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THE EFFECT OF THEOPHYLLINE ON THE KINETICS OF cAMP-DEPENDENT PROTEIN KINASE CATALYTIC SUBUNIT, cAMP, PROTEIN KINASE INHIBITOR AND THEIR RELATIONSHIP IN LUNG TISSUE

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ABSTRACT

We have investigated the effect of theophylline on the kinetics of the catalytic subunit of protein kinase and related factors in lung tissue. The results show that the point of highest concentration of the C subunit of protein kinase which is active in casein phosphorylation is at 3h of incubation time, but in the presence of 100 µg/mL and 10µg/mL theophylline, this is shifted to 1.5 and 2.5 hrs, respectively. Also the maximum concentration of cAMP for the control is at 2.5 h of incubation time, but in the treated samples shifts to 2.15 and 1.15 hrs, respectively. Inhibitor protein content also changes considerably in the presence of 10µg/mL theophylline. The results suggest an effect of theophylline on the function of cAMP-dependent protein kinase.

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Key words: Theophylline; cAMP; catalytic subunit; inhibitor protein; protein kinase

INTRODUCTION

Cyclic AMP-dependent protein kinases are a group of proteins in eukaryotic cells whose activity are directly regulated by cAMP.[1] Each protein consists of two regulatory and two catalytic subunits that together constitute an inactive holoenzyme. The activation occurs via the binding of intracellular cAMP to the regulatory subunits and subsequently releases the catalytic subunits.[2]

It has been shown that the two free subunits mediate the effect of cAMP on the expression of a number of genes and synthesis of proteins.[3] The regulatory proteins of cAMP-inducible genes have a cluster of potential phosphorylation sites for protein kinases that are necessary for their transcriptional activity.[4]

Theophylline (1,3-dimethylxanthine) is one of the naturally occurring methylxanthines which is used therapeutically to alleviate neonatal apnea and to control asthmatic manifestations. This drug changes the balance between cyclic 3′,5′-AMP formation from ATP and its degradation toward AMP by the inhibition of cAMP phosphodiesterase.[5,6] It has been reported that the addition of theophylline at a concentration of 10µg/mL to the lung conditioned medium enhances granulocyte-macrophage colony stimulating factor (CSF) production and at higher concentrations shows an inhibitory effect.[7] In addition, the transcription of the c-fos proto-oncogene in CV-1 cells can be regulated by theophylline indirectly.[8] In this study we have investigated the role of theophylline on cAMP levels and its relationship to the kinetics of cAMP-dependent protein kinase catalytic subunit and its protein inhibitor in lung tissue.

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MATERIALS AND METHODS

Materials: DEAE-Cellulose (DE-32) and QAE-Sephadex were obtained from Whatman Chemical Co. Casein and cAMP were from Sigma. Reagents for tissue culture were obtained from Gibco. Inorganic materials were from Merck and all reagents were analytical grade. Balb/c mice weighing about 40g and 20-25 of either sex were obtained from Hesarak Institute (Tehran) and used for the lung conditioned medium and biological assay respectively. Theophylline (Boehringer-Ingelheim) was freshly dissolved in distilled water as a stock solution (2.5 mg/mL) and then diluted to the desired concentration with DMEM before use.

Normal and treated condition medium: Mice were anesthetized by ether and the lungs were removed from the chest cavity under sterile conditions. The lungs were washed with normal saline, minced and incubated in 5mL of Dulbecco's Modified Eagle's Medium (DMEM) for 48 hours at 37°C in a fully humidified atmosphere of 5% CO_2 in the air. Drug-treated samples were made essentially as described for normal lung conditioned medium except that after lung tissue was minced in DMEM, theophylline at two concentrations, 10 and 100µg/mL, was added to the cultures and mixed thoroughly immediately before incubation. The volume of all samples was kept constant. At the end of each time interval, lung tissues were used for assays and extractions.

Isolation and measurement of protein kinase subunits: Catalytic (C) and regulatory (R) subunits of protein kinase were isolated and purified employing the method of Schwoch, et al.[9] The lung tissue was homogenized in a cold solution of 5mM Tris/HCl, pH 7.0 containing 4 mM-EDTA, with a Teflon pestle (4000 r.p.m.) in a glass homogenizer. NaCl was added to a final concentration of 140 mM and the resulting suspension was centrifuged for 20 min at 20,000g (4°C). The supernatant fluid was diluted to 15 mM-NaCl, 4 mM-EDTA, 5mM-Tris/HCl, pH 7.0 and a portion containing the equivalent of 1 g of lung tissue was applied to a DEAE-cellulose (DE-32) column (0.9 × 6.5cm) pre-equilibrated with the same buffer. The proteins were eluted with 45 mL of buffer and 1.5 mL fractions were collected at a flow rate of 17 mL/h at 5°C. To elute the R subunit, a gradient of 0.01-0.4 M NaCl was applied. Absorbance of all fractions was monitored at 280 nm and accordingly, curves were depicted and the area under the peaks was determined.

