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GTPase and Adenylate Cyclase Desensitize at Different Rates in NG108-15 Cells

LUC VACHON,[1] TOMMASO COSTA, and ALBERT HERZ

Department of Neuropharmacology, Max-Planck-Institut für Psychiatrie, Am Klopferspitz 18A, D-8033 Planegg-Martinsried, Federal Republic of Germany

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SUMMARY

The time course of opioid receptor binding disappearance and loss of responsiveness of the opioid-controlled GTPase and adenylate cyclase were compared in membranes derived from NG108-15 cells pretreated with the opioid peptide agonist [D-Ala^2,D-Leu^5]enkephalin (DADLE). Upon pretreatment with DADLE, a rapid desensitization of the opioid-stimulated GTPase occurred with a time course distinguishable as two exponential components having respective half-lives of 5–9 and 60–80 min. Opioid receptor binding activity, as assessed using [^{3}H]diprenorphine, also decayed as two exponential components whose half-lives were similar to those for GTPase desensitization (7 and 120 min). However, when [^{3}H]diprenorphine binding was measured in the presence of sodium and GTP, only the second, slow component was apparent. In contrast, desensitization of the opioid-controlled adenylate cyclase occurred as only one exponential decaying process, displaying a half-life of 57 min. Whereas the loss of responsiveness of GTPase to DADLE was entirely accounted for by a reduction in the maximal stimulation produced acutely by DADLE, desensitization of adenylate cyclase was characterized by both a decrease in maximal inhibition and a shift to the right of the EC_{50} of the agonist in inhibiting acutely the enzyme. In addition, after 1 hr of pretreatment with DADLE, the opioid-stimulated GTPase was desensitized by 65%, whereas 80% of maximal inhibition of adenylate cyclase could still be achieved. We suggest that: 1) the rapid loss of responsiveness of the opioid-GTPase system results from an uncoupling between the receptor and the nucleotide-binding regulatory protein (N); 2) the fast decaying GTPase activity appears to be not directly related to the opioid-mediated inhibition of adenylate cyclase; and 3) the slow decaying GTPase activity, as well as the desensitization of the opioid-adenylate cyclase, is most likely accounted for by down-regulation of the opioid receptor. These findings may indicate that part of the opioid-stimulated GTPase in the membrane is not involved in inhibition of the cyclase and could reflect the activity of a regulatory protein which couples opioid receptors to another membrane effector. Alternatively, they might be interpreted on the basis of a model which involves a tight coupling between receptor activation and N protein and a large amplification mechanism between N protein and adenylate cyclase.

Opioid receptors in NG108-15 cells inhibit adenylate cyclase activity and cAMP formation (1, 2). As for many other inhibitory receptors (3, 4), this inhibition is mediated through an N protein and coincides with the stimulation of a high affinity membrane-bound GTPase (5).

Two closely related N proteins, N_i and N_o, both endowed with intrinsic GTPase activity and both substrates of pertussis toxin, are present in many tissues (6–8) and can interact with inhibitory receptors (9). In NG108-15 cells, the levels of N_o are much higher than those of N_i (10), and it is not yet clear whether both proteins are involved in opioid-mediated inhibition of adenylate cyclase, or at which extent the opioid-dependent GTPase activity measured in the membrane reflects their relative abundance.

It has been clearly shown by reconstitution studies that the interaction between pure receptors and pure N proteins promotes the activation of GTPase activity, regardless of the presence of the catalytic subunit of adenylate cyclase (11, 12). Therefore, the agonist-stimulated GTPase activity measured in intact membranes most likely reflects the primary event which follows receptor activation upon agonist binding. Yet, very few studies have investigated the desensitization of receptor-dependent GTPase activity upon exposure of intact cells to an agonist.

We have previously reported that the opioid-controlled GTPase undergoes desensitization after treatment of intact

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[1] Present address: Centre de Recherche en Sciences Neurologiques, Faculté de Médecine, Université de Montréal, C.P. 6128, succursale A, Montréal, Québec, Canada, H3C 3J7.

ABBREVIATIONS: N proteins, nucleotide-binding regulatory proteins, mediating the stimulation (N_s) or the inhibition (N_i) of adenylate cyclase; App(NH)p, 5-adenylylimidodiphosphate; DADLE [D-Ala^2, D-Leu^5]enkephalin; DMEM, Dulbecco's modified Eagle's medium; RIA, radioimmunoassay; Ro 20-1724, DL-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

NG108-15 cells with the opioid peptide agonist DADLE (13). More recently, we have observed[2] that exposure of intact cells to this agonist for 1 hr induces similar levels of down-regulation of opioid receptor binding and loss of responsiveness of GTPase, and that these effects can be reversed also to a similar extent by morphine, a partial agonist in these cells (14–16). Here, we have further investigated the temporal relationship between down-regulation of the opioid receptor and desensitization of opioid-controlled GTPase and adenylate cyclase. It is shown that GTPase and adenylate cyclase desensitize at different rates in NG108-15 cells.

Experimental Procedures

Materials. DMEM (4.5 g of glucose/liter), hypoxanthine, aminopterin, thymidine, and phosphate-buffered saline were from Gibco, Karlsruhe, FRG. Fetal calf serum was from Gibco or Boehringer Mannheim GmbH Biochemica, Mannheim, FRG. [γ-^{32}P]GTP (10–50 Ci/mmol) and [^{3}H]diprenorphine (38 Ci/mmol) were from Amersham Buchler GmbH & Co. KG, Braunschweig, FRG. DADLE was from Bachem Feinchemikalien AG, Bubendorf, Switzerland. Diprenorphine was from Reckitt & Colman, Hull, U.K. Naloxone hydrochloride was from E. I. Dupont de Nemours & Co., Garden City, NY. Ro 20-1724 was from Hoffmann-La Roche, Basel, Switzerland. ATP (disodium), App(NH)p, GTP (Tris), cAMP, and dithiothreitol were from Sigma Chemie GmbH, Taufkirchen, FRG.

Cell culture, treatment, and harvesting. NG108-15 cells (passage number 21; stored in liquid nitrogen), kindly provided by D. M. Nirenberg (National Institutes of Health, Bethesda, MD), were grown at 37° in DMEM containing 5% fetal calf serum, 10% HAT (100 μM hypoxanthine, 1 μM aminopterin, and 16 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 of medium, which was changed on day 2 or 3 of subculture, and every subsequent day (up to day 6). Confluent monolayers (10–15 × 10^6 cells) were treated as follows. Upon reaching confluency, medium was changed (30 or 40 ml) at least 12 hr before the beginning of the pretreatment. The vehicle or DADLE, prepared in DMEM, was added to the flasks in a volume of 1 ml (to a final concentration of 100 nM) starting with the longest time of pretreatment. At the end of pretreatment, the medium was aspirated and cells were washed three times with ice-cold medium. The duration of the pretreatment was defined as the time elapsed between the addition of the vehicle or DADLE to the flasks and the time at which the monolayer was put in contact with cold medium. Flasks were then kept on ice and cells were harvested, centrifuged (800–1000 × g, 5–10 min, 4°), and stored as pellets at −70°.

Harvesting of cells, preparation of membranes, and GTPase and adenylate cyclase assays were performed either in the absence or presence of the 100 nM concentration of DADLE used in the pretreatment. These two conditions are referred to as deprived and nondeprived, respectively.

