Negative Cooperativity Exhibited by Prostaglandin E_1 Stimulated Adenylate Cyclase in LM Cells†

Victor H. Engelhard,‡ Dan R. Storm,*,§ and Michael Glaser¶

ABSTRACT: Adenylate cyclase in LM cell membranes is stimulated by prostaglandin E_1 (PGE_1) in the presence of GTP. At saturating concentrations of GTP, the ratio of PGE_1 + GTP stimulated activity to basal activity increased with ATP concentration in the range of 0.1–1 mM ATP. This phenomenon was due to the apparent negative cooperativity for ATP expressed by the PGE_1 + GTP stimulated adenylate cyclase activity which was not observed with basal activity. Basal adenylate cyclase activity had a K_m for ATP of 70 μM, whereas PGE_1 + GTP stimulated activity could be characterized by K_m values of 210 and 490 μM. Therefore, the basal activity was saturated with respect to ATP at concentrations of ATP where PGE_1 + GTP stimulated activity was still increasing with ATP concentration. There are several possible interpretations of this data which include the presence of two distinct adenylate cyclase species, one enzyme under negative cooperative control or the existence of an ATP regulatory site distinct from the GTP binding or catalytic sites. Regardless, of which molecular mechanism is operative, the basic observation suggests a mode of regulation for adenylate cyclase which has not been previously reported. It is proposed that the apparent negative cooperativity for PGE_1-stimulated adenylate cyclase activity may kinetically buffer cyclic AMP concentrations within the cell.

Adenylate cyclase in animal cell membranes is stimulated by peptide hormones, catecholamines, and prostaglandins. It has also been demonstrated that GTP is required for hormonal stimulation of adenylate cyclase (Rodbell et al., 1971; Londos and Rodbell, 1975; Leray et al., 1972; Krishna et al., 1972). Although initial studies suggested that GTP was an obligatory requirement for hormonal stimulation (Rodbell et al., 1971), it was discovered that ATP, at millimolar concentrations, would substitute for GTP (Goldfine et al., 1972; Birnbaumer et al., 1972; Krishna and Harwood, 1972; Rodbell et al., 1975). These observations suggested that ATP could also bind at the GTP regulatory site, but with lower affinities than GTP. More recently, however, it was discovered that commercial ATP samples are contaminated with trace amounts of GTP and that highly purified ATP cannot substitute for GTP (Kimura et al., 1976; Kimura and Nagata, 1977).

While studying PGE_1[1] stimulation of adenylate cyclase in LM cell membranes, it was discovered that stimulation by PGE_1 is strongly dependent upon ATP concentrations, even at saturating GTP concentrations. Therefore, the GTP and ATP concentration dependence for basal and PGE_1-stimulated adenylate cyclase activities were examined in greater detail. This kinetic study indicates that PGE_1 + GTP stimulated adenylate cyclase activity, but not basal activity, exhibits apparent negative cooperativity with respect to ATP. It is proposed that this phenomenon may kinetically buffer intracellular cAMP pools and provide a mechanism for regulating cAMP concentrations within the cell.

Materials and Methods

Materials. All chemicals were reagent grade. Organic solvents were redistilled. Prostaglandin E_1 (PGE_1) was kindly supplied by Dr. John Pike, Upjohn Co.

Growth and Supplementation of Cells. Mouse LM cells were grown in suspension culture in Higuchi's medium (Higuchi, 1970) containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.4), 1 g/L methylcellulose, and 0.02 g/L sodium dextran sulfate. Growth and harvesting of cells were carried out as previously described (Glaser et al., 1974; Engelhard et al., 1976).

Preparation of Plasma Membranes. Plasma membranes were prepared by a method previously described (Esko et al., 1977). The cells were lysed in a hypotonic medium and gently sheared to separate the nuclei from the plasma membrane. In some experiments, the cells were homogenized with a Tissumizer (Tekmar Co.). This resulted in a lower yield of plasma membranes but did not otherwise affect the results. The nuclei were removed by centrifugation at 3000*g* for 10 min, and the supernatant was centrifuged at 48 000*g* for 1 h. The membrane pellet was layered into a discontinuous sucrose gradient, and the bands were isolated after centrifugation. Examination of the adenylate cyclase activities in the different fractions showed a similar purification to the (Na^+,K^+)ATPase and indicated that the adenylate cyclase was localized in the plasma membrane of the cells (data not shown). The 48 000*g* membrane pellet was routinely used for adenylate cyclase assays.

