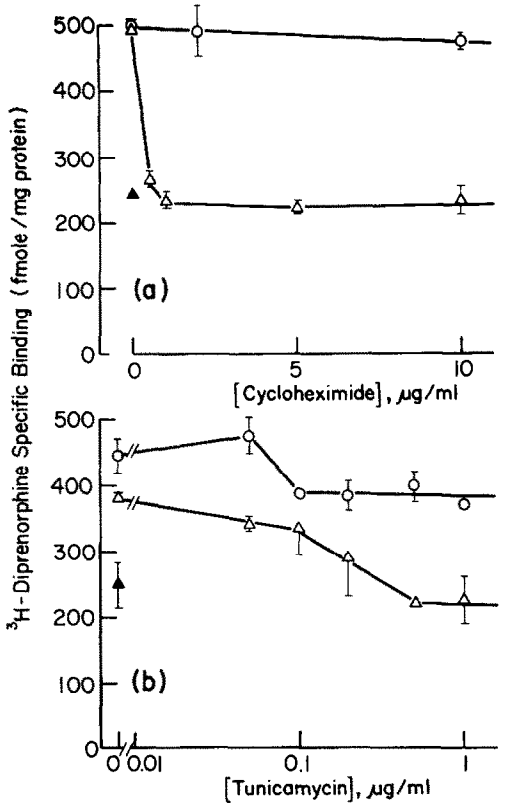

Fig. 7. Cycloheximide and tunicamycin concentration-dependent inhibition of recovery of opiate receptor binding. NG108-15 cells from three separate passage numbers were treated with 10 nM etorphine for 24 hr. Afterwards, 10 μM naloxone was added and the chronic etorphine effect was reversed by challenging cells with 10 μM naloxone for 5 min. Recovery of opiate receptor binding was measured after 8 hr (△). In (a) various concentrations of cycloheximide were present during the recovery period, and in (b) various concentrations of tunicamycin were present during the recovery period. Key: (-○-○-) effect of cycloheximide or tunicamycin on [^{3}H]diprenorphine binding in control cells; (▲) amount of [^{3}H]diprenorphine bound in receptor down-regulated state.

of 10 μg/ml of cycloheximide concurrent with etorphine to the incubation medium did not antagonize the chronic effect of etorphine. Similar results could be obtained with 0.1 μg/ml tunicamycin treatment. These two inhibitors did not potentiate etorphine-induced desensitization either. When opiate receptor was partially desensitized with 2 nM etorphine for 18 hr, addition of tunicamycin, 0.1 μg/ml, did not alter the chronic effect of etorphine. Hence, etorphine-induced opiate receptor desensitization was not related to protein synthesis or protein glycosylation.

Cycloheximide and tunicamycin effects on opiate receptor recovery. Opiate receptor down-regulation and desensitization in NG108-15 cells can be reversed by complete removal of opiate agonist [1]. Since a period of several hours is required [1], *de novo* synthesis of receptor protein could possibly be involved in the recovery process. To test this, hybrid cells were treated with cycloheximide during the recovery period. As shown in Fig. 7a, chronic exposure of hybrid cells to 10 nM etorphine for 24 hr produced a 50% decrease in [^{3}H]diprenorphine specific binding. The amount of [^{3}H]diprenorphine bound returned to the control level 6 hr after removal of etorphine from incubation medium. However, when various concentrations of cycloheximide were added to the medium during the 6-hr recovery period, the amount of [^{3}H]diprenorphine bound to cells washed free of etorphine was close to that in the down-regulated cells. Blockade of opiate receptor binding recovery could be observed also when NG108-15 cells were treated with actinomycin D (data not shown). Incubation of hybrid cells not down-regulated with various concentrations of actinomycin D for 6 hr decreased the amount of [^{3}H]-diprenorphine bound maximally by 50%. The EC_{50} value of actinomycin D was determined to be 5 μg/ml medium. Addition of actinomycin D to the incubation medium during 6 hr of the recovery prevented the increase of opiate receptor binding, with an actinomycin EC_{50} value of 10 μg/ml.

Increase in opiate receptor binding in the down-regulated cells after agonist removal could be blocked by tunicamycin treatment also. As shown in Fig. 7b, there was a tunicamycin concentration-dependent decrease in opiate receptor recovery, with a tunicamycin ED_{50} value of 0.2 μg/ml. At this concentration of tunicamycin, measurable inhibition of protein synthesis was not observed (Fig. 1).

