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Regulation of phospholipid synthesis in *Mycobacterium smegmatis* by cyclic adenosine monophosphate

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Abstract. Forskolin, an adenylate cyclase activator and a cyclic AMP analogue, dibutyryl cyclic AMP have been used to examine the relationship between intracellular levels of cyclic AMP and lipid synthesis in *Mycobacterium smegmatis.* Total phospholipid content was found to be increased in forskolin grown cells as a result of increased cyclic AMP levels caused by activation of adenylate cyclase. Increased phospholipid content was supported by increased [^{14}C] acetate incorporation as well as increased activity of glycerol-3-phosphate acyltransferase. Pretreatment of cells with dibutyryl cyclic AMP had similar effects on lipid synthesis. Taking all these observations together it is suggested that lipid synthesis is being controlled by cyclic AMP in mycobacteria.

Keywords. Phospholipids; cAMP; mycobacteria; forskolin.

1. Introduction

The occurrence of cyclic AMP in different mycobacterial species has been reported (Padh and Venkitasubramanian 1976). Since this nucleotide is present in large amounts both in cells and extracellular fluid, studies are in progress to assign a definite role to it in these organisms. In prokaryotes, a large number of microbial activities are affected either by exogenous supplementation of cAMP or by manipulations of endogenous levels of cAMP (Rickenberg 1974). Cyclic AMP levels in an organism depend upon the balance between the activities of adenylate cyclase and phosphodiesterase. Of these two enzymes, adenylate cyclase, which synthesizes cAMP, is thought to be the major control point of cAMP metabolism.

Cyclic AMP has been shown to affect lipid metabolism in mast cells (Kennerly *et al* 1979) and *Microsporum gypseum* (Bindra and Khuller 1992). In *Mycobacterium smegmatis,* this nucleotide has been shown to enhance the incorporation of [^{14}C] acetate into lipids (Ahmad *et al* 1981). However, no information is available whether a direct correlation between cAMP and lipid biosynthesis exists in *M. smegmatis* or not. In the present study, in order to gain insight into the role of cAMP in the regulation of biosynthesis of lipids in *M. smegmatis,* we have examined the effects of forskolin, an activator of adenylate cyclase, and of direct incubating of cells with dibutyryl cAMP.

2. Materials and methods

[1-^{14}C] acetate (specific activity 56·2 mCi mmol^{-1}), [^{14}C]-glycerol-3-phosphate (specific activity 4·75 mCi mmol^{-1}) , [^{3}H] ATP (specific activity 13·5 Ci mmol^{-1})

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were obtained from the Bhabha Atomic Research Centre, Bombay. [^{3}H] adenosine-3'-5'-monophosphate was purchased from Amersham, UK. Forskolin, acetyl coenzyme A and dibutyryl cAMP were obtained from Sigma (USA). Other chemicals used were of analytical grade.

2.1 *Organism and growth conditions*

M. smegmatis ATCC 607 originally obtained from NCTC, London was grown in modified Youman's medium as a stationary culture at 37°C. Cells were harvested in their mid log phase and processed for different studies.

2.2 *Uptake studies*

To measure the incorporation of [1-^{14}C] acetate into lipids, log phase cells were harvested, washed and resuspended in Kreb's Ringer buffer (pH 7·2). The cell suspension was incubated with 5 μM forskolin for 3 h at 37° C with shaking and then labelled with [1-^{14}C] sodium acetate (1 μCi/500mg cells/10 ml of buffer, pH 7·2) for 1 h. To study the effects of dibutyryl cAMP, the cell suspension was incubated with 4 mM dibutyryl cAMP, 1 μCi [^{14}C] acetate, 4 mM EDTA in a final volume of 5 ml for 3 h at 37° C. The reaction was stopped by adding 1 ml of 1 M NaCN. Cells were separated by filtration and processed further.

2.3 *Quantitation of lipids*

Lipids were extracted by the method of Folch *et al* (1957). Phospholipids were quantitated by the method of Marinetti (1962). Total phospholipids from uptake studies were separated by thin-layer chromatography in acetone. The respective spots were scrapped off the plate and transferred into vials containing toluene based scintillation fluid for radioactivity determination.

