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SHORT COMMUNICATIONS

# Loss of Opiate Receptor Activity in Neuroblastoma × Glioma NG108-15 Hybrid Cells after Chronic Opiate Treatment

## A Multiple-Step Process

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SUMMARY

Treatment of NG108-15 cells with 1 nM or 10 nM etorphine for 24 hr produced an identical magnitude of compensatory increase in prostaglandin $E_1$-stimulated adenylate cyclase activity. Activity of etorphine was retained, albeit reduced, in NG108-15 cells treated with 1 nM etorphine but not in cells treated with 10 nM etorphine. Exposure to 100 μM morphine for 72 hr produced a complete loss of morphine and levorphanol but not $Leu^5$-enkephalin activity in NG108-15 hybrid cells. Apparently, the loss of opiate activity involves a multiple-step process. Short-term incubation with 10 nM etorphine (<3 hr) produced loss of opiate activity with minimal alteration in [$^3$H]diprenorphine specific binding. Long-term exposure to 10 nM etorphine (≥24 hr) produced down-regulation of the receptor. A mechanism similar to that of *beta*-adrenergic receptor desensitization may be involved in opiate receptor desensitization in NG108-15 cells.

The adenylate cyclase activity in neuroblastoma × glioma NG-108-15 hybrid cells has been demonstrated to be inhibited by a high-affinity stereospecific opiate receptor (1–8). As with other receptors which regulate adenylate cyclase (9), opiate agonist affinity for the receptor and opiate receptor coupling to adenylate cyclase are themselves regulated by the guanine nucleotide GTP (10, 11). Similar to the adenylate cyclase-activating receptors which activate membrane-bound GTPase activity (9), opiate agonists also activate GTPase activity (12). Studies of the relative potencies of various opiate agonists suggest that the receptor in NG108-15 cells belongs to the *delta* subclass. Enkephalin and its analogues possess the greatest affinity for the receptor and the greatest potency with respect to inhibition of adenylate cyclase (3, 13).

Upon chronic exposure to opiate agonists, there is a gradual decline in opiate inhibition of adenylate cyclase activity in NG108-15 cells (5–8). Both basal and $PGE_1$[2]-activated adenylate cyclase activities in the presence of opiate agonists return to control levels after 12–24 hr of exposure to opiate agonists. Concomitantly, after removal of the opiate agonist, a 2-fold increase in the basal adenylate cyclase activity above control levels is observed in cells chronically treated with opiates. Sharma *et al.* (5, 6) and Lampert *et al.* (8), have suggested that this compensatory increase in adenylate cyclase activity is the cause of the failure to observe opiate inhibitory activity after chronic treatment. The opiate receptor remained coupled to the adenylate cyclase. This was concluded from the observations that (a) naloxone could induce the compensatory increase in the presence of opiate agonists, and (b) this induced enzymatic activity could be inhibited by opiates (5, 6). However, only the adenylate cyclase activity induced after chronic opiate treatment was inhibited by opiate agonists (6). It is plausible that this loss of opiate regulation reflects a desensitization of the opiate-sensitive adenylate cyclase. Therefore, it is the purpose of the present communication to establish whether the failure of opiates to inhibit adenylate cyclase activity in NG108-15 cells after chronic opiate treatment is due to receptor desensitization. The compensatory increase in adenylate cyclase activity cannot explain the loss of opiate receptor activity. We also wish to illustrate that the desensitization of opiate-sensitive adenylate cyclase, analogous to the adenylate cyclase stimulated by the *beta*-adrenergic receptor (14, 15), involves a multiple-step process.