Adenylate Cyclase Assay. Adenylate cyclase was assayed according to the method of Salomon et al. (1974) in 50 μL containing 1 mM $\alpha[^{32}P]$ATP (300 cpm/mol), 2 mM cAMP, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM β-mercaptoethanol, 5 mM theophylline, 1.1% bovine serum albumin and 20 mM K_2HPO_4, pH 7.5 (Salomon et al., 1974). Creatine kinase (1 mg/mL) and phosphocreatine (20 mM) were present as a ATP regenerating system. ATP used in assays was purified by DEAE-Sephadex A-25 chromatography, followed by Dowex AG-50 chromatography. This ATP was completely free of contaminating GTP, as judged by thin-layer chromatography (Kimura and Nagata, 1977). Each sample contained 90–110 μg of membrane protein. Samples were incubated at 30 °C for

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[1] Abbreviations used: PGE_1, prostaglandin E_1; DEAE, diethylaminoethyl.

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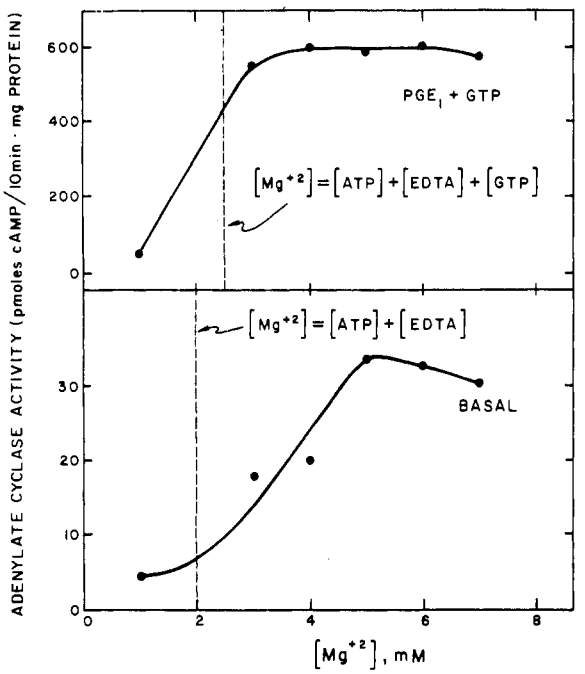

FIGURE 1: Dependence of adenylate cyclase activity on Mg^{2+} concentration. Adenylate cyclase activity was assayed as outlined under Materials and Methods, varying the Mg^{2+} concentration. GTP was present at a final concentration of 0.5 mM.

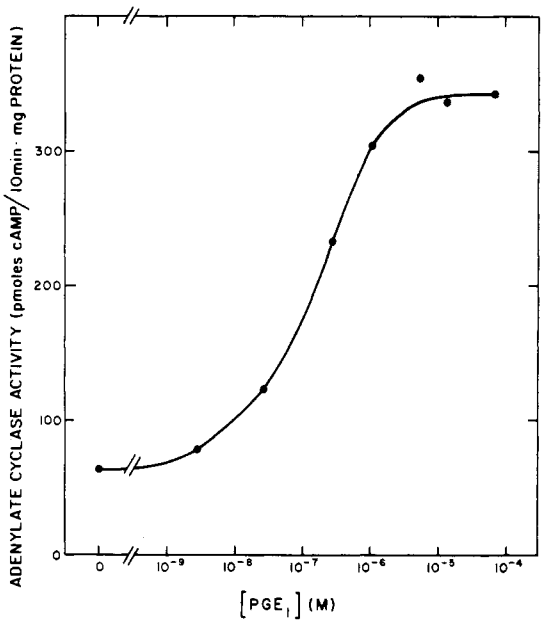

FIGURE 2: Dose-response curve of PGE_1-stimulated adenylate cyclase. Adenylate cyclase was assayed as outlined under Materials and Methods on membranes prepared from cells grown in the presence of choline. PGE_1 concentrations in the assay varied from 2.8×10^{-9} to 7.0×10^{-5} M. ATP was present at 1.0 mM.