2.4 *Determination of intracellular cAMP levels*

Cells were suspended in 10 volumes of 0·1 N HCl and heated at 95° C for 10 min. (Hylemon and Phibbs 1974). Each sample was then centrifuged at 12,000 *g* for 10 min and supernatant fluid was adjusted to pH 7·0 with NaOH. Supernatant fluid was again centrifuged at 12,000 *g* for 10 min to remove precipitated material. Intracellular cAMP was then measured by the method of Takeda *et al* (1989).

2.5 *Enzyme assays*

A homogenate was prepared by harvesting and sonicating the cells at 4°C in 40 mM Tris HCl buffer (pH 7·5). The homogenate was centrifuged at 5000 *g* for 20 min to remove cell debris and the supernatant was used for various enzyme assays. The activities of adenylate cyclase and acyltransferase were determined as described earlier (Bindra and Khuller 1992).

3. Results and discussion

The role of cAMP in phospholipid biosynthesis of *M. smegmatis* was studied by using forskolin, an adenylate cyclase activator and an analogue of cAMP. Forskolin has been found to activate nearly all mammalian adenylate cyclases by a mechanism independent of surface receptors (Award *et al* 1983). The concentration dependence for the activation of adenylate cyclase by forskolin was determined (figure 1) by using different concentrations ranging from 0·05 to 20 μ M. At concentrations below 1 μM, there was no significant increase in the activity of adenylate cyclase. However, with 5 and 10 μM forskolin, there was 50% and 40% increase in activity respectively. Hence 5 μM forskolin was used throughout this investigation.

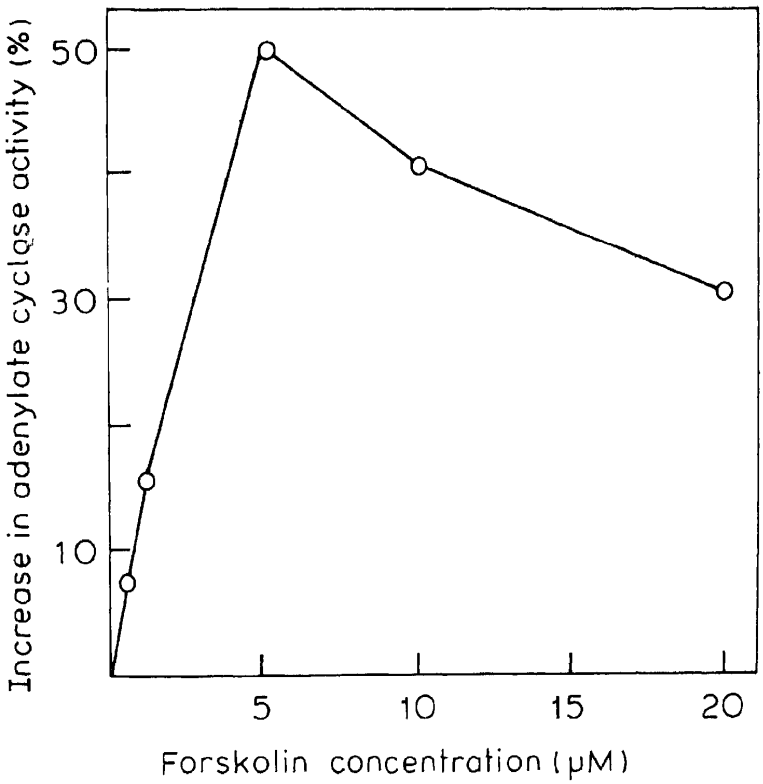

Figure 1. Concentration dependent effects of forskolin treatment on per cent increase in adenylate cyclase activities of *M. smegmatis*.

Incorporation of [^{14}C] acetate into total lipids and total phospholipids was examined in the presence of forskolin (5 μM). There was increased incorporation of acetate (approx. two-fold) into total lipids and phospholipids in the presence of forskolin (table 1). Imai *et al* (1984) also observed enhanced [^{3}H] glycerol incorporation into lipids by forskolin in a dose related fashion and have attributed this to rise in cAMP levels. Intracellular cAMP levels of *M. smegmatis* were also found to be increased (40%) when cells were grown in the presence of forskolin

Table 1. Effect of forskolin on the incorporation of [14C] acetate into total lipids and phospholipids.

