Effects of GTP,GDP[βS] and glucose on adenylate cyclase activity of *E. coli* B

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Adenylate cyclase activity was measured in suspensions of *E.coli* B, rendered permeable with toluene. The enzyme was activated in a dose-dependent manner by GTP and by its non-hydrolysable analogue, GTP[γS]. In contrast, incubation with GDP[βS], a non-phosphorylatable analogue of GDP, caused a dose-related inhibition of adenylate cyclase; this was partially overcome by addition of GTP. GTP did not relieve, and GDP[βS] augmented, the non-competitive and dose-related inhibition of *E.coli* adenylate cyclase by glucose.

E.coli *Adenylate cyclase* *Catabolite repression* *GTP* *GTP[γS]* *GDP* *GDP[βS]*

1. INTRODUCTION

Changes in the intracellular concentration of cyclic 3′,5′-adenosine monophosphate (cAMP) exert a variety of metabolic effects in *Escherichia coli*. These include the initiation of transcription of some inducible operons, the utilization of one substrate in the presence of another, and control of growth rate (reviews [1,2]). The synthesis of cAMP from ATP is effected by adenylate cyclase, the cellular activity of which is inhibited by good growth substrates ('catabolite repression' [3,4]). However, this effect requires only the uptake and not the metabolism of these substrates: for example, not only glucose, but also its non-catabolizable analogue methyl α-glucoside inhibit cAMP formation in intact *E. coli* [1,5] and inhibit the adenylate cyclase activity of cells rendered permeable with toluene [6,7]. That this inhibition of adenylate cyclase by substrates of the main glucose transport system of *E. coli* is of physiological importance is shown by mutation of a component (factor III^{glc}) of that uptake system. Mutants that lack factor III^{glc} activity require cAMP for growth on many substrates [8] and have greatly reduced adenylate cyclase activity [9]; introduction into such mutants of a plasmid carrying a gene specifying factor III^{glc} also restores adenylate cyclase activity (H.L. Kornberg and L.G. Lee, unpublished).

It is not known how factor III^{glc} affects the activity of the membrane-associated adenylate cyclase. However, this mode of regulation appears to suggest a fundamental difference between the enzyme in *E. coli* and that present in vertebrate cells. In the latter, some hormones react with specific receptors on the cell membrane; this in turn potentiates the activation of adenylate cyclase, mediated by the binding of GTP. Hydrolysis of the GTP (to GDP and P_i) terminates this activation [10].

To test whether there is indeed such a fundamental difference between the enzyme in prokaryotic and eukaryotic cells, we studied the effects of guanine nucleotides on the adenylate cyclase of *E. coli*. We had, perforce, to measure the activity of the enzyme in cells rendered permeable with toluene [6] since, so far, all attempts at purifying the enzyme have resulted in the loss of regulatory properties. Moreover, since rather concentrated suspensions of toluenized *E. coli* are used for assays of the enzyme, such assays are performed in the presence of unspecified quantities of endogenous guanine nucleotides. We therefore used

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not only GTP but also its analogue GTP[γS], which cannot be hydrolysed, to test for activation; similarly, we used the non-phosphorylatable GDP analogue GDP[βS] to test for inhibition [11] of adenylate cyclase activity. The results show that, like the enzyme of vertebrate cells, that of *E. coli* is significantly activated by GTP and inhibited by GDP. This is consistent with the work of Roy et al. [12] in which *cya*, the structural gene for adenylate cyclase in *E. coli*, was cloned in a plasmid and two functional domains of the adenylate cyclase were defined. One of these is concerned with regulation by certain sugars, and is believed to be membrane-associated; the other domain exhibits catalytic activity, and is not membrane-associated.

2. MATERIALS AND METHODS

2.1. *Materials*

ATP, cAMP, GTP, GTP[γS], GDP[βS], creatine kinase and creatine phosphate were supplied by Boehringer (Lewes, East Sussex, UK).

Cyclic [^{3}H]AMP and $^{32}P_i$ were obtained from Amersham International (Amersham, Bucks, UK). [α-^{32}P]ATP was prepared by the method of Martin and Voorheis [13].

