

Agonist regulation of the expression of the delta opioid receptor in NG108-15 cells

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Abstract Exposure of neuronal cells to the chronic presence of opiates leads to a complex series of biochemical events which reflect the changes that result in tolerance and dependence in animals. To achieve a better understanding of the molecular mechanisms underlying these processes, we have examined the effect of agonist efficacy on the regulation of the δ -opioid receptor mRNA in NG108-15 cells. Incubation with various opiates decreased receptor numbers in the order of their efficacy. Northern blot analysis showed that there are 4 size classes of mRNA coding for the δ -opioid receptor in NG108-15 cells even though only one known protein species is found. Moreover, the amount of each transcript is coordinately decreased by long-term etorphine treatment, but not necessarily to the same extent. The etorphine-induced decrease in receptor mRNA was found to be slow in onset, whereas a much more rapid loss of receptor number was observed. This disparity suggests that the down-regulation induced by etorphine can occur both at the levels of receptor protein modification and receptor gene expression, and that the mechanisms of the two processes may be different.

Key words: δ -Opioid receptor; Opiate; Binding activity; Transcription; Down-regulation

1. Introduction

Receptor activation can stimulate or inhibit a variety of cellular processes which range from basic metabolic activities to certain highly specialized functions in differentiated tissues. Although receptors serve as regulators of these cellular activities, they are themselves subject to considerable regulation. For many G-protein-linked receptors, stimulation is followed by a period of reduced responsiveness or desensitization in the continued presence of agonists, which usually involves some combination of receptor phosphorylation, sequestration and loss of receptor number, and uncoupling of the receptor from the effector [1–3]. In addition to this regulation at the protein level, the expression of the receptor gene itself can be modulated as a consequence of activation. In several cases mRNA levels for the receptor are rapidly reduced following stimulation, thereby further contributing to the loss in receptor number and responsiveness [4–7]. Clearly, this diversity of receptor regulation must depend on different cell signaling pathways which themselves serve to regulate gene expression.

There is a long history of studies aimed at measuring the possible down-regulation of opiate receptors by their ligands. After some initial confusion caused by the unsuspected presence of several types of opiate receptor and a range of agonist

efficacies for a given receptor type, it became clear that down-regulation of receptor number by pure agonists is a property of opiate receptors just as it is of adrenergic and other related receptors [8–10]. The recent success of a number of laboratories in cloning opiate receptors [11–17] provided the first opportunity to investigate some of the unique aspects of the mechanism of down-regulation of opiate receptors at the mRNA level. The delta opiate receptor found as the sole type in NG108-15 mouse neuroblastoma x rat glioma hybrid cells is coded for by a single gene in the parental mouse and yet exhibits at least 4 identifiable size species of mRNA in the cell line [17]. The study described here was designed to determine whether any or each of these RNA species is affected by the presence of opiate agonists.

2. Materials and methods

2.1. Preparation of membrane fractions

NG108-15 membranes were prepared according to a previously described procedure [18] with slight modifications. Frozen-packed NG108-15 cells were suspended in 9 volumes of cold 0.32 M sucrose, 10 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 10 μ M GTP, 50 mM NaCl, and disrupted with 25 strokes of a ground-glass tissue-grinder. The homogenate was centrifuged at 20,000 rpm for 10 min at 4°C. The pellet was washed 3 times with the same buffer and resuspended in 0.32 M sucrose. Protein was measured by the method of Lowry et al. [19] with bovine serum albumin as the standard.

2.2. Ligand binding assay

Delta opiate receptor numbers were measured in membrane fractions by equilibrium radioligand binding analysis with 2 nM [³H]diprenorphine as previously described [20]. Specific binding is defined as the difference between the amount of radioactive ligand bound in the presence and absence of 10 μ M non-radioactive ligand after 10 min incubation at 37°C.

2.3. Northern hybridization analysis

Six μ g aliquots of poly(A)⁺ RNA [17] from differently treated NG108-15 cells were separated by electrophoresis on denaturing 1.2% agarose/3.0% formaldehyde gels. RNA was transferred to a nylon membrane and hybridization and washes were performed as described previously [17]. The blots were dried and subjected to autoradiographic exposure for 7 days. For the delta opiate receptor probe, a 980 bp *Hind*III to *Eco*47III fragment of TB27 cDNA [17] was radiolabelled by the random hexamer-priming method. The glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene was used as a control for RNA loading with a probe corresponding to the mouse gene. For quantitation, films were scanned into a Macintosh computer and analyzed with NIH Image [21]. Integrated values were normalized to the G3PDH loading control. The results of the quantitation were confirmed by scanning gels with a Fuji BAS3000 Bio-Imaging Analyzer.

