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OPIOID RECEPTOR DESENSITIZATION IN NG 108-15 CELLS

DIFFERENTIAL EFFECTS OF A FULL AND A PARTIAL AGONIST ON THE OPIOID-DEPENDENT GTPase

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Abstract—Opioid-receptor binding and the opioid-mediated stimulation of low K_m GTPase and inhibition of adenylate cyclase were studied in membranes derived from NG 108-15 cells pretreated with either the opioid peptide [D-Ala^2, D-Leu^5]enkephalin (DADLE) or morphine. Pretreatment with DADLE resulted in a concentration-dependent loss of responsiveness of GTPase to the peptide; this effect was entirely accounted for by a reduction in the maximal stimulation produced acutely by DADLE, without changes in the EC_{50} of the peptide, indicating a non-competitive type of desensitization. The degree of desensitization of GTPase was similar after one and 24 hr of pretreatment with DADLE, indicating that the process occurs rapidly. In contrast, morphine, which was 70–80% as potent as DADLE in stimulating GTPase and inhibiting adenylate cyclase in acute conditions, induced only a minimal desensitization of the opioid–GTPase system and, in contrast to DADLE, did not desensitize adenylate cyclase. Pretreatment with DADLE for one hour led to a decrease in opioid receptor density which was quantitatively similar to the degree of desensitization of GTPase: both these effects of DADLE were antagonized to a similar extent when morphine was also present in the pretreatment. Thus, desensitization of the opioid-stimulated GTPase appears to be correlated with down-regulation of the opioid receptor. Moreover, these findings suggest that partial agonists cannot induce this process.

Neuroblastoma × glioma NG 108-15 hybrid cells bear the δ-type of opioid receptor [1], which mediates inhibition of adenylate cyclase activity and cyclic AMP (cAMP) formation [2, 3]. Upon prolonged exposure of intact cells to morphine, there is a time-dependent increase in the specific activity of the enzyme, which, however, can still be inhibited by the alkaloid [4, 5] with a potency comparable to that observed in controls [6]. This compensatory increase in enzymatic activity, which counteracts the initial acute inhibition produced by the opioid, has been proposed as a model of tolerance and dependence at the cellular level [4].

More recently, it has been shown that opioid agonists with higher intrinsic activity than morphine can, upon chronic exposure to the cells, induce an attenuation of the opioid-mediated inhibition of adenylate cyclase and down-regulation of the opioid receptor [7, 8]. On the basis of time-course and temperature requirements, it has been suggested that desensitization of the opioid–adenylate cyclase system and down-regulation of the opioid receptor are two distinct processes, the former occurring before the latter [7].

Opioid receptors also stimulate a membrane-bound low K_m GTPase in NG 108-15 cells [9]. It is now generally accepted that this receptor-mediated reaction reflects an interaction between the receptor and the inhibitory nucleotide binding-regulatory protein (*N*), which couples the receptor and adenylate cyclase [10, 11]. This GTP hydrolysis step, which is a feature of many hormone- and neurotransmitter-receptors linked to adenylate cyclase, appears to be intrinsic to the receptor–*N* protein interaction, since reconstitution studies have shown that it does not require the catalytic subunit of adenylate cyclase [12].

We have previously shown that the opioid–GTPase system undergoes desensitization in NG 108-15 cells upon chronic exposure to the δ-opioid peptide [D-Ala^2, D-Leu^5] enkephalin (DADLE)† [13]. Here, we have further investigated this process by studying the mechanism of the loss of responsiveness of the enzyme to DADLE after pretreatment with this agonist. In addition, since morphine, a partial agonist in NG 108-15 cells [14], has been reported not to induce desensitization of the opioid–adenylate cyclase system [6, 15], it was of interest to compare the ability of morphine and DADLE to induce down-regulation of the opioid receptor, and desensitization of GTPase and adenylate cyclase.

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† Abbreviations used: App(NH)p, 5-adenylylimidodiphosphate; cAMP, adenosine 3: 5-cyclic monophosphate; DADLE, ([D-Ala^2,D-Leu^5]enkephalin; DMEM, Dulbecco's modified Eagle's medium; *N* proteins, nucleotide binding-regulatory proteins, mediating the stimulation or the inhibition of adenylate cyclase; PBS, phosphate buffered saline; PGE, prostaglandin E_1; RIA, radioimmunoassay; RO 20-1724, DL-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone.

Enzymes: Adenylate cyclase (EC 4.6.1.1.); GTPase (EC 3.6.1.-).