The recovery of GTPase responsiveness upon removal of the agonist was studied as follows. Cells were pretreated with DADLE for 60 min; at the end of the incubation the monolayers were washed three times with ice-cold medium to remove the agonist and arrest the process. Some cells were harvested at this stage to assess the level of desensitization produced (time 0), whereas the remaining flasks were reincubated at 37° with agonist-free medium and allowed to recover for different times from 10 to 480 min. The duration of resensitization was expressed as the time elapsed from the addition of agonist-free medium to the harvesting of the cells.

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 (hereafter referred to as the buffer), with a Dounce homogenizer (pestle A, 20 strokes). The homogenate was centrifuged at 1,000 × 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 1,000 × g for 10 min. The two supernatants were pooled and centrifuged at 22,500 × g for 20 min. The pellet was resuspended in the original volume of the buffer (without sucrose) and centrifuged at 22,500 × g for 30 min. The pellet was finally resuspended in buffer (without sucrose) to have a final concentration of 1 mg of 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 (17), 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, 10 mM $MgCl_2$, 0.5 mM ATP, 1 mM App(NH)p, 5 mM phosphocreatine, 5 units of 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 of protein, in a final volume of 100 μl. The reaction mixture was prewarmed at 37° for 2–3 min, the reaction was started by adding the membranes in a volume of 20 μl and was conducted at 37° for 10 min, after which 100 μl of ice-cold 40 mM H_3PO_4 were added and tubes were placed on ice. Then, 750 μl of an ice-cold suspension of 5% activated charcoal in 20 mM H_3PO_4 were added 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 (5, 17), the low K_m GTPase activity was calculated after the subtraction of the amount of hydrolysis (in cpm) observed with 50 μM GTP (due to "high" K_m GTPase) from that measured with 0.25–0.5 μM GTP (unless otherwise stated); the former 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. GTPase activity is expressed as pmol of GTP hydrolyzed per mg of protein per min.

Adenylate cyclase assay. Adenylate cyclase activity was assayed under 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. The reaction was started by adding 3–10 μg of protein, as for the GTPase assay, and was conducted at 37° for 10 min, after which 500 μl of ice-cold 0.01 N HCl were added and tubes placed on ice. Samples were stored at 5° or −20° 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 activity was linear with time and protein concentration. Assays were run in triplicate or quadruplicate, 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) (hereafter 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, and the pellet was resuspended in the same volume of Tris-buffer and centrifuged a second time. The pellet was finally resuspended in 5 mM Tris-buffer to obtain a final concentration of 0.5–1.0 mg of protein (1–2 × 10^6 cells) per ml and immediately used for the binding assay.

The binding reactions were conducted for 90 min at 20° in a total volume of 2 ml in 15 mM potassium phosphate (pH 7.5), 1.25 mM Tris-buffer, 10 mM $MgCl_2$ in the presence or absence of GTP (100 μM) and NaCl (100 mM) as indicated. The concentration of [^{3}H]diprenorphine was 150–250 pM and the final concentration of membrane proteins was 250–500 μg ml^{-1}. Nonspecific binding was defined as the amount of radioactivity bound in the presence of 1 μM diprenorphine and accounted for 10–20% of the total radioactivity bound. Saturation binding isotherms were constructed using a constant concentration of tracer (0.15 nM) and increasing concentrations (0.21–1000 nM) of unlabeled ligand. The reaction was initiated by the addition of the membrane

[2] L. Vachon, T. Costa, and A. Herz, submitted for publication.

suspension and terminated by filtration under vacuum on GF/B Whatman glass fiber filters. The filters were washed with two aliquots (4 ml) of ice-cold Tris-buffer, equilibrated overnight in Beckman HP/b scintillation cocktail (3 ml), and counted at an efficiency of 55%.

In some experiments the binding was determined in membranes prepared for GTPase and adenylate cyclase assays. In this case, the reaction was conducted under identical conditions, except that the total volume was reduced to 1 ml, the concentration of [^{3}H]diprenorphine was 1 nM, and 100 μg ml^{-1} of membrane proteins were used.

Determination of opioid-mediated inhibition of cAMP accumulation in intact cells. Confluent monolayers of cells were harvested and resuspended in DMEM containing 25 mM Na-HEPES and no $NaHCO_3$ (DMEM-HEPES) at 0.5–1 × 10^6 cells ml^{-1}. Aliquots of the cell suspension (2.5–5 × 10^5 cells) were distributed in Eppendorf 1.5-ml plastic vials in the presence or absence of Ro 20-1724 (100 μM) to a final volume of 90 μl. After 30 min of preincubation at 37°, DADLE (10 μl to achieve a final concentration of 100 nM) or its vehicle was added to triplicates tubes, and the incubation was continued for different times and terminated by the addition of 1 ml of ice-cold 0.05 N HCl. "Time 0" points were obtained by the addition of the acid immediately before that of peptide.

At the end of the experiment, the vials were centrifuged (8000 × *g*, 5 min at 0°) and the supernatants used for the determination of cAMP levels by RIA.

RIA of cAMP. 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 (16).

Protein determination. Proteins were determined using a modification of the Lowry method, as described by Peterson (18), using bovine serum albumin as standard.

Analysis of data. Concentration-response curves for the stimulation of GTPase activity or the inhibition of adenylate cyclase by DADLE were analyzed with the computer program ALLFIT (19), which was also used for the statistical comparison between the EC_{50} values or the maximal effects of different curves.

The time-dependent decline in binding activity and the desensitization of GTPase and adenylate cyclase activities were analyzed with the computer program EXPFIT, which allows weighted nonlinear least squares fitting of families of multiexponential curves[3] (20). Two alternative models, both involving a component of nondecaying activity, and, respectively, one or two exponential components of decaying activity were used to fit the data according to the following equations:

$$\text{model 1: } A/A_o = a_1 \exp^{-K_1 t} + a_o$$
$$\text{model 2: } A/A_o = a_1 \exp^{-K_1 t} + a_2 \exp^{-K_2 t} + a_o$$

where A_o is the activity at time 0, A is the remaining activity measured at each time point, K_1 and K_2 are the time constants (reported as half-lives, $t_{1/2} = 0.693/K$), t is the time in min, a_1, a_2, and a_o are the amplitudes of the components. The resensitization of GTPase activity was analyzed similarly as decline of the total desensitized activity measured at time 0 of recovery.

Decay curves obtained in different experiments of the same type were fitted simultaneously, first, to estimate the best-fitting model and the respective parameters. Second, the hypothesis that the curves shared common parameters was statistically tested by forcing the curve to share the parameter of interest and examining the effect of this constraint on the "goodness of fit." The F-test statistic based on the "extra sum of squares" principle was used to compare different fits and to choose between models of differing complexity (21).

Binding isotherms for diprenorphine were analyzed with the computer program LIGAND (21).

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.

[3] V. Broccardo, P. Munson, and D. Rodbard, manuscript in preparation.

Results

Cells were treated for different times with 100 nM DADLE, a concentration of agonist previously shown to induce maximal desensitization of the opioid-stimulated GTPase after 24 hr (13). The harvesting and preparation of membranes were done in the absence (deprived) or presence (nondeprived) of the same concentration of agonist, as detailed in Experimental Procedures.

