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RECEPTOR-MEDIATED STIMULATION OF BRAIN GTPase BY OPIATES IN NORMAL AND DEPENDENT RATS

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In membranes from rat brain striatum, opiate agonists stimulated low-K_m GTPase. Half-maximal enhancement of enzyme activity was obtained with 0.09 μM morphine and 3.8 μM levorphanol. This order of potency corresponded to that of the affinities of these compounds in binding to opiate receptor. The effect was inhibited by the antagonist naloxone. As shown by the use of the enantiomers levorphanol and dextrorphan, only the pharmacologically active stereoisomer stimulated GTPase. In membranes isolated from morphine-dependent rats, the activity of GTPase was reduced 20-40% relative to that in control rats. After the precipitation of morphine abstinence by naloxone, brain GTPase activity was intermediate between the respective values for naive and dependent animals.

In NG108-15 neuroblastoma x glioma hybrid cells, opiates inhibited adenylate cyclase, and the involvement of the cAMP system in the development of opiate tolerance and dependence in these cells has been suggested (1,2). We have previously described the inhibition by opiates of prostaglandin-induced formation of cAMP in striatal slices and in primary cultures of neurons from rat brain (3,4). The effect was stereospecific and was blocked by opiate antagonists. In contrast, in primary cultures of glia, which lack opiate receptor, prostaglandin-induced cAMP formation was insensitive toward inhibition by opiate agonists (4). These results suggested that the interaction of opiates with adenylate cyclase in brain is mediated by ligand occupancy of the opiate receptor.

Recently, opiate stimulation of membrane-bound GTPase in the NG108-15 hybrid cells was reported (5), and discussed as the mechanism by which these drugs inhibit adenylate cyclase (6). According to the forwarded hypothesis, the stimulation leads to the enhanced hydrolysis of GTP, thus increasing the

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rate of adenylate cyclase inactivation. The present study was carried out to investigate the functioning of such a mechanism in mammalian brain.

MATERIALS AND METHODS

[γ-^{32}P]GTP was obtained from New England Nuclear Corporation, Boston MA. The biochemicals for the enzyme assay, including GTP, ouabain, creatine phosphate, creatine kinase, and the nonhydrolyzable adenosine derivative p[NH]ppA, were purchased from the Sigma Chemical Company, St. Louis, MO. Other chemicals were of reagent grade.

Opiate dependence in rats (180 g male Sprague-Dawley) was induced by the implantation of 75 mg pellets of morphine base according to published procedures (7,8). The state of opiate dependence was assessed by precipitation of the withdrawal syndrome by i.p. injection of naloxone (2 mg/kg). The onset of diarrhea and the frequency of wet-dog shakes were followed, quantitated, and compared to those previously reported (7).

Animals were sacrificed either at 72 hours post-implantation (maximal dependence), or 45 minutes after the i.p. administration of naloxone to the dependent rats (precipitated abstinence). The procedure for the isolation of membranes from brain striatum differed in some details depending on the use of the preparation on the day of isolation (fresh membranes) or after storage at -70° (frozen membranes). In essence, the membranes were isolated as described previously (9). The tissue was dissected at 4° and disrupted in 10 mM Tris.HCl-0.1 mM EDTA, pH 7.5, with a Potter-Elvehjem type glass-glass homogenizer at 2,500 rpm (fresh membranes), or by using a Polytron homogenizer, model PT-10, for 60 sec at power output 6.5 (frozen membranes). The homogenate was centrifuged at 20,000 x g for 20 min at 4°, and the pellet suspended in the original volume of the buffer. Aliquots of this suspension were either frozen at -70° until use (frozen membranes), or centrifuged as described above. The pellet was suspended in the Tris.HCl-EDTA buffer to yield a protein concentration of 2-5 mg per ml (fresh membranes). In experiments with frozen membranes, aliquots were thawed, centrifuged, and the pellet suspended with Tris.HCl-EDTA as described above for the freshly isolated membranes.

Protein was determined according to Lowry *et al.* (10), using bovine serum albumin as standard.

GTPase was assayed as described (5). The assay is based on the release of inorganic phosphate (P_i) from [γ-^{32}P]GTP (11). The reaction mixture included 12.5 mM Tris.HCl, pH 7.5, an ATP-regenerating system, p]NH]ppA, and ouabain to inhibit Na,K-ATPase. Each tube contained 2-5 µg membrane protein. After incubation at 37°, the tubes were placed in ice, and a suspension of activated charcoal in phosphoric acid was added to absorb excess nucleotides. Released $^{32}P_i$ was measured by liquid scintillation counting.

