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EFFECTS OF THE DESENSITIZATION BY MORPHINE OF THE OPIATE-DEPENDENT ADENYLATE CYCLASE SYSTEM IN THE RAT STRIATUM ON THE ACTIVITY OF THE INHIBITORY REGULATORY G PROTEIN

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Abstract—Opiates act through a specific receptor to inhibit the striatal adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] and stimulate a high-affinity GTPase (EC 3.6.1). The present study analyzes the functions of the striatal adenylate cyclase complex following chronic morphine treatment in the rat. The inhibitory effects of GTP on basal adenylate cyclase activity, between 10^{-6} and 10^{-4} M, were reduced. Moreover, the half-maximal inhibitory concentration of the opiate receptor agonist (D-Ala^2-Met^5)-enkephalinamide (DAME) on striatal adenylate cyclase activity was increased by about four times, whereas the maximal effect was reduced in membranes from treated rats. In parallel, the half-maximal stimulatory concentration of DAME on GTPase was increased by two times, and the maximal stimulation was reduced from 60 to 25%. Binding studies performed with [3,5-^{3}H]DAME (saturation curves) and with [^{3}H]naloxone (competition curves) did not show any change in opiate receptor numbers and affinity. Moreover, the kinetics of the activation of the inhibitory GTP binding protein (G_i) which transduces the opiate receptor effect on adenylate cyclase showed a small but significant delay. Therefore, hypofunction of G_i can be, at least in part, responsible for the observed desensitization by morphine of the opiate-dependent GTPase and adenylate cyclase.

The inhibition of adenylate cyclase activity by opiates [1] has been implicated as a critical factor in the development of tolerance to and dependence on these drugs [2, 3].

The adenylate cyclase complex is composed of a neurotransmitter binding site coupled to the catalytic moiety of the enzyme (C) by either a stimulatory (G_s) or an inhibitory (G_i)† guanine nucleotide regulatory protein which is activated once GTP is bound to it [4]. Hydrolysis of the bound GTP to GDP by a high-affinity GTPase terminates this activation [5, 6]. We have shown recently that this model is operative also in striatal membranes [7].

The function of this complex appears, essentially from studies in cell lines, to undergo three types of adaptation during chronic opiate treatment, which are considered the biochemical correlates to the tolerance of opiate action:

(1) Opiate receptor densitization, which is attributed to the uncoupling of the opiate receptors from G_i [8, 9].
(2) Opiate receptor down-regulation that follows opioid peptide administration but not alkaloid agonist administration and is caused by the internalization of the ligand–receptor complex [10].
(3) Compensatory increases in both basal and stimulated adenylate cyclase activities [11, 12].

Withdrawal or displacement of the opiate from the receptor by an antagonist leads to an increase in basal and stimulated adenylate cyclase activity, as observed in NG 108-15 cells [11]. This rebound effect, likened to dependence *in vivo*, is, however, a matter for discussion [8, 13, 14], and no "unitary theory" about all these events has yet been defined [15]. Moreover, there is little information about the events occurring in the CNS, where the neighboring circuits may greatly influence enzyme complex function.

We have observed recently that, in striatal membranes from rats chronically exposed to morphine, the adenylate cyclase complex has less basal GTPase activity, suggesting the occurrence of profound changes in G protein function [16]. Now we report evidence suggesting that densensitization of the opiate sensitive receptor-adenylate cyclase system following chronic morphine treatment may be related to a reduction of the intrinsic activity of G_i.‡

MATERIALS AND METHODS

Reagents. Adenylyl-5′-*O*-imidodiphosphate (App(NH)p), ATP, and guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) were purchased from Boehringer Mannheim (Mannheim, FRG). Dithiothreitol (DTT), creatine phosphate, creatine kinase,

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† The nature of the inhibitory G protein coupling the brain opiod receptors has not been established, and the term G_i, when referred to the striatal adenylate cyclase, is general.

‡ A partial account of this work has appeared: F. Tirone, M. Parenti, P. Misiano and A. Groppetti, *J. Neurochem.* **44** (Suppl.), S89 (1985).

