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Opioid Receptors Are Coupled Tightly to G Proteins but Loosely to Adenylate Cyclase in NG108-15 Cell Membranes

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SUMMARY

Opioid receptors in intact NG 108-15 cells were irreversibly inactivated with increasing concentrations of the alkylating antagonist β-chlornaltrexamine (CNA). The consequence of the reduction in density of opioid binding sites (quantified by saturation analysis of opioid binding in membranes) was studied at two steps of opioid receptor-mediated responses, (a) stimulation of high affinity GTPase and (b) inhibition of basal adenylate cyclase. Both agonist-mediated stimulation of GTPase and inhibition of adenylate cyclase activities were progressively reduced as the concentration of CNA in the pretreatment was increased. However, the loss of responsiveness for the two enzymes differed in two aspects. First, the diminution of GTPase responsiveness was in agreement with the loss of binding sites and took place at concentrations of CNA that were lower than those necessary to reduce responsiveness of adenylate cyclase. Second, the loss of responsiveness of GTPase occurred simply as reduction of maximal stimulation, whereas that of adenylate cyclase involved an initial reduction of apparent agonist affinity (10-fold) that was followed by a decrease in maximal effect. We next examined the loss of responsiveness of both GTPase and adenylate cyclase in membranes prepared from cells that had been exposed to increasing concentrations of pertussis toxin (PTX) to inactivate PTX-sensitive G proteins *in vivo*. Also in this case, the extent of reduction in responsiveness was more pronounced for GTPase than for adenylate cyclase, especially in membranes treated with high concentrations of PTX. However, the pattern of loss was identical for the two enzymes and involved a main reduction in maximal effect of the agonist that was followed only after a large degree of inactivation (>60%) by a diminished apparent affinity for the agonist. Opioid receptor-mediated inhibition of cAMP accumulation in intact cells exhibits an IC_{50} for the agonist that is 30–10 times lower than that measured in membranes for stimulation of GTPase or inhibition of cyclase, respectively. Treatment of cells with either CNA (1 μM) or various concentrations of PTX altered the concentration-response curves for agonist-mediated inhibition of cAMP accumulation in a manner similar to that observed for adenylate cyclase in membranes, inasmuch as both maximal inhibition and apparent affinities for the agonist were decreased. However, this decrease in affinity (5-fold) was not sufficient to eliminate the discrepancy in agonist potency between membranes and intact cells. We conclude that (a) the mechanism of signal transmission between opioid receptors and adenylate cyclase involves tight coupling between receptor and G protein but a loose one between G protein and catalytic subunit of the cyclase and (b) receptor-mediated inhibition of intracellular cAMP levels requires an amplification factor that is not operating under the conditions of adenylate cyclase assay in isolated membranes.

Activation of opioid receptors in NG 108-15 cells results in the inhibition of adenylate cyclase activity (1) and of voltage-dependent Ca^{2+} channels (2). Both these events are mediated via activation of G proteins that are substrates of PTX (2, 3). PTX treatment abolishes opioid-mediated stimulation of low K_m GTPase activity in membranes isolated from NG 108-15 cells (4), indicating that this stimulation reflects the interaction between opioid receptors and PTX-sensitive G proteins in native membranes.

Exposure of intact cells to an opioid agonist results in loss of responsiveness of both GTPase (5) and adenylate cyclase (6) and in marked down-regulation of opioid receptors (5–7). It is believed that the agonist-induced loss of responsiveness occurs through two distinct mechanisms; one is an apparent loss of the ability of the receptor to interact with the effector system (desensitization), which occurs more rapidly, the other is the reduction of receptor density in the cell membrane (down-regulation) (6, 8).

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ABBREVIATIONS: PTX, pertussis toxin (also known as islet-activating protein); AppNHp, 5′-adenylylimidodiphosphate; CNA, β-chlornaltrexamine; Ro 20-1724, DL-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; DMEM, Dulbecco's modified Eagle medium; PBS, Dulbecco's phosphate-buffered saline; G protein, GTP-binding regulatory proteins mediating stimulation (G_s) or inhibition (G_i) of adenylate cyclase; G_o, a closely related G protein of unknown function; NG108-15 cells, neuroblastoma × glioma hybrid cells; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; DADLE, [D-Ala^2,D-Leu^5]enkephalin; IC_{50}, half-maximal inhibitory concentration; EC_{50}, half-maximal effective concentration; *K*, equilibrium affinity constant; *R*, maximal binding capacity.

We recently reported that the opioid-induced loss of responsiveness of GTPase occurs at a faster rate and exhibits a different pattern than that of adenylate cyclase (8). The agonist-induced disappearance of GTPase stimulation was manifested as reduction of maximal stimulation with no changes in apparent agonist potency, whereas the corresponding decrease in the ability of the agonist to inhibit the cyclase occurred initially as reduction of agonist apparent affinity that was followed by reduction of maximal inhibitory effect (8). As an explanation, we proposed that the "reserve" in the multiple steps coupling opioid receptor occupancy by the agonist to final biological response might be present at the step of interaction between activated G protein and catalyst of the cyclase, rather than between activated receptor and G protein.

Evidence for a large receptor reserve in opioid receptor-mediated cAMP response in NG 108-15 cells has been documented previously, either by the use of the irreversible antagonist chlornaltrexamine (9) or by the analysis of occupancy-activity relationships in intact cells (10, 11). In all those studies, however, receptor occupancy was directly compared with agonist-mediated inhibition of cAMP accumulation in intact cells rather than with opioid responses measured in the membrane. Thus, those data do not indicate which step of signal transmission is responsible for the lack of linearity between binding and activity.

In this study we have examined the pattern of loss of responsiveness to opioid receptors for GTPase and adenylate cyclase in membranes after inactivation, in intact cells, of either opioid binding sites, [using the alkylating antagonist chlornaltrexamine (9)] or G proteins (using pertussis toxin). Similarly, we have studied and compared the effect of these two manipulations on the ability of opioid agonist to inhibit cAMP accumulation in intact cells.

Here we show results that are consistent with the notion that opioid receptor-mediated inhibition of adenylate cyclase involves a tight relationship between receptor number and G protein activation but a loose one between receptor number and cyclase inhibition. We also show, however, that the lack of linearity between receptor-mediated stimulation of GTPase and inhibition of cyclase observed in isolated membranes is insufficient to account for the receptor reserve that is apparent in intact cells between agonist binding and inhibition of cAMP accumulation. This suggests that additional factors that are missing or perhaps functionally inhibited under the conditions of the enzymatic assays *in vitro* are necessary to amplify opioid receptor-mediated regulation of cAMP levels *in vivo*.

Experimental Procedures

Materials. DADLE was purchased from Bachem (Bubendorf, Switzerland). [^{3}H]DADLE, [^{3}H]diprenorphine (40 and 36 Ci/mmol, respectively), [^{32}P]NAD$^+$ (0.8–1 Ci/μmol), and [γ-^{32}P]GTP (6 Ci/μmol) were obtained from Du Pont de Nemours (Dreieich, FRG). Chlornaltrexamine·HCl was obtained from RBI (Anawa, Wangen, Switzerland). Naltrexone·HCl was a gift from Du Pont de Nemours, Ro20-1724 was donated by Hoffmann-La Roche (Basel, Switzerland). Pertussis toxin was purchased from List Biologicals (CA). GTP, AppNHp, ATP, 2′-*O*-monosuccinyladenosine 3′:5′-cyclic monophosphate tyrosyl methyl ester, creatine kinase, and phosphocreatine were purchased from Sigma (Muenchen, FRG). Media and serum for cell culture were from Gibco BRL (Eggenstein, FRG), whereas disposable sterile plasticware was purchased from Nunc (Wiesbaden, FRG).

