THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 252, No. 22, Issue of November 25, pp. 7964-7966, 1977
Printed in U.S.A.

GTP Stimulates and Inhibits Adenylate Cyclase in Fat Cell Membranes through Distinct Regulatory Processes*

(Received for publication, March 23, 1977)

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GTP and hormones activate, synergistically, adenylate cyclase in purified plasma membranes from rat adipocytes. Addition of chelating reagents (EDTA or ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid) or thiol-reducing reagents (dithiothreitol or 2-mercaptoethanol) results in marked inhibition of enzyme activity without altering the synergistic stimulatory effects of GTP and hormones. The inhibitory effects of the reagents required the presence of GTP, indicating that inhibition involves a GTP-dependent process. This process is separate from the GTP-dependent process responsible for activation of the enzyme since it is selectively abolished by pretreatment of fat cell membranes with trypsin. It is suggested that inhibition and activation of fat cell adenylate cyclase by GTP occur through distinct regulatory processes.

Several studies have shown that GTP or Gpp(NH)p exert both stimulatory and inhibitory effects on the multireceptor adenylate cyclase system in fat cells (1-4). Both effects of the nucleotides were found to be sensitive to assay conditions, including temperature, magnesium ion concentrations, pH, and hormones. As an explanation for the effects of the nucleotides, it was proposed recently (4), that the enzyme system is accessible to three states having differing V_{max} and K_i for uncomplexed ATP ($HATP^{3-}$ or ATP^{4-}), postulated to be a potent inhibitor at the active site (5, 6). The guanine nucleotides, acting through a common site, affect the distribution of the states such that, depending on the incubation conditions (pH, Mg^{2+}, hormone, temperature), the activity expressed will be inhibitory or stimulatory relative to the basal state of the enzyme.

During the course of investigating the stimulatory effects of GTP under conditions which minimized the inhibitory effects of the nucleotide, we found that the addition of thiol-reducing agents or chelators resulted in decreases in enzyme activity. The effects of these agents were dependent on the concentration of GTP in the medium and the type and concentration of divalent cation present in the incubation medium. This report describes these findings and provides evidence that the inhibitory process is due to a regulatory protein which is distinct from the regulatory components responsible for GTP and hormonal activation of adenylate cyclase.

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‡ Recipient of Public Health Service International Fellowship F05TW-0221-02.

EXPERIMENTAL PROCEDURES

Materials—ACTH[1-24] [1] (Synacthen) was a gift of Ciba-Geigy. Crystalline porcine glucagon was obtained from Eli Lilly and Co. Secretin was a gift of Dr. V. Mutt (Karolinska Institute, Stockholm). Epinephrine bitartrate, epinephrine/HCl, dithiothreitol, and 2-mercaptoethanol were purchased from Sigma. Trypsin (266 units/mg) and soybean trypsin inhibitor (1 mg inhibits 1.53 mg of trypsin) were purchased from Worthington. All radioactive materials and other reagents were obtained from sources previously described (4).

Preparation of Fat Cell Membranes—Fat cell membranes were prepared as described previously (1). The final membrane pellet was suspended in 1 mM EDTA and 20 mM Tris/HCl, pH 7.5, to give a concentration of 1 mg/ml of membrane protein. Aliquots were frozen and stored in liquid nitrogen.

Assay of Adenylate Cyclase—The method of Salomon *et al.* (7) was used for assaying the production of cyclic AMP (2). The standard assay medium contained in 100 μl, 30 mM Tris/HCl, pH 7.5, 0.05% bovine serum albumin, 1 mM cyclic AMP, 0.1 mM [α-^{32}P]ATP (50 to 200 cpm/pmol), 5 mM creatine phosphate, 50 units/ml of creatine phosphokinase, 1 mM ascorbic acid, and, except when indicated, 10 mM $MgCl_2$. Reactions were initiated by the addition of membranes to give a final concentration of 3 to 5 μg of protein/ml. EDTA present in the membrane suspension was no higher than 10 μM in the final assay medium. Incubations were carried out for 8 min at 37°. All experiments were carried out with at least two preparations of fat cell plasma membranes and each assay was run in duplicate or triplicate.