3-isobutyl-1-methyl-xanthine (IBMX), ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA), (D-Ala^2-Met^5) enkephalin-amide (DAME) and other compounds or reagents were from Sigma (St. Louis, MO). [α-^{32}P]ATP (40–50 Ci/mmol), [γ-^{32}P]GTP (10–50 Ci/mmol), [2,8-^{3}H]cyclic AMP (33.5 Ci/mmol), [3,5-^{3}H]DAME (33.4 Ci/mmol) and [^{3}H]naloxone (35 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Forskolin was from Calbiochem-Behring (La Jolla, CA).

Membrane preparation and protein assay. Partially purified synaptic plasma membranes were obtained from striata of male Sprague–Dawley rats weighing 150–180 g (Charles River, Calco, Italy), prepared as described previously [16]. Membrane suspensions were stored under liquid nitrogen in buffered medium (buffer A: 50 mM imidazole–HCl buffer, pH 7.4, containing 0.32 M sucrose, 1 mM DTT, 1 mM EGTA and 0.1 mM IBMX) to give a protein concentration of about 2 mg/ml. Membranes were thawed on ice just before the assay. Protein concentration was evaluated by the micromethod of Bradford [17].

GTPase assay. GTPase activity was assayed by measuring the $^{32}P_i$ hydrolyzed from [γ-^{32}]GTP, according to the method of Cassel and Selinger [18] and as previously described [16]. The assay medium contained 25 nM [γ-^{32}P]GTP, 0.75 μM GTP, 1 mM ATP, 5 mM $MgCl_2$, 100 mM NaCl, 0.375 mM ouabain, 4 mM App(NH)p, 1 mM EGTA, 1 mM DTT, 0.1 mM IBMX, 10 mM creatine phosphate, 50 units/ml creatine phosphokinase and 50 mM imidazole–HCl buffer, pH 7.4, in a final volume of 100 μl. Reactions were started by the addition of membrane suspension (4–6 μg protein) and continued for 10 min at 25°. They were stopped by adding 0.9 ml of ice-cold 50 mM imidazole–HCl buffer, pH 7.4, containing 5% (w/v) of activated charcoal. The samples were centrifuged at 10,000 *g* for 10 min. A 500-μl sample of the supernatant fraction was transferred into scintillation vials, and the radioactivity was counted. Release of $^{32}P_i$ in the absence of membrane preparations was 1 to 1.5% of added [γ-^{32}P]GTP. The enzyme activity was linear with time (up to 10 min). Less than 10% of the labeled nucleotide was hydrolyzed under the standard assay conditions. High-affinity GTPase activity was calculated from the difference between the counts of $^{32}P_i$ released in the presence of 0.75 μM GTP and 30 μM GTP. This calculation enables us to subtract the low-affinity GTPase activity present in the membranes [19]. The concentration of the half-maximally stimulatory dose (EC_{50}) was calculated by linear regression analysis of the percentage of effects transformed in probits. The EC_{50} values were then tested for their differences by Student's paired *t*-test.

Adenylate cyclase assay. Enzyme activity was assayed in a 100-μl reaction mixture under the same conditions used for the GTPase assay, except that App(NH)p and ouabain were omitted and [α-^{32}]ATP was present instead of [γ-^{32}P]GTP. [2,8-^{3}H]Cyclic AMP (cAMP) was included to monitor recovery. Membrane protein (20–30 μg) was present in each sample. Reactions were terminated by adding 200 μl of a solution containing 2% sodium dodecyl sulfate (w/v), 45 mM ATP and 1.3 mM cAMP. cAMP was isolated according to the method of Salomon *et al.* [20]. For the time course of cyclic AMP accumulation, membranes were preincubated for 5 min at 25° in the complete reaction mixture, with 10 μM forskolin. At 0 time [α-^{32}P]ATP and 40 nM GTPγS (final concentration) were added, in 10% of the final volume of incubation. This procedure gave linear production of cAMP. The time courses were determined in single experiments. The lag time was calculated by extrapolating the straight lines calculated by linear regression analysis (correlation coefficient was never less than 0.998), as described by others [21].