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle Medium (DMEM) (4.5 g glucose/l), HAT (hypoxanthine, aminopterin, thymidine) and phosphate buffered saline (PBS) were from Gibco, Karlsruhe, F.R.G. Fetal calf serum was from Gibco or Boehringer Mannheim GmbH Biochemica, Mannheim, F.R.G. [γ-^{32}P]GTP (10–50 Ci/mmol) and [^{3}H]diprenorphine (38 Ci/mmol) were from Amersham Buchler GmbH & Co. KG, Braunschweig, F.R.G. DADLE was from Bachem Feinchemikalien AG, Bubendorf, Switzerland. Diprenorphine was from Reckitt & Colman, Hull, U.K. Morphine HCl from Merck, Darmstadt, F.R.G. Naloxone HCl was from E.I. Dupont de Nemours & Co, Garden City, NY, U.S.A. RO 20-1724 was from Hoffmann-La Roche, Basel, Switzerland. ATP (disodium), 5-adenylylimidodiphosphate (App(NH)p), GTP (Tris), cAMP and dithiothreitol were from Sigma Chemie GmbH, Taufkirchen, F.R.G.

Cell culture, treatment and harvesting. NG 108-15 cells (passage number 21; initially stored in liquid nitrogen), kindly provided by D.M. Nirenberg (National Institute of Health, Bethesda, MD, U.S.A.), were grown at 37° in DMEM containing 5% fetal calf serum, 10% HAT (100 μM hypoxanthine, 1 μM aminopterin and 17 μM thymidine), in a humidified atmosphere of 5 or 7% CO_2 and 93–95% air. Cells were grown in 75 cm^2 flasks (Falcon, NUNC or Costar) in the presence of 30–40 ml medium which was changed on day 2 or 3 of subculture, and every subsequent day (up to day 6). Confluent monolayers ($10–15 \times 10^6$ cells) were treated for 1 or 24 hr with the vehicle, DADLE or morphine, Following the pretreatment, cells were washed 2–3 times with 10 ml of either cold PBS or cold DMEM, detached, centrifuged (800–1000 *g*, 5–10 min) and frozen as pellets at −70°. Cell viability (>90%) was assessed by the trypan blue exclusion test. Cells of passage number 21 to 31 were used in this study.

Harvesting of cells, preparation of membranes, GTPase and adenylate cyclase assays were performed either in the absence or the presence of the respective concentrations of DADLE or morphine used in the pretreatment. These two conditions are referred to as deprived and non-deprived, respectively.

Preparation of membranes for GTPase and adenylate cyclase assays. All procedures were carried out at 0–4°. Cells were thawed (10×10^6/ml) and homogenized in 5 mM Tris–HCl, pH 7.4 (25°), containing 1 mM EGTA, 1 mM dithiothreitol and 0.32 mM sucrose (thereafter referred to as the buffer), with a Dounce homogenizer (pestle A, 20 strokes), The homogenate was centrifuged at 1000 *g* for 10 min. The supernatant was kept, and the pellet was shortly vortexed in the presence of the original volume of buffer and centrifuged at 1000 *g* for 10 min. The two supernatants were pooled and centrifuged at 22,500 *g* for 20 min. The pellet was resuspended with the original volume of buffer (without sucrose) and centrifuged at 22,500 *g* for 30 min. The pellet was finally resuspended in buffer (without sucrose) so as to have a final concentration of 1 mg protein/ml, rehomogenized with the Dounce homogenizer (10 strokes), and frozen in fractions at −70°.

GTPase assay. GTPase activity was assayed according to Cassel and Selinger [16], with minor modifications. The reaction mixture included: 41 mM Tris–HCl (pH 7.5), 0.2 mM EGTA, 0.2 mM dithiothreitol, 100 mM NaCl and 10 mM $MgCl_2$ (unless otherwise stated), 0.5 mM ATP, 1 mM App(NH)p, 5 mM phosphocreatine, 5 units creatine phosphokinase, 0.25–0.50 μM GTP (including 0.5–2.5×10^5 CPM from [γ-^{32}P]GTP), and 2–8 μg protein, in a final volume of 100 μl. The reaction mixture was prewarmed at 37° for 2–3 min and the reaction started by adding the membranes in a volume of 20 μl and conducted at 37° for 10 min, after which was added 100 μl of ice-cold 40 mM H_3PO_4 and tubes placed on ice. Then was added 750 μl of an ice-cold suspension of 5% activated charcoal in 20 mM H_3PO_4 and tubes centrifuged at 5° for 10 min in an Eppendorf table centrifuge. Radioactivity was counted in 650 μl aliquots in the presence of 3 ml of scintillation fluid.

As described by others [9, 16], the low K_m GTPase activity was calculated by subtracting the activity obtained with 50 μM GTP ("high" K_m GTPase) from the one observed with 0.25–0.50 μM GTP, which accounted for 40–60% of total $^{32}P_i$ released. Blanks (no membranes or heated membranes) accounted for 0.2–0.5% of total radioactivity added. Under these conditions, the enzymatic activity was linear with time and protein concentration. Assays were run in duplicates or triplicates, with a variability routinely less than 5% of the mean. GTPase activity is expressed as pmol of GTP hydrolysed per mg of protein per min.