Cells. NG 108-15 neuroblastoma × glioma cells were grown in DMEM containing (in 100 ml) 0.37 g of Na_2HCO_3, 0.45 g of glucose, 5 ml of fetal calf serum, no sodium pyruvate, and no antibiotics but supplemented with HAT (100 μM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine) and maintained at 37° in a humidified atmosphere of 7.5% CO_2 and 92.5% air, as described previously (8, 10). Confluent 80-cm^2 flasks were harvested in Ca^{2+}/Mg^{2+}-free PBS by gently tapping the flasks on the palm of the hand. Cell clumps were dispersed by repipetting and the cells were sedimented in a table-top centrifuge (240 × *g* for 5 min at room temperature). Cell pellets were frozen at −70° before membrane preparation.

Treatment of intact cells with CNA or pertussis toxin. Confluent cell monolayers were washed three times with PBS to remove any trace of contaminating serum; subsequently, 10 ml of PBS prewarmed at 37° and containing various concentrations of CNA were added and the flasks were returned to the incubator. Stock solutions of CNA were prepared in ethanol at a concentration of 10 mM so that, with the highest concentration of CNA (10 μM), the final ethanol added was 0.1%. Appropriate controls indicated that this concentration of ethanol did not alter GTPase or adenylate cyclase activities. The incubation with CNA lasted for 45 min and was arrested by aspiration of the incubation medium and addition of ice-cold Ca^{2+}/Mg^{2+}-free PBS containing 0.1% bovine serum albumin. The cells were harvested in this medium, subjected to three cycles of low speed centrifugation to remove unreacted CNA, and finally frozen for membrane preparation or used immediately for experiments on the accumulation of cAMP.

PTX was resuspended in sterile PBS at 100 μg/ml and directly added to the cell medium. Confluent monolayers of cells were incubated with the toxin in DMEM for 18 hr. At the end of the incubation, the medium was removed and the cells were harvested as described before.

Preparation of membranes for GTPase, adenylate cyclase, and binding assays. Membranes from frozen cells were prepared as detailed elsewhere (8). The membranes were stored as aliquots in 5 mM Tris·HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EGTA, at a protein concentration of 2–3 mg/ml. In a few experiments, CNA treatment was done on membranes rather than on intact cells. For these experiments, membrane aliquots were thawed, pelleted at 20,000 × *g* for 15 min, and resuspended in 15 mM sodium phosphate, pH 7.5 at 3°. This procedure was repeated twice before a final resuspension in the same buffer containing various concentrations of CNA. Incubations lasted for 30 min at 20° and were arrested by the addition of the same volume of sodium phosphate buffer supplemented with 0.2% bovine serum albumin. Membranes were centrifuged (20,000 × *g* for 15 min), resuspended three times in the same buffer, and immediately used for GTPase assay.

GTPase and adenylate cyclase assays. GTPase activity was assayed according to the method of Cassel and Selinger (12) with minor modifications as described previously (8). The reaction mixture included 0.2 mM EGTA, 0.2 mM dithiothreitol, 10 mM $MgCl_2$, 0.5 mM ATP, 1 mM AppNHp, 5 mM phosphocreatine, 50 units/ml creatine kinase, 200–500 nM GTP, 100 mM NaCl, and 51 mM Tris·HCl (pH 7.5) in a total volume of 0.1 ml. [γ-^{32}P]GTP was added to a final specific activity of 20,000–30,000 cpm/pmol except in some experiments in which the specific activity was increased to 90,000–100,000 cpm/pmol (see Fig. 7).

In order to study the effect of opioid receptor-mediated inhibition on basal adenylate cyclase activity in the absence of any stimulatory ligand and in the presence of saturating concentrations of ATP, it was necessary to monitor the formation of cAMP by radioimmunoassay, because any other protocol based on cyclization of radiolabeled ATP did not provide enough sensitivity. The reaction mixture was identical to that used for GTPase activity, except for the omission of AppNHp, inclusion of 100 μM Ro20-1724, and use of 1 μM GTP (8). Neither the removal of AppNHp from nor the addition of Ro20-1724 to the GTPase assay had any significant effect on the concentration-response curve for DADLE.

Both reactions were started by the addition of membrane suspensions (5–10 μg of protein), lasted 10 min at 37°, and were arrested by the addition of either 0.1 ml of ice-cold 40 mM H_3PO_4 (for GTPase) or

0.1 ml of ice-cold 0.05 N HCl (for adenylate cyclase). Determination of P_i released or cAMP formed were carried out as described (8).

Binding assay. Saturation analysis of opioid receptors was carried out with a membrane preparation cruder than that used for GTPase and adenylate cyclase assay, because the construction of binding isotherms that extend to the sub-nanomolar range of agonist concentrations requires a "tracer" concentration of radiolabeled ligand and a large reaction volume. However, in some experiments the reduction of binding as a function of the concentration of CNA in the pretreatment was also examined in purified membranes, using a single concentration (2 nM) of [^{3}H]DADLE, and was found to agree with the decrease in B_{max} determined in less-purified membranes. Membrane preparation and the binding reaction was performed as described extensively elsewhere (8), except that the reaction mixture (2 ml) included 50 mM Tris·HCl (pH 7.5) and 10 mM $MgCl_2$. [^{3}H]DADLE or [^{3}H]diprenorphine were used as monitoring ligands. Saturation isotherms were obtained using a fixed concentration of radiotracer (0.2 nM) and increasing concentrations of unlabeled ligand.

ADP-ribosylation assay. PTX-catalysed ADP-ribosylation of G proteins in membranes prepared from cells exposed to PTX was carried out as described earlier (13). The reaction was arrested by the addition of 2× concentrated sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and the samples were heated for 5 min at 95° before electrophoresis. ADP-ribosylated membrane proteins were separated using the discontinuous system of Laemmli (14). Slab gels (1.5 mm thick) consisted of a 10% separating gel (120 mm long) and a 5% stacking gel (10 mm long). ^{14}C-methylated proteins (Amersham) were used as molecular weight standards. Separations were conducted at 15° until the band of bromophenol blue reached the bottom of the gel. For autoradiography, gels were dried and exposed to Hyperfilm-βmax (Amersham).

Determination of opioid-mediated inhibition of cAMP accumulation in intact cells. NG 108-15 cells pretreated (as indicated) with either CNA or PTX were harvested as described under Cells. Cell pellets were resuspended in HEPES-buffered saline (referred to as HEPES buffer) of the following composition: 20 mM HEPES/NaOH, 113 mM NaCl, 1 mM EGTA, 2 mM $CaCl_2$, and 1 mM Mg_2SO_4. Aliquots (5×10^4 cells) of the cell suspension were transferred to prewarmed Eppendorf 1.5-ml plastic tubes containing HEPES buffer supplemented with 100 μM Ro 20-1724 (final concentration) and various concentrations of DADLE, to achieve a final total volume of 0.1 ml. A final concentration of 0.1% ethanol (necessary to solubilize Ro 20-1724) was present in all tubes. The incubations were carried out for 30 min at 37° and arrested by the addition of ice-cold 0.05 N HCl. The tubes were centrifuged (8000 × g for 5 min at 4°), and the supernatant was used for the determination of cAMP levels.

Radioimmunoassay of cAMP. The levels of cAMP were determined by radioimmunoassay after dilution of the samples in sodium acetate buffer (0.1 M, pH 6.5) and acetylation as described previously (8, 10).

Protein determinations. Proteins were determined as described by Peterson (15), using bovine serum albumin as standard.

Presentation and analysis of data. GTPase determinations were carried out in triplicate and exhibited an intraassay variability of usually less than 1.5% (based on a mean joint estimate of the variance for each assay). Adenylate cyclase determinations were carried out in quadruplicate and exhibited an intraassay error of less than 5%. The specific activity of the two enzymatic activities (pmol × 1/min × 1/mg of protein) varied as much as 2.5-fold from one membrane preparation to another. Except where indicated otherwise, most of the experiments presented are representative; each of them was repeated at least three times in different membrane preparations to verify the reproducibility of the results. Concentration-response curves for opioid-mediated stimulation of GTPase or inhibition of adenylate cyclase were analysed with the computer program ALLFIT (16) to obtain estimates of IC_{50} values and maximal (inhibitory or stimulatory) effect and of the statistical significance for their differences. Binding isotherms for opioid binding were analyzed by the computer program LIGAND (17). The covariances and joint 95% confidence ellipses of the estimated binding parameters were computed as described by Munson and Rodbard (18).