Fig. 1 shows the desensitization of GTPase studied in deprived conditions. The *upper panel* of illustrates the actual GTPase activity in the absence or presence of either 100 μM DADLE, a supramaximal effective concentration, or 100 μM naloxone. The basal activity did not change with the time of pretreatment, nor was it affected by naloxone. In contrast, the activity measured in the presence of DADLE declined rapidly during the first 30 min and more slowly during the next hours of pretreatment. After 24 hr (not shown in Fig. 1, *upper panel*), we consistently measured a residual amount of stimulation, in agreement with previous reports (13). When cells incubated in the absence of the agonist were harvested with cold medium containing 100 nM DADLE and subjected to the same washing procedures as for the pretreated cells (indicated as "time 0" in Fig. 1), GTPase activity measured in the absence or presence of either DADLE or naloxone was identical to that of controls; this indicates that the assessment of desensitization is not affected by contamination with the desensitizing agonist carried over in the course of membrane preparation. The *lower panel* in Fig. 1 shows the exponential analysis of the disappearance of the net DADLE-stimulated GTPase activity, which is the difference between the activity observed in the presence of 100 μM DADLE and the one measured in its absence. The activity which is affected by the process (80%) can be distinguished as two components present in equal proportions with half-lives differing by a factor of 10 (see Table 1 for the decay constants).

In contrast, a difference is evident when the time course of desensitization of GTPase is studied without altering the concentration of agonist present during the pretreatment (nondeprived conditions) (Fig. 2). The basal activity is here only apparent, since it is measured in the presence of 100 nM DADLE and therefore reflects the residual acute stimulation produced by this concentration of agonist. Time 0 was obtained by harvesting control cells in cold medium containing 100 nM DADLE, which was thereafter present during the preparation of membranes and the GTPase assay, as for pretreated cells. The *upper panel* in Fig. 2 shows that the activity measured at time 0 was higher than in controls, could be further stimulated by 100 μM DADLE to the same extent as for untreated cells, and was decreased to the control level by the addition of naloxone. Thus, even in the presence of agonist, no further loss of responsiveness occurs in the course of the preparative procedures. With increasing time of pretreatment, the apparent basal activity and that measured in the presence of 100 μM DADLE decreased in parallel to the control level within the first hour. However, upon increasing the time of pretreatment, the addition of naloxone to the GTPase assay (which had no effect in controls) decreased the apparent basal activity to a level slightly lower than the control activity. This "overshooting" effect of naloxone was reproducible and stereospecific, as illustrated in the *inset* in Fig. 2, *upper panel*. Therefore, the assessment of the net DADLE-stimulated GTPase activity is

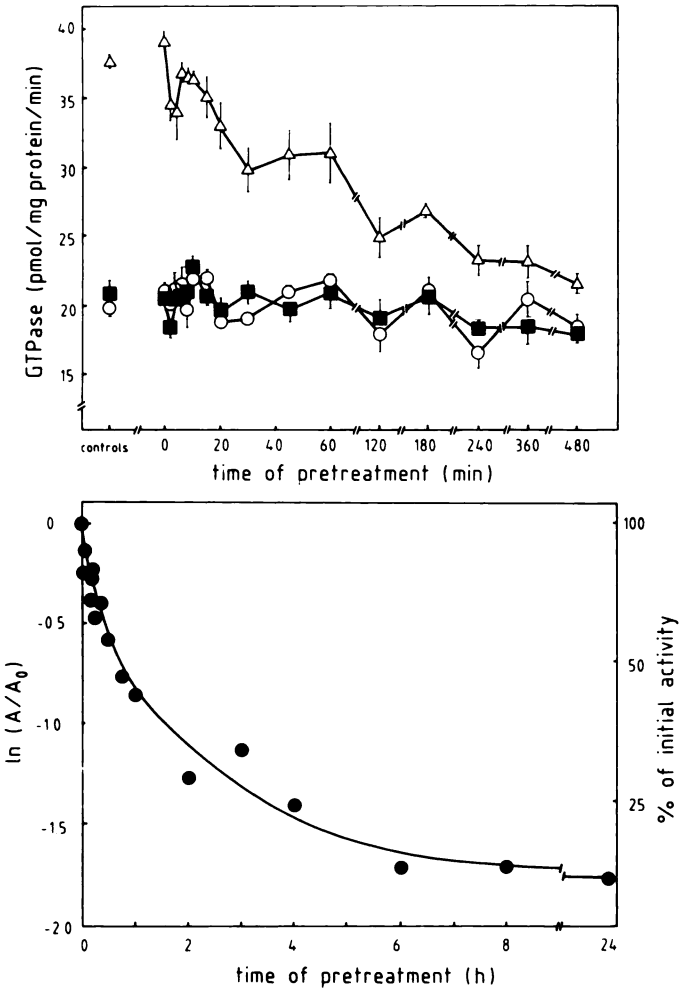

Fig. 1. Time course of desensitization of the opioid-stimulated GTPase in deprived membranes upon pretreatment of intact cells with DADLE. Intact cells were pretreated with the vehicle (controls) or with 100 nM DADLE for the indicated times. Cells were then washed with cold medium and processed in the absence of DADLE as described in Experimental Procedures. *Upper panel:* GTPase activity was assayed in the absence (○) or presence of either 100 μM naloxone (■) or 100 μM DADLE (△). Time "0" represents the activity observed in membranes derived from cells that were not pretreated with DADLE but were washed in its presence (100 nM) and then processed as the controls (data are the means ± standard errors of triplicate determinations of a representative experiment which was repeated five times with similar results). *Lower panel:* The net DADLE-stimulated GTPase activity (activity in the presence minus activity in the absence of 100 μM DADLE) in membranes derived from cells pretreated for different times with the agonist was computed for five experiments performed on four different batches of cells (including the one presented in the *upper panel*). The resulting five decay curves were analyzed simultaneously with the computer program EXPFIT, sequentially, using a model based on one or two exponential components of decreasing activity, as indicated in Experimental Procedures (see Data analysis). The second model provided a statistically significantly better description of the data ($p = 0.012$), and the five curves appeared to differ only in the relative proportion of the fast and slow component ($p < 0.01$) rather than in rates ($p = 0.92$), as indicated by the effect of sharing, the first and second set of parameters, respectively, on the quality of the fit. The data are presented in logarithmic coordinates following their normalization as ratios of the activity measured at each time point versus that measured at time 0 (A/A_0). The experimental points (●) are means of three (time points between 2 and 8 min) and five experiments. —, the mean of the five best-fitting theoretical curves. The average of the computed parameters with their standard error is given in Table 1. The mean net GTPase activity measured at time 0 is (in pmol × mg^{-1} × min^{-1}) 16.6 ± 2.5, assayed with 500 nM substrate (two experiments) and 9.7 ± 0.81 with 250 nM (three experiments).

profoundly affected whether we take as a baseline the activity in controls or that measured in the presence of naloxone, as shown in the *lower panel* of Fig. 2: when the net-stimulated activity is evaluated over the naloxone baseline, the proportion of nondecaying activity is smaller and that of rapidly decaying activity is greater than in deprived conditions, whereas when the control activity is used as the baseline (Fig. 2, *inset*), most of the net activity is rapidly decaying, and a remaining component of slow decay cannot be determined accurately. In both cases, however, the time constants are comparable to those measured in deprived conditions (Table 1).