The ability of cycloheximide and tunicamycin to inhibit the recovery of opiate inhibition of [^{3}H]cAMP production was also measured. As reported previously [1] and observed in current studies (Table 3), chronic treatment of hybrid cells with 50 nM etor-

Table 3. Effects of treatment of neuroblastoma X glioma NG108-15 hybrid cells with cycloheximide or tunicamycin on the recovery of etorphine inhibition of cAMP production after desensitization*

	Intracellular [^{3}H]cAMP level (cpm/mg protein/20 min) 10^{-5}		
		Chronic etorphine	
	Control	−Naloxone wash	+ Naloxone wash
Control	0.75 ± 0.04 (66.7%)	2.25 ± 0.02 (0%)	0.74 ± 0.02 (67.1%)
+ Tunicamycin			
0.1 μg/ml	0.95 ± 0.03 (58.0%)	2.39 ± 0.03 (−6.2%)	0.91 ± 0.02 (59.6%)
0.5 μg/ml	1.10 ± 0.03 (51.1%)	2.77 ± 0.02 (−23.1%)	1.41 ± 0.03 (49.1%)
+ Cycloheximide			
0.1 μg/ml	0.81 ± 0.01 (64.0%)	2.38 ± 0.07 (−5.8%)	0.89 ± 0.02 (62.6%)

* Neuroblastoma X glioma NG108-15 hybrid cells, cultured in 17 mm plates, were treated with 50 nM etorphine for 24 hr. To half of these plates, 20 μM naloxone was added after 24 hr of etorphine treatment. These cells, designated as + naloxone wash cells, were treated with naloxone for 5 min, and all opiate alkaloids were removed by repeated washings with growth media. Hybrid cells were then cultured for another 24 hr. In the remaining half of the culture plates, designated as − naloxone wash cells, 50 nM etorphine remained in the media during the second 24-hr culturing. The ability of 5 μM PGE_1 to stimulate [^{3}H]cAMP production in the presence of 50 nM etorphine was then determined as described in Methods. Values represent average ± S.E.M. determinations from four separate plates. The numbers in parentheses are percentages of cAMP production inhibited by etorphine. The specific activity of PGE_1-stimulated [^{3}H]cAMP production in control cells in the absence of any added drug was 2.24 ± 0.03 × 10^5 cpm/mg/20 min.

phine completely abolished the ability of 50 nM etorphine to inhibit cAMP production. However, after exposing hybrid cells to 20 μM naloxone for 5 min and culturing the cells for another 24 hr after the removal of the antagonist, the ability of 50 nM etorphine to inhibit cAMP production fully recovered (Table 3). Treatment of the hybrid cells with 0.1 μg/ml of cycloheximide during the recovery period did not alter resensitization of opiate receptor to etorphine. Parallel treatment of the hybrid cells with cycloheximide indicated a complete blockade of protein synthesis and inhibition in recovery of opiate receptor binding (Fig. 1 and 7). Similar treatment of hybrid cells during the recovery period with 0.1 μg/ml of tunicamycin also indicated no effect in the receptor resensitization to etorphine (Table 3). The ability of etorphine to regulate cAMP production in the resensitized cells treated with 0.1 and 0.5 μg/ml tunicamycin was identical to that in control cells treated with tunicamycin (Table 3). Thus, inhibition of protein synthesis or protein glycosylation did not affect opiate receptor resensitization.

DISCUSSION

Prolonged treatment of NG108-15 hybrid cells with either cycloheximide or tunicamycin produced a reduction in opiate receptor number. From our isotope incorporation studies, one could conclude that these two antibiotics exerted their effect by reducing protein glycosylation. Both cycloheximide and tunicamycin inhibited [^{3}H]glucosamine incorporation by the hybrid cells (Fig. 1). However, the cycloheximide effect on opiate receptor binding activity most likely is due to inhibition of protein synthesis. As shown in Table 1, treatment of hybrid cells with cycloheximide produced a dramatic reduction in total cellular proteins. With the reduction of nascent polypeptide chains, a decrease in the amount of protein being glycosylated should be observed. Alteration in both K_d and B_{max} values of [^{3}H]diprenorphine binding to cycloheximide-treated cells, as compared to alteration in B_{max} value only in tunicamycin-treated cells, further suggested that the sites of action of these two antibiotics are not identical.

The ability of tunicamycin to reduce opiate binding sites further indicated that the opiate receptor is a glycoprotein. The glycoprotein nature of the opiate receptor has been implied by its ability to be adsorbed by wheat germ lectin columns [7]. In studies with other receptors which are known glycoproteins, e.g. the nicotinic and insulin receptors, the effect of tunicamycin is due to the acceleration of receptor degradation or processing. Tunicamycin accelerated nicotinic receptor degradation in primary cultures of chicken embryonic skeleton muscles [5]. Glycosylation of insulin receptor in 3T3-L1 cells [4] or the α-subunit of nicotinic receptor in mouse clonal cell line BC3H-1 [13] is essential for activation of these receptors. The tunicamycin reduction of opiate binding could be analogous to its effect on these other receptors.