3. Results and discussion

One of the interesting features of the delta opiate receptor system of NG108-15 cells that was discovered as a result of the

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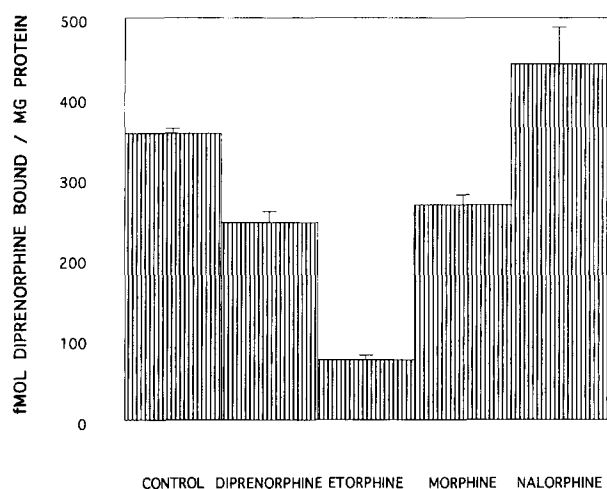


Fig. 1. Effect of treatment with various opiates on δ -opioid number in NG108-15 cell membranes. Cells were incubated in diprenorphine, etorphine, morphine, nalorphine, or vehicle for 4 days. All opiates were administered at a dose of $10 \mu\text{M}$. The membrane receptor number was measured by radioligand binding using [^3H]diprenorphine as ligand. The data shown are representative of three separate experiments. The error bars show the S.E.M. of the measurements.

recent cloning experiments is the fact that at least 4 sizes of mRNA are found, even though there is only a single gene in the mouse and probably in the cells as well. Several size species of mRNA presumably coding for the delta opiate receptor are found in the rat as well. What is the functional significance of these several species of RNA and how are their levels controlled? These questions prompted the present studies and are partially answered by the results.

Challenging NG108-15 cells with various opiate receptor agonists and antagonists revealed that etorphine, a pure and powerful agonist reduced the number of receptor to 30% of control (Fig. 1). Diprenorphine and morphine, both of which are only partial agonists at the delta receptor [18], had much smaller effects, whereas nalorphine, an almost pure antagonist [8], had almost no effect on the number of receptors. To explore the basis of this loss of opiate receptors in the cells, we measured receptor mRNA levels by Northern hybridization analysis. NG108-15 cells expressed δ -opiate receptor transcripts of 9.5, 7.5, 4.4 and 3 kb, with the smallest mRNA as the most abundant (Fig. 2) [11,17]. These experiments showed that of the drugs tested, only etorphine caused a remarkable decline in the amounts of each of the transcripts. Furthermore, scanning densitometry of the blots showed that the differently sized transcripts were coordinately decreased, although there appears to be a preferential reduction of the larger transcripts. Interestingly, Jehab and Inturrisi showed that exposure of NG108 cells to the pure opiate antagonist, naloxone, produces a increase in the level of expression of opiate receptor mRNA [22].

Incubation of the cells with etorphine led to a time-dependent decrease in receptor density to 30% of control after 1 day which persisted for at least 4 days (Fig. 3). This decline was already evident after one hour of incubation but not after only several minutes (data not shown). In contrast to the rapid loss of opiate receptor numbers upon etorphine treatment, receptor mRNA levels were unchanged during the first 2 days of treatment. Receptor mRNA levels only begin to decline on day 3 and the loss was maximal by day 5 (Fig. 4).

Interestingly, each of the opiate receptor mRNA species is decreased after 3 or more days of culture with etorphine. This result strengthens the conclusion that each of these size classes of mRNA is indeed coding for the opiate receptor. The amounts of the larger members of this family of RNAs, those at 7.5 and 9.5 kb, are decreased to a significantly greater extent than are the smaller species. This result could suggest that the rate of synthesis of the mRNA is decreased since the larger species would likely be the first products of transcription. However, the rate of degradation of the transcripts could also be size dependant and direct measurements of opiate receptor mRNA turnover will be needed to answer these questions.