Adenylate cyclase assay. Adenylate cyclase activity was assayed in conditions identical to those of the GTPase assay, except that no App(NH)p was present, the concentration of GTP was 10 μM, and the reaction mixture included 100 μM Ro 20-1724 and, when specified, 10 μM prostaglandin E_1 (PGE). The reaction was started by adding 3–10 μg protein as for the GTPase assay, and conducted at 37° for 10 min, after which 500 μl of ice-cold 0.01 N HCl was added and tubes placed on ice. Samples were stored at 5° until the RIA determination of cAMP (0–7 days). Blanks (no membranes or heated membranes) accounted for 4–10% of total cAMP measured. Under these conditions, the enzymatic acivity was linear with time and protein concentration. Assays were run in triplicates or quadruplicates, with a variability routinely less than 5% of the mean. Adenylate cyclase activity is expressed as pmol of cAMP formed per mg of protein per min.

Binding studies. Frozen cell pellets were thawed and homogenized in 50 mM Tris–HCl (pH 7.5) (herein referred to as Tris-buffer) at 0°, using a Polytron homogenizer (setting 7, 20 sec) and 0.2 ml of Tris-buffer for 10^6 cells. The homogenate was centrifuged at 28,000 *g* for 15 min, the pellet resuspended in the same volume of Tris-buffer and centrifuged a second time. The pellet was finally resuspended in fresh Tris-buffer to obtain a final concentration of 0.5–1.0 mg of protein ($1–2 \times 10^6$ cells) per ml, and immediately used for the binding assay. Binding reactions were conducted at 20° in a

total volume of 2 ml in Tris-buffer for 90 min. They were initiated by adding membranes to a final concentration of 200–300 μg/ml to reaction tubes (glass, 12 × 75 mm) and terminated by rapid filtration on glass fiber filters (Whatman, GF/B, 2.5 cm), under vacuum. Binding isotherms for diprenorphine were obtained using a constant concentration of [^{3}H]diprenorphine (0.15 nM) and ten increasing concentrations (0.1–1000 nM) of the unlabelled ligand. After filtration, the GF/B filters were washed twice with 5 ml of cold Tris-buffer, and the radioactivity retained on them counted at an efficiency of 55% in a Beta counter (Beckman, LG 2800), after overnight equilibration in Beckman HP/b scintillation fluid.

cAMP radioimmunoassay. cAMP was measured by RIA after dilution (1:10 to 1:20) of the samples in sodium acetate buffer (0.1 M, pH 6.5) and acetylation of the samples, as previously described [17].

Protein determination. Proteins were determined using a modification of the Lowry method, as describe by Peterson [18], using bovine serum albumin as standard.

Analysis of data. Concentration–response curves for the stimulation of GTPase activity by DADLE or morphine were analysed with the computer program ALLFIT [19]. Sets of curves obtained in a single experiment (for example, concentration–response curves on membranes derived from cells pretreated with different concentrations of agonist) were simultaneously fitted, firstly to estimate their respective parameters (maximal effect, slope, EC_{50}). Then, the hypothesis that certain curves shared common parameters was statistically tested by forcing the curves to share the parameter of interest and examining the effects of this constraint on the "goodness of fit" [20]. F-test statistics based on the "extra sum of squares" principle was used to compare different fits. A significant value of the test indicates that the shared parameters are different from each other. In this way we evaluated the statistical significance of the differences in EC_{50} or maximal effect, and the P-values are reported in the text or in the legends. Binding isotherms were analysed with the computer program LIGAND [20].

The data presented here are, as indicated, either the means of two or more experiments, or the results of a representative experiment which was repeated at least twice in different membrane preparations.

RESULTS

It has been shown that desensitization of the opioid–adenylate cyclase system in NG 108-15 cells is a time-dependent process which is also time-dependently reversed upon withdrawal of the agonist [7]. In order to evaluate whether a part of the mechanism is dependent upon the continuous occupancy of the receptor by the agonist, we have studied in parallel membranes maintained either in the presence (non-deprived) or the absence (deprived) of the respective concentrations of opioid used in the pretreatment, as described in Methods.

Figure 1 shows that pretreatment with DADLE for 24 hr induced a concentration-dependent loss of responsiveness of GTPase to the opioid either in deprived or non-deprived conditions (upper panels). As previously reported [13], the pretreatment produced also a small decrease in the basal GTPase activity, which was not affected by the presence of naloxone in the assay (insets, upper panels). The analysis (see Methods) of the concentration–response curves for the acute stimulation produced by DADLE in membranes derived from control or pretreated cells indicated that the loss of responsiveness was entirely accounted for by a reduction in the maximum effect of DADLE, without significant changes in its EC_{50} for stimulating acutely the enzyme (panel C). The concentration-dependent increase in the EC_{50} of DADLE (panel C, closed circle) observed in non-deprived conditions is conceivably accounted for by the presence of the agonist in the assay. Thus, the mechanism of desensitization of the opioid-controlled GTPase appears to be non-competitive in respect to the DADLE-mediated stimulation of the enzyme. A replot of the maximal effect of DADLE as a function of the concentration of agonist used in the pretreatment showed that after 24 hr of pretreatment, the maximal desensitization of GTPase was 86 ± 3%. This replot also revealed that the potency of DADLE (EC_{50}) in desensitizing the opioid-GTPase system (5.0 ± 1.1 nM) was higher ($P < 0.01$) than its potency for stimulating acutely the enzyme (17 ± 1 nM). The presence of 100 μM naloxone during the pretreatment completely antagonized the desensitization induced by 100 nM DADLE, while naloxone by itself had no effect (data not shown).