Animal treatment. Chronic morphine treatment of rats was performed by implantation of two morphine pellets (75 mg base/pellet) beneath the skin in the interscapular region. Control rats were sham operated. Rats were decapitated 72 hr after pellet implantation or sham operation.

Opiate receptor binding. All the binding studies were performed at equilibrium (1-hr incubation) under the same conditions used for adenylate cyclase assay, with the omission of the ATP-regenerating system and GTP in the assay medium. The saturation studies were performed with increasing concentrations of [3,5-^{3}H]DAME, defining the non-specific binding as the amount of [3,5-^{3}H]DAME bound to membranes after filtration through Whatman GF/B filters when the incubations included 4 μM DAME. The data from three experiments in duplicate ranging over six concentrations of [3,5-^{3}H]DAME (from 0.1 to 20 nM) were analyzed by Scatchard analysis [22]. Statistics of receptor number (B_{max}) and affinity (K_D) were performed by Student's paired *t*-test (df = 2). The drug competition studies were performed with 1 nM [^{3}H]naloxone, displaced by increasing concentrations of morphine. Displacement curves have been analyzed with computer-assisted non-linear regression analysis by LIGAND program [23]. The affinity constants (K_I) obtained for both control and treated membrane data sets of three experiments in duplicate have been statistically tested for their difference with a three-step procedure. Initially control and treated data sets were fitted either simultaneously, obtaining a common K_I value, or separately, obtaining two different K_I values. The sum of squares obtained from both data fitting were then tested for their statistical difference [24].

RESULTS

Increasing concentrations of GTP elicited a biphasic curve of striatal adenylate cyclase activity (Fig. 1). Following *in vivo* chronic morphine treatment the response to GTP was increased significantly along the entire curve. Moreover, the inhibitory effects of GTP observed from 10^{-6} to 10^{-4} M were significantly less in treated membranes.

To assess the desensitization (tolerance) of the opiate receptor after *in vivo* chronic morphine treatment, we evaluated the inhibitory concentration–response curves to the μ-δ opiate DAME on striatal adenylate cyclase. As shown in Fig. 2, where the values are reported as percent changes from basal activity, the concentration–response curve was

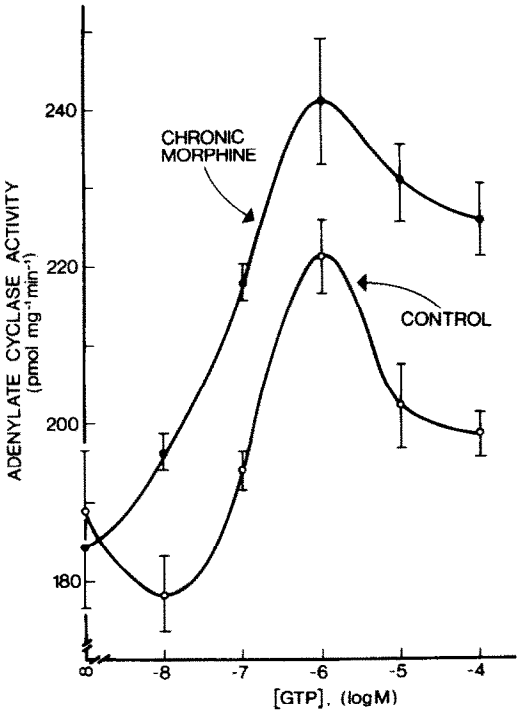

Fig. 1. Effects of chronic morphine pretreatment of the rat on GTP-dependent striatal adenylate cyclase activity. Membranes from control and pretreated animals, obtained as described in Materials and Methods, were diluted in 4 vol. of ice-cold buffer A without sucrose, then centrifuged 12 min at 14,000 *g* and resuspended in buffer A. Data are means ± SE of duplicate determinations from four experiments with different membrane preparations. The two curves differed at each point with $P < 0.05$ at least (paired *t*-test). The inhibitory effect of GTP between 10^{-6} and 10^{-4} M was significant ($P < 0.05$, df = 7, paired *t*-test) only for control membranes.