The culturing of neuroblastoma × glioma NG108-15 hybrid cells was carried out as described (1). Initial stock

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[2] The abbreviations used are: $PGE_1$, prostaglandin $E_1$; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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cultures of NG108-15 hybrid cells were generous gifts of Dr. B. Hamprecht, Physiologischchemisches, Institut des Universität Wurzburg (Wurzburg, Federal Republic of Germany). The NG108-15 cells were chronically exposed to either etorphine for 24 hr or morphine for 72 hr. In the experiments in which the correlation between the opiate receptor binding and opiate inhibition of adenylate cyclase after chronic opiate treatment was determined, NG108-15 cells were exposed to 10 nM etorphine for various periods of time. Adenylate cyclase activity was measured by labeling the intracellular ATP pools with [$^3$H]adenine in the presence of an identical opiate concentration which was used in the chronic opiate treatment, and in the presence of the phosphodiesterase inhibitors, 3-isobutyl-1-methyl-xanthine (0.5 mM) and 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (0.1 mM) (13). After a 1-hr labeling period, the cells were mechanically detached from the growth surface and excess radioactivity was removed. NG108-15 cells were then resuspended in a Krebs-Ringer-Hepes buffer at pH 7.4 (13) in a concentration that delivered 3–4 × $10^5$ cells per assay. An identical concentration of opiate used in the chronic treatment was included in the cell suspension in order to prevent the compensatory increase in adenylate cyclase activity. The cells were then incubated at 37° for 20 min at the final volume of 0.5 ml in the presence of 3-isobutyl-1-methylxanthine, 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone, $PGE_1$ (10 μM), and various concentrations of opiate agonists. The reactions were terminated by the addition of 50 μl of 3.3 N perchloric acid. After the addition of 10,000 cpm of standard $^{32}$P-labeled cyclic AMP, $^3$H-labeled cyclic AMP was separated from other radioactive nucleotides by the method of White and Karr (16).

Opiate receptor binding with viable cells was carried out by incubating $10^6$ cells in 1.0 ml of Krebs-Ringer-Hepes buffer at pH 7.4 at 37° for 20 min with various concentrations of [$^3$H]diprenorphine in the presence or absence of 10 μM levorphanol. It has been determined that [$^3$H]diprenorphine binding attains equilibrium within 5 min at 37°. The incubations were terminated by collecting the cells on Whatman GF-B filters, and excess radioactivity was removed by washing the filters with 3 × 5 ml of Krebs-Ringer-Hepes buffer at 0°. Specific binding was defined as the difference in average radioactivity bound to sets of triplicate samples of cells in the presence or absence of levorphanol.

When the neuroblastoma × glioma NG108-15 hybrid cells were treated with an opiate concentration which produced maximal inhibition of adneylate cyclase activity, loss of opiate activity appeared to correlate with the compensatory increase in adenylate cyclase activity (5). However, if NG108-15 cells were treated with opiate concentrations which did not elicit maximal inhibition, the loss of opiate inhibitory activity did not coincide with the compensatory increase. As shown in Fig. 1, after NG108-15 cells were exposed to 1 nM and 10 nM etorphine for 24 hr and adenylate cyclase activity was measured in the presence of 10 μM naloxone, the cells treated with both etorphine concentrations exhibited an identical 19% increase in $PGE_1$-stimulated adenylate cyclase activity. This magnitude of increase in $PGE_1$-stimulated adenylate cyclase activity is similar to that reported by Sharma

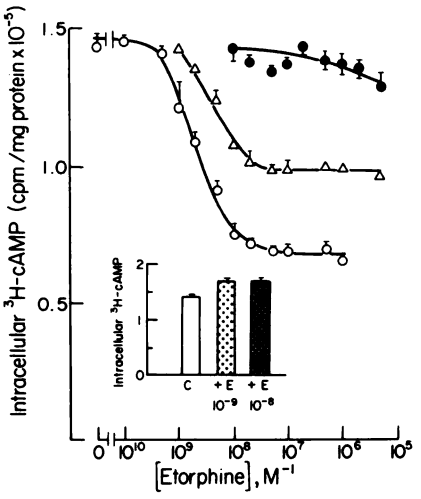


FIG. 1. *Opiate receptor activity after chronic etorphine treatment*
The ability of various concentrations of etorphine to inhibit the $PGE_1$ (10 μM)-stimulated increase in intracellular $^3$H-labeled cyclic AMP production in control cells (○), in cells treated with 1 nM etorphine for 24 hr (△), or in cells treated with 10 nM etorphine for 24 hr (●) was determined as described in the text. The *inset* represents $PGE_1$-activated adenylate cyclase activity in the presence of 10 μM naloxone in control (*open bar*), 1 nM etorphine-treated (*light stippled bar*), or 10 nM etorphine-treated (*dark stippled bar*) cells, which was determined to be 1.43 ± 0.06 × $10^5$ cpm/mg of protein, 1.70 ± 0.03 × $10^5$ cpm/mg of protein, and 1.70 ± 0.06 × $10^5$ cpm/mg of protein, respectively.