Toluene (special for chromatography) was supplied by BDH. Nutrient broth was from Oxoid (Basingstoke, Hants, UK). All other chemicals used were of analytical reagent grade, or the purest available commercially.

2.2. *Methods*

2.2.1. Growth of bacteria

Cultures of *E. coli* B were grown for 6–8 h at 37°C with vigorous aeration in tubes containing 2 ml of 2.6% (w/v) nutrient broth. The cells were then diluted 100-fold into a defined salts medium [14] with 15 mM glucose as carbon source and grown overnight to stationary phase. This stock preparation could be kept for about 5 days. For each experiment, cells from the stock preparation were diluted 10-fold into fresh salts medium containing 8 mM glucose and were grown aerobically at 37°C (doubling time, 1 h). Growth was estimated turbidimetrically at 680 nm; under these conditions, 1 ml of a suspension of bacteria at A_{680nm} of 1.0 contained 0.68 mg dry mass of cells [15]. When the cultures had grown to approx. 0.4 mg dry mass · ml^{-1}, they were harvested by centrifugation at 4°C and kept at this temperature. The cells were washed twice by resuspension in 50 mM Tris-HCl buffer (pH 7.5) and centrifugation. Finally they were resuspended in this buffer at a concentration of 10 mg dry mass · ml^{-1}.

2.2.2. Permeabilization of cells

Samples (3 ml) of cells and 30 μl toluene were placed in a 25-ml conical flask and covered with foil. The flask was shaken in an orbital shaker at 200 strokes · min^{-1} at room temperature for 1 min, and then immediately placed on ice.

2.2.3. Adenylate cyclase assay

The method of Salomon et al. [16] was used, with minor modifications. Samples were incubated at 30°C in a volume of 0.1 ml. Each assay contained toluenized cells (130–160 μg protein), 20 mM Tris-HCl buffer (pH 7.5), 0.5 mM ATP, 0.5 μCi [α-^{32}P]ATP, 0.5 mM cAMP, 1 mM dithiothreitol, 26 mM $MgCl_2$, 5 mM phosphocreatine, 17 units of creatine kinase and 16 mM K_2HPO_4. We confirm the observation [7] that adenylate cyclase activity is enhanced by the

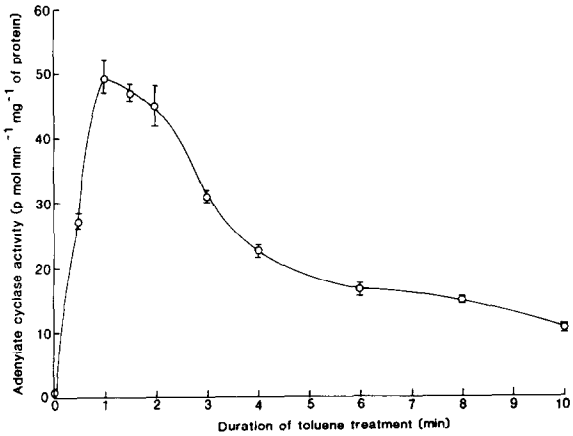

Fig.1. Adenylate cyclase activity in *E. coli* B, treated with toluene for various lengths of time. Washed cells were resuspended in glucose-free buffer (pH 7.5) and shaken with toluene as described in section 2. At each time point 200 μl cells were withdrawn and kept on ice. Each sample was then mixed with a solution of K_2HPO_4, and portions of this mixture, containing 136 μg protein, were added to the assay cocktail. They were incubated at 30°C for 15 min, and adenylate cyclase activity was measured. Each point recorded is the mean of 4 separate incubations ± SE.

addition of potassium phosphate. In our experiments, this salt was added separately to the toluenized cells (all on ice), and the resultant mixture was added to the remainder of the assay components so that the final concentration of K_2HPO_4 in the assay was 16 mM.