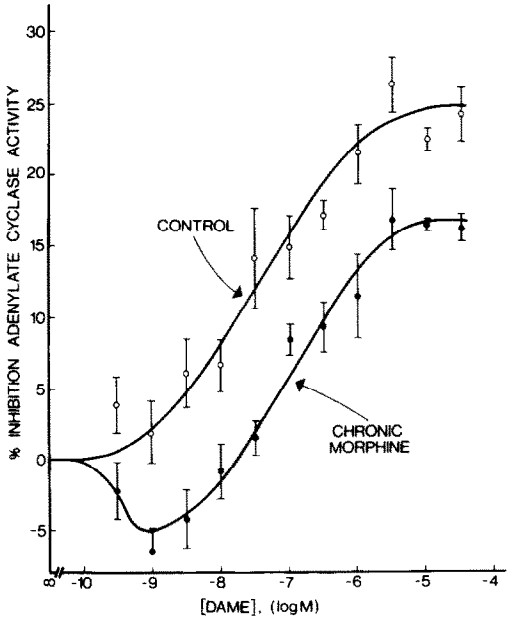

Fig. 2. Concentration-dependent inhibition by DAME of striatal adenylate cyclase in membranes from control and morphine-pretreated rats. The assay was performed, as described in Materials and Methods, at 0.75 μM GTP. Values are expressed as the means ± SE of the percentage of basal activity, from duplicate determinations of four experiments. The two curves differed at each point (except 3×10^{-10} M DAME) with $P < 0.05$ at least (df = 7, paired *t*-test). The values ± SEM of the enzyme activity without DAME were: 242.5 ± 8.5 pmol/mg/min, control animals; 285.2 ± 10.3 pmol/mg/min, morphine-treated animals.

shifted toward the right following morphine treatment, with the half-maximal concentration of DAME being about 45 nM for the control and 180 nM for the treated. The maximal inhibition of basal activity differed significantly, being about 25% in control membranes and 16% in membranes from treated animals, indicating that desensitization of the opiate receptor sensitive adenylate cyclase had occurred. The basal levels of enzyme activity were increased following chronic morphine, as shown above (control animals: 242.5 ± 8.5; morphine-treated animals: 285.2 ± 10.3 pmol/mg/min). Moreover, the chronic morphine treatment apparently induced a stimulatory effect of DAME, at concentrations between 10^{-9} and 10^{-8} M. We do not have any explanation for this stimulation and we do not exclude its possible contribution in the observed shift of the half-maximal concentrations of DAME.

The opiate receptor-mediated inhibition of adenylate cyclase is associated with stimulation of a high-affinity GTPase [16]. Therefore, we also evaluated the responsivity of the opiate-sensitive adenylate cyclase system by the level of GTPase activity (Fig. 3). The concentration of DAME which half-maximally stimulated the enzyme activity was shifted slightly, from 20 to about 40 nM in treated membranes, whereas the maximal stimulation of DAME expressed as percent increase of basal activity was reduced significantly from about 60 to 25%. We verified if this effect was a consequence of changes occurring in the specific, low-affinity GTPase activity (see Materials and Methods) during chronic morphine treatment. No differences of activity were observed in membranes from control or chronically treated animals at a concentration of 30 μM GTP, either in the absence or presence of a maximal concentration of DAME (1 μM, Table 1). This indicates, in agreement with others [19], that only the GTPase activity that we measured at a lower concentration of GTP (0.75 μM) can be considered specifically sensitive to the opiate receptor and responsible for the above effects. To evaluate if the observed changes in enzyme activities were due to changes in receptor function, we studied the opiate receptor binding. Scatchard analysis of saturation curves obtained in the presence of increasing concentrations of [3,5-^{3}H]DAME showed no significant difference in either the number of receptors (B_{max}) or affinity (K_D) after morphine treatment (control: B_{max} = 500 fmol/mg, K_D = 4.87 nM, morphine-treated: B_{max} = 492 fmol/mg, K_D = 5.02 nM). Similar results were obtained with drug competition studies, where increasing concentrations of morphine were used to displace [^{3}H]naloxone (Fig. 4). The