Cells	Total lipids (cpm × 10^3/g dry wt of cells)	Total phospholipids
Control	483·82 ± 32·51	253·82 ± 15·46
Forskolin	954·55 ± 16·46**	392·60 ± 40·02*

Values are mean ±SD of three different batches
*P<0·01; **P<0·001

(5 μM) (table 2). The total phospholipid content of forskolin grown cells was also found to be elevated significantly *(P* <0·01) as compared to control cells (table 2). Increased phospholipid synthesis upon treatment with forskolin (table i) accounts for the significant increase in the net amount of phospholipid in forskolin grown cells.

Table 2. Effect of forskolin on phospholipid content, intracellular cAMP levels and activity of glycerol-3-phosphate acyltransferase.

Cells	Total phospholipids (mg/g dry wt)	cAMP (nmol/g dry wt)	Glycerol-3-phosphate acyltransferase (nmol/mg protein/h)
Control	24·41 ± 1·88	18·16 ± 0·39	116·92 ± 6·87
Forskolin grown	30·79 ± 0·99*	25·94 ± 1·98*	255·33 ± 23·53**

Values are mean ±SD of three different batchs
*P<0·01; **P<0·001

Cyclic AMP does not effect the metabolic pathways directly but through enzymes involved in these pathways. Therefore the activity of one of key enzymes of phospholipid biosynthetic pathway *i.e.* glycerol-3-phosphate acyltransferase was examined to find a correlation between cAMP levels and phospholipid synthesis (table 2). The activity of this enzyme was found to increase significantly *(P*< 0·001) in the presence of forskolin which suggests a link between cAMP levels and activity of enzyme. Similar observations were reported by us earlier for *M. gypseum* (Bindra and Khuller 1992) in which a direct correlation between cAMP levels and enzyme activity was observed. Furthermore dependence of glycerol-3-phosphate acyl-transferase on cAMP has also been shown by other workers in rat liver (Argilaga *et al* 1978).

After establishing a correlation between cAMP levels and lipid synthesis, we further confirmed it by preincubating the cells with dibutyryl cAMP, an analogue of cAMP for 3h. On monitoring the incorporation of [^{14}C] acetate into total lipids and total phospholipids in the presence of dibutyryi cAMP (4 mM) and EDTA (4mM), enhanced incorporation was observed in both lipids and phospholipids fraction (table 3). EDTA was added along with cAMP to alter the membrane permeability and hence to expedite influx of dibutyryl cAMP into cells (Ahmad *et al* 1981). Observed enhancement of lipid biosynthesis in *M. smegmatis,* by dibutyryl cAMP is comparable to effect of cAMP as such on lipid synthesis (Ahmad *et al* 1981). This enhanced synthesis can also be attributed to increased levels of

Table 3. Effect of dibutyryl cAMP on [^{14}C] acetate incorporation into total lipids and phospholipids.

Cells	[^{14}C]-acetate (incorporation cpm × 10^3/g dry wt)	[^{14}C] acetate + EDTA + dibutyryl cAMP (incorporation cpm × 10^3/g dry wt)
Total lipids	1501 ± 208	2640 ± 270*
Total phospholipids	444 ± 50	817 ± 27*

Values are mean ±SD of 3 different batchs
*P<0·01; **P<0·001

intracellular cAMP which were found to be increased drastically (table 4). There are numerous reports available indicating stimulation of biosynthesis of phospholipids by cAMP modulators or cAMP analogues (Gross and Rooney 1977; Pelech *et al* 1982). The activity of glycerol-3-phosphate acyltransferase was also found to be increased in dibutyryl cAMP exposed cells (table 4) which further explain the increased synthesis of phospholipids.

Table 4. Effect of dibutyryl cAMP on intracellular levels of cAMP and activity of glycerol-3-PO_4 acyltransferase.

Cells	cAMP (nmol/g dry wt)	Gly-3-phosphate acyltransferase (nmol/mg protein/h)
Control	18·16 ± 0·39	116·92 ± 6·87
Dibutyryl cAMP†	117·79 ± 10·61*	183·70 ± 5·64*

Values are mean ± SD of 3 different batches.
†After incubation with dibutyryl cAMP, cells were washed thoroughly to remove adhering cAMP and only then intracellular levels were monitored *P<0.001.

In brief our results using a cAMP analogue and its modulator clearly demonstrate that cAMP levels influences the phospholipid synthesis in *M. smegmatis* by regulating the activity of glycerol-3-phosphate acyltransferase.

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