*et al.* (5, 6). If the compensatory increase in adenylate cyclase activity were the cause of the loss of opiate activity, then the cells treated with 1 nM and 10 nM etorphine would have identical decreases in opiate activity. As shown in Fig. 1, etorphine retained its inhibitory activity in cells chronically treated with 1 nM etorphine but not in those treated with 10 nM. The $IC_{50}$ value of etorphine increased from 2.1 nM in the control cells to 5.9 nM in the cells chronically treated with 1 nM ertorphine. The apparent cooperativity of etorphine (Hill coefficient = 1.5) in the control cells was retained in the chronically opiated-treated cells. There was a decrease in the maximal opiate inhibitory level in the chronically opiate-treated cells (Fig. 1). In cells treated with 10 nM etorphine for 24 hr, etorphine concentrations up to 10 μM failed to attenuate the $PGE_1$-stimulated adenylate cyclase activity. Therefore, the degree of attenuation of opiate receptor activity by chronic opiate treatment was not accounted for by the compensatory increase in adenylate cyclase activity.

It has been reported that after exposure of NG108-15 cells to 10 μM morphine for 72 hr, complete loss of opiate activity is observed (5, 6). Similar results were obtained in the current studies. After 72 hr of exposure to 100 μM morphine, the $PGE_1$-stimulated adenylate cyclase activity in chronically opiate-treated cells in the presence of agonist was identical with that of the control cells in the absence of any opiate ligand (Fig. 2a). The ability of morphine and other *mu* agonists (e.g., levorphanol) to inhibit adenylate cyclase activity disappeared. Levorphanol ($IC_{50}$ = 164 nM) is more potent than morphine ($IC_{50}$ = 3.5 μM) in NG108-15 cells. As shown in Fig. 2a, after chronic morphine treatment, levorphanol concentrations up to 200 μM failed to inhibit adenylate cyclase

activity. If this failure is due to a compensatory increase in adenylate cyclase activity, then all opiate agonists should show the same lack of activity. However, as shown in Fig. 2b, Leu$^5$-enkephalin retained partial activity in cells which had been treated with morphine for 72 hr. There was an increase in the $IC_{50}$ value of enkephalin from the control 1.8 nM to 4.4 nM and a concomitant decrease in the maximal inhibitory level after chronic opiate treatment. Nevertheless, the NG108-15 cells remained sensitive to enkephalin even though they were completely desensitized to *mu* agonists.