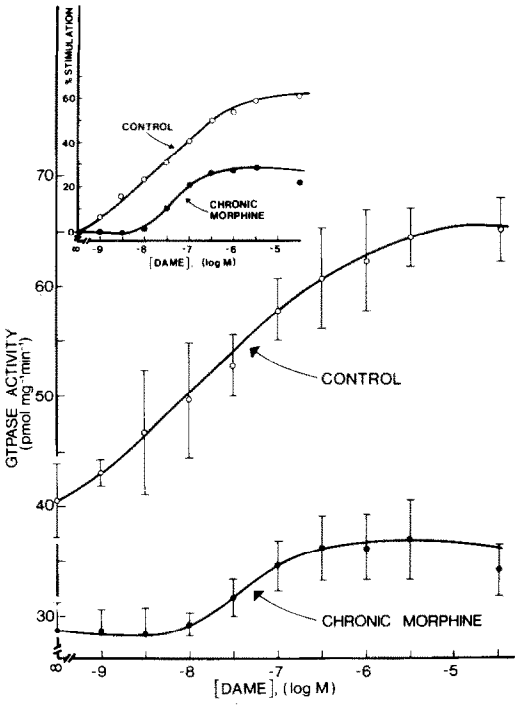

Fig. 3. Concentration-dependent stimulation by DAME of striatal GTPase activity in membranes from control and morphine-pretreated rats. GTPase activity of control and pretreated membranes was assayed at 0.75 μM GTP. Values are the means ± SE of duplicate determinations from three experiments with different membrane preparations. The inset shows the same curve as percent of basal activity. The two percent curves differed from 10^{-8} M DAME, with $P < 0.05$ at least (df = 5, paired t-test).

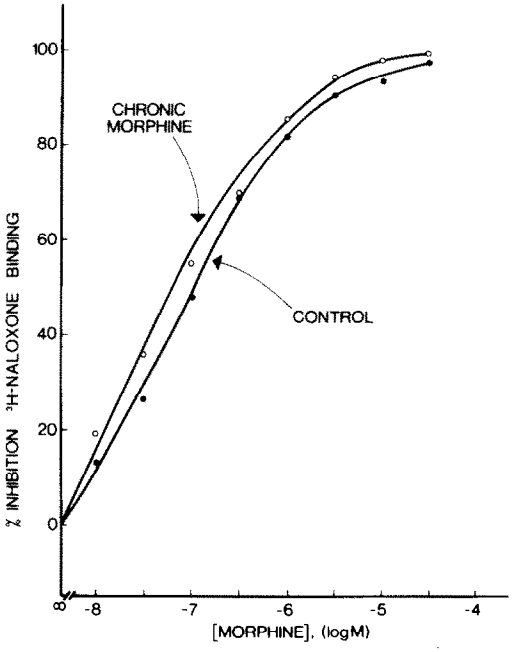

Fig. 4. Displacement curve by morphine of [^{3}H]naloxone binding from striatal membranes of control and morphine-pretreated rats. This plot represents the amount of [^{3}H]naloxone displaced by morphine, over the total added, versus the log of morphine concentrations. The points represent the values of a representative of three experiments, performed in duplicate. Binding values ± SEM in the absence of morphine were: 165 ± 10 fmol/mg protein, control; 170 ± 12 fmol/mg protein, treated. The K_I values (see Results) were estimated from the data of all three experiments, as described in Materials and Methods. The statistical difference between K_I values of control and treated rats had a $P > 0.05$ (df = 39, 36).

affinity constants (K_I) were obtained fitting to the data an equilibrium binding model with one class of specific binding sites, using the computer program termed LIGAND [23]. Statistical analysis of K_I values [24] in membranes from control (64 nM) and morphine-treated rats (73 nM) showed no significant difference.