In non-deprived membranes the maximal degree of desensitization (100 ± 1%) induced by a 24 hr pretreatment was more pronounced ($P < 0.001$) than in deprived conditions (panel D). A small decrease in basal GTPase activity was also observed in non-deprived membranes, and the addition of naloxone to the assay had no effect on this activity, indicating that there was no residual acute effect of the agonist (data not shown). A high level of desensitization of GTPase was already observed after one hour of pretreatment with DADLE, as depicted in Fig. 2. The basal activity was not affected by the pretreatment, in contrast with what was observed after a 24 hr pretreatment; the presence of naloxone in the assay had no effect on the basal activity. The acute net stimulation produced by DADLE was concentration-dependently decreased by the pretreatment (inset), the maximal loss of responsiveness of GTPase was 68 ± 4%. The potency of DADLE in desensitizing the opioid-stimulated GTPase after one hour of pretreatment was lower (<0.02) than the potency observed after 24 hr of pretreatment. As observed after 24 hr of pretreatment, the loss of responsiveness of GTPase induced by the one hour pretreatment was entirely accounted for by a reduction in the maximal stimulation produced acutely by DADLE, without changes in the EC_{50} of the peptide, revealing again a non-competitive type of desensitization (data not shown).

In Fig. 3 the effect on GTPase activity of the pretreatment with morphine, a partial agonist in NG 108-15 cells [14], is shown in deprived membranes. After 24 hrs, there was a minimal reduction in the maximal stimulation produced acutely by morphine