The ability of cycloheximide to potentiate agonist-

induced down-regulation implied that receptor down-regulation was not due to an acceleration of receptor degradation. With the inhibition of receptor synthesis by cycloheximide, decrease in detectable receptor should reflect the rate of receptor degradation. Chronic muscarinic agonist treatment increased the muscarinic receptor degradation rate in NG108-15 cells [3], for the cycloheximide's and muscarinic agonist inhibition of receptor binding were additive. On the other hand, a blockade of receptor synthesis with cycloheximide did not accelerate the opiate receptor down-regulation rate. Thus, it can be concluded that opiate receptor down-regulation was not caused by the agonist inhibition of receptor synthesis or the agonist-induced acceleration of receptor degradation.

Although in the presence of tunicamycin opiate agonist-induced receptor down-regulation was not potentiated, the level of [^{3}H]diprenorphine binding was lowest in cells treated with tunicamycin and etorphine concurrently. Tunicamycin, the inhibitor of protein glycosylation, could be inhibiting the assembly and hence the activation of newly synthesized opiate receptors. An opiate receptor model composed of oligomeric units has been suggested from its apparent molecular weight (200K to 350K dalton) and molecular weight of the putative binding sites (58K dalton) [14]. Because opiate agonist could further decrease the receptor level in cells treated with tunicamycin, probably the site of opiate agonist-induced receptor down-regulation is not the same as that of tunicamycin, the assembly of active receptor. The site of opiate agonist action must lie after the synthesis and processing of the receptor and must lie before the degradation of the receptor by the lysosomes.

If one examines the putative itinerary of the life cycle of a membrane receptor [15, 16], there are several sites where opiate agonists could exert their actions. In this probable itinerary, the receptor is synthesized in the rough endoplasmic reticulum and is transported to the Golgi apparatus where the receptor is processed. The receptor is then packaged and inserted into the plasma membrane. Upon the binding of agonist, clustering of the receptors at the coated pits is observed. Endocytosis of the receptors in the coated vesicles will then follow. At this point, the ligand–receptor complexes could either dissociate with the receptors being recycled back to the plasma membrane or the ligand–receptor complexes will be delivered to the lysosomes where degradation of the ligand and/or receptor occurs. In this chain of cellular events, an opiate agonist could regulate the plasma membrane receptor level by controlling the synthesis rate, the degradation rate, the processing rate, or the recycling rate of the receptor. From our current data, it is clear that opiate agonist-induced receptor down-regulation is not due to the decrease in the synthesis rate or to the increase in the degradation rate of the opiate receptor for, in the presence of cycloheximide, where protein synthesis in the hybrid cells was minimal, the rate of receptor down-regulation did not increase. If the opiate agonist effect is at the regulation of receptor synthesis, then the cycloheximide and chronic effect of etorphine should be additive. This was not observed. In addition, tunicamycin treatment did not potentiate the etorphine-induced receptor down-regulation also. Hence, the processing of the receptor or the glycosylation of the receptor was not involved in the agonist-induced receptor down-regulation also.

The internalization of the opiate receptor in neuroblastoma N4TG1 cells during chronic opiate treatment has been reported by Blanchard *et al.* [17]. Our recent studies with chloroquine, in which the amount of [^{3}H]D-Ala^2-D-Leu^5-enkephalin accumulated by the hybrid cells was potentiated by this lysosomotropic agent,* suggested that the receptor in NG108-15 hybrid cells also internalized during chronic treatment. Thus, it is likely that the site of opiate agonist-induced receptor down-regulation must lie within the receptor recycling process, i.e. opiate agonists exert their effects by regulating the internalization of the receptor and/or regulating the insertion of recycled receptors or the newly synthesized and processed receptors into the plasma membrane. The exact site of opiate action remains to be elucidated.

The current data further substantiate our previous conclusion that receptor down-regulation and receptor desensitization are two different cellular adaptation processes [1]. Although cycloheximide and tunicamycin could abolish the recovery of opiate binding sites (Fig. 7), these two antibiotics could not prevent the recovery of receptor coupling to adenylate cyclase molecules. Desensitization to opiate agonist after chronic opiate treatment is not the consequence of reduction in receptor number. Rather receptor desensitization is due to an alteration in the coupling between receptor and adenylate cyclase. This coupling process does not involve protein synthesis or protein glycosylation, for cycloheximide or tunicamycin treatment did not alter opiate receptor desensitization.

In conclusion, by using cycloheximide and tunicamycin, we deduced that the site of etorphine-induced receptor down-regulation was not at *de novo* protein synthesis, assembly or processing of active receptor. Because of the inability of these two antibiotics to attenuate resensitization of cellular response to etorphine after removal of agonist, receptor desensitization and receptor down-regulation clearly seem to be two separate cellular adaptation processes.

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