To evaluate directly the intrinsic activity of the G_i protein in the desensitization of striatal adenylate cyclase, we attempted to study the activation reaction of G_i. To do this, we measured the inhibition of the forskolin-stimulated formation of cAMP, observed in the presence of nanomolar concentrations of

Table 1. Effects of DAME on the high- and low-affinity striatal GTPase

	$^{32}P_i$ released (dpm/μg striatal membranes)	
	GTP (30 μM)	GTP (0.75 μM)
Control		
No DAME	2760 ± 197	3870 ± 237
1 μM DAME	2790 ± 191	4750 ± 158*
Chronic morphine		
No DAME	2670 ± 206	3830 ± 220
1 μM DAME	2600 ± 92	4090 ± 301

The assay was performed as described under Materials and Methods. The data are means ± SE of triplicate determinations from three experiments with different membrane preparations. The values are expressed as dpm of inorganic [^{32}P] measured in the supernatant fraction after charcoal precipitation.

* Significant difference, within each concentration of GTP, computated with ANOVA (df = 3, 32, $P < 0.05$).

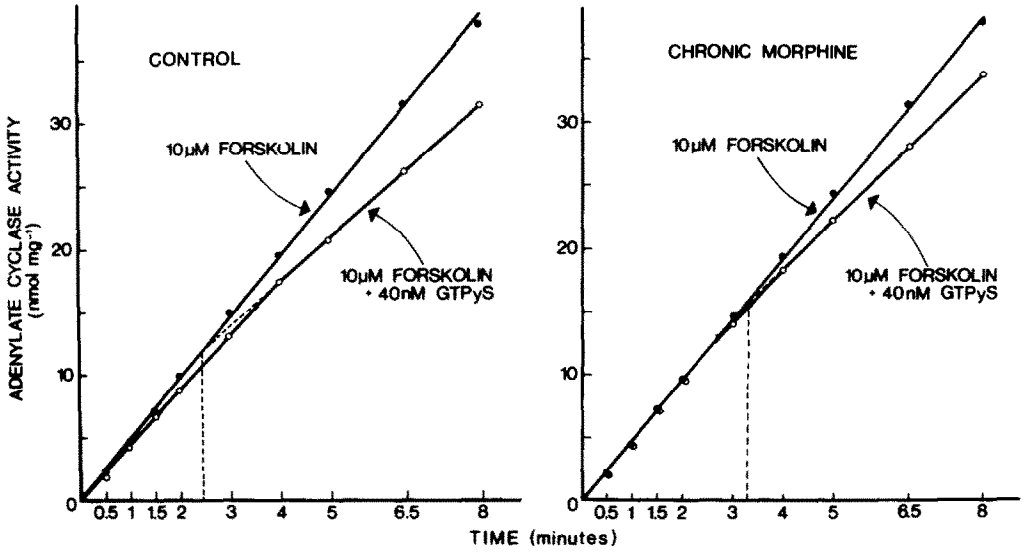

Fig. 5. Time–course of GTPγS inhibition of cyclic AMP formation in striatal membranes of control and morphine-pretreated rats. [α-^{32}P]ATP, with or without 40 nM GTPγS, was added at 0 time to membranes from control and treated animals preincubated in the complete reaction mixture, containing 10 μM forskolin, for 5 min at 25°. The figure shows a representative experiment. The time lags reported in the Results are the means ± SE of six experiments with different membrane preparations. The value differed with $P < 0.05$ (df = 5, paired t-test).

GTPγS, a stable GTP analogue (Fig. 5). This effect has been reported in other adenylate cyclase systems and considered as an expression of G_i activation by GTPγS [21, 25]. The forskolin-stimulated cAMP formation measured at 8 min was reduced by 40 nM GTPγS to a greater extent in control (20%) than in morphine-treated membranes (10%) (Fig. 5). Moreover the inhibition was delayed in membranes from treated animals, with the time lag necessary for the reaction to occur significantly increased from 2.19 ± 0.26 min in control to 3.44 ± 0.28 min in treated membranes, suggesting that *in vivo* chronic morphine treatment delays its appearance.