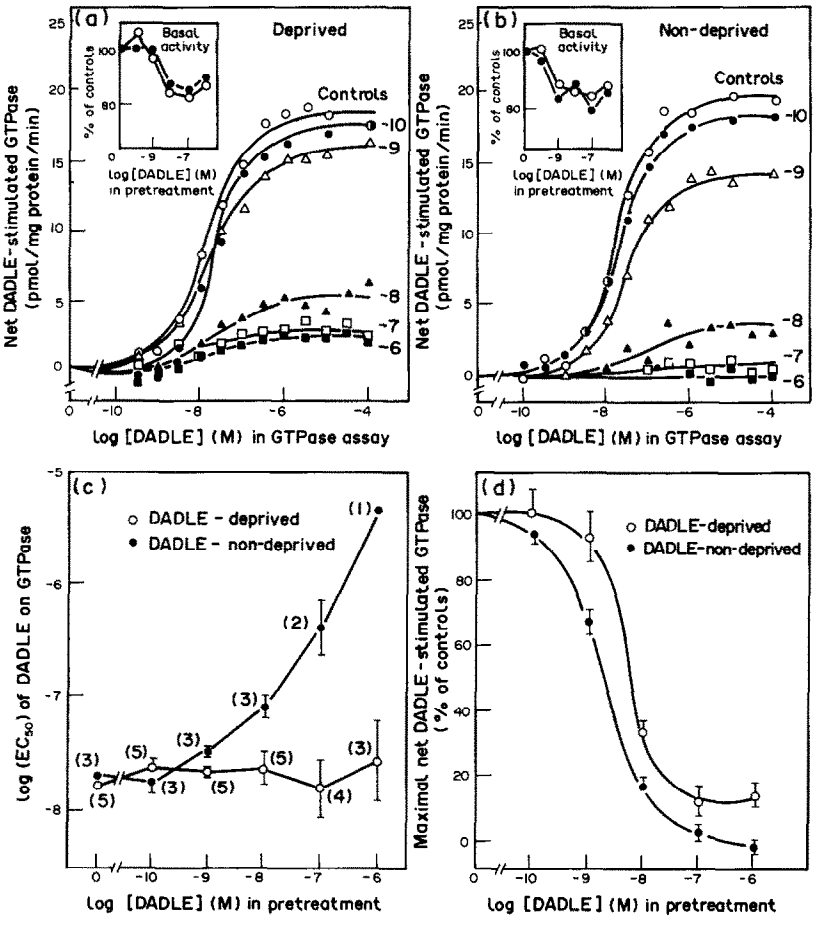

Fig. 1. Effects of pretreatment of intact cells with DADLE for 24 hr on the opioid-stimulated GTPase. Intact cells were pretreated for 24 hr with the vehicle (controls) or with different concentrations of DADLE (10^{-10} to 10^{-6} M), as indicated. *Panel a and b:* GTPase activity was assayed in the presence of DADLE at the indicated concentrations in deprived (a) and non-deprived (b) membranes. The enzymatic activity is expressed as net DADLE-stimulated GTPase, which is the difference between the activity observed in the presence of DADLE and the one measured in its absence (data are the mean of three experiments). Insets: basal GTPase activity as a function of the concentration of DADLE used in the pretreatment, assayed in the absence (○) or presence (●) of 100 μM naloxone. The absolute basal GTPase activity in controls was 32 ± 2 pmol/mg protein/min (data are the means ± SEM of five experiments). *Panel c:* Replot of the EC_{50} of DADLE for stimulating acutely GTPase, as a function of the concentration of DADLE used in the pretreated. The EC_{50} values were computed (program ALLFIT) by simultaneously fitting the concentration–response curves; data are the mean ± SEM of the log value of the EC_{50}s or ± the standard error of the computer fit when N = 1 (N is given in parentheses for each point). In non-deprived membranes, the EC_{50} for DADLE in cells treated with the highest concentration of the agonist were not measurable in all the experiments. *Panel d:* Replot of the maximal net DADLE-stimulated GTPase activity as a function of the concentration of DADLE used in the pretreatment (data are the means ± SEM of 5 experiments).

(5–15%), with no significant change in the EC_{50} of the alkaloid for stimulating GTPase (controls = 1.9 ± 0.4 μM; pretreated = 2.6 ± 0.4 μM). Furthermore, the maximal acute stimulation of GTPase produced by DADLE (100 μM) was, after the pretreatment, 91 ± 1% of the one observed in controls (data not shown). In order to determine whether this lack of effect of morphine pretreatment on the opioid–GTPase system might be accounted for by a reversal of desensitization occurring during the preparative procedures, the effects of a 24 hr pretreatment with morphine were studied in non-deprived membranes. Figure 4 shows that in such conditions, the basal activity (morphine present) was concentration-dependently increased after 24 hr of pretreatment, and reversed to the control level when naloxone was added to the GTPase assay. Both morphine and DADLE, at maximally effective concentrations, could still stimulate the enzymatic activity to levels comparable to those observed in control membranes. Thus, morphine can induce only a slight desensitization of the opioid-controlled

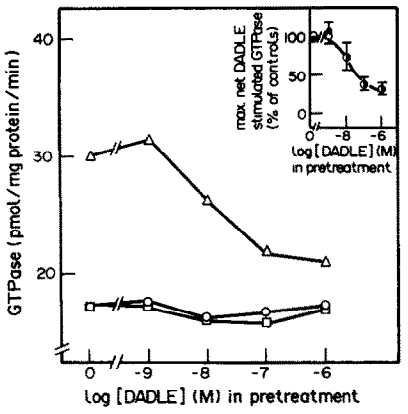

Fig. 2. Effects of pretreatment of intact cells with DADLE for one hour on the opioid-stimulated GTPase. GTPase activity was assayed in the absence (○) or presence of either 100 μM naloxone (□) or 100 μM DADLE (△), in deprived membranes derived from cells pretreated with DADLE for one hour at the indicated concentrations (data are the means of three experiments). Insets: Replot of the maximal net DADLE-stimulated GTPase activity, as the difference between the activity observed in the presence of 100 μM DADLE and the activity measured either in the absence (○) or the presence (●) of 100 μM naloxone (data are the means ± range of 2 experiments). See legend to Fig. 1 for details.

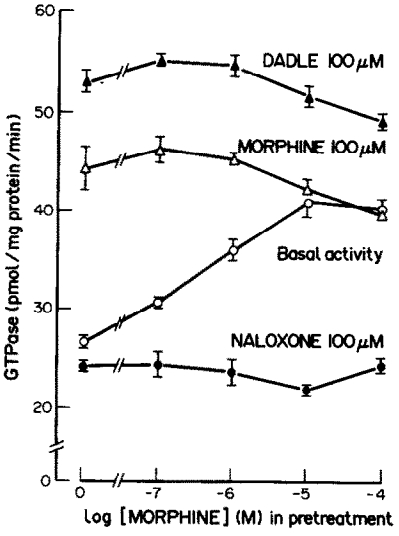

Fig. 4. Effects of pretreatment of intact cells with morphine for 24 hr on the opioid-stimulated GTPase in non-deprived membranes. Intact cells were pretreated for 24 hr with morphine at the indicated concentrations and GTPase assayed in non-deprived membranes in the absence (○) or presence or either 100 μM naloxone, 100 μM morphine or 100 μM DADLE, as indicated (data are the means ± SEM of triplicate determinations of an experiment repeated three times with similar results). See legend to Fig. 1 for details.

GTPase, even after 24 hr of pretreatment with a concentration 100 times higher than its EC_{50} for stimulating acutely the enzyme.