Similar to *beta*-adrenergic receptor desensitization (14, 15), the loss of opiate activity in NG108-15 cells appears to involve a multiple-step process. When the ligand-receptor interaction was monitored by [$^3$H]diprenorphine binding, a correlation between the decrease in the [$^3$H]diprenorphine binding and the decrease in the ability of 10 nM etorphine to inhibit adenylate cyclase activity did not exist (Fig. 3). During the initial period, i.e., after 2 hr of exposure to 10 nM etorphine, there was a pronounced reduction in etorphine's ability to inhibit adenylate cyclase activity but no measurable alteration of [$^3$H]diprenorphine specific binding. After continuous exposure to 10 nM etorphine for 24 hr, there was a complete loss of opiate activity (Figs. 1 and 3). However, 40% of the control [$^3$H]diprenorphine binding remained after 24 hr of etorphine treatment. Scatchard analysis of the binding data obtained after 24 hr of chronic etorphine treatment revealed a reduction of the total number of [$^3$H]diprenorphine binding sites without any alteration of binding affinity (Fig. 4). The apparent $K_{diss}$ values of [$^3$H]diprenorphine for control and chronically etorphine-treated cells in the presence of 10 mM etorphine were 4.9 nM and 5.1 mM, respectively. These $K_{diss}$ values were greater than the $K_{diss}$ values obtained when 10 nM etorphine was omitted from the reaction mixture, which was determined to be 1.96 ± 0.04 nM ($n$ = 4). The $B_{max}$, with the 95% confidence limit in parentheses, for the control cells was 597 fmoles/10$^6$ cells (544–645 fmoles/10$^6$ cells); for chronically etorphine-treated cells it was 221 fmoles/10$^6$ cells (187–255 fmoles/10$^6$ cells). This down-regulation of the opiate receptor was not observed with cells treated with etorphine for a short period of time (less than 3 hr). However, during the time period when a minimal reduction of [$^3$H]diprenorphine binding was observed, the affinity of the agonist for the receptor was reduced. The amount of D-Ala$^2$-D-Leu$^5$-enkephalin required to displace 50% of [$^3$H]diprenorphine (2 nM) specifically bound to membrane preparations in the presence of 5 mM $MgCl_2$ increased from the control value of 7.3 nM to 74.5 nM in membrane preparations isolated from cells treated with 10 nM etorphine for 3 hr.

From the present studies, it is concluded that the loss of opiate agonist inhibitory activity in NG108-15 cells after chronic opiate treatment is not caused by the observed compensatory increase in adenylate cyclase activity, as suggested by Sharma *et al.* (5, 6). Furthermore, the compensatory increase in adenylate cyclase activity and the loss of opiate activity might involve two separate cellular adaptation processes. In neuroblastoma N18TG2 cells, one of the parents of NG108-15 cells, chronic ex-

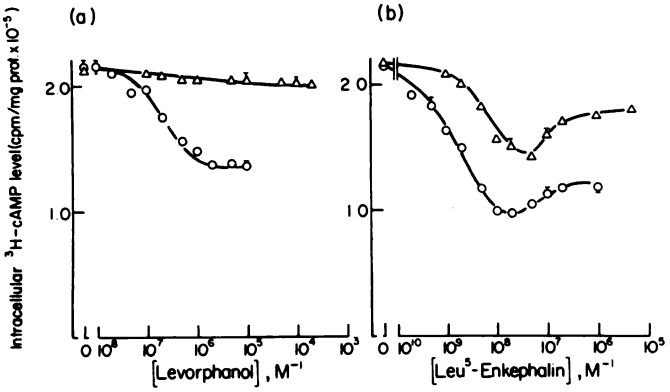


FIG. 2. *Inhibition of $^3$H-labeled cyclic AMP production by levorphanol and Leu$^5$-enkephalin in NG108-15 cells*

The ability of various concentrations of levorphanol (a) and Leu$^5$-enkephalin (b) to inhibit 10 μM $PGE_1$-activated adenylate cyclase activity in control cells (○) and in cells treated with 100 μM morphine for 72 hr (△) was determined as described in the text.

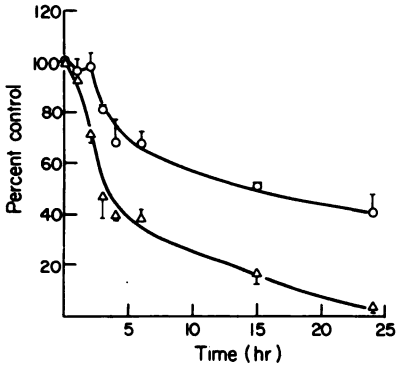


FIG. 3. *Time course of opiate receptor desensitization*

The amount of [$^3$H]diprenorphine (4 nM) specifically bound to the viable cells (○) and the ability of 10 nM etorphine to inhibit $PGE_1$-stimulated adenylate cyclase activity (△) in cells treated with 10 nM etorphine for various time periods was determined. Etorphine (10 nM) was added to both control and chronically etorphine-treated cells in the binding assays to normalize the concentration of nonradioactive opiate agonist in each assay. The values represent the average of three separate etorphine pretreatments.