The resensitization of GTPase activity was studied in cells exposed for 1 hr to DADLE (100 nM). Following extensive washing from the agonist, the cells were allowed to recover in DADLE-free medium and harvested at various times starting 10 min up to 8 hr from the withdrawal of the peptide. The recovery of stimulated activity proceeded in a biphasic manner (data not shown): of the total desensitized activity (60–65% of control), approximately half was restored within the first 30 min, whereas the remaining half appeared to recover slowly during the next hours of incubation so that, still, 20% of GTPase activity was desensitized after 8 hr. Computer analysis of the data is consistent with two exponential components and indicates that the half-time for the rapid recovery is similar to the time constant of the fast decaying component of desensitization (Table 1), whereas the half-time of the slow recovery largely exceeds that measured for the slow component of desensitization. Whether a complete recovery of the desensitized activity is possible cannot be established in this type of experiment since, even if the observation time were extended beyond 8 hr, the rapid mean doubling time of the cells (22–25 hr under these growth conditions) would complicate the interpretation.

We also studied the activity of adenylate cyclase in nondeprived membranes derived from cells pretreated for different times with DADLE (100 nM); this is shown in Fig. 3. After increasing the time of exposure to the agonist, the maximal inhibition of adenylate cyclase (determined in the presence of 100 μM DADLE) and the apparent basal activity (that is, the inhibition produced by 100 nM DADLE), were slowly attenuated in parallel within the first 2 hr of exposure. However, a substantial inhibition could still be observed for the remaining times of pretreatment. The addition of naloxone to the assay increased the apparent basal activity to levels comparable to those of the control, indicating a reversal of the acute inhibition due to the presence of the agonist. The analysis of the maximal inhibition measured in the presence of 100 μM DADLE (Fig. 3, *inset*) was consistent with a single exponential, which decreased with a time constant similar to that measured for the slow component of GTPase desensitization, and reached an asymptotic lower level of the initial responsiveness (Table 1).

In order to compare the respective mechanism of desensitization of GTPase and adenylate cyclase, we studied the concentration-effect relationship of the responsiveness of both enzymes to DADLE in deprived membranes derived from cells pretreated for either 1 or 24 hr with the agonist. The results are shown in Fig. 4. Two differences are clearly apparent: first, the degree of desensitization, measured as a reduction of the maximal acute effect of the agonist in the two enzymatic assays, was much higher for GTPase (66%) than for adenylate cyclase (19%) after 1 hr of pretreatment; second, the concentration-response curve of DADLE for cyclase inhibition not only exhibited a reduction in maximal effect compared to that obtained

TABLE 1

Time constants for desensitization of GTPase and adenylate cyclase activity, and resensitization of GTPase activity following 1 hr of desensitization

	Components[a]				
	Rapid		Slow		Plateau (a_0)
	t	a_1	t	a_2	
GTPase					
Deprived	8.9 (1.8)[b]	38 (19)	81 (20)	40 (18)	17 (6)
Nondeprived[c] (over naloxone)	4.9 (1)	64 (7)	60 (29)	26 (6)	9 (2.5)
Nondeprived (over control)	6 (.9)	82 (12)	55 (60)	17 (5)	ND[d]
Adenylate cyclase (non deprived)	ND		57 (21)	68 (5)	32 (4)
GTPase resensitization	5.6 (2)	45 (6)	500 (98)	54 (4)	

[a] t indicates the $t_{1/2}$ in minutes and a_1, a_2, and a_0 are the amplitudes of the components expressed in percentage of total activity. See analysis of data in Experimental Procedures for the equations.
[b] Values in parentheses, ±standard error.
[c] The net stimulation of GTPase activity in "nondeprived" conditions was computed both over control and over the naloxone baseline. See the legend of Fig. 2 for details.
[d] Nondetectable, i.e., model 1 fitted the data better than model 2.

in control, but also showed a shift to the right of approximately 10-fold for the EC_{50} of the agonist, which was evident following either 1 or 24 hr of exposure to the agonist. In contrast, the reduction in responsiveness of GTPase activity was at both times of pretreatment solely accounted for by a diminished maximal stimulation, without changes in the EC_{50} of DADLE. Thus, although the desensitization of GTPase is consistent with a "noncompetitive" mechanism, a more complex pattern is observed for adenylate cyclase.

It has been shown that exposure of intact NG108-15 cells to opioid agonists results in a reduction of opioid receptor binding activity (22, 23). In order to correlate the time-dependent loss of responsiveness of GTPase to DADLE with down-regulation of the opioid receptor, the properties of opioid receptor binding were studied using [^{3}H]diprenorphine in membranes derived from cells pretreated for different times with 100 nM DADLE. Fig. 5 shows that pretreatment with the peptide produced a time-dependent decrease in [^{3}H]diprenorphine binding measured in the absence of sodium and GTP, which closely resembles the time course of GTPase desensitization studied in deprived conditions: 40% (95 ± 13 fmol/mg of protein) of the initial activity declined rapidly ($t_{1/2}$ = 6.8 ± 1.9 min), whereas a further 36% (87 ± 14 fmol/mg of protein) decreased more slowly ($t_{1/2}$ = 121 ± 45 min) to reach a final plateau of 23% (56 ± 12 fmol/mg of protein), which was not further affected by the pretreatment. In contrast, when [^{3}H]diprenorphine binding was assayed in the corresponding membranes but in the presence of sodium (100 mM) and GTP (100 μM), only the slower component of the decay was detectable, whose rate was indistinguishable from the slow decrease measured in the absence of sodium and GTP; this component accounted for 65% (66 ± 4 fmol/mg of protein) of the initial binding activity. Thus, upon pretreatment of cells with DADLE, a fast decrease in opioid receptor binding, whose rate agrees with the rapid loss of opioid-stimulated GTPase activity, can be observed only in the absence of sodium and GTP (24). Fig. 6 shows that, either in the absence or presence of sodium and GTP, the decrease in binding activity measured with [^{3}H]diprenorphine was accounted for mainly by a reduction in apparent binding capacity. Only minor changes in affinity were produced by the pretreatment. Both in the absence or presence of the ion and nucleotide, the Scatchard plots of [^{3}H]diprenorphine binding were consistent with a single class of sites.

To compare the time courses of desensitization described before with the kinetics of opioid-induced reduction of cAMP levels in intact cells, we have studied the time course of the inhibition of cAMP levels in suspensions of intact cells incubated with 100 nM DADLE in the absence or presence of a phosphodiesterase inhibitor (Ro 20-1724, 100 μM). As illustrated in Fig. 7, cAMP levels were time-dependently reduced upon exposure to the agonist, and reached a plateau after approximately 30 min of treatment; this effect was observed whether Ro 20-1724 was present or absent during the incubation, although in the second condition, a much lower level of maximal inhibition was found (40% versus 75% in the presence of Ro 20-1724). In both cases, however, the decrease in cAMP concentrations occurred with a half-life of 5–7 min. This value closely correlates with the rate of the fast decaying component of GTPase desensitization and opioid receptor binding.

Discussion

We have studied in detail the time course of desensitization of GTPase and adenylate cyclase to opioids and compared it with the decline in opioid receptor binding activity resulting from the exposure of intact cells to an agonist.

The existence of rapidly reversing mechanisms complicates the analysis of desensitization. For instance, the desensitization of the β-adrenoceptor, which is the system more extensively studied to date, is a complex multistep process and involves a rapid loss of responsiveness followed by a slower down-regulation of receptors (for a review see Ref. 25). In NG108-15 cells, Green and Clark (26), for muscarinic, and Law *et al.* (23), for opioid receptors, have shown that a similar pattern may also account for the desensitization of adenylate cyclase to inhibitory receptors.