Down-regulation of opiate receptor numbers is observed in NG108-15 cells after culture by agonists of high efficacy like etorphine, but not with agonists of only moderate or low efficacies at this receptor, like morphine and diprenorphine [18] as shown in Fig. 1. The down-regulation observed with etorphine is very rapid in onset as shown in Fig. 4 and is even observed after only 1 h of treatment (data not shown). In contrast to the very rapid loss of receptors measured by ligand binding, Changes in mRNA levels associated with culture in the pres-

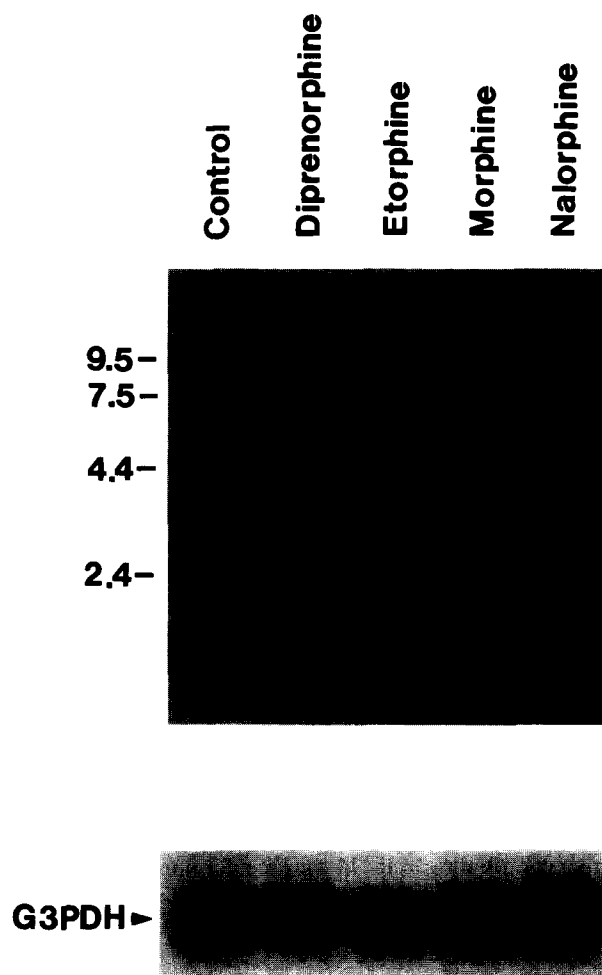


Fig. 2. RNA blot analysis of δ -opioid receptor mRNA in NG108-15 cells treated with various opiates. Poly(A)⁺ RNA was prepared from NG108-15 cells incubated for 4 days with no addition, diprenorphine ($10 \mu\text{M}$), etorphine ($10 \mu\text{M}$), morphine ($10 \mu\text{M}$), and nalorphine ($10 \mu\text{M}$). Each lane contained 6 mg of poly(A)⁺ RNA. Transcript size was determined by comparison to the mobility of RNA standards.

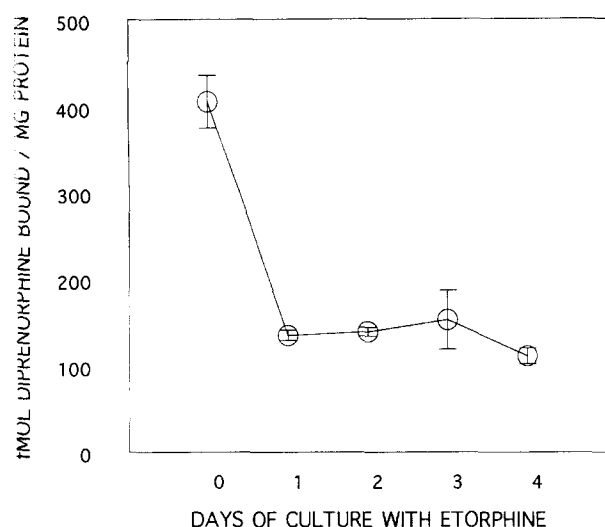


Fig. 3. Time course of the etorphine-induced decrease in δ -opioid receptor number. NG108-15 cells were incubated for the indicated times with $10 \mu\text{M}$ etorphine. Cells were harvested and receptor number was determined by binding of [^3H]diprenorphine on the membrane fraction. Data shown are the mean S.E.M. of three experiments.