Other Determinations—Protein was determined by the method of Lowry *et al.* (8) using bovine serum albumin as standard.

RESULTS

Effects of GTP on Basal and Hormone-stimulated Activities—Fig. 1 illustrates the stimulatory effects of GTP on both basal and hormone-stimulated activities; half-maximal stimulation required about 0.1 μM GTP in all cases. Previously determined (1) saturating concentrations of the hormones were added in these experiments. Note that, with the exception of epinephrine, stimulation by hormones was marginal in the absence of GTP. These results demonstrate the synergistic effects of GTP and hormones on the fat cell adenylate cyclase system under the conditions of assay (0.1 mM ATP, 10 mM Mg^{2+}, pH 7.6, 37°) employed in this study.

Effects of Chelators in Presence of GTP and Hormones—As shown in Fig. 2, EGTA inhibited both basal and hormone-

[1] The abbreviations used are: ACTH, adrenocorticotropic hormone; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

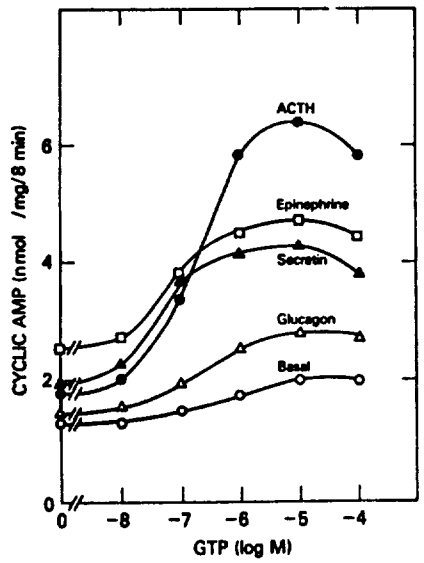

FIG. 1 (*left*). Effects of GTP on basal and hormone-stimulated adenylate cyclase activities. Adenylate cyclase activity was assayed under the standard conditions described in the text. The concentrations of hormones are 10 μM ACTH, 10 μM epinephrine, 20 μM secretin, and 2 μM glucagon.

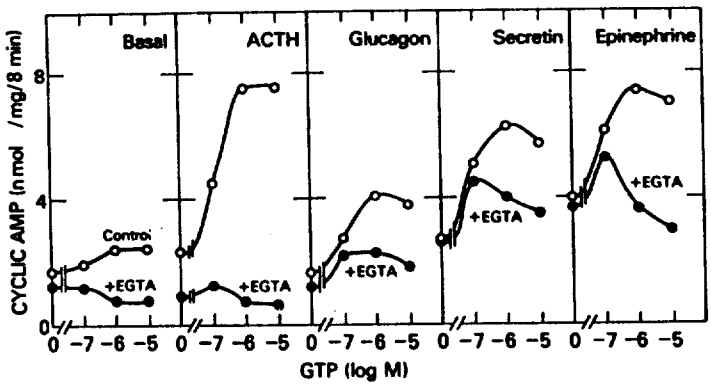

FIG. 2 (*right*). Dependence of EGTA inhibition of adenylate cyclase on concentration of GTP. Adenylate cyclase activity was assayed under standard assay conditions in the absence or presence of 1 mM EGTA and the indicated concentrations of GTP. Concentrations of hormones are the same as in the legend to Fig. 1.