In order not to overlook some aspects of this process due to a rapid reversal which could conceivably take place during the preparative procedures, we have studied in parallel deprived and nondeprived membranes. In the latter, the constant presence of the agonist should stabilize any labile state of the desensitized receptor.

The agonist-induced loss of responsiveness of GTPase can be distinguished as two decaying processes regardless of whether desensitization is studied in "deprived" or "nondeprived" conditions, although, in the second case there is a

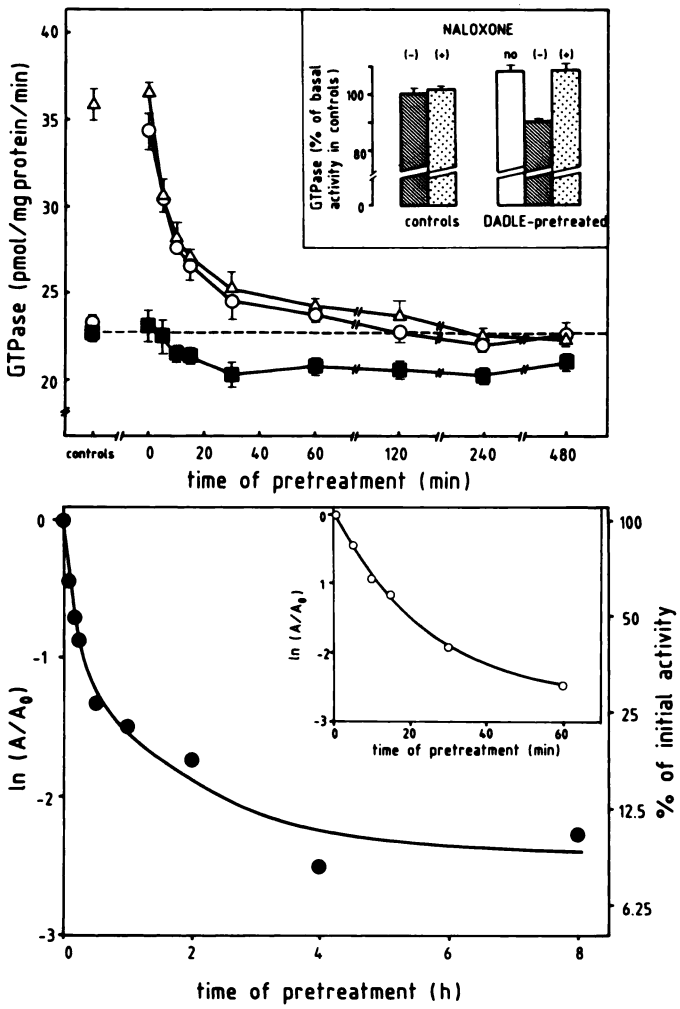

Fig. 2. Time course of desensitization of the opioid-stimulated GTPase in nondeprived membranes upon pretreatment of intact cells with DADLE. Intact cells were pretreated with the vehicle (controls) or with 100 nM DADLE for the indicated times. Cells were then washed with cold medium containing (except in controls) 100 nM DADLE, which was thereafter present throughout all experimental procedures, including the GTPase assay. *Upper panel:* GTPase was assayed without (○) or with the further addition of either 100 μM naloxone (■) or 100 μM DADLE (△). – – –, the activity measured in controls in the presence of 100 μM naloxone. Time "0" represents the activity observed in membranes derived from cells which were not pretreated with DADLE but for which 100 nM DADLE was present during washing and during all the experimental procedures thereafter, including the GTPase assay (data are the means ± standard errors of triplicate determinations of an experiment which was repeated twice with similar results). *Inset:* Effect of (−)- and (+)-naloxone on the apparent basal activity in nondeprived membranes. GTPase was assayed in nondeprived membranes derived from cells pretreated with 100 nM DADLE for 1 hr, without (□) or with the further addition of either 100 μM (−)-naloxone (▨) or 100 μM (+)-naloxone (□). Results are expressed as percentage of the basal GTPase activity measured in controls (22 ± 1 pmol mg^{-1} min^{-1}) (data are the means ± standard errors of five experiments). *Lower panel:* EXPFIT analysis of the decay of net stimulated GTPase activity under nondeprived conditions. The net activity is estimated as the difference between the activity measured upon the addition of DADLE (100 μM) and either the activity in the presence of 100 μM naloxone (*lower panel*, ●) or the activity of the control untreated cells (*inset*, ○). The data obtained from two independent experiments performed on two different batches of cells were analyzed as detailed in the legend of Fig. 1. Model 2 provided a better fit than model 1 ($p < 0.05$) for the net activity over the naloxone baseline (●). For the data in the *inset* the exponential models used did not include an asymptotic component, since the net activity was 0 after 4 hr of pretreatment in both experiments. Although in this case the comparison between the two- and one-component models yielded a $p = 0.08$, the former model is clearly more appropriate to describe the data, as is evident from the inspection of the computer-generated line (see *inset*), and the low level of significance is due to the paucity of available experimental points rather than to a true monoexponential decay. Time constants are tabulated in Table 1. The mean values (pmol × mg^{-1} × min^{-1}) for the net GTPase activity measured at time 0 are: over the naloxone baseline, 17.7 ± 1.31; over control, 12.6 ± 1.15. The naloxone baseline was taken as the mean of the GTPase activity measured in the presence of 100 μM naloxone starting after 30 min of treatment.

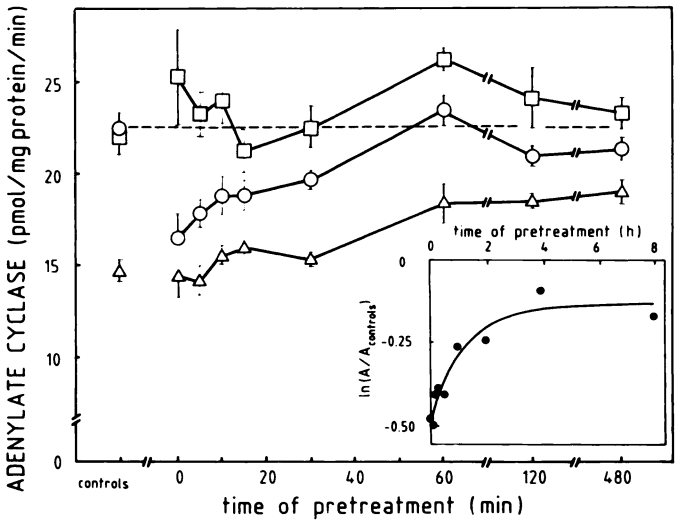

Fig. 3. Time course of desensitization of adenylate cyclase in nondeprived membranes upon pretreatment of intact cells with DADLE. Intact cells were pretreated with the vehicle (controls) or with 100 nM DADLE for the indicated times. Cells were then processed in the presence (except in controls) of DADLE, as described in the legend of Fig. 2. Adenylate cyclase was assayed without (○) or with the further addition of either 100 μM naloxone (□) or 100 μM DADLE (△). – – –, the basal activity measured in controls. Time "0" represents the activity observed in membranes prepared as described for time 0 in the legend of Fig. 2 (data are the means ± standard errors of quadruplicate determinations of an experiment which was repeated twice with similar results). The *inset* is the exponential analysis of the attenuation of opioid-mediated inhibition of adenylate cyclase activity as a function of the time of pretreatment. The data are means of two independent experiments and indicate the activity measured in the presence of 100 μM DADLE expressed as percentage of control. —, best-fitting theoretical curve averaged from the simultaneous fitting of the two experiments and consistent with the monoexponential ($t_{1/2}$ = 57 ± 21 min) reduction of the initial inhibition (38 ± 4.7%) which approaches a baseline of remaining inhibition of 12.5 ± 0.44%. No further improvement of the fit can be obtained by the introduction of a second, slower exponential component of decrease of inhibition for the baseline ($p = 0.99$).