To investigate whether the desensitization of the opioid-stimulated GTPase was paralleled by a similar loss of responsivness to opioid of adenylate cyclase, membranes derived from NG 108-15 cells pretreated with DADLE or morphine for 24 hr were assayed for adenylate cyclase activity. Figure 5 shows that

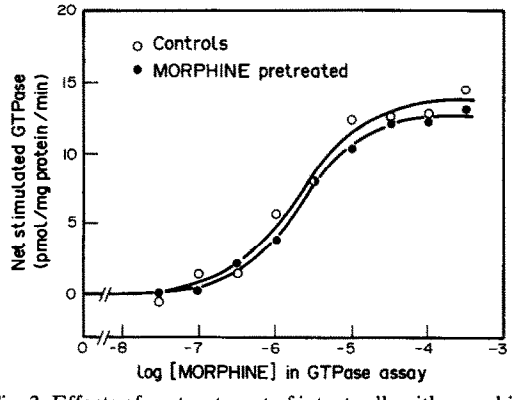

Fig. 3. Effects of pretreatment of intact cells with morphine for 24 hr on the opioid-stimulated GTPase in deprived membranes. Intact cells were pretreated for 24 hr without (○) or with (●) 100 μM morphine and GTPase assayed in deprived membranes in the presence of morphine at the indicated concentrations. GTPase activity is expressed as net morphine-stimulated GTPase. The basal enzymatic activity was 24 ± 1 and 21 ± 1 in membranes derived from control and morphine-pretreated cells, respectively (data are the means of two experiments). See legend to Fig. 1 for details.

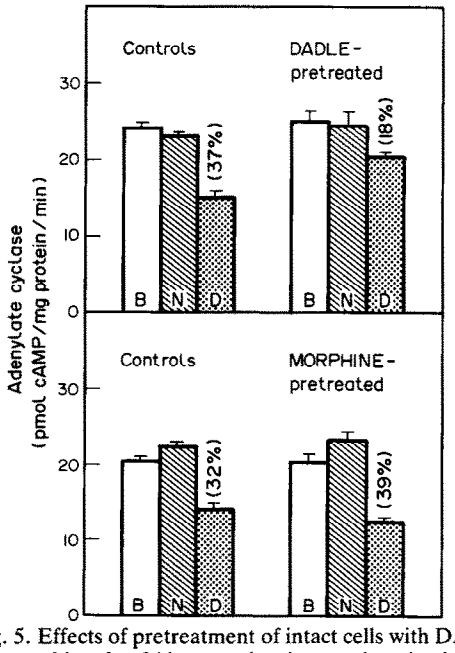

Fig. 5. Effects of pretreatment of intact cells with DADLE or morphine for 24 hr on adenylate cyclase in deprived membranes. Intact cells were pretreated for 24 hr with the vehicle (controls) or with either 100 nM DADLE (upper panel) or 100 μM morphine (lower panel). Adenylate cyclase activity was assayed in deprived membranes in the absence (B) or the presence of 100 μM naloxone (N) or 100 μM DADLE (D). The percentage of inhibition produced by DADLE is indicated in parenthesis (data are the means ± SEM of quadruplicate determinations of an experiment repeated three (upper panel) or two times (lower panel) with similar results. See legend to Fig. 1 for details.

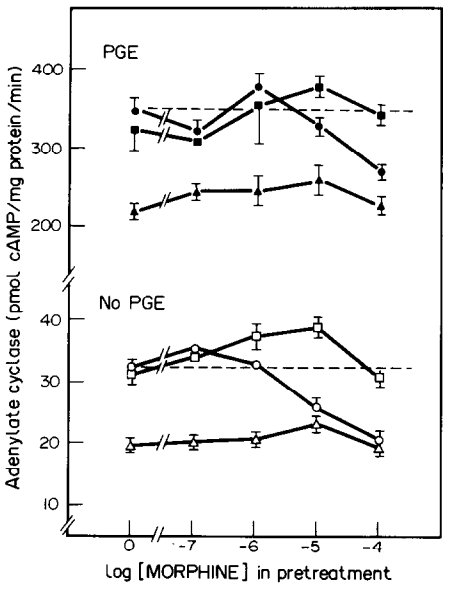

Fig. 6. Effects of pretreatment of intact cells with morphine for 24 hr on adenylate cyclase in non-deprived membranes. Intact cells were pretreated for 24 hr with morphine at the indicated concentrations. Adenylate cyclase activity was assayed in non-deprived membranes without (open symbols) or with 10 μM PGE, in the absence (○, ●) or presence or either 100 μM naloxone (□, ■) or 100 μM DADLE (△, ▲) (data are means ± SEM of quadruplicate determinations of an experiment repeated twice with similar results). See legend to Fig. 1 for details.

pretreatment for 24 hr with 100 nM DADLE resulted in about a 50% loss of responsivess of adenylate cyclase to the opioid, without altering the basal enzymatic activity or its sensitivity to naloxone (higher panel). In contrast, pretreatment for 24 hr with morphine was unable to desensitize the opioid–adenylate cyclase system, neither did it alter the basal enzymatic activity or its sensitivity to naloxone (lower panel). The inability of morphine to desensitize the opioid-controlled adenylate cyclase was confirmed in non-deprived conditions, as shown in Fig. 6. Upon pretreatment for 24 hr with different concentrations of morphine, the apparent basal enzymatic activity (morphine present) was concentration-dependently lower than in control membranes, regardless of whether the activity was studied in the absence (empty symbols) or presence (closed symbols) of 10 μM prostaglandin E_1 (PGE). This indicates that morphine was still inhibiting adenylate cyclase after the pretreatment: in fact, the addition of naloxone to the assay increased the basal activity to levels comparable to, or higher (up to 20%), than the control activity. Moreover, the addition of 100 μM DADLE could further inhibit the enzyme to the control level. Therefore, morphine does not induce any substantial desensitization of adenylate cyclase or GTPase.