RESULTS

The experiments in the first phase of this study were designed to test the hypothesis that the interaction of opiates with brain GTPase is mediated by ligand occupancy of the opiate receptor. The GTPase assay was carried out for 10 min with 2-5 µg brain membrane protein, representing linear analytical conditions (Fig. 1). Average values for the K_m and V_{max} of basal GTPase activity were 0.4 µM and 90 pmole/min per mg protein, respectively. At GTP concentrations below 50 µM, opiate agonists stimulated GTPase activity

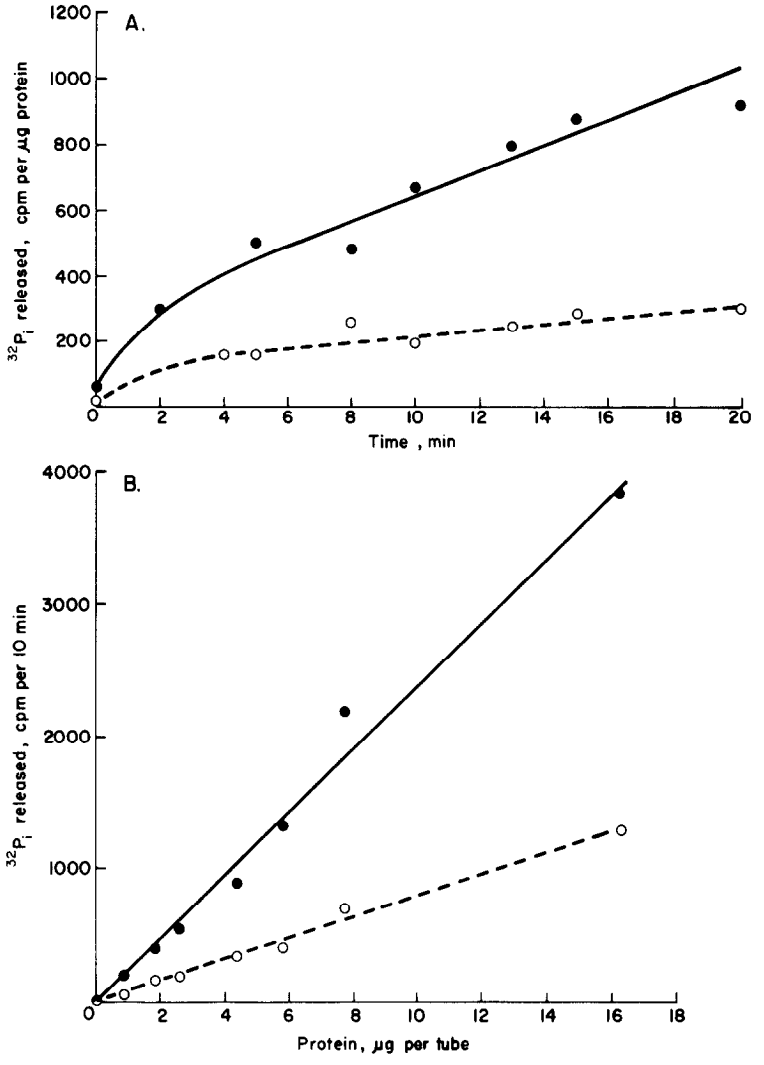

Fig. 1. GTPase activity in brain membranes as a function of time and protein concentration. Aliquots of striatal membranes, corresponding to 4.5 µg protein, were incubated for various lengths of time in the GTPase assay medium (graph A). In other experiments the incubation was conducted for 10 min with different concentrations of membrane protein (graph B). Plotted are results of experiments carried out with fresh membranes at GTP concentrations of 10^{-5} M (●) and 10^{-8} M (○). Linear relationships were also obtained with GTP concentrations between those depicted. Throughout this study, freshly isolated and previously frozen membranes yielded similar results.

(Fig. 2). At higher concentrations of the nucleotide, the stimulation was masked by the contribution of an opiate-insensitive GTP-hydrolyzing activity of apparently high K_m. Maximal enhancement of the low-K_m GTPase by 10 µM levorphanol was 28%. The concentration for half-maximal stimulation was 0.09 µM for levorphanol (Fig. 2, inset) and 3.8 µM for morphine (data not shown). This order of potency is similar to that of the EC_{50} values displayed