Protein kinase assay: The method of Torphy, et al. was used.[10] The assay was started by the addition of 25 µL of the purified C subunit solution to a 200 µL reaction mixture containing 40 mM Tris/HCl pH 7.4, 20 mM magnesium acetate, 0.2 mM ATP and 2 mg/mL casein as a substrate. After incubation at 30°C for 20 min, the reaction was stopped with 10% trichloroacetic acid (TCA). Precipitated proteins were dissolved in SDS-sample solvent and loaded onto SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

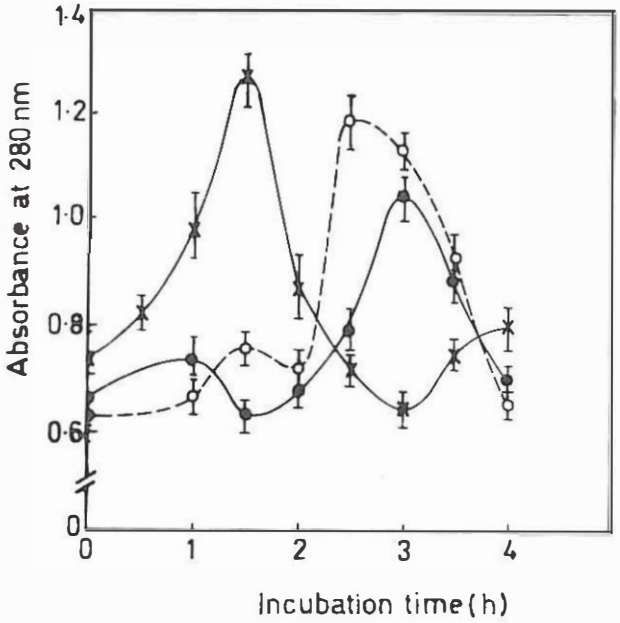


Fig 1: The effect of theophylline on catalytic subunit content in lung tissue at different incubation time intervals. (×----×) 100 µg/mL, (○----○) 10 µg/mL, (●----●) control. Mean ±SD of four experiments.

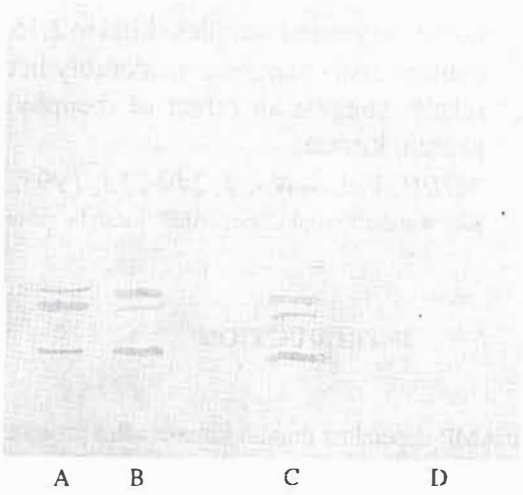


Fig. 2: SDS-polyacrylamide gel electrophoresis of phosphorylated casein by catalytic subunit. Incubation times were 35 min (A) and 20 min(B), phosphorylation by a mixture of R and C subunits at 20 min incubation (C), standard casein (D).

R and C subunit interaction: Interaction was carried out according to the method of Hofmann, et al.[11] The samples containing C and R subunits were dialyzed overnight against 10 mM potassium phosphate buffer, pH 7.4. Reaction was

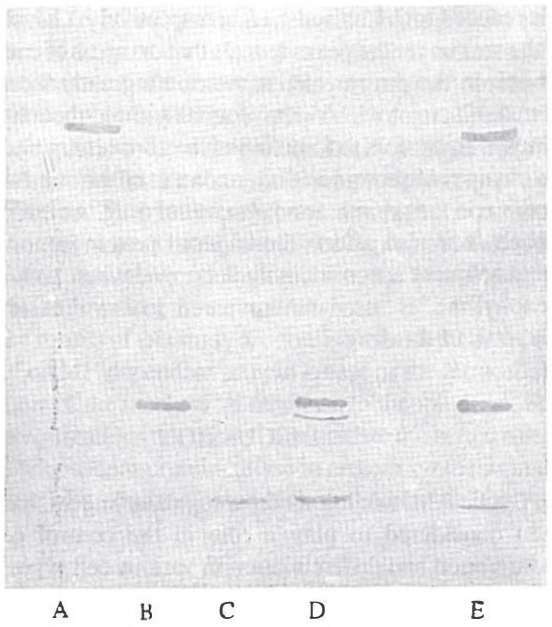


Fig. 3: The effect of inhibitor protein on casein phosphorylation as determined by SDS-polyacrylamide gel electrophoresis. Extracted inhibitor protein (A), standard casein (B), catalytic subunit (C), phosphorylation of casein by catalytic subunit in the absence (D) and presence (E) of inhibitor protein.