10 min. In some experiments, the concentration of ATP was less than 1 mM. In these cases, the cAMP concentration was maintained at 200% of the ATP concentration, and the incubation time was reduced to 2 min. Formation of cAMP was linear with time during this incubation period. Concentrations of fluoride and PGE_1 were 15 mM and 4 μM, unless otherwise specified. Each data point is the mean of triplicate determinations with a standard deviation of less than 5%. The data presented in this report were obtained with one membrane preparation; however, identical results were obtained with two other different preparations.

Protein. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Results

Mg^{+2} Dependency for Adenylate Cyclase Activity. The actual substrate for adenylate cyclase is Mg·ATP (Drummond and Duncan, 1970; Robison et al., 1968), and the association constant for the formation of the Mg·ATP complex is 1.74×10^4 M at pH 8 (Khan and Martell, 1966). Therefore, it was important, as a preliminary study, to determine the optimum Mg^{2+} concentrations for basal and PGE_1 + GTP stimulated activities. The Mg^{2+} dependence for basal and PGE_1-stimulated adenylate cyclase activities are reported in Figure 1. Basal activity reached a maximum at 5 mM Mg^{2+}, which corresponds to a free Mg^{2+} concentration of approximately 3 mM. In contrast, PGE_1 + GTP stimulated activity was maximal at 3 mM total Mg^{2+}, which represented a free Mg^{2+} concentration of approximately 0.5 mM. Thus, the PGE_1 + GTP stimulated state was much less dependent upon excess Mg^{2+} than the basal activity. In the experiments described below, Mg^{2+} at a concentration of 5 mM was used in order to obtain a maximum expression of basal and PGE_1-stimulated adenylate cyclase activities.

Stimulation by PGE_1. The dose-response curve of adenylate cyclase activity as a function of PGE_1 concentration is given in Figure 2. A significant increase in adenylate cyclase activity occurred in the presence of as little as 2.8×10^{-9} M, the lowest

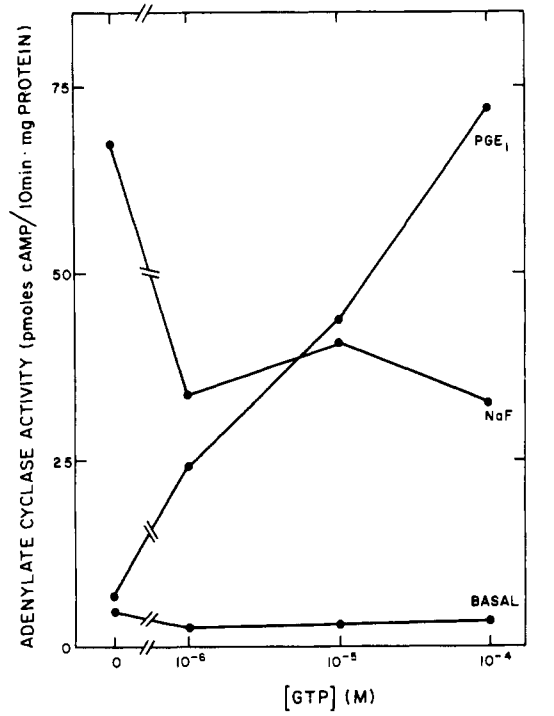

FIGURE 3: Effect of GTP on basal-, fluoride-, and PGE_2-stimulated adenylate cyclase. Adenylate cyclase was assayed in the presence of 50 μM ATP and varying concentrations of GTP. Incubation time was 2 min. Other experimental details are given under Materials and Methods.

PGE_1 concentration tested. Half-maximal activation was observed at 1.8×10^{-7} M, and activity was maximal at concentrations of PGE_1 above 4.5×10^{-6} M. In most experiments, a concentration of 1.4×10^{-5} M was used to achieve maximal activation.