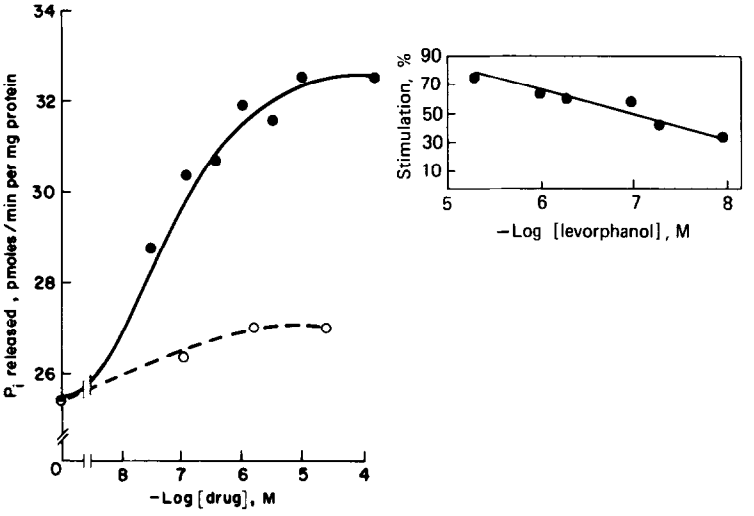

Fig. 2. Stereospecificity of GTPase stimulation by opiates. Aliquots of the membrane preparation were incubated as described in the text at 0.12 μM GTP with different concentrations of levorphanol (●) and dextrorphan (○). The values for enzyme activity were calculated by subtracting the cpm of $^{32}P_i$ released at high levels (≥50 μM) of GTP (representing opiate-insensitive GTP-hydrolyzing activity), and by correcting for the specific radioactivity of [γ-^{32}P]GTP at each concentration of the nucleotide (5,11). The inset depicts the log-probit plot of GTPase stimulation by levorphanol. Shown as results of a typical experiment run in duplicates. The log-probit plot represents mean values of 4 experiments. The standard deviation around the mean was in all instances less than 15%.

by these opiates in displacing the opiate receptor binding of [^{3}H]etorphine in brain membranes (12). During maximal enhancement of GTPase activity its K_m and V_{max} were increased by 19% and 26%, respectively, thus suggesting that the rate of hydrolysis was primarily responsible for the observed stimulation. The dependence of GTPase stimulation on the concentration of GTP, and increased values for the kinetic constants have previously been observed with the NG108-15 hybrid cells (5,6). As shown by the lacking effect of dextrorphan, the dextrarotatory isomer of levorphanol, the stimulation of GTPase by opiates was stereospecific (Fig. 2). Furthermore, the stimulation of GTPase by levorphanol was blocked by the antagonist naloxone (Fig. 3). The concentration of naloxone that produced half-maximal inhibition of GTPase stimulation, caused by 10 μM levorphanol, was 3.0 μM (Fig. 3, inset).

In view of previous reports on the involvement of adenylate cyclase in morphine dependence induced in NG108-15 hybrids in culture (1,2), it was of interest to examine the interaction of opiates with GTPase in brain membranes

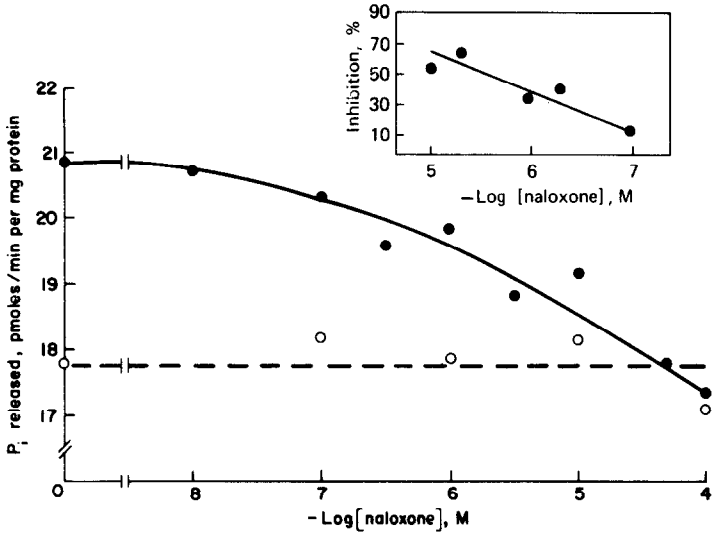

Fig. 3. Inhibition by naloxone of GTPase stimulation by opiate agonists. Aliquots of the membrane preparation were incubated as described in the text at 0.12 μM GTP with (●) and without (○) 10 μM levorphanol, and with increasing concentrations of naloxone. The inset depicts the log-probit plot of naloxone inhibition. Shown are averages of 2 experiments. The corresponding data of the two experiments varied by less than 5%.

isolated from morphine-dependent rats. In striatal membranes isolated from rats made dependent to morphine by pellet implantation, GTPase activity was lower than in comparable membranes of naive animals (Fig. 4). Determined at various concentrations of GTP, the decrease ranged between 20% and 40%. After the precipitation of morphine abstinence by naloxone, GTPase activity was intermediate between the values obtained for naive and dependent animals (Fig. 4). Furthermore, enzyme activity in each of these three membrane preparations was enhanced to a similar extent (mean value 20%) by 10 μM levorphanol added *in vitro*. The removal of residual morphine, deriving from the implanted pellet, by the extensive dilutions and washing of the particulate material in the course of membrane isolation was ascertained by the identical EC_{50} values obtained for morphine in displacing [^{3}H]etorphine in brain membranes isolated from normal and morphine-dependent rats (data not shown).