greater proportion of initial activity which decreases rapidly and a smaller component of nondecaying activity can only be identified when the shift of baseline caused by naloxone is considered. The biphasic desensitization of GTPase activity calls for two explanations. One is that there are two components of stimulated GTPase activity possibly related to two different enzymes both activated from the receptor. The other is that the inactivation of the same enzyme follows two mechanistically distinct processes. The fact that the relative percentage of these two components is significantly different whether or not the agonist is maintained during the assay procedure suggests that the second explanation is more likely. This difference may indicate that part of the desensitization process undergoes reversal in the course of the preparative procedures. Since the rapidly decaying component is likely to be also the one which

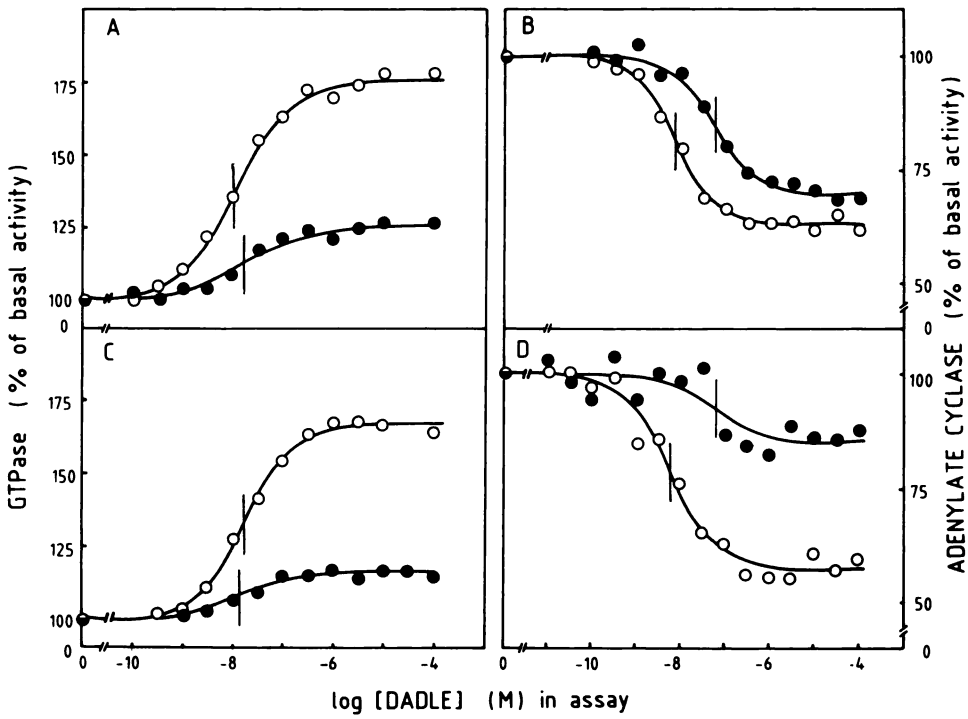

Fig. 4. Effects of pretreatment of intact cells with DADLE on the concentration-effect relationship of DADLE on GTPase and adenylate cyclase. Intact cells were pretreated without (○) or with (●) 100 nM DADLE for 1 (A and B) or 24 (C and D) hr. GTPase (A and C) and adenylate cyclase (B and D) were assayed in deprived membranes, in the presence of DADLE at the indicated concentrations. Concentration-response curves were analyzed using the computer program ALLFIT (see Experimental Procedures). *Bars* crossing the curves indicate the computed EC_{50} values of DADLE, which were: A, controls = 11 ± 0.1 nM, DADLE pretreated = 16 ± 4 nM, $p = 0.127$; B, controls = 7.0 ± 0.1 nM, DADLE pretreated = 57 ± 8 nM, $p < 0.001$; C, controls = 17 ± 1 nM, DADLE pretreated = 14 ± 0.3 nM, $p = 0.46$; D, controls = 5.8 ± 1.5 nM, DADLE pretreated = 60 ± 47 nM, $p = 0.006$. Results are expressed as percentage of basal enzymatic activity observed, respectively, in controls and membranes derived from DADLE-pretreated cells. Basal GTPase activities were: controls = 24 ± 2, DADLE pretreated for 1 hr (A) = 24 ± 4, DADLE pretreated for 24 hr (C) = 18 ± 0.3 pmol/mg of protein/min. Basal adenylate cyclase activities were: controls = 30 ± 4, DADLE pretreated for 1 hr (B) = 27 ± 7, DADLE pretreated for 24 hr (D) = 30 ± 4 pmol of cAMP/mg of protein/min. Data are the means of two (B and D) or three (A and C) experiments.

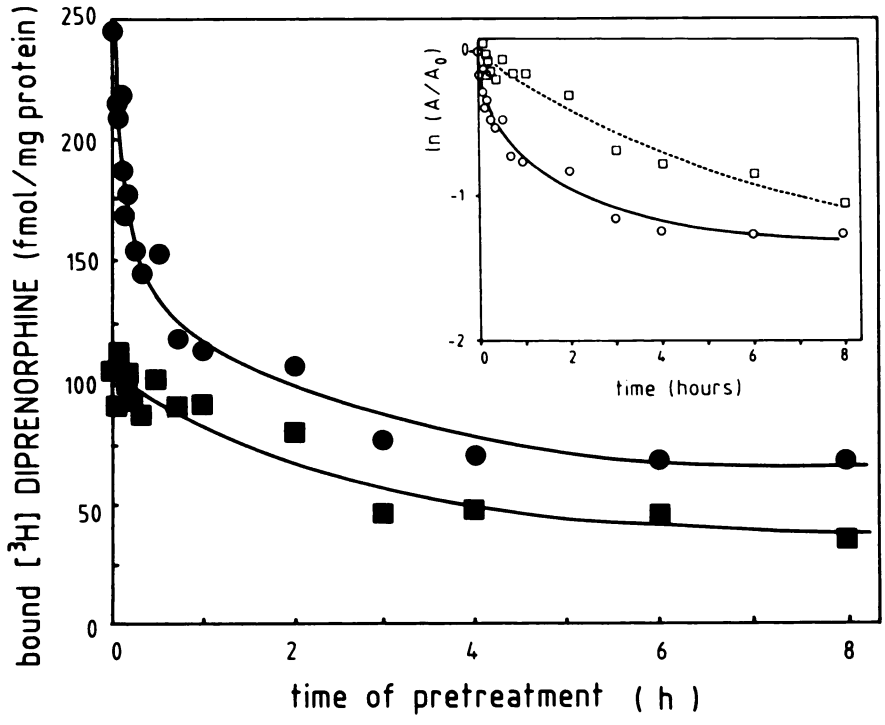

Fig. 5. [^{3}H]Diprenorphine binding in membranes prepared following exposure of intact cells to DADLE. Cells were treated for the times indicated with 100 nM DADLE, membranes were prepared, and the binding of [^{3}H]diprenorphine (0.25 nM) was determined as described in Experimental Procedures, in the presence (■, □) or absence (●, ○) of GTP (100 μM) and NaCl (100 mM). The data are the means of three independent experiments, each assayed with quadruplicate determinations. —, the best-fitting model of the data computed with the program EXPFIT. Model 2 (see Experimental Procedures) was significantly better in describing the data than model 1 for the binding measured in the absence of Na^+ and GTP ($p = 0.027$), but not for the binding measured in their presence ($p = 0.81$). The best-fitting parameters are given in the text. The *inset* is a replot of the main panel in logarithmic scaling following normalization to the respective initial binding for each curve.