Effect of GTP and ATP on Adenylate Cyclase Activity. The influence of GTP on basal-, fluoride-, and PGE_1-stimulated adenylate cyclase activities at an ATP concentration of 50 μM is reported in Figure 3. In the absence of GTP, PGE_1 produced

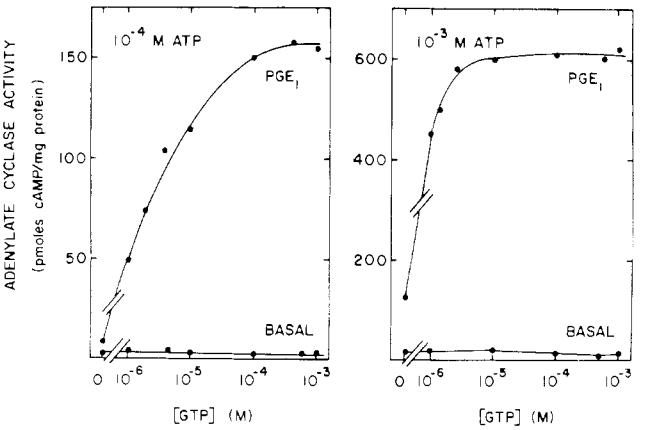

FIGURE 4: Effect of GTP on basal and PGE_1-stimulated activity of 0.1 and 1 mM ATP. Adenylate cyclase was assayed as outlined under Materials and Methods, using either 0.1 or 1 mM ATP and varying concentrations of GTP.

only a 1.3-fold increase in basal activity, in contrast to the 4.4-fold increase produced at 1 mM ATP. However, addition of as little as 1 μM GTP produced a 350% increase in PGE_1-stimulated activity, and additions of up to 0.1 mM GTP increased activity still further. In contrast, basal activity was slightly inhibited by GTP. Thus, GTP and PGE_1 were acting synergistically rather than additively. Fluoride-stimulated activity was inhibited 50% by 1 μM GTP, while further increases had no effect.

The effects of GTP on basal and PGE_1-stimulated activity at two ATP concentrations are compared in Figure 4. In the presence of 0.1 mM ATP, increasing the GTP concentration produced corresponding increases in PGE_1-stimulated activity, which were maximal at 0.5 mM GTP. In the presence of 1 mM ATP, GTP enhancement of PGE_1-stimulated activity was already maximal at 5 μM GTP. The difference in concentrations of GTP producing a maximal effect at different ATP concentrations indicated that either the purified ATP was contaminated by trace amounts of GTP or higher concentrations of ATP enhanced the GTP + PGE_1 stimulated activity. However, the ATP used in these assays was shown by thin-layer chromatography to be free of GTP. In addition, other evidence discussed below illustrated that the ratio of PGE_1 + GTP stimulated activity to basal activity increased with ATP concentration in the presence of saturating concentrations of GTP.

At low ATP concentrations, PGE_1 stimulated the enzyme only slightly, and the degree of stimulation increased with increasing ATP (Figure 5A). The curve represented in Figure 5A did not reach a maximum with ATP concentrations as high 1 mM, and PGE_1 stimulation increased quite rapidly with ATP concentration in the range of 0.1-1 mM.

The data presented thus far indicated that stimulation by PGE_1 or PGE_1 + GTP increased as a function of ATP concentration. The data presented in Figure 5B illustrate that the increase in PGE_1 + GTP stimulated activity relative to basal activity could not possibly be due to GTP contamination. The GTP binding site was saturated at GTP concentrations of 0.5 mM (Figure 4). However, as illustrated in Figure 5B, ATP enhanced the degree of stimulation by PGE_1 + GTP at saturating concentrations of GTP. This was not due to an additive increase in the degree of stimulation seen in the presence of PGE_1 alone which was reported in Figure 5A. The dotted line in Figure 5B indicates the expected response if the data in Figure 5A were added to the low limit of tenfold stimulation produced by PGE_1 + GTP at low concentrations. Rather, ATP