Results

Incubation of intact NG 108-15 cells with various concentrations (0.03–10 μM) of CNA resulted in a concentration-dependent loss of opioid receptors measured in membranes. Fig. 1 shows that the decrease of opioid binding activity in membranes obtained from cells treated with CNA was completely accounted for by a reduction of receptor density, with no significant changes in binding affinity. Identical results were obtained when the binding was monitored with the radiolabeled partial agonist diprenorphine. Thus, CNA appears to produce an irreversible inactivation of opioid receptors, which is consistent with a covalent interaction of this molecule with nucleophilic groups present on the binding site of the receptor.

The effect of exposure of intact cells to different concentrations of CNA on opioid-mediated stimulation of GTPase activity is shown in Fig. 2. GTPase activity stimulated by a maximal concentration (10 μM) of DADLE was markedly reduced by CNA pretreatment. Examination of net stimulated activity (i.e., activity measured in the presence of agonist minus that measured in its absence) as a function of the concentration of CNA used in the pretreatment (Fig. 2, *inset*) indicated that CNA pretreatment of intact cells suppressed up to 85% of DADLE-mediated stimulation, with an apparent IC_{50} of 14 nM. The apparent basal GTPase activity (measured in the absence of agonist) was also slightly reduced (up to 25%) by the treatment, suggesting that a portion of this activity may be accounted for by a spontaneously coupled opioid receptor. Preincubation of intact cells with 10 μM naltrexone together with 1 μM CNA prevented the inactivating effect of CNA on both stimulated and basal GTPase activity (Fig. 2). This indicates that the CNA-mediated reduction of both stimulated and basal activities required the interaction of the drug with opioid receptors and was not due to nonspecific alkylation of membrane proteins.

To examine the mechanism underlying the loss of receptor-mediated stimulation of GTPase induced by pretreatment with the alkylating antagonist, we analyzed concentration-response curves for DADLE-mediated stimulation of GTPase activity in membranes prepared from cells that had been treated with increasing concentrations of CNA. Fig. 3 shows that the reduction of opioid-mediated stimulation was entirely accounted for by a reduction of maximal effect of the agonist with no changes in its apparent affinity. In fact, a replot of the EC_{50} for DADLE as a function of the concentration of CNA used in the pretreatment (Fig. 3, *inset*) indicates that these values were randomly scattered around that observed in control membranes and did not exhibit any trend for an increase as the degree of receptor inactivation was progressively increased. Thus, the data indicate that the suppression of opioid-mediated GTPase responsiveness is "noncompetitive" with respect to the concentration of CNA in the pretreatment.

Treatment of membranes with CNA (1 μM) also resulted in the reduction of DADLE-mediated maximal stimulation of the enzyme with no change in agonist apparent affinity and this effect was also prevented by coincubation of CNA with 10 μM naltrexone (Fig. 4). However, the ability of CNA to inactivate opioid-mediated stimulation appeared to be much lower under these conditions. In fact, 1 μM CNA was an almost maximally

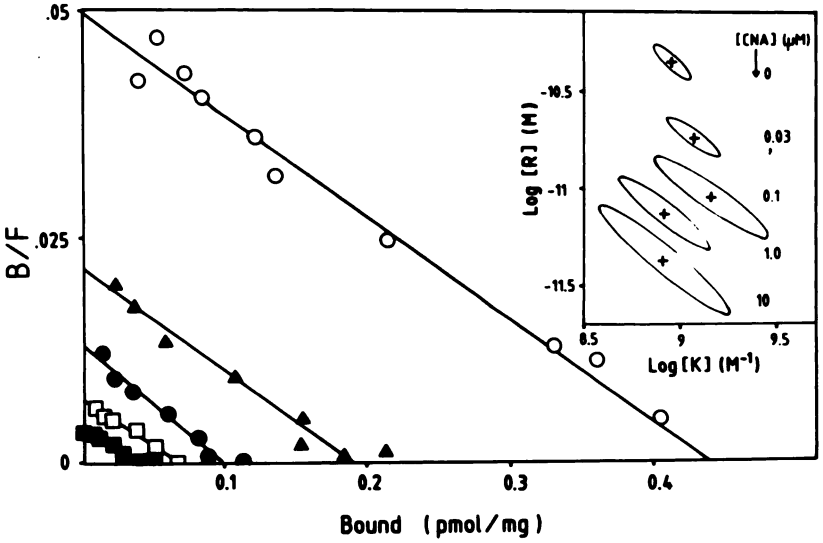

Fig. 1. Effect of CNA pretreatment on [^{3}H]DADLE binding. Membranes were prepared from cells pretreated with different concentrations of CNA as detailed in Experimental Procedures. Binding isotherms, obtained as competition of different concentrations of DADLE for a constant concentration of [^{3}H]DADLE (0.25 nM), were analysed with the computer program LIGAND and are displayed in Scatchard's coordinates. The concentrations of CNA (μM) used in the pretreatment were 0 (○), 0.03 (▲), 0.1 (●), 1 (□), and 10 (■). The K_d of DADLE measured in control was 1.1 (± 0.09 nM and B_{max} was 0.43 ± 0.03 pmol/mg of protein. *Inset*, *K–R* plot (18) of the same data. The log of the equilibrium binding affinity (*K*; M^{-1}) and total binding capacity (*R*, M) corresponding to the curves of the main panel are plotted together with the 95% confidence region for the parameter estimates. Confidence ellipses were computed from the covariance matrix of the computer fit (17). They show that the binding capacity is the only parameter significantly affected by CNA pretreatment.

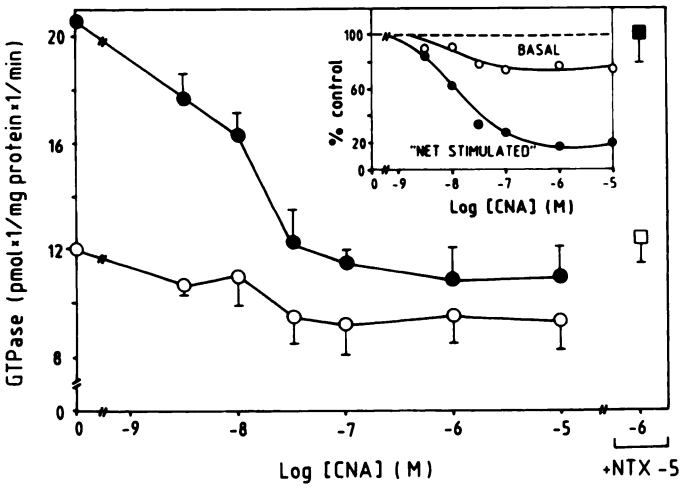

Fig. 2. Effect of CNA pretreatment on opioid-mediated stimulation of GTPase. Cells were pretreated with vehicle or different concentrations of CNA as indicated. Washing, harvesting of the cells, and preparation of the membranes was done as detailed in Experimental Procedures GTPase was assayed in the absence (○) or presence (●) of 10 μM DADLE. The high K_m activity was not altered by CNA and was subtracted from the data. On the *right* of the graph is shown the GTPase activity in the absence (□) and presence (■) of DADLE measured in membranes prepared from cells that were exposed simultaneously to 1 μM CNA and 10 μM naltrexone (*NTX*). The data are means ± standard errors of three independent experiments, which were assayed in triplicate. *Inset*, basal and net stimulated GTPase (activity in the presence minus activity in the absence of 10 μM DADLE) are replotted as percentage of their respective controls.

effective concentration in intact cells (Fig. 2) but produced only 32% inhibition of DADLE stimulation when used in incubations of membranes. Proteins present at higher concentration in membrane suspensions and acting as nonspecific scavengers or the lower temperature of reaction used for CNA pretreatment of membranes may be some of the factors responsible for this reduced efficiency of the alkylating agent, when inactivation of receptor is performed on membranes rather than intact cells. Nonetheless, CNA produced qualitatively similar effects on the concentration-response curve of DADLE for GTPase stimulation when receptors were inactivated either on isolated membrane preparations or on intact cells.