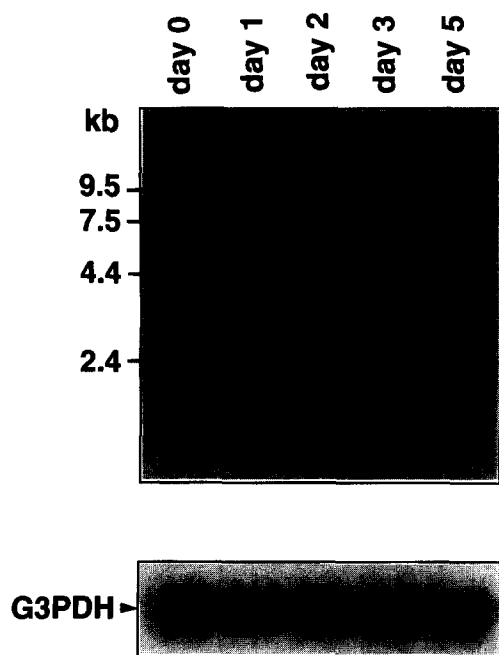
ence of etorphine are slow in onset and occur over the course of days rather than hours. Therefore, the initially rapid decrease in receptor numbers is likely to reflect a process unre-

lated to the slower decrease in mRNA level. By analogy with other G-protein-coupled receptors, it is likely that the rapid down-regulation induced by etorphine is due to a phosphorylation and internalization of the receptors. How this process leads to the reduction of mRNA levels several days later is not known. The fact that down-regulation and reduction in mRNA levels are both correlated with agonist efficacy suggests that they are both initiated by interaction with G-proteins.

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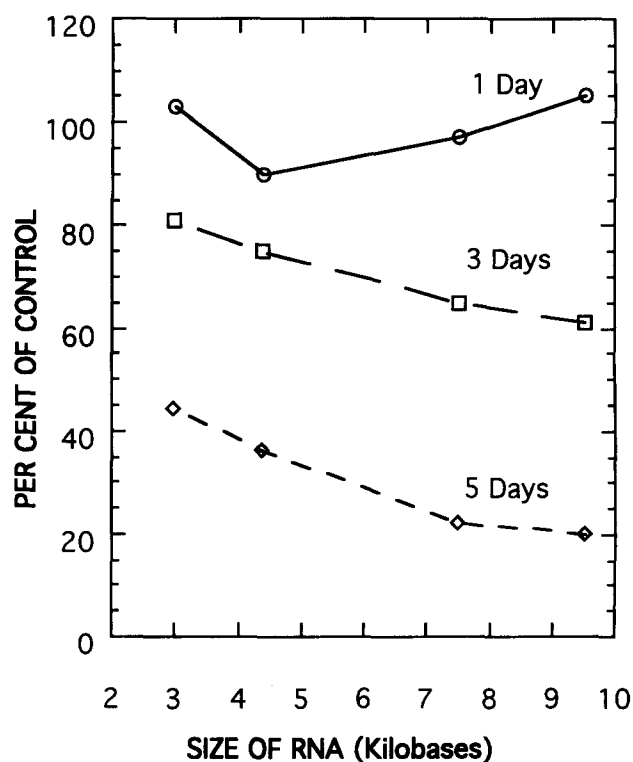


Fig. 4. Time course of etorphine down-regulation of δ -opioid receptor mRNA. Poly(A)⁺ RNA was prepared from untreated NG108-15 cells or at the indicated times after addition of $10 \mu\text{M}$ etorphine. (A) Northern blots were performed, and the levels of δ -opioid receptor and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were analyzed by scanning autoradiograms with the NIH Image program. (B) δ -Opioid receptor mRNA levels (with day 0 defined as 100%) are expressed relative to GAPDH mRNA, since GAPDH expression is unaffected by etorphine treatment. The experiment was performed two times with similar results. Shorter times of exposure were used to quantitate the GAPDH RNA, and the results were confirmed by phosphorimager analysis.

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