It has been reported that morphine can antagonize the down-regulation of the opioid receptor induced by opioid peptides agonists [8]. We have therefore evaluated whether this antagonism by morphine could be observed for the desensitization of the opioid-GTPase system. The data are reported in Table 1: pretreatment of intact cells with DADLE (100 nM) for one hour produced comparable levels of desensitization of GTPase (64%) and opioid receptor down-regulation (57%). Morphine, after one hour of pretreatment, produced no reduction in binding and minimal desensitization of the opioid-stimulated GTPase (9%). However, when both DADLE and morphine were present in the pretreatment, the levels of desensitization (46%) and down-regulation (28%) were both lower than those induced by DADLE alone. Thus, the degree of desensitization of the opioid-stimulated GTPase correlates with the degree of down-regulation of the opioid receptor, at least after one hour of pretreatment.

DISCUSSION

The main features of the agonist-mediated desensitization of the opioid-dependent GTPase are: (1) a loss of responsiveness which correlates, at least after one hour of pretreatment, with down-regulation of the opioid receptor, and which is entirely accounted for by a reduction in maximal stimulation, without changes in the potency of the agonist; (2) a process which occurs rapidly, since the degree of desensitization observed after one hour of pretreatment is only slightly lower than the one produced after 24 hr; (3) the inability of morphine to induce desensitization of the enzyme.

As previously reported [13], the DADLE-induced loss of responsiveness of GTPase was observed regardless of the presence of the agonist throughout all the experimental procedures, although the maximal degree of desensitization was higher in non-deprived than in deprived membranes. In fact, only in the non-deprived condition could an almost complete loss of responsiveness of GTPase to DADLE be observed. The fact that the addition of naloxone to the assay had no effect on the basal GTPase activity, either in non-deprived or in deprived membranes, indicates that there was no residual effect on the enzyme. Thus, a likely explanation for this difference is that a certain degree of reversal of the process occurs in the course of the preparative procedures, and only the continuous presence of the agonist can prevent this reversal. Since the assessment of receptor down-regulation cannot be performed in non-deprived conditions, the comparison between desensitization of a pharmacological effect and the disappearance of receptor must take this fact into account. On the other hand, the results obtained with morphine demonstrate that the continuous presence of the agonist in the assay can influence the estimation of the desensitization if there is still stimulation. A glaring example is offered by Fig. 4. There, morphine produces only a small, if any, desensitization of GTPase, when the net effect is correctly estimated as the difference between the opioid-stimulated activity and the naloxone baseline (open triangles minus closed circles), but it would appear as a potent desensitizing agent if one considers the difference between the stimulated activity and the apparent basal activity (open triangles minus open

Table 1. Antagonism by morphine of DADLE-induced desensitization of GTPase and down-regulation of the opioid receptor

Pretreatment (1 hr)	GTPase (pmol/mg protein min)						[^{3}H]diprenorphine binding parameters	
	Additions to the assay				Net stimulated GTPase*			
	none	naloxone (100 μM)	DADLE (100 μM)	morphine (100 μM)	DADLE	morphine	B_{max} (fmol/10^6 cells)	K_d (nM)
Controls	22.6 ± 0.6	22.1 ± 0.6	40.8 ± 0.6	36.6 ± 1.2	18.2 (100%)	14.0 (100%)	465 ± 33 (100%)	0.43 ± 0.04
DADLE (100 nM)	20.7 ± 0.7	20.4 ± 0.6	27.4 ± 1.2	24.1 ± 0.8	6.6 (36%)	3.4 (24%)	201 ± 12 (43%)	0.59 ± 0.06
Morphine (100 μM)	21.8 ± 0.7	22.1 ± 0.6	38.4 ± 1.2	33.8 ± 1.2	16.6 (91%)	11.9 (85%)	482 ± 29 (104%)	0.65 ± 0.07
DADLE + morphine	20.4 ± 0.9	19.5 ± 0.7	30.2 ± 0.8	26.5 ± 1.1	9.8 (54%)	6.1 (44%)	336 ± 47 (72%)	0.51 ± 0.13

* The net opioid-stimulated GTPase activity was calculated as the difference between the activity observed in the presence of either DADLE or morphine, minus the activity measured in their absence.

Intact cells were pretreated for one hour with either the vehicle, 100 nM DADLE, 100 μM morphine, or both.