Examination of the ability of a maximal concentration of DADLE (10 μM) to inhibit adenylate cyclase activity in membranes prepared from cells treated with different concentrations of CNA (Fig. 5A) indicated that a reduction of responsiveness to opioid receptors of adenylate cyclase activity was obtained at concentrations of CNA that were systematically higher than those sufficient to markedly reduce GTPase stimulation. In fact, a clear decrease of DADLE-mediated inhibition (5A, *inset*) was only observed in membranes of cells pretreated with 1 and 10 μM CNA. Fig. 5B shows that CNA-induced inactivation of opioid receptors affected the concentration-response curves for DADLE-mediated inhibition of adenylate cyclase activity in a manner different from that observed for stimulation of GTPase. In membranes treated with 0.1 μM CNA, DADLE produced a maximal inhibition of adenylate cyclase (45%) that was identical to that observed in nontreated cells; however, the IC_{50} for DADLE was shifted 10-fold to the right. Upon treatment with higher concentrations of CNA, there was no further shift in the IC_{50} for DADLE, but a reduction in maximal inhibition became also apparent (Fig. 5B). Thus, the change in responsiveness to the agonist for adenylate cyclase after receptor inactivation involves an initial loss of sensitivity (i.e., an increase of apparent IC_{50}), which is then followed by a reduction of efficacy (i.e., decrease of maximal inhibitory effect) and differs from the pattern of loss of responsiveness observed for GTPase (compare Fig. 5B with Fig. 3).

Another means to inactivate opioid receptor-mediated responses in NG 108-15 cells is PTX. This toxin catalyses the ADP-ribosylation of G proteins in intact cells, which results in their irreversible inactivation and in loss of their ability to transduce signals from activated receptors to adenylate cyclase (19). To compare the loss of opioid responsiveness induced by inactivation of binding sites (CNA treatment) with that caused by inactivation of G proteins (PTX treatment), we examined DADLE-mediated stimulation of GTPase and inhibition of adenylate cyclase activities in membranes prepared from cells that had been exposed (18 hr) to different concentrations (0.01–100 ng/ml) of PTX. Fig. 6 shows that in these membranes there was a marked reduction of GTPase activity measured in the presence of 10 μM DADLE and a parallel (although less

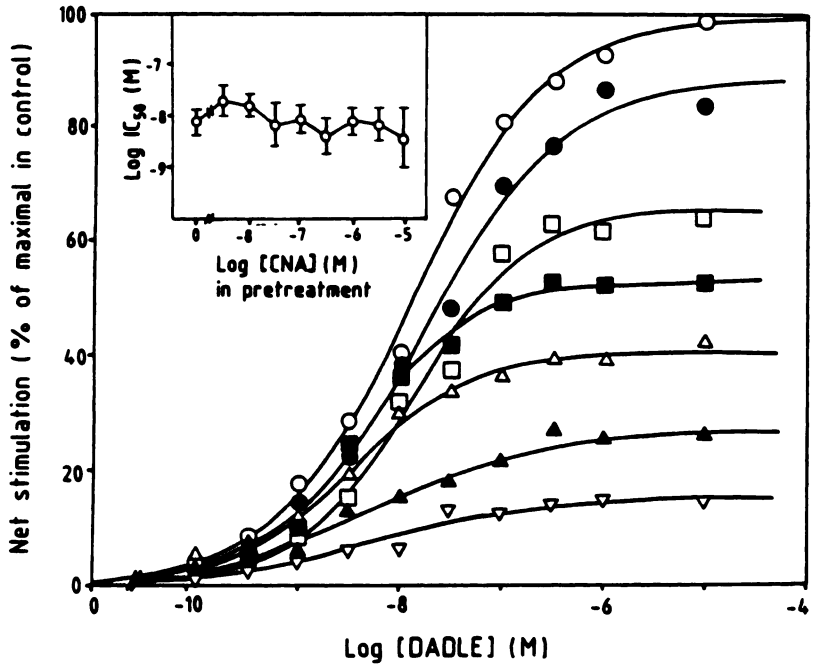

Fig. 3. Effects of pretreatment of intact cells with different concentrations of CNA on the concentration-response curve of DADLE for stimulation of GTPase. Intact cells were pretreated with the following concentrations (μM) of CNA: 0 (○), 0.003 (●), 0.01 (□), 0.03 (■), 0.1 (△), 1 (▲), and 10 (▽). Net (DADLE-stimulated) GTPase activity is expressed as percentage of the value measured in control membranes in the presence of 10 μM DADLE (19.2 pmol × 1/mg × 1/min). The family of curves was simultaneously fitted with the computer program ALLFIT; the hypothesis that the curves did not differ in IC_{50} was statistically checked by forcing all the curve to share an identical IC_{50}. This procedure did not alter significantly the "goodness of fit" ($p = 0.3$). Data points are means of triplicate determinations from a representative experiment in which the same batch of cells was treated with different concentrations of CNA. Similar results were obtained in two additional experiments. *Inset*, logarithms of the IC_{50} for DADLE-mediated stimulation of GTPase plotted as a function of the concentration of CNA in the pretreatment. The data are means ± standard error from three independent experiments.

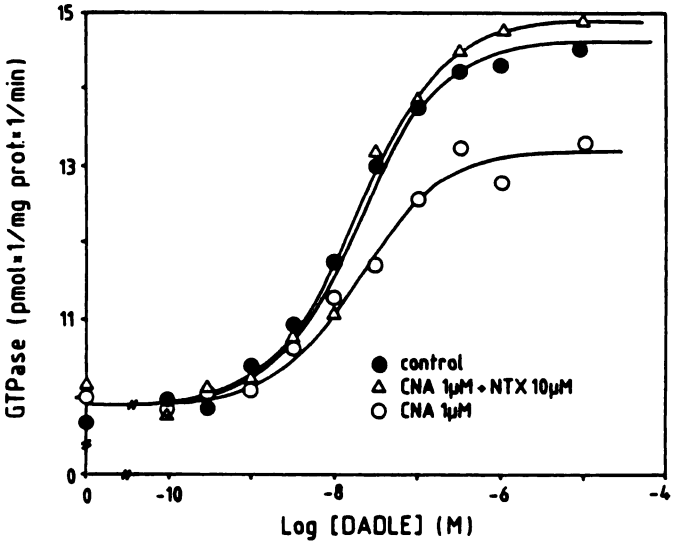

Fig. 4. Effect of CNA pretreatment of membranes on the concentration-response curves of DADLE for stimulation of GTPase. Membranes prepared from NG 108-15 cells (2 mg of protein per ml) were incubated at 25° for 45 min in the presence of 0.1% ethanol (control) (●), 1 μM CNA (○), or 1 μM CNA and 10 μM naltrexone (*NTX*)(△). After washing (see Experimental Procedures), GTPase was assayed in the presence of different concentrations of DADLE.