DISCUSSION

The results described above indicate that the enhancement of brain GTPase by opiates fulfills the criteria for mediation by opiate receptor (i.e., the

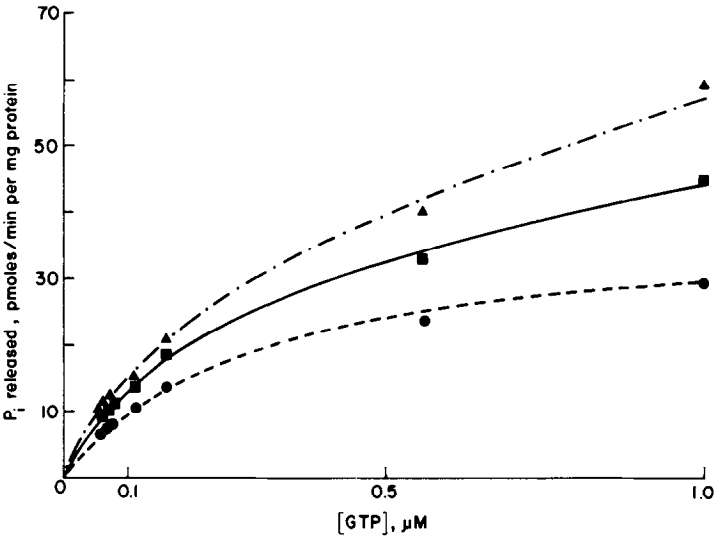

Fig. 4. GTPase activity in brain membranes from naive, opiate-dependent and opiate-abstinent rats. Opiate dependence in rats was induced by morphine pellet implantation as described under Materials and Methods. Animals were sacrificed either at 72 hours post-implantation (maximal dependence), or 45 minutes after the i.p. administration of naloxone to the dependent rats (precipitated abstinence). The membranes were isolated from naive (▲), morphine-dependent (●), and opiate-abstinent (■) rats, and GTPase activity was determined at different concentrations of GTP. Shown are results of a representative experiment run in duplicate. In 4 separate experiments, GTPase activity in membranes from normal and dependent animals differed by 20-40% and was significant at the $p<0.001$ level.

phenomenon exhibited agonist-selectivity with appropriate order of potency, stereospecificity, and antagonism by naloxone), in analogy to the inhibition of brain adenylate cyclase by these compounds (3). Considering that a similar reciprocal relationship between GTPase stimulation and adenylate cyclase inhibition by opiates exists in NG108-15 neuroblastoma x glioma hybrid cells (5,6), our results suggest a common mechanism for these phenomena in brain and in the transformed neural cells.

In view of the data on striatal GTPase in opiate dependence, it is tempting to assume the functioning of a reciprocal mechanism between adenylate cyclase and GTPase during chronic exposure to opiate agonists. Similarly to the relationship observed in the neuroblastoma-glioma hybrids (1,2), long-term presence of morphine leads to a decreased activity of brain GTPase, apparently compensating for the acute stimulatory effect of the opiate agonist (Fig. 4). During precipitated abstinence, the enzyme activity indicates a recovery

toward normal levels. However, a functional relationship between opiate dependence and high-affinity GTPase has yet to be established.

In light of the results presented in this paper, it is of interest to note the limited extent by which adenylate cyclase, both basal and prostaglandin-stimulated, is affected by opiate agonists. Maximal inhibition of basal and induced enzyme activity was about 27% and 50% in striatal homogenates (13) and brain slices (3), respectively. This plateau of adenylate cyclase inhibition corresponds to the limited stimulation of GTPase by opiate agonists in brain membranes (Fig. 2). Such findings suggest that opiate-sensitive GTPase and adenylate cyclase represent specific, opiate receptor-linked, subtypes of these enzymes (14). Indeed, further characterization of the underlying molecular mechanism of receptor-effector coupling may largely depend on the successful enrichment of the opiate-sensitive adenylate cyclase and GTPase from neural tissue. Notwithstanding the questions raised about the use of the NG108-15 hybrids cells as models in elucidating the mechanisms of narcotic action in brain (15), our studies on opiate interaction with adenylate cyclase and GTPase in brain from normal and dependent rats provide evidence for the similarity of these events in the two biological systems.

After the completion of this work a brief report appeared describing, in essence, observations similar to ours (16).

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