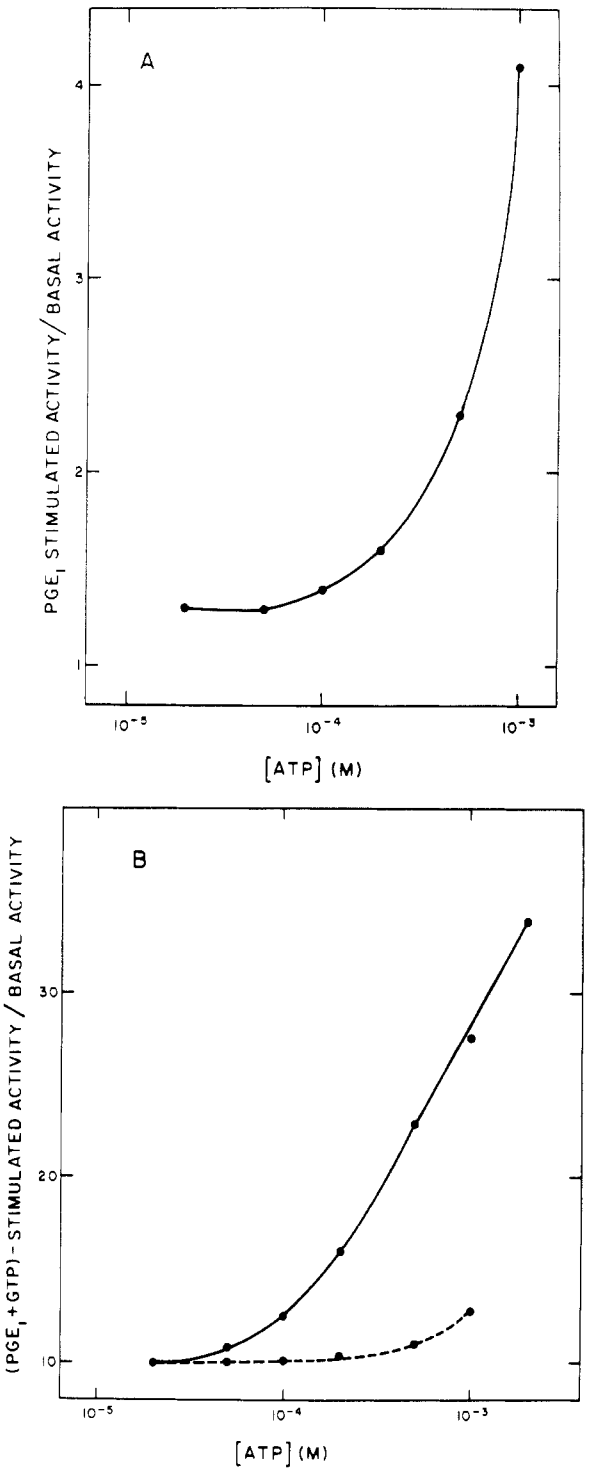

FIGURE 5: Influence of ATP concentration on the degree of stimulation by PGE_1 (A) in the absence of GTP and (B) in the presence of 0.5 mM GTP: (●—●) experimental data; (●---●) calculated by adding the data of A to tenfold stimulation produced by PGE_1 + GTP at low ATP concentrations. Adenylate cyclase was assayed as outlined under Materials and Methods using varying concentrations of ATP + cAMP. Incubation times were 2 min.

enhanced the degree of stimulation by either PGE_1 or PGE_1 + GTP to the same extent. This is illustrated by a comparison of the ratios of activation produced at 1 mM ATP to that obtained with 5×10^{-5} M ATP. This number was 3.2 for PGE_1-stimulated activity and 2.5 for PGE_1 + GTP stimulated activity. This indicates that ATP has similar effects on both states of the enzyme.