marked) reduction of the activity measured in the absence of agonist (basal). In the same membranes there was a concurrent reduction of the PTX-catalyzed incorporation *in vitro* of [^{32}P]-ADP-ribose into a 40-kDa polypeptide corresponding to the α subunit of G proteins (Fig. 6, *upper inset*), indicating that the loss of GTPase was correlated with the ability of the toxin to ADP-ribosylate G proteins in intact cells. The net (DADLE-stimulated) GTPase activity was reduced up to 97% by toxin treatment with an IC_{50} for the toxin of approximately 0.15 ng/ml (range, 0.08–0.3 in four independent experiments); the concentration-response curves for PTX-induced suppression of net stimulated GTPase were steep (IC_{80}/IC_{20} ratios ranged between 18 and 25). The apparent basal activity was also reduced under these experimental conditions to a maximum of 50% (range, 40–60% in four experiments) and with the same IC_{50} for the toxin (Fig. 6, *lower inset*). Concentration-response curves for DADLE-mediated stimulation of GTPase activity in membranes treated with progressively increasing concentrations of PTX are shown in Fig. 7A. In membranes in which the loss of responsiveness was lower than or equal to 50% (i.e., prepared from cells that had been exposed to PTX concentrations between 0.01 and 0.1 ng/ml) the reduction of DADLE-mediated stimulation was explained by a diminished maximal effect with no significant changes in the EC_{50} for the agonist. However, upon exposure of cells to larger concentrations of PTX (0.3–10 ng/ml), a 10-fold shift to the right of the EC_{50} for DADLE became evident (Fig. 7A, *inset*). The relationship between diminution of maximal effect and reduction of apparent affinity for DADLE induced by toxin treatment is illustrated in Fig. 7B, in which these two parameters are plotted together as a function of the concentration of PTX in the pretreatment. It is clear that the loss of sensitivity (increase in EC_{50} of DADLE) becomes detectable only when the maximal effect drops below 20–30% of the control.

Opioid receptor-mediated inhibition of adenylate cyclase activity was also decreased in membranes treated with pertussis toxin (Fig. 8A). The basal activity was not, or only slightly, changed by treatment with toxin up to 1 ng/ml and was only slightly reduced at higher concentrations; in contrast, the activity inhibited by 10 μM DADLE did increase as the concentration of toxin added to intact cells was increased, so that the inhibition of enzymatic activity induced by DADLE (Fig. 8A, *inset*) was reduced from a value of 45% in membranes from nontreated cells to about 4% in membranes from cells treated with 100 ng/ml PTX. The apparent IC_{50} for PTX-induced suppression of DADLE-mediated inhibition of adenylate cyclase (0.46 ng/ml; range, 0.2–0.6 in three experiments) was only slightly larger than that measured for the suppression of GTPase stimulation, but the concentration-response curves for

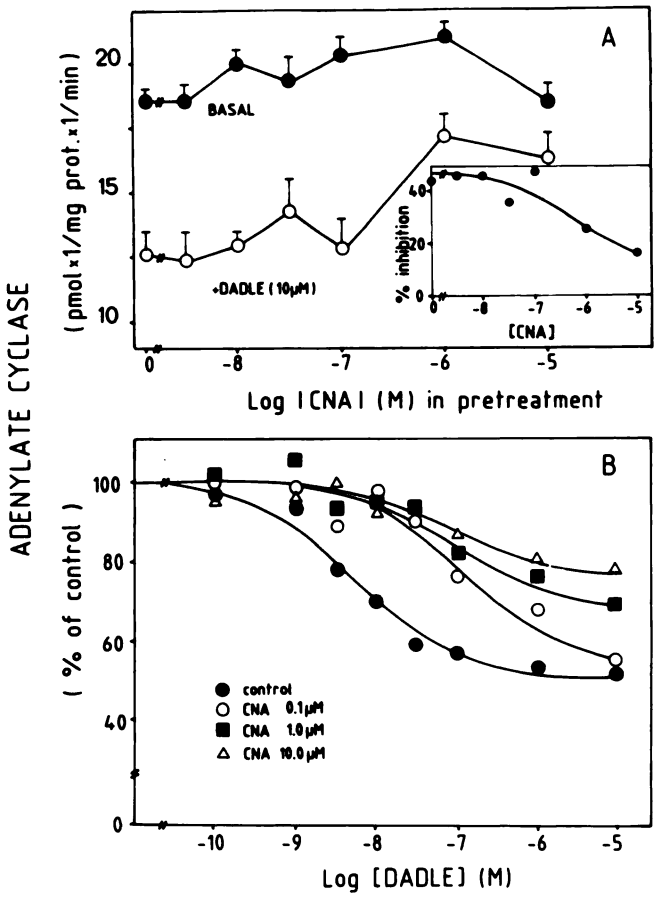

Fig. 5. Effect of CNA pretreatment of intact cells on DADLE-mediated inhibition of basal adenylate cyclase activity. A, Adenylate cyclase activity was assayed in the absence (●) or presence (○) of 10 μM DADLE in membranes prepared from cells that had been exposed to different concentrations of CNA (as indicated). *Inset*, the degree of opioid-mediated inhibition of adenylate cyclase (as percentage of basal activity) is replotted as a function of the concentration of CNA in the pretreatment. B, Concentration-response curves for DADLE-mediated inhibition of cyclase activity in membranes obtained from cells pretreated with different concentrations of CNA. Data are presented as percentage of the activity measured in the absence of agonist, the absolute value of which for each membrane is given in A.

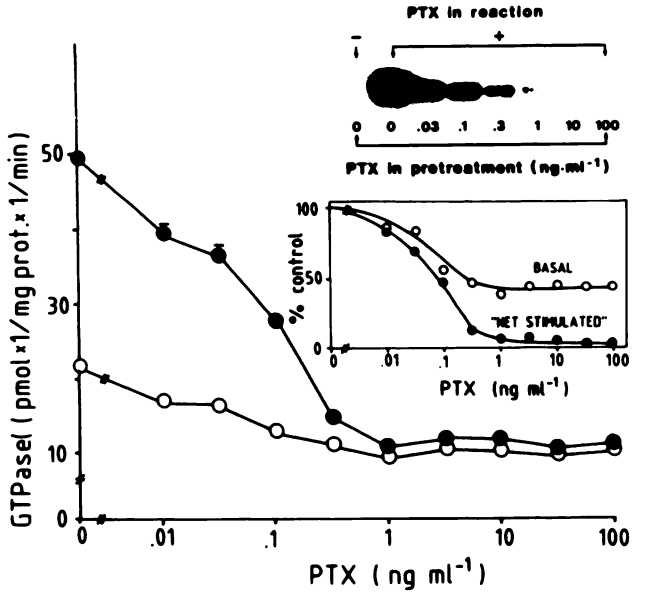

Fig. 6. Effect of PTX treatment of intact cells on opioid-mediated stimulation of GTPase activity. Cells were exposed to different concentrations of PTX (as indicated) and membranes were prepared as detailed in Experimental Procedures. GTPase activity in the membranes was measured in the absence (○) or presence (●) of 10 μM DADLE. *Lower inset*, replot of basal and net-stimulated activity as detailed in Fig. 2, *inset*. *Upper inset*, PTX-catalyzed incorporation of [^{32}P]ADP-ribose into the 40-kDa peptide corresponding to the α subunit of G protein, measured in the same membranes of the main panel.

the toxin were not as steep (IC_{80}/IC_{20} ratio was 128 ± 30, three experiments). The concentration-response curves for DADLE-mediated inhibition of adenylate cyclase activity (Fig. 8B) in membranes obtained from cells treated with different concentrations of PTX exhibited a reduction of maximal inhibition, with no changes in IC_{50} for the agonist, at low degrees of G protein inactivation (0.01 and 0.1 ng/ml PTX). However, after a greater extent of inactivation (1 ng/ml PTX), not only was the reduction of maximal inhibition correspondingly enhanced, but a rightward shift of the IC_{50} for the agonist became also evident (Fig. 8B). Hence, the pattern of opioid receptor-mediated responsiveness for adenylate cyclase activity after inactivation of G proteins is very similar to that observed for GTPase activity.