DISCUSSION

Several types of evidence obtained with striatal membranes point to an altered sensitivity of the adenylate cyclase complex to GTP after *in vivo* chronic morphine treatment. There was increased responsivity of basal adenylate cyclase activity along the entire biphasic GTP concentration-dependent curve (Fig. 1) as well as reduced responsivity to GTP of a low K_m basal GTPase activity [16, 26]. It has been proposed that activation of adenylate cyclase is turned off by enzyme hydrolysis of GTP bound to G_s [5]. According to this model, which seems to be operative for striatal adenylate cyclase too [7], the observed increase in basal enzyme activities following chronic morphine treatment could be due to an alteration in the G_s effect on GTP hydrolysis. However, the inhibitory effect of the higher concentrations of GTP (between 10^{-6} and 10^{-4} M), which has been proposed to be the expression of the G_i protein effect [7, 27], also was reduced significantly in striatal membranes of rats treated chronically with morphine (Fig. 1). Since a G_i protein is responsible for the opiate receptor-mediated effect on striatal adenylate cyclase and GTPase activities [7, 28], this may indicate that the prolonged treatment with an opiate agonist also affects the G_i function.

Reduced activity of G_i is also suggested by the finding that densensitization to DAME following chronic morphine treatment, observed for adenylate cyclase (Fig. 2), was also seen for the opiate-sensitive GTPase (Fig. 3). In fact, intrinsic GTPase activity has been demonstrated in purified G_i protein [29]. It is possible, therefore, that the desensitization of the opiate-sensitive GTPase is secondary to a more intrinsic change in G_i (e.g. the ability of G_i to bind GTP or the rate of exchange of guanine nucleotides from G_i binding site) rather than an expression of uncoupling between the opiate receptor and G_i, as seen in NG 108-15 cells [8, 9, 30, 31]. In fact, the analysis of the opiate receptor binding, either with saturation or displacement curves, does not indicate any change in the affinity of the receptor after *in vivo* treatment. Moreover, the observed decreases of GTP inhibition of adenylate cyclase activity (Fig. 1) and of basal GTPase activity [16] after morphine indicate the existence of alterations in the adenylate cyclase complex independent of opiate receptor coupling. From the binding data it can also be excluded that the desensitization in treated membranes is due to receptor down-regulation or to incomplete removal of morphine during the extensive washing in the membrane isolation procedure. It seems, therefore, to be the true expression of a tolerance to the opiate effect on the adenylate cyclase system.

An additional analysis of the function of G_i after chronic morphine treatment was performed by studying the kinetics of the turn-on of G_i by measuring

adenylate cyclase inhibition by the non-hydrolyzable GTP analogue, GTPγS. In fact, not only G_s, but also G_i, once bound to stable GTP analogues, is persistently activated, since the inactivation of G_i is due to hydrolysis of GTP bound to G_i [6]. In the presence of a maximally effective concentration of forskolin, which stimulates the catalytic subunit of adenylate cyclase [32], we have seen that nanomolar concentrations of GTPγS produce an inhibitory effect on the enzyme, which can be considered a measure of G_i activation (Refs. 21 and 25 and Fig. 5). *In vivo* treatment with morphine reduced this inhibition by 50% (from 20 to 10%) as measured on the total amount of cAMP produced after 8 min. Moreover, a significant, although low, delay of this inhibition was observed, suggesting that prolonged stimulation of G_i by morphine leads to partial impairment of G_i activation by GTPγS. This can be due to either a delay in the formation of G_i–GTPγS active complex or a delayed coupling between the active G_i and C. Both could account for the adenylate cyclase and GTPase desensitization of DAME after morphine pretreatment. Attempting to explain the limited extent of the observed delay, this could be a consequence of the ability of our assay to measure the activation of G_i molecules of all neuronal populations in striatum, whereas the opiate receptor(s) is possibly coupled to only a few percent of the total G_i proteins. Further studies are necessary, therefore, to clarify this aspect and the participation of the neighboring circuits to the altered responsivity of the opiate system.

In conclusion, the present study demonstrated desensitization of the opiate-dependent adenylate cyclase and GTPase activities after chronic morphine treatment. We surmise that G_i hypoactivity was partially responsible for this effect. The extent of the involvement of G_i in this process is still under investigation.

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