K_m Determinations for Basal and PGE$_1$ + GTP Stimulated Adenylate Cyclase Activities. If indeed there exists a separate ATP regulatory site or, alternatively, cooperativity with respect to ATP, then this should be reflected in the ATP concentration dependence for the enzyme-catalyzed reactions. Lineweaver–Burke and Eadie–Hofstee plots of basal adenylate cyclase activity were linear over two orders of magnitude in ATP concentration (0.02–2 mM), and there was no indication of cooperativity or site heterogeneity (Figure 6A). The apparent K_m for ATP was 70 μM. In contrast to the simple behavior of basal activity, PGE$_1$ + GTP stimulated activity did not obey Michaelis–Menton kinetics. Lineweaver–Burke and Eadie–Hofstee plots could not be fit by a single line (Figure 6B). In order to estimate values of K_m at high and low ATP concentrations, the data was fit by two lines (correlation coefficient 0.975). At ATP concentrations less than 0.1 mM, the K_m for PGE$_1$ + GTP stimulated activity was 210 μM and at higher ATP concentrations the K_m was 490 μM. This kinetic analysis illustrates quite clearly why the ratio of PGE$_1$ + GTP stimulated activity to basal activity increased with ATP concentration between 0.1 and 1 mM ATP. The basal activity had a K_m of 70 μM, and the high K_m for PGE$_1$ + GTP stimulated activity was seven times greater. Thus, the basal activity was saturated with respect to ATP at concentrations where PGE$_1$ + GTP stimulated activity was still increasing with ATP concentration. The ratio of these two activities would, therefore, necessarily increase with ATP in the range of 0.1–1 mM.

Discussion

The LM cell adenylate cyclase system shares many common features with adenylate cyclase activities found in other animal cells. The enzyme is associated with the plasma membrane, it is stimulated by NaF and PGE$_1$, and it utilized Mg·ATP as a substrate. However, a more detailed study of PGE$_1$ stimulation of LM cell adenylate cyclase revealed an effect of ATP on hormone stimulation which has not been previously reported. The ratio of PGE$_1$ + GTP stimulated activity to basal activity increased significantly with ATP concentration at saturating concentrations of GTP. Thus, there is an effect of ATP on PGE$_1$-stimulated adenylate cyclase activity that is clearly distinct from GTP.

A kinetic analysis of the PGE$_1$ + GTP stimulated activity demonstrated two apparent K_m values for ATP. At ATP concentrations less than 0.1 mM, the K_m was 210 μM. At higher ATP concentrations, the apparent K_m was 490 μM. In contrast, Eadie–Hofstee plots of basal activity were readily fit by one line with an apparent K_m of 70 μM. Therefore, stimulation by PGE$_1$, in the presence of saturating concentrations of GTP, increased with ATP concentrations in the range of 0.1–1 mM ATP.

All kinetic studies that have been reported for animal cell adenylate cyclase activities have used either membranes, solubilized membrane preparations, or partially purified enzymes. Thus, interpretation of the kinetic results reported in this study and others is necessarily subject to the uncertainties associated with heterogeneous systems. However, there are several reasonable interpretations of the data presented in this study which may be of considerable significance for the regulation of cAMP levels in LM cells. The Lineweaver–Burke and Eadie–Hofstee plots for PGE$_1$ + GTP stimulated adenylate cyclase activity suggested negative cooperativity with respect to ATP. The observation that basal activity did not exhibit this phenomena is quite significant and suggests that coupling between ATP bound to catalytic sites occurs only in the presence of bound PGE$_1$. The apparent negative cooperativity could reflect the existence of two adenylate cyclases differing in K_m for ATP or interactions between ATP binding sites on a single enzyme species. Alternatively, the phenomena reported in this study could also be due to the existence of an ATP binding site distinct from the catalytic and GTP regulatory sites which is only operative when PGE$_1$ is bound to the enzyme.