To evaluate how the loss of opioid receptor-mediated responsiveness upon inactivation of either receptors or G proteins was manifested *in vivo*, we examined agonist-induced inhibition of cAMP accumulation in intact cells that had been exposed either to CNA (1 μM) or to various concentrations of PTX. Fig. 9 shows that treatment with 1 μM CNA did not alter the accumulation of cAMP induced by the phosphodiesterase inhibitor Ro 20-1724 but affected the concentration-response curve for DADLE-mediated inhibition of this accumulation, in a manner similar to that observed for inhibition of adenylate cyclase activity; both maximal inhibitory effect and apparent affinity were reduced by CNA. The shift in IC_{50} (5-fold), however, was not as pronounced as that measured for inhibition of adenylate cyclase in the membranes. In cells treated with increasing concentrations of PTX (Fig. 10), there was a concentration-dependent enhancement of the accumulation of cAMP induced by Ro 20-1724 (Fig. 10, *inset*). The maximal inhibitory effect of DADLE (83% inhibition in control) was unchanged in cells exposed to 0.1 ng/ml toxin and then more clearly reduced at higher concentrations of toxin. The IC_{50} of DADLE was not increased at 0.1 and 0.3 ng/ml PTX but it was increased at 1 and 10 ng/ml.

Discussion

In this study we produced varying degrees of opioid receptor inactivation by exposing intact cells to the irreversible antagonist CNA. The consequence of the reduction of free receptor concentration in the membrane (quantified by saturation analysis with the radiolabeled opioid ligand) was studied at two levels of message transmission, (a) agonist-mediated stimulation of high affinity GTPase activity and (b) inhibition of adenylate cyclase activity in the presence of NaCl and GTP but in the absence of stimulatory ligands. The disappearance of agonist-mediated GTPase stimulation was correlated with the reduction of receptor density and occurred as a main diminution of maximal stimulated activity with no significant changes in apparent agonist affinity. This pattern of decrease is in conflict with that expected for a system in which agonist-

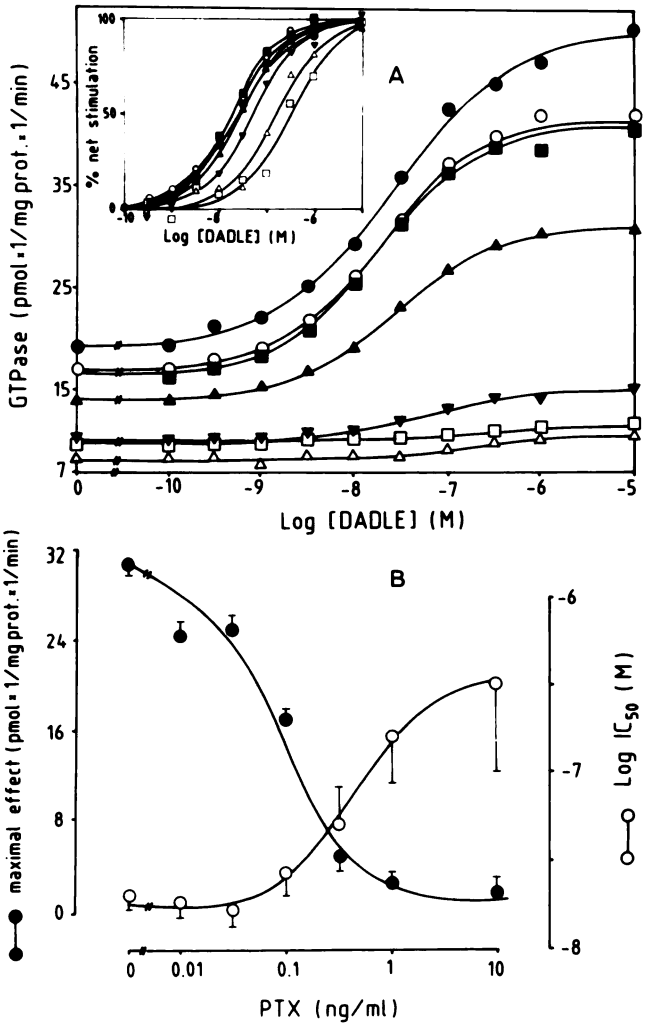

Fig. 7. Effect of exposure of intact cells to different concentrations of PTX on the concentration-response curves for DADLE-mediated stimulation of GTPase activity. A, GTPase was measured in membranes obtained from cells treated with vehicle (●), or the following concentrations (ng/ml) of PTX: 0.01 (■), 0.03 (○), 0.1 (▲), 0.3 (▼), 1 (△), and 10 (□). The concentration of GTP was kept constant at 250 nM but the specific activity was 15,000 cpm/pmol in the first four curves (●, ■, ○, ▲) and 90,000 cpm/pmol for the curves obtained in membranes treated with the highest concentrations of PTX (▼, △, □). Concentration-response curves were analyzed with the computer program ALLFIT as described in the legend to Fig. 3. *Inset*, the same curves of the main panel are replotted as percentage of the net maximal stimulated activity observed in each membrane, to demonstrate the shift in IC_{50}. B, Replot of the parameter estimates for the curves in A. Maximal effect (*left ordinate*) is expressed as net stimulated activity observed in each membrane. Logarithms of IC_{50} for each curve are shown on the *right ordinate*. The parameters are plotted with standard errors derived from the computer fit.

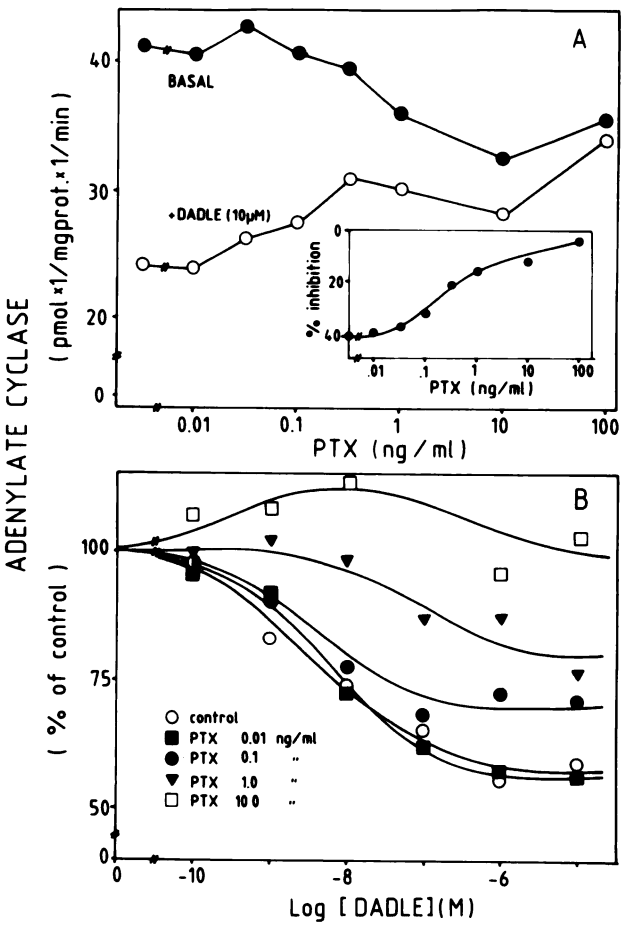

Fig. 8. Effect of PTX on DADLE-mediated inhibition of adenylate cyclase activity. A, Adenylate cyclase activity was assayed in the absence (●) or presence (○) of 10 μM DADLE in membranes prepared from cells exposed to different concentrations of PTX. *Inset*, replot of the percentage of inhibition produced by DADLE as a function of PTX concentration. B, Concentration-response curves for DADLE-mediated inhibition of adenylate cyclase activity in membranes prepared from cells exposed to different concentrations of PTX (as indicated). The data are normalized as percentages of the activity measured in the absence of agonist; values corresponding to 100% for each membrane are reported in A. This is a representative experiment. Two additional experiments yielded similar results.

receptor complexes catalytically activate a correspondingly larger population of G proteins, because in this case the diminution of receptor sites in the membrane would first result in loss of efficiency (i.e., a decrease in agonist apparent affinity) that would precede the decrease in maximal stimulated activity. However, such a pattern of decrease upon CNA treatment was observed for agonist-mediated inhibition of adenylate cyclase activity, which, thus, differed from the pattern of decrease observed for GTPase.