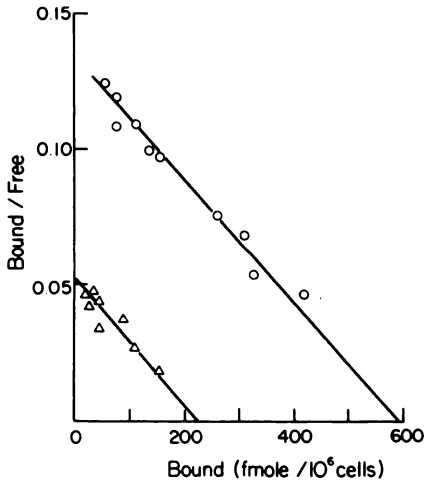


FIG. 4. *Scatchard analysis of [$^3$H]diprenorphine binding to control cells (○) and cells treated for 24 hr with 10 nM etorphine (△)*

Binding conditions were as described in the legend to Fig. 3 and as described in the text.

posure to etorphine produced receptor desensitization without any measurable compensatory increase in adenylate cyclase activity (13).

The data summarized in Fig. 2 suggested the possible existence of multiple opiate receptors in NG108-15 cells. It has been reported by Schulz and his co-workers (17) that cross-tolerance between opiate agonist subclasses (e.g., *delta* and *mu* agonists) does not exist in guinea pig ileum and mouse vas deferens. The fact that enkephalin still inhibits adenylate cyclase activity in chronically morphine-treated cells suggests the existence of putative *mu* and *delta* receptors in NG108-15 cells. However, these observations could be explained equally well by postulating that the loss of opiate activity, which is due to opiate receptor desensitization, is proportional to the intrinsic activity or efficacy of the opiate agonist. With desensitization of the *beta*-adrenergic receptor, the degree of receptor desensitization was related to the intrinsic activity of the ligands (14). The intrinsic activity of a *beta*-adrenergic ligand has been shown by computor analysis to be proportional to the ability of the ligand to induce the receptor into a high-affinity state (15, 18). An analogous situation could exist for opiate receptor inhibition of adenylate cyclase. In the inhibition of adenylate cyclase activity in NG108-15 cells, morphine and levorphanol are less efficacious than etorphine and enkephalin.[3] Therefore, those receptors which are not desensitized upon chronic treatment with morphine, a ligand with low intrinsic activity, could be utilized by enkephalin and etorphine, ligands with high intrinsic activity. However, from the present data, this model could not be distinguished from the multiple opiate receptor model in explaining the observations reported in Fig. 2.

Apparently, the opiate receptor desensitization process is similar to that reported with the *beta*-adrenergic receptor (14, 15). The *beta*-adrenergic specific desensitization involves at least two steps (14, 15). The first step involves a rapid-onset desensitization of the receptor without any alteration in the receptor density. During this phase, agonist binding affinity is reduced, and agonist binding is no longer under the regulation of GTP. The second step, slower in onset, involves a decrease in receptor density, defined as down-regulation of the receptor. The *beta*-adrenergic receptor density in the plasma membrane is irreversibly decreased (14). The desensitization of opiate-sensitive adenylate cyclase apparently follows this two-step process. Our binding data show that after 24 hr of etorphine treatment, a decrease in opiate receptor density occurs, but not after the shorter opiate treatment. During the short incubation period, the affinity of the agonist for the receptor was reduced. However, at present, whether or not agonist binding in the desensitized receptor remains under GTP control has not been established. If during opiate receptor desensitization the GTP effect on agonist binding is lost, then the mechanism for opiate receptor desensitization could be identical with that of *beta*-receptor desensitization. Possibly, similar to the *beta*-adrenergic receptor, desensitization of the opiate receptor is due to the inability of the agonist to induce the opiate receptor to an activated form which could couple with adenylate cyclase. Whether this is the mechanism for opiate receptor desensitization is under current investigation.

[3] P. Y. Law, D. S. Hom, and H. H. Loh, manuscript in preparation.

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