Incubations were stopped by adding 0.2 ml of 1 N perchloric acid; 0.1 ml of [^{3}H]cAMP and 0.6 ml of water were added, the tubes were vortexed and then centrifuged. The supernatant solutions were processed as in [17], except that the presence of perchloric acid in our samples caused some retardation of both ATP and cAMP on the Dowex 50 columns. ATP was therefore eluted (and discarded) with 3.5 ml water and cAMP was eluted with a further 7 ml water onto the alumina columns.

2.2.4. Protein

Protein was determined by the method of Lowry et al. [18].

3. RESULTS AND DISCUSSION

3.1. *Permeabilization of* E. coli *suspensions*

To determine the optimum duration of the toluene treatment of *E. coli* B suspensions, they were shaken with the organic solvent for various periods, and the adenylate cyclase activity was measured in cells sampled at each time point. Only negligible amounts of cAMP were formed by suspensions that had not been exposed to toluene; the greatest amounts were observed after toluenization for 1 min. The apparent adenylate cyclase activity, as thus measured, decreased with more prolonged shaking with toluene and, after 10 min, had fallen to less than a quarter of that measured after 1 min (fig.1). This behaviour may well be strain-dependent and may also be affected by the growth conditions. For example, the maximal adenylate cyclase activity of *E. coli* K12, grown and treated in the same manner as *E. coli* B, was not observed until the suspensions had been shaken with toluene for about 5 min; it is also known [19] that growth conditions affect the ease with which the membranes of any one strain are rendered permeable by toluene. For these reasons, a toluenization time of 1 min, and (as far as practicable) standardization of growth conditions, were adopted for all experiments here reported.

3.2. *Effects of guanine nucleotides on adenylate cyclase activity*

Suspensions of *E. coli* B, treated with toluene for 1 min, formed about 43 pmol cAMP $min^{-1} \cdot mg^{-1}$ protein and did so at a rate linear with time for at least 30 min (fig.2). When the incubation mixture was supplemented with 0.3 mM GTP, this rate of cAMP formation increased by about 40%, to 62 $pmol \cdot min^{-1} \cdot mg^{-1}$ protein; in 12 experiments of this kind, an average stimulation of 37% was observed (with SE of ± 6%). This stimulatory effect was dependent on the GTP concentration used: in 6 experiments, the rate of cAMP formation in the presence of 0.1 mM GTP was 32% (± 4%) higher than that observed in the absence of added nucleotide, and some stimulation was produced even by 0.01 mM GTP (fig.3). Indeed, this typical dose-response curve of GTP on the observed adenylate cyclase activity suggests

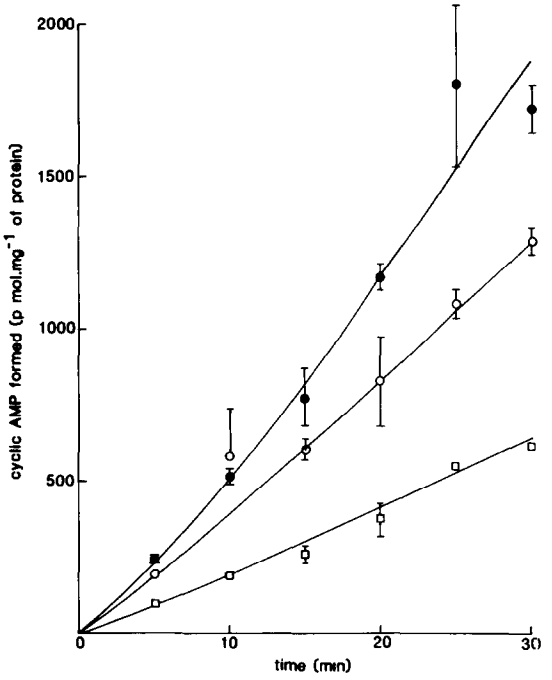

Fig.2. Time course of effect of GTP and of GDP[βS] on cAMP production by toluenized *E. coli* B suspensions. Toluenized cells (170 μg protein per assay) were incubated in glucose-free buffer (pH 7.5) with the following additions: (○) none; (●) 0.3 mM GTP; (□) 1 mM GDP[βS]. Adenylate cyclase activity was measured as described in section 2. Each point recorded is the mean of 3 separate incubations ± SE.

that half maximal activation occurs at about 0.08 mM GTP. Since GTP[γS], the non-hydrolysable analogue of GTP, exerted a virtually identical effect on adenylate cyclase activity (fig.3), it appears that the stimulation observed is not the result of a process involving transfer of the terminal phosphate from GTP.