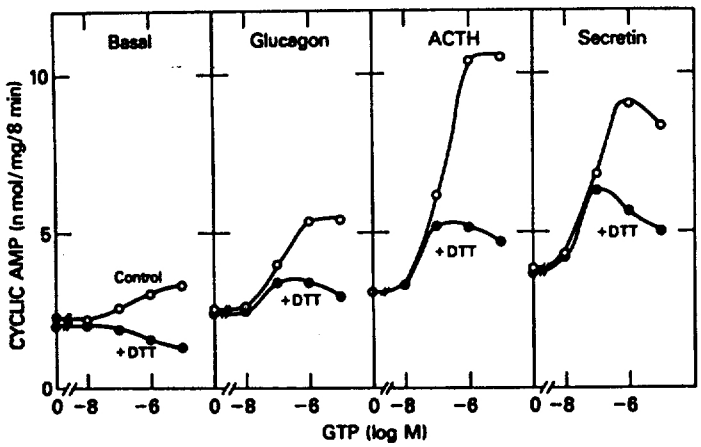

FIG. 3 (*left*). Dependency of dithiothreitol inhibition on the concentration of GTP. Adenylate cyclase activities were assayed under the conditions described in the legend to Fig. 1. The concentration of dithiothreitol (*DTT*) was 1 mM.

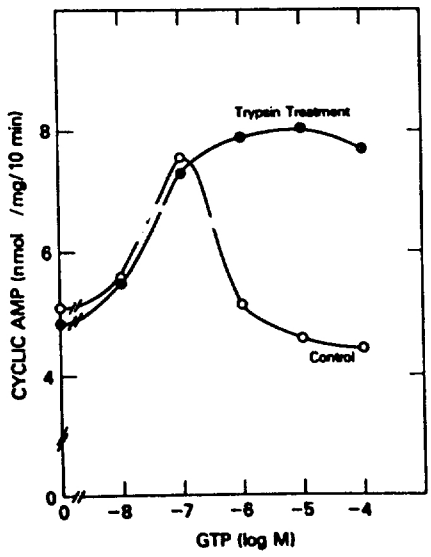

FIG. 4 (*right*). Effects of trypsin pretreatment on the actions of GTP and dithiothreitol. Fat cell membranes (100 μg/ml) were incubated in the absence (control) or presence of 0.2 μg/ml of trypsin for 5 min at 30°. The incubation medium (0.5 ml) contained 0.1% bovine serum albumin and 20 mM Tris/HCl, pH 7.6. Soybean trypsin inhibitor was added to both control and trypsin-treated membranes at a final concentration of 2 μg/ml. Adenylate cyclase activities were assayed under standard assay conditions in the presence of 1 mM dithiothreitol, 10 μM GTP, and 1 mM epinephrine.

stimulated activities. Inhibition by the chelator required the presence of GTP at concentrations in excess of 0.1 μM. With the exception of ACTH, EGTA did not selectively inhibit basal or hormone-stimulated activities. The selective loss of ACTH response with EGTA has been noted previously (9, 10) and has been attributed to removal of calcium ion which is thought to be essential for the actions of ACTH. Addition of 10 μM calcium ion in the presence of 10 μM EGTA resulted in near complete recovery of ACTH action and abolished the inhibitory effect of EGTA on both basal activity as well as the activities obtained with the other hormones (data not shown).

A similar pattern of inhibition was observed with EDTA but 10-fold higher concentrations were required to give inhibition comparable to that observed with EGTA. Based on the stability constants for MgEDTA and MgEGTA (11), the effects of the chelators on enzyme activity cannot be explained by

chelation of sufficient Mg^{2+} to alter the concentrations of free Mg^{2+}, $MgATP^{2-}$ (as substrate), or of uncomplexed forms of ATP (ATP^{4-}, $HATP^{3-}$).

Effects of Thiol-reducing Agents—As observed with the chelators, dithiothreitol also inhibited both basal and hormone-stimulated activities provided that GTP was present in the medium at concentrations exceeding 0.1 μM (Fig. 3). Half-maximal inhibition required 0.5 mM dithiothreitol under all incubation conditions, indicating that the thiol reagent did not selectively inhibit basal or hormone-stimulated activities. Similar inhibitory effects were observed also with 2-mercaptoethanol; half-maximal inhibition required 1 mM 2-mercaptoethanol. It should be noted in Fig. 3 that the thiol reagents did not alter the ability of GTP and hormones to synergistically stimulate adenylate cyclase activity.