GTPase activity was assayed in deprived membranes (data are the means ± SEM of triplicate determinations of an experiment repeated twice with similar results). Saturation isotherms for [^{3}H]diprenorphine binding, obtained as described in methods, were analyzed with the computer program LIGAND and were consistent with a single class of sites. The binding parameters are shown ± the standard error of the computer fit.

circles). Furthermore, the same data could be interpreted as indicating a lack of cross-desensitization between morphine and DADLE, since at concentrations of morphine producing an apparent total loss of responsiveness to the alkaloid, a substantial degree of stimulation by DADLE would still be measurable (closed triangles minus open circles). Thus, if the degree of desensitization is evaluated as the net stimulation produced over the apparent basal activity, this degree will be overestimated at an extent which is the larger the smaller is the level of desensitization produced.

The comparison between the desensitization induced by one and 24 hr of pretreatment indicates that most of the process takes place within the first hour of exposure of the cells to the agonist. It is interesting that after 24 hr of pretreatment, the EC_{50} of DADLE in inducing desensitization of GTPase (5 nM) is lower than its EC_{50} in stimulating acutely the enzyme (17 ± 1 nM). It appears that the potency of the agonist in desensitizing the enzyme is more closely related to the high affinity agonist-specific state of the opioid receptor (2–4 nM) which is lost after pertussis toxin treatment and hence, most likely represents a receptor-nucleotide binding regulatory protein complex [21, 22, 23].

The fact that the opioid-stimulated GTPase desensitization in a "non-competitive" way in aspect to the agonist would be consistent with a mechanism reflecting receptor disappearance. This interpretation is supported by the correlation found in deprived conditions between the degree of desensitization of the opioid-controlled GTPase and the level of down-regulation of the opioid receptor. After one hour of pretreatment with DADLE both GTPase responsiveness to DADLE and opioid receptor density were decreased to degrees which were quantitatively similar, and the diminutions were reversed also to a similar degree upon simultaneous exposure of cells to morphine and DADLE. Since the affinity for [^{3}H]diprenorphine was not effected by the pretreatment, the decrease in binding sites appears as a true loss of receptors. This is in line with the report of Chang *et al.* [8], who have shown that morphine and other partial agonists in NG 108-15 cells can attenuate the down-regulation of the opioid receptor induced by opioid peptide agonists. The non-competitive mechanism by which desensitization of GTPase occurs also speaks in favour of a close association between the loss of responsiveness of the enzyme and receptor disappearance. This implies the existence of a tight coupling between receptor binding and the GTP hydrolysis step. In contrast, there is evidence for the existence of a large receptor reserve for the opioid-mediated inhibition of cAMP levels in NG 108-15 cells [17, 24, 25]. Furthermore, a dissociation between desensitization of adenylate cyclase and down-regulation of opioid receptors has been documented [7, 26]. Therefore, we may suggest that the amplification mechanism responsible for the discrepancy between receptor occupancy and modulation of cAMP levels is at the site of the *N* protein–adenylate cyclase rather than the receptor–*N* protein interaction.

The desensitization of GTPase observed here not only correlates with down-regulation of the opioid receptor, but is also paralleled by a loss of responsiveness of adenylate cyclase to the agonist. In fact, after 24 hr of pretreatment with DADLE, the ability of the agonist to inhibit the enzyme was impaired, although a substantial responsiveness was still present. Analogously, morphine did not induce down-regulation, had minimal effects on the opioid-stimulated GTPase and did not desensitize adenylate cyclase. This was observed in deprived as well as in non-deprived conditions, and therefore, cannot be attributed to a reversal of the process during the preparative procedures, which would be faster for morphine, due to its lower dissociation constant. The inability of morphine to desensitize adenylate cyclase is in perfect agreement with the early reports of Sharma *et al.* [4, 6] and Traber *et al.* [5] who have shown that exposure of intact NG 108-15 cells to morphine for up to 72 hr did not alter the ability of the alkaloid to inhibit the enzyme nor its potency in producing this effect, although a compensatory increase in the specific activity of the enzyme was apparent.

Although the inability of morphine to induce desensitization of the opioid-GTPase-adenylate cyclase system could be attributed to the fact that this opioid is a partial agonist in NG 108-15 cells [14], morphine can acutely produce as much as 70–80% of the effect of DADLE on both GTPase and adenylate cyclase [14]; yet it produced a degree of desensitization of GTPase which was barely 10% of that induced by DADLE. Since the apparent receptor reserve for morphine in intact cells is only a fraction of the one exhibited for DADLE [17], it appears that the ability of an opioid agonist to produce desensitization is related to its coupling efficiency rather than to its intrinsic activity. In other words, the process would depend upon the way whereby the system is activated rather than on the extent of the effect produced. Although it remains to be investigated whether this lack of morphine is shared by other partial opioid agonists, it is interesting that, for β-adrenoceptors, partial agonists can induce, although at a slower rate than full agonists, complete desensitization of adenylate cyclase [27]. We may speculate that this phenomenon reflects basic differences with regard to the mechanistic relationship between inhibitory and stimulatory receptors and adenylate cyclase.

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