In contrast to these stimulatory effects of GTP and GTP[γS], GDP[βS], the non-phosphorylatable analogue of GDP, inhibited adenylate cyclase activity: at 1 mM, this substance reduced the rate of cAMP formation from 43 to 21 pmol·min^{-1}·mg^{-1} protein (fig.2); in 12 experiments, the average inhibition observed was 50% (± 2%). To test whether this inhibition was perhaps the result of competition with endogenous GTP, increasing concentrations of GTP were added to a toluenized cell suspension to which either no, or one of three fixed quantities of GDP[βS] had also been added.

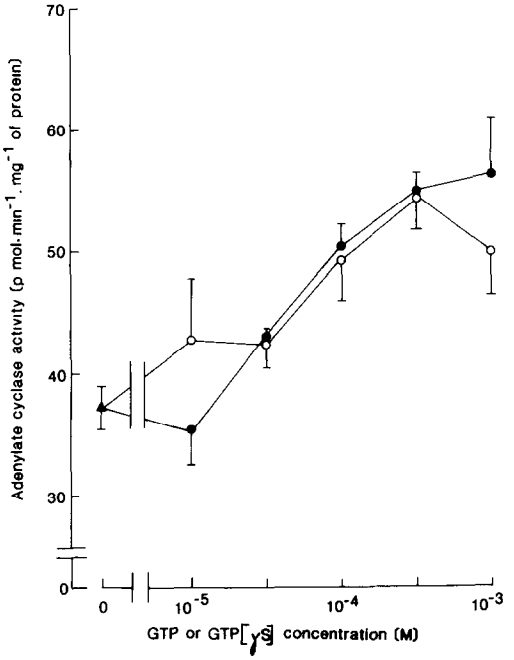

Fig.3. Effect of GTP and of GTP[γS] on adenylate cyclase activity in toluenized *E. coli* B suspensions. Toluenized cells (156 μg protein per assay) were incubated in glucose-free buffer (pH 7.5) for 15 min with the following additions: (▲) none; (○) GTP; (●) GTP[γS] at the concentrations stated. Adenylate cyclase activity was measured as described in section 2. Each point recorded is the mean of 3 separate incubations ± SE.

In the absence of GTP, increasing amounts of GDP[βS] increased the degree of inhibition of adenylate cyclase observed, from about 20% at 0.3 mM GDP[βS] to about 53% at 1 mM (fig.4). However, GTP tended to overcome this inhibition: at 1 mM, the activity of adenylate cyclase in the presence of 0.3 mM GDP[βS] was nearly the same as that measured in its absence. These findings suggest that GTP and GDP[βS] compete for the same site on the adenylate cyclase.

3.3. *Effect of glucose on adenylate cyclase activity*

Incubation of *E. coli* suspensions with 1 mM glucose results in complete inhibition of adenylate cyclase activity [6,7]. We confirm this (fig.5) and further note that glucose, between 0.01 and 1 mM, inhibits adenylate cyclase activity in a dose-