G proteins act as biochemical transducers of membrane receptors (R), because by transmitting signals to the effector system(s), they also provide a first amplification step of the weak stimulus generated upon receptor recognition by the agonist (see review in Ref. 20 and references therein). For G_s-R interactions, two mechanisms are responsible for G protein-mediated amplification. First, as shown in reconstituted systems (21), agonist-receptor complexes act catalytically in activating nucleotide exchange in G_s; accordingly, the EC_{50} of the agonist for activation of G_s is much lower than its half-maximal receptor occupancy despite a stoichiometric excess of G_s over receptors (21). Second, the molar turnover number of the intrinsic GTPase activity of purified G proteins, even under maximal stimulation, is considerably lower than that of the effector enzyme adenylate cyclase; hence, the relatively long half-time of the activated state of each molecule of activated G protein can result in a large response of the catalyst (22). According to the subunit dissociation model of G_s-mediated activation of adenylate cyclase, activated α subunits shuttle between receptor (R) and catalytic moiety (C) (20); thus, both mechanisms of amplification mentioned above may cooperate

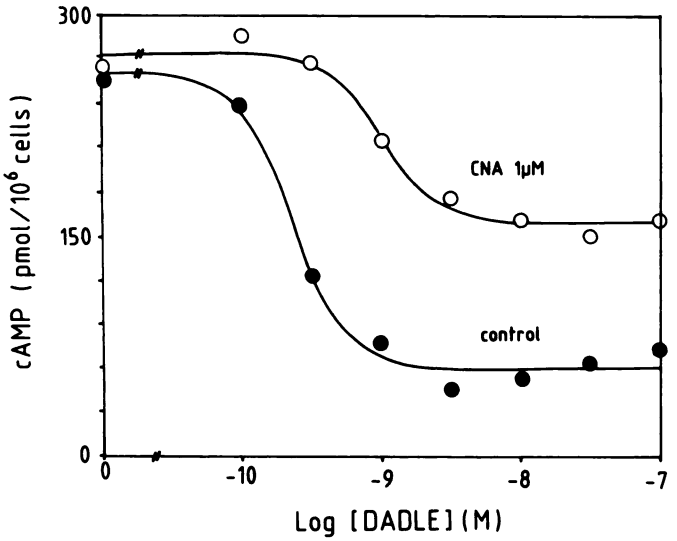

Fig. 9. Effect of CNA (1 μM) on the ability of DADLE to inhibit cAMP accumulation in intact cells. Cells were treated with CNA and washed. Accumulation of cAMP induced by Ro 20-1724 (100 μM) was measured as described in Experimental Procedures. The IC_{50} of DADLE computed for the two curves (ALLFIT) were significantly different ($p = 0.001$). Their value was 0.22 ± 0.026 nM in control and 1.1 ± 0.24 nMin cells treated with CNA, respectively.

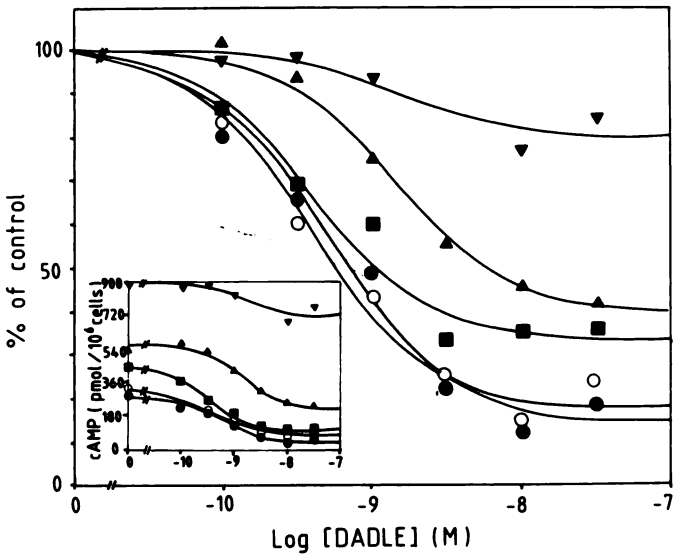

Fig. 10. Effect of PTX pretreatment on the concentration-response curves for DADLE-mediated inhibition of cAMP accumulation in intact cells. Experimental details are given in Fig. 9. Cells were treated with vehicle (●), 0.1 (○), 0.3 (■), 1 (▲), or 10 (▼) ng/ml PTX respectively. The estimates (ALLFIT) for the IC_{50} of DADLE were not significantly different ($p = 0.68$) from control (0.34 nM) in cells treated with the first two concentrations of PTX (○, ●) but were 1.44 and 1.6 nM in cells treated with 1 (▲) and 10 (▼) ng/ml PTX, respectively. *Inset*, the same data are shown in absolute values to illustrate the increase of cAMP accumulation produced by PTX pretreatment.

to produce substantial gain and provide a molecular basis for the large receptor reserve in β-adrenoceptor stimulation of cyclase observed in native membranes (23) and in whole cells (24). However, kinetic analysis of this activation of cyclase either in native membranes after receptor inactivation by an irreversible antagonist (25) or, more recently, in reconstituted systems (26) indicates that activation of C is a linear function of [R] and suggests that G_s exists as a tight complex with C; biochemical evidence in support of this notion has been provided (27). Thus, a modification of the subunit dissociation model was suggested, which assumes tight coupling between G_s and C and loose coupling between R and G_s (28). For receptors that mediate inhibition of adenylate cyclase (R_i) and interact with the G_i/G_o group of G proteins, the mechanism of signal transduction is less well understood. However, an attractive model is still that involving G protein subunit dissociation. According to this model, R_i-mediated activation of G_i results in dissociation of β-γ subunits, increase of their intramembranous concentration, and indirect inhibition of $G_{s-\alpha}$ (20). Thus, in contrast to the stimulatory pathway, the mechanism of signal transduction for the inhibitory pathway predicts loose coupling between G_i and C and fits the experimental observations showing either lack of direct interaction (29) or very weak interactions (30) between G_i and cyclase in reconstitution experiments.

The data presented here are also consistent with the notion that G_i/G_o-mediated inhibition of adenylate cyclase activity via the opioid receptor in NG 108-15 cells occurs through a mechanism that involves tight coupling between G protein and receptor but loose coupling between G protein-receptor complexes and catalytic subunits of the adenylate cyclase. Thus, they support the idea that dissociation of β subunits from receptor-activated α subunits might be the most important mechanism of cyclase inhibition in native membranes (20). In addition, these data suggest that there is little if any amplification between receptor activation by the agonist and stimulation of nucleotide hydrolysis onto the α subunit of G proteins in native membranes. One possible explanation for these findings is that the mechanism of receptor-mediated activation of the G_i/G_o group of G proteins differs substantially from that extensively characterized for β-adrenoceptors and G_s. The difference may be in the stability of the agonist-occupied receptor-G protein complex. This ternary complex is believed to occur only as a transient intermediate during β-adrenergic activation of G_s at physiological concentrations of GTP (25, 31, 32). However, it is possible that opioid receptors (and other receptors that also interact with PTX-sensitive G proteins) form much more stable complexes with the α subunit of G protein, so that dissociation of R_i-G_α during the cycle of activation either does not occur or occurs much more slowly than dissociation of the β subunit. A number of additional pieces of evidence support this contention. First, there are observations that α_2-adrenoceptors and D_2-dopamine receptors (two other receptors that interact with PTX-sensitive G proteins) can be purified by affinity chromatography as stable complexes with functional G proteins (33, 34). Second, inactivation studies with irreversible antagonists of D_2 dopamine receptors in native membranes and subsequent analysis of agonist competition for antagonist binding suggest that R_i and G_i behave as a tight complex whereupon binding of each respective ligand to either component can regulate allosterically the affinity of the other (35, 36). Third, electron inactivation studies of opioid receptors in NG 108-15 cells suggest that receptor and G protein (presumably its α subunit) are inactivated as a complex rather than as two easily exchangeable components (37). Fourth, purified muscarinic receptors reconstituted in liposomes with either G_i or G_o exhibited good agreement between receptor occupancy and activation of G protein by the agonist (19). In contrast, for β-adrenoceptor and G_s the difference between potency of activation and half-maximal occupancy was 2 orders of magnitude

(21), although both reconstitution protocols involved a similar excess of G protein over receptors (19, 21). This discrepancy may reflect intrinsic differences between inhibitory and stimulatory receptors in the mechanism of activation of G proteins.