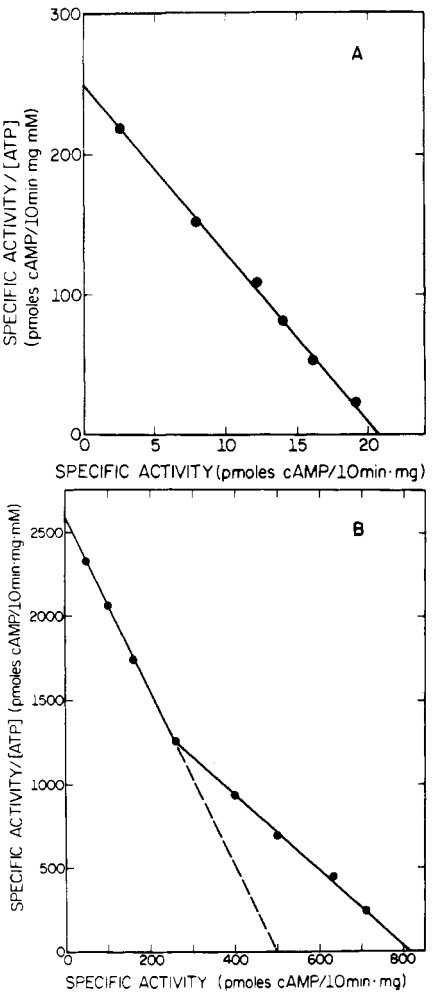

FIGURE 6: Eadie–Hofstee plots of adenylate cyclase activities: (A) basal adenylate cyclase activity. (B) PGE$_1$ + GTP stimulated adenylate cyclase activity. Adenylate cyclase was assayed as described under Materials and Methods using varying concentrations of ATP + cAMP. Incubations were for 2 min. Lines are best linear least-square fits of the data.

Regardless of the molecular interpretation applied to this data, the basic observations are significant and suggest a mode of regulation for adenylate cyclase activity which has not been previously reported. At ATP concentrations in excess of 0.1 mM, the K_m for PGE$_1$ or PGE$_1$ + GTP stimulated activities was significantly increased. This apparent negative cooperativity may effectively buffer cAMP concentrations within the cell when ATP concentrations are high and PGE$_1$ is present. Conway and Koshland have proposed that negative cooperativity may effectively buffer cAMP concentrations within the cell when ATP concentrations are high and PGE$_1$ is present. Conway and Koshland have proposed that negative cooperativity may serve as a mechanism for protection against drastic changes in the chemical environment of an organism (Conway and Koshland, 1968). In contrast to positive cooperative systems (Koshland et al., 1966; Monod et al., 1965) which may provide a switch to modify the regulatory state of the organism, negative cooperativity can serve as a kinetic buffer. It is in-

teresting in this respect that negative cooperativity has also been reported for a cyclic adenosine 3′:5′-monophosphate phosphodiesterase (Russell et al., 1972). The kinetic data presented in this study and that reported by Russell et al. (1972) are both consistent with either one enzyme under negative cooperative regulation or two separate enzymes. It is clear, however, that either situation provides for substantial regulatory control and suggests that regulation of intracellular cAMP concentrations may be controlled not only by hormones and GTP but also by substrate levels.

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Hormonal Induction of α_{2u}-Globulin Synthesis in Isolated Rat Hepatocytes†

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ABSTRACT: Hepatocytes freshly prepared with collagenase synthesize α_{2u}-globulin and other hepatic proteins in vitro at approximately the same rate throughout 30 h of incubation. The newly synthesized proteins are efficiently secreted into the medium throughout this period. That the secretion of proteins by hepatocytes is not due to cell leakage is shown by the fact that 30 μM colchicine prevents the appearance of labeled α_{2u}-globulin and other proteins in the medium. Hepatocytes isolated from animals in different endocrine states synthesize α_{2u}-globulin in vitro at rates consistent with the hormonal effects upon its in vivo biosynthesis. In vitro addition of androgens to hepatocytes isolated from castrated male rats evokes an elevation of general protein synthesis in these hepatocytes. Glucocorticoids, added in vitro, specifically induce an elevated rate of α_{2u}-globulin synthesis.

The α_{2u}-globulin was originally found to be in the urine of male rats and absent from the urine of female rats (Roy & Neuhaus, 1966). Both liver extirpation and immunofluorescent studies have shown that α_{2u}-globulin is synthesized in the parenchymal cells of male rat liver, secreted into the serum, and excreted in the urine (Roy & Raber, 1972; Kurtz et al., 1976b). The rate of in vivo hepatic synthesis of α_{2u}-globulin is under multiple endocrine control: androgens, thyroid hormone, pituitary growth hormone, and glucocorticoids each stimulate α_{2u}-globulin synthesis, while estrogens have been shown to be specific anti-inducers in vivo (Sippel et al., 1975; Kurtz et al., 1976a,b). Previous reports from this laboratory have indicated that the regulation of the synthesis of α_{2u}-globulin by glucocorticoids (Sippel et al., 1975), thyroid hormones (Kurtz et al., 1976a), and sex hormones (Kurtz et al., 1976b) occurs via

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