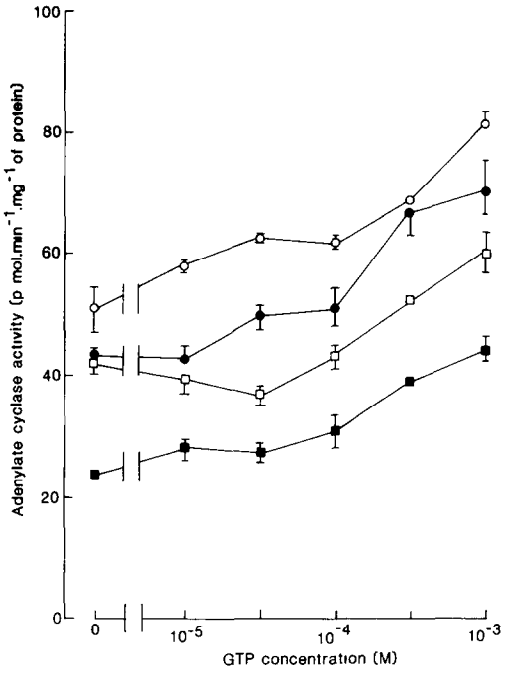

Fig.4. Effect of GDP[βS] and of various concentrations of GTP on adenylate cyclase activity in toluenized *E. coli* B suspensions. Toluenized cells (140 μg protein per assay) were incubated in glucose-free buffer (pH 7.5) for 15 min with GTP at the concentrations indicated. The concentrations of GDP[βS] also added were: (○) none; (●) 0.3 mM; (□) 0.5 mM; (■) 1 mM. Adenylate cyclase activity was measured as described in section 2. Each point recorded is the mean of 3 separate incubations ± SE.

dependent manner. However, the presence of glucose does not alter qualitatively the stimulation of adenylate cyclase by GTP, and its inhibition by GDP[βS]. It thus appears that glucose and guanine nucleotides act at different sites on the enzyme.

4. CONCLUDING REMARKS

Guanine nucleotide-binding proteins are closely involved with regulation of adenylate cyclase in membranes of vertebrate cells [20–23]. We do not know whether the effects of GTP and GDP[βS] that we have observed on the adenylate cyclase of *E. coli* are exerted similarly via a guanine nucleotide-binding protein, and whether GTP and GDP are the natural substrates for it. For example, the intracellular concentration of guanosine 5′-diphosphate 3′-diphosphate (ppGpp) in *E. coli* rises dramatically during glucose starvation, and during a variety of other changes in growth conditions that also favour increased adenylate cyclase activity [23]. Through the generosity of Dr A. Travers, we were able to test whether 1 mM ppGpp either inhibited or stimulated the adenylate cyclase activity of toluenized *E. coli* B suspensions to a significant extent. It did neither. It is also noteworthy that, whereas the adenylate cyclase in toluene-treated *E. coli* is activated by GTP and inhibited by glucose, the enzyme removed from its association with the membrane and purified is inhibited by GTP and unaffected by glucose [24]. This again emphasizes that the purified enzyme exhibits behaviour quite different from that in vivo.

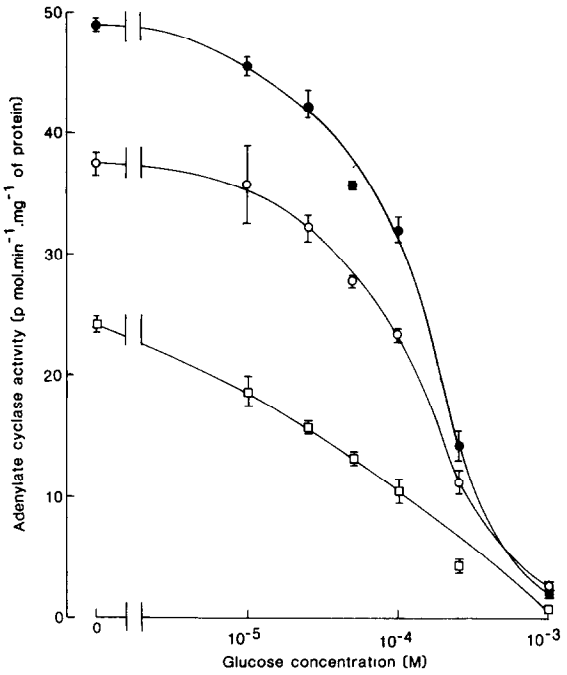

Fig.5. Effect of GTP, GDP[βS] and glucose on adenylate cyclase activity in toluenized *E. coli* B suspensions. Toluenized cells (130 μg protein per assay) were incubated for 15 min at 30°C in buffer (pH 7.5) with glucose added to the concentrations indicated, together with the following additions: (○) none; (●) 0.3 mM GTP; (□) 1 mM GDP[βS]. Adenylate cyclase activity was measured as described in section 2. Each point recorded is the mean value of 3 separate incubations ± SE.

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