Inactivation of opioid receptors by CNA in intact NG 108-15 cells did not only result in loss of net agonist-stimulated GTPase in the membrane but also produced a small but significant reduction of "basal" activity. This reduction of basal activity was specific, inasmuch as it was prevented (as it was the net stimulated activity) by coincubation with the reversible antagonist naltrexone, and suggests that a small proportion of the basal GTPase activity measured in the membrane of this cell type may be accounted for by a spontaneous (non-agonist-mediated) interaction between opioid receptors and G proteins. This point is of major interest with respect to the general theory of drug-receptor interactions and certainly deserves more detailed studies.

In the attempt to gain further understanding of the relationship between receptor-mediated stimulation of GTPase and inhibition of adenylate cyclase in membranes from NG 108-15 cells, we also examined the loss of responsiveness that follows inactivation of G proteins in intact cells by exposure to PTX. This loss differed in two main features from that produced by alkylation of receptor sites. First, the reduction of agonist-mediated GTPase stimulation induced by PTX exhibited a more complex pattern than that observed for the reduction induced by CNA. In fact, whereas the bulk of stimulated activity disappeared as loss of maximal effect of the agonist with no changes in apparent affinity, further reductions in responsiveness were also accompanied by a reduced apparent affinity for DADLE. The apparent IC_{50} of PTX for the reduction of maximal GTPase stimulation was 1 order of magnitude lower than the IC_{50} for the reduction of DADLE apparent affinity (Fig. 7B), indicating that the latter requires extensive diminution of functional G proteins in the membranes to become detectable. Second, the patterns of loss of agonist-mediated GTPase stimulation and adenylate cyclase inhibition were in reasonable agreement. In fact, for both enzymes the reduction of responsiveness occurred initially as loss of maximal effect that was followed by reduction of apparent agonist affinity.

Although the apparent IC_{50} of PTX for the reduction of DADLE-mediated inhibition of cyclase was only slightly larger (2–3-fold) than the IC_{50} for the abolition of GTPase sitmulation, there was no close relationship between the extent of loss of agonist-mediated regulation of these two enzymatic activities, inasmuch as the concentration-response curves of the toxin were very steep in quenching GTPase stimulation and shallow in reducing cyclase inhibition. For example, in membranes of cells exposed to 1 ng/ml PTX the residual GTPase stimulation was only 5%, whereas inhibition of adenylate cyclase was still 40% of that obtained in controls. On the other hand, the reduction of GTPase stimulated activity was in close agreement with the degree of modification of G protein, as assessed by PTX-catalysed incorporation of [^{32}P]ADP-ribose *in vitro*. These data raise the possibility that the complex relation in loss of responsiveness between the two enzymes after PTX treatment might in part reflect the heterogeneity of PTX-sensitive G proteins present in the membranes. Using site-specific antibodies we found, in agreement with previous reports (38), that at least two types of PTX substrates (G_o and $G_{i\text{-}2}$) are present in NG 108-15 cells (39). Thus, it is possible that opioid receptors interact preferentially with G_i and that this interaction accounts for a major proportion of stimulated GTPase activity and requires a lower concentration of agonists than the interaction with G_o; in contrast, both proteins may contribute to inhibition of cyclase activity. Assuming that G_i is more efficiently ADP-ribosylated by PTX than G_o in the intact cells, as it is in membranes (40), then the reductions of PTX-dependent ADP-ribosylation and agonist-mediated GTPase stimulation would be related to each other more closely than either of them to the diminution of agonist-mediated inhibition of adenylate cyclase. Because the α subunits of G_o and $G_{i\text{-}2}$ (39 and 40 kDa, respectively) cannot be easily resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (41), it is difficult to test this hypothesis directly. However, the discrepancy described here between the extent of decrease in ability of the agonist to stimulate GTPase and to inhibit adenylate cyclase upon PTX treatment is similar to that described in liver membranes between the degree of PTX-catalyzed modification of G protein and the reduction of the ability of angiotensin II to inhibit adenylate cyclase (42). Thus, this phenomenon seems to be general rather than a peculiar characteristic of membranes prepared from NG 108-15 cells. Whether this phenomenon might be interpreted as a further indication that the mechanism of inhibition in native membranes does not involve a direct interaction between inhibitory α subunits and catalyst (20), or rather as a reflection of the presence of multiple G proteins interacting with the same receptor, cannot be established without additional studies.

Exposure to PTX did not only abolish agonist-stimulated GTPase activity but also caused a substantial decrease of apparent basal activity. Because ADP-ribosylation by PTX does not alter the intrinsic GTPase activity of purified G proteins (19), this decrease in basal activity indicates that a considerable proportion of the low K_m activity measured in the absence of agonist under these assay conditions is due to preactivated PTX-sensitive G proteins. This preactivation may result in part from a spontaneous (not mediated by agonist) interaction with opioid receptors, as suggested by the effect of CNA on basal activity mentioned before and by the fact that down-regulation of opioid receptors also results in a naloxone-irreversible decrease in basal activity (5). Although GTPase and adenylate cyclase were assayed under almost identical conditions, we did not observe any corresponding increase in basal adenylate cyclase activity upon PTX treatment. In contrast, the stimulation of cyclase by prostaglandin E_1 (which acts via G_s) was clearly potentiated by PTX (data not shown). This lack of PTX effects on basal cyclase activity is in agreement with data reported by others (20, 42, 43), and its discordance with the PTX effect on basal GTPase noted here might be a further indication of the loose coupling between G protein activation and cyclase inhibition in native membranes.

In the final part of this study, we have examined the effect of both receptor and G protein inactivations on the ability of the agonist to inhibit the accumulation of cAMP induced by a phosphodiesterase inhibitor in intact cells. Although, under the experimental conditions used here, the accumulation of cAMP in intact cells should mainly reflect the activity of the adenylate cyclase, there were two notable differences in the ability of DADLE to inhibit the enzymatic activity in membranes, as compared with its ability to lower cAMP levels in intact cells. First, the extent of inhibition (as percentage of basal activity)

was only 35–45% in membranes but as much as 70–85% in intact cells. Second, the agonist was 10–20 times more potent in preventing cAMP accumulation (IC_{50}, 0.2–0.4 nM) than in inhibiting membrane-bound cyclase activity (IC_{50}, 4–6 nM). This difference in potency of the agonist most likely reflects the existence of a large amplification mechanism. Neither CNA treatment nor PTX exposure of intact cells produced more than a 5-fold shift in the potency of DADLE to inhibit cAMP accumulation and, therefore, neither abolished the discrepancy in agonist responsiveness between membranes and intact cells. Similarly, Fantozzi *et al.* (9) showed that CNA treatment did not abolish the discrepancy between receptor occupancy and effect measured in intact cells. This indicates that the amplification between opioid receptor occupancy and final inhibition of cAMP levels in NG 108-15 cells is not completely accounted for by the mechanism of adenylate cyclase inhibition at the membrane level and suggests that multiple additional factors may be involved synergistically with G protein-dependent cyclase inhibition in amplifying receptor-mediated reductions of cAMP levels in the intact cell. We have obtained preliminary evidence that the accumulation of cAMP levels mediated by Ro 20-1744 in NG 108-15 cells is markedly reduced by the removal of extracellular Ca^{2+} (in the presence of EGTA) and that under these conditions the potency of DADLE in inhibiting cAMP accumulation is decreased.[2] Additional studies are necessary to elucidate the multiple steps involved in the reduction of cAMP levels mediated by inhibitory receptors.

Acknowledgments

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