recovers quickly upon removal of the agonist, a larger proportion of initial activity is expected to be found in this component when the constant presence of the agonist (nondeprived conditions) inhibits such reversal. Therefore, although the time constants of desensitization measured under deprived and nondeprived conditions are comparable, it is only under nondeprived conditions that the loss of responsiveness of GTPase activity more closely reflects the situation of the membrane in an intact cell exposed to the agonist. This indicates that the major part (80%) of the initial receptor responsiveness is lost within the first 10–20 min with a half-time of only 5–7 min. Such a rapid desensitization of opioid receptor function has

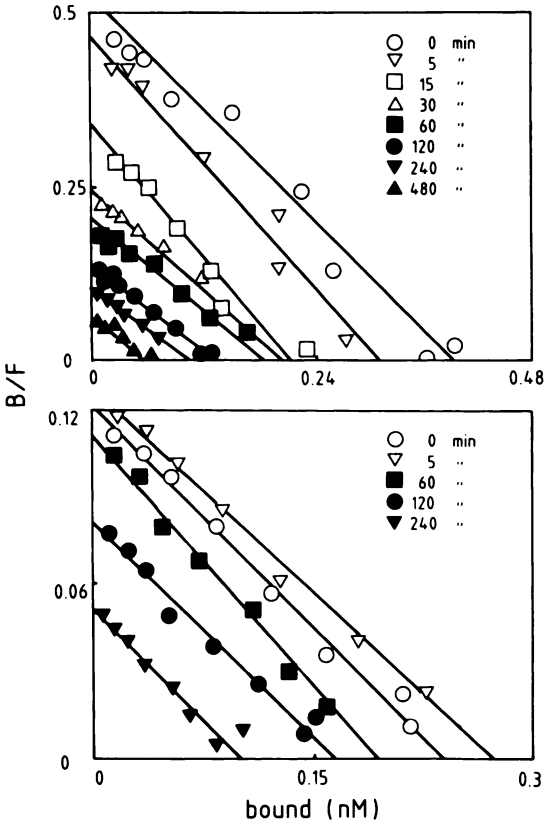

Fig. 6. Scatchard replots of the binding isotherms of [^{3}H]diprenorphine measured in the absence (*upper panel*) or presence (*lower panel*) of Na^+ and GTP in membranes prepared from cells exposed for different times to 100 nM DADLE. Binding isotherms were obtained as described in Experimental Procedures and analyzed with the computer program LIGAND. A model based on a single class of sites plus a nonsaturable component (nonspecific binding) described the data satisfactorily, since for none of the curves shown could a further statistical improvement of the fit be obtained using a two-site model. The binding affinities ($\times 10^9$ M^{-1}) measured in the absence of Na^+ and GTP were: 0 time, 1.51 ± 0.3; 5 min, 2.11 ± 0.35; 15 min, 2 ± 0.23; 30 min, 1.3 ± 0.4; 60 min, 1.1 ± 0.2; 120 min, 1.2 ± 0.09; 240 min, 1.2 ± 0.4; 480 min, 1.32 ± 0.5. In the presence of Na^+ and GTP: 0 time, 0.52 ± 0.03; 5 min, 0.49 ± 0.03; 60 min, 0.59 ± 0.05; 120 min, 0.49 ± 0.05; 240 min, 0.51 ± 0.07. The data are presented in Scatchard coordinates, with *solid lines* derived from the computer fit.

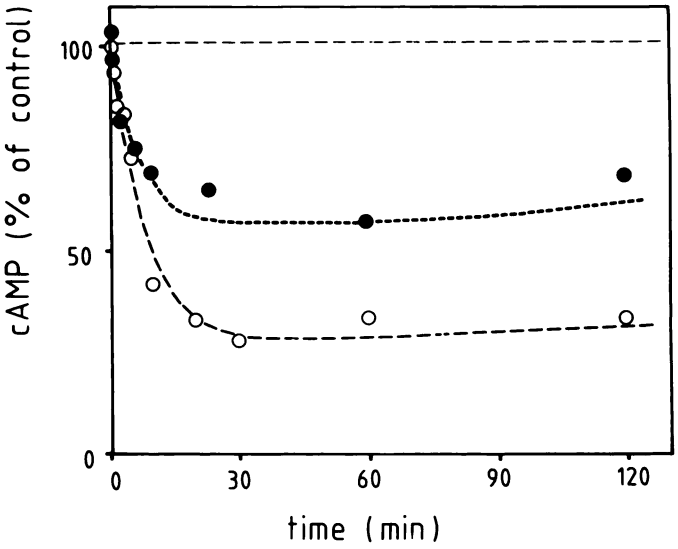

Fig. 7. Time course of the decrease of cAMP levels produced by DADLE in intact NG108-15 cells. Intact cells were preincubated for 30 min at 37° in the presence (○) or absence (●) of Ro 20-1724 (100 μM). DADLE (100 nM) or vehicle was added to triplicate tubes and the incubation was arrested at the indicated times as described in Experimental Procedures. The levels of cAMP were measured by RIA and represent the sum of intracellular and extracellular levels. The data points are means of four (○) and three (●) experiments, respectively, and are reported as percentage of the levels of cAMP measured in cells incubated for the corresponding times in the absence of the agonist. The mean values of cAMP levels measured at 0 time (incubation arrested immediately before the addition of DADLE) were (pmol/mg of protein) 368 ± 74 in the presence and 21.2 ± 3.2 in the absence of Ro 20-1724, respectively. The cAMP levels in the absence of the agonist remained within 10% of the control value during the time of the incubation for the experiments performed without phosphodiesterase inhibitor, whereas they tended to increase (to a maximum of 30–50% over control) when Ro 20-1724 was present.

not been described in the literature before. However, the fact that the antagonist only in "nondeprived" conditions can inhibit the apparent basal activity to a level lower than the control baseline is intriguing. Although this phenomenon is steroselective and, thus, clearly receptor mediated, any explanation, at the moment, would not be beyond the realm of speculation. Cerione *et al.* (11), using purified β-adrenoceptors reconstituted with purified N_s in liposomes have reported that the antagonist alprenolol produces a small but significant and stereospecific reduction of the basal GTPase activity obtained in the absence of the agonist. They suggest that the antagonist, upon binding, may induce a form of the receptor with a reduced ability to interact with N; such a mechanism could account for the effect of naloxone reported here, although it would not explain why this effect can only be observed in desensitized membranes at a time when the rapidly decaying portion of GTPase activity is lost.