Effects of Dithiothreitol and Chelators in Presence of Mn^{2+}—The degree of inhibition in the presence of GTP, chelators, and thiol reagents was dependent on the type and concentration of divalent cation present in the incubation medium. In all experiments reported above, inhibition was observed in the presence of 10 mM Mg^{2+}. However, increasing the concentration of Mg^{2+} to 50 mM abolished the inhibitory effect of dithiothreitol (data not shown, but see Ref. 4). When 10 mM Mg^{2+} was replaced with 3 mM Mn^{2+}, only slight inhibition was observed even with 10 mM dithiothreitol in the presence of GTP alone (basal) or plus hormones (epinephrine could not be tested because of oxidation of catecholamines in the presence of Mn^{2+}).

Effects of Trypsin Treatment on Actions of GTP—The biphasic stimulatory and inhibitory effects of GTP observed in the presence of chelators and dithiothreitol raised the possibility that these effects were exerted through independent processes. As one means of testing this possibility, fat cell membranes were pretreated with varying concentrations of trypsin for 5 min at 30°, followed by addition of trypsin inhibitor to stop the reaction. Trypsin-treated and control membranes were then tested for epinephrine-stimulated adenylate cyclase activity under standard assay conditions in the presence of dithiothreitol (1 mM) and GTP (10 μM). A typical result, obtained with an optimal concentration of trypsin (0.2 μg/ml), is shown in Fig. 4. The control (nontrypsin-treated) membranes showed the typical biphasic effects of GTP on adenylate cyclase activity in the presence of dithiothreitol whereas the trypsin-treated membranes showed only the stimulatory effect of GTP. These results demonstrate that the GTP-inhibitory process is particularly sensitive to trypsin and clearly distinguishes this process from the GTP-stimulatory process observed in the presence of hormones.

DISCUSSION

Inhibitory effects of GTP have been reported with the fat cell system in the absence of chelators of thiol reagents when the incubation temperature was below 37° (1) or when the pH and the concentration of Mg^{2+} in the incubation medium were reduced (4). We have shown in this study that two different types of reagents, thiol-reducing agents and chelators, cause inhibition of adenylate cyclase activity in fat cell membranes. Inhibition by both types of reagents was dependent on the presence of GTP and became particularly evident at concentrations of the nucleotide in excess of 0.1 mM. With the exception of the effects of EGTA on ACTH action, the reagents did not alter the ability of GTP and hormones to activate the enzyme.

Earlier it had been suggested that the biphasic effects of GTP could be accounted for by the formation of a transition state which is highly susceptible to inhibition by $HATP^{3-}$ (4). However neither the chelators (present below 1 mM) or thiol-reducing agents (which do not form stable complexes with Mg^{2+} or other cations) are likely to affect the concentration of $HATP^{3-}$ under the incubation conditions described here. Other explanations for the effects of GTP must therefore be sought.

The finding that trypsin treatment of the membranes results in loss of inhibition by GTP (in the presence of dithiothreitol) without affecting the synergistic response of adenylate cyclase to GTP and hormones provides strong evidence that the inhibitory process is distinct from the GTP-regulatory component involved in hormonal activation of the enzyme. The fact that the inhibitory process is trypsin-sensitive suggests that it is a protein that interacts with GTP. Previous studies (1) have shown that Gpp(NH)p mimics the stimulatory and inhibitory actions of GTP on the fat cell systems; GDP does not inhibit the enzyme. Therefore, it is unlikely that this putative protein has phosphohydrolase or phosphotransferase activities.

Since the inhibitory effect of GTP was not specific for a particular hormone and basal activity was affected to about the same extent, it is unlikely that either hormone receptors or the "coupling" between receptors and catalytic unit are involved. The simplest explanation is that inhibition occurs through a discrete regulatory protein which alters the catalytic unit of the enzyme and that this interaction is affected by temperature, pH, metal ions, chelators, and thiol-reducing agents. The selective effects of trypsin treatment on GTP inhibition and the fact that the process involved in the synergistic actions of GTP and hormones can be preserved under all these conditions now provide a means of distinguishing between the two GTP-dependent effects on enzyme activity and of selectively examining the mechanisms underlying these processes. Such studies are in progress.

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