Further information about the nature of the two agonist-induced processes which cause the loss of opioid receptor function can be obtained from the comparison between desensitization of GTPase activity and the decline of opioid receptor binding activity. Here we show that the decline of binding activity is biphasic and has decay constants close to those recorded for GTPase desensitization when it is monitored in the absence of sodium and GTP, whereas the same decline is monophasic and accounts for only the slow component of GTPase desensitization when it is monitored in the presence of ion and nucleotide. This apparently is a paradox, since the stimulation of GTPase activity is measured in the presence of Na and GTP. However, it has been suggested recently, using kinetic studies, that [^{3}H]diprenorphine primarily labels receptor-N protein complexes in the absence and free receptor units in the presence of Na and GTP (24). Thus, when sodium and GTP are present in the assay, a condition under which the tracer is bound to free receptor units, the agonist-induced decline of binding activity reflects their disappearance from the membrane, a process known as down-regulation. In contrast, in the absence of sodium and GTP, under which condition the tracer is bound to a complex between receptor and N protein, the agonist-induced loss of activity is multiexponential because it reflects not only down-regulation, but also a more rapid process which, most likely, represents the loss of coupling between receptor and N protein; both processes would lead to the disappearance of receptor-stimulated GTPase activity and this explains why the latter only agrees with the decline of binding activity monitored in the absence of Na and GTP: it is

only under those conditions that the coupled form of the receptor can be detected.

Upon removal of the agonist, a proportion of GTPase activity similar to that rapidly desensitized recovers with an analogously rapid time constant, whereas the remaining activity recovers at a rate much slower than the corresponding slow component of desensitization. This brings further support to the idea that the rapid and slow components of GTPase desensitization represent uncoupling and down-regulation, respectively. In fact, down-regulation can be expected to reverse a rate even slower than its onset since its recovery would depend upon receptor synthesis and turnover in the cell. The agreement found between the decline of binding activity and desensitization of GTPase is closer when the enzyme is studied under deprived conditions; this is not surprising since binding can only be measured after washing the cells from the agonist (i.e., under deprived conditions). Conversely, it can be extrapolated from the difference between deprived and nondeprived conditions observed for GTPase that the rapid decline of coupling ability may account for the major loss of receptor functionality in an intact cell exposed to a constant concentration of opioid agonist.

Surprisingly, however, there is no evidence for a quick loss of responsiveness of adenylate cyclase to the agonist, even in nondeprived membranes. In fact, the time course of desensitization of this enzyme more closely resembles the slow component of the loss of responsiveness of GTPase and correlates with the rate of disappearance of opioid receptor binding assessed in the absence of sodium and GTP, which, as previously discussed, would reflect down-regulation of the receptor. Although GTPase and adenylate cyclase are assayed in almost identical conditions, the possibility has to be considered that differences in the assay conditions may account for the different time courses of desensitization of the two enzymes. In particular, the relatively high concentration of GTP (10 μM), necessary to observe opioid-mediated inhibition of adenylate cyclase, might accelerate the reversal of the fast component of desensitization, despite the continuous presence of the agonist in the assay. Although we cannot rule out this possibility, we consider it unlikely for two reasons: 1) an analogous discrepancy between the extent of impairment of opioid-mediated stimulation of GTPase and inhibition of adenylate cyclase is also observed in membranes derived from NG108-15 cells treated with pertussis toxin (27), which produces a covalent modification of N which, therefore, cannot be reversed in the assay; and 2) the intracellular concentration of GTP, likely to be higher than 10 μM, would preclude, if it were able to accelerate a reversal process, the detection of the fast decaying component even for the GTPase and the binding assays. It is rather conceivable that the difference between the time course of desensitization of GTPase and adenylate cyclase reflects differences between their respective relationship with the receptor. Two alternative interpretations may be proposed.

One is that a substantial proportion of the opioid-stimulated GTPase activity measured in membranes is not involved in the inhibition of adenylate cyclase. The finding that NG108-15 cell membranes contain far larger amounts of N_o than of N_i would be in line with this hypothesis (10). In fact, it has been suggested recently that, in contrast to N_i, the purified α subunit of N_o cannot inhibit directly the catalytic component of adenylate cyclase (28); it has been proposed that this GTP-regulatory protein might be involved in coupling inhibitory receptors to other membrane signal-transducing systems (28). An interesting implication of the data presented here would then be that the opioid receptors in NG108-15 cells are also linked to an effector other than adenylate cyclase and which could be, for instance, an ion channel whose activation or inhibition occurs very rapidly upon the receptor-agonist interaction. Indeed, recent data suggest that the opioid receptor in NG108-15 cells may be coupled in an inhibitory fashion via N_o to a voltage-dependent calcium channel (29). Such an interpretation might also resolve the apparently puzzling relationship between the rate at which the fast component of GTPase desensitization takes place and the kinetics of inhibition of cAMP levels measured in intact cells. Under the experimental conditions used here (presence of phosphodiesterase inhibitor; monitoring of total, intracellular, and extracellular cAMP), the rate of change of cAMP concentration in intact cells is conceivably not unaffected either by *degatative* processes or by active mechanism of secretion and, thus, largely reflects the rate of activation of the receptor system following acute exposure to the agonist. As is shown here, the time constant of this activation is indistinguishable from that recorded for the rapid component of desensitization, which implies that the receptor is activated and desensitized by the agonist at identical rates and that the maximal inhibition coincides with the maximal loss of responsiveness of the system.

A second interpretation is that a tight relationship exists between the receptor and the N protein, whereas the interaction between GTPase (N protein) and adenylate cyclase involves an amplification mechanism. Such an interpretation is supported by the present findings showing that desensitization of GTPase occurs as a "noncompetitive" process with regard to the agonist, whereas the loss of responsiveness to adenylate cyclase exhibits a more complex pattern. According to this interpretation, a rapid uncoupling between the receptor and the N protein, followed by the disappearance of the receptor, would invariably lead to a reduction of the maximal stimulation of GTPase produced by the agonist. In contrast, a shift in the EC_{50} of the agonist in inhibiting adenylate cyclase would be initially observed as a consequence of a loss of efficiency of the amplification mechanism and, then, would be followed by reduction in the maximal inhibition produced by the agonist. Since the time course of desensitization of adenylate cyclase was studied here as the decrease in the inhibition produced by a supramaximal concentration of DADLE (100 μM), it is possible that only the slow component of the process (decrease in maximal effect) was apparent, whereas the rapid one (shift in the EC_{50} of the agonist) could not be detected but would in fact correlate with the fast decaying GTPase activity and the rapid disappearance of opioid receptor binding measured in the absence of sodium and GTP. This would explain why the concentration-response curves of DADLE, in acutely inhibiting adenylate cyclase, displayed similar shifts in EC_{50} after 1 and 24 hr of pretreatment with DADLE, whereas the decrease in maximal inhibition was much more pronounced after 24 hr than after 1 hr of exposure of the cells to the agonist. Current studies are in progress to differentiate between these two possibilities.

In conclusion, the present study shows that, upon treatment of intact NG108-15 cells to an opioid agonist, the opioid-GTPase system undergoes a rapid loss of responsiveness which does not correlate with the rate at which desensitization of adenylate cyclase occurs and appears to reflect an uncoupling between the receptor and the N protein. Conversely, the slow

component of GTPase desensitization and the loss of responsiveness of adenylate cyclase seem to be accounted for by a down-regulation of the opioid receptor.

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Send reprint requests to: Dr. Tommaso Costa, Department of Neuropharmacology, Max-Planck-Institut fur Psychiatrie, Am Klopferspitz 18A, D-8033 Planegg-Martinsried, Federal Republic of Germany.