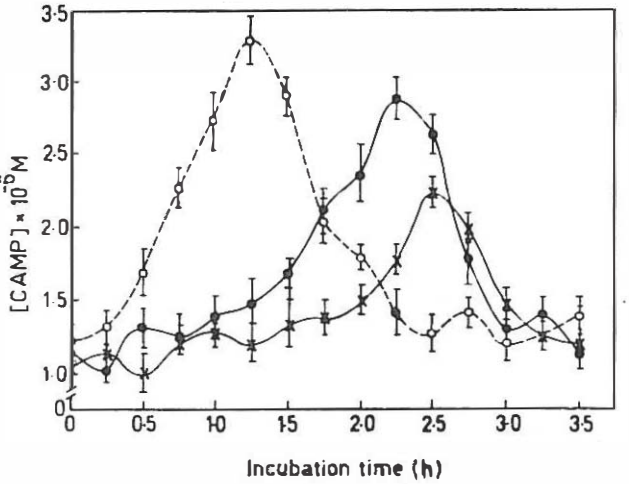


Fig. 4: The effect of theophylline on cAMP concentration in lung tissue at different time intervals. (x----x) control, (●----●) 10 μg/mL, (○----○) 100 μg/mL. Mean ±SD of five experiments.

carried out in 50 mM Tris/HCl, 10 mM $MgCl_2$, and 10 mM mercaptoethanol. After 20 min incubation at 37°C the reaction was stopped by the addition of TCA solution to 7% and the precipitated proteins were subjected to electrophoresis.

Purification of protein inhibitor: The method of Walsh, et al. was used.[12] The lung tissue was homogenized in 2 volumes of buffer A (4 mM EDTA, 0.5 M glycerol phosphate pH 6.8). Then the pH was adjusted to 7.0 by the addition of 6M ammonium hydroxide. The neutral solution was heated and brought to 95°C over a 20 min period. The resultant suspension was cooled to 10°C in an ice bath and centrifuged at 2000g for 10 min. The supernatant fluid was made 15% TCA with respect to 100% TCA and after centrifugation the precipitate was resuspended in 25 mL of 0.05 M glycerol-phosphate buffer, pH 6.8 containing 2 mM EDTA. The resultant turbid solution was dialyzed exhaustively against 1mM EDTA and 5mM potassium phosphate buffer, pH 7.0. The retentate was centrifuged at 34,000g for 20 min and the supernatant fluid containing the inhibitor protein was retained.

Electrophoresis: 15% SDS-polyacrylamide slab gel (15%) electrophoresis of the eluted proteins was carried out as described by Laemmli.[13] After loading 10μg of the protein, electrophoresis was carried out at 180 V(RT), the gels were stained with coomassie brilliant blue (R-250) and destained in 7% acetic acid and 45% methanol in water.

Extraction and measurement of cAMP: Cyclic AMP from the control and theophylline-treated samples was purified by ion exchange resin column chromatography as described by Schultz, et al.[19] The lung tissues were rapidly homogenized in a cold solution of 0.1 M zinc acetate and 0.1M disodium carbonate pH 7.0 and after incubation in boiling water for 2 min, it was centrifuged at 20,000g for 5 min. The clear supernatant fluid was loaded onto a QAE-Sephadex column (0.7×10 cm) in its formate form. After application of 10 mL of distilled water followed by 6mL of 0.1 M ammonium formate, pH 9.0, cAMP and cGMP were eluted with 0.1 M ammonium formate pH 6.0. The fractions (2 mL) were collected and their absorbance measured at 260 nm. The concentration of cAMP was estimated using a standard curve.

RESULTS

To study the effect of theophylline on the kinetics of protein kinase, two concentrations of drug used were 10 and 100μg/mL. The reason for the selection of these two concentrations was based on our previous results obtained from the effect of theophylline on colony stimulating factor (CSF) production by lung tissue.[7] We have shown that theophylline enhances CSF production during 48h of incubation and at higher concentrations (e.g. 100 μg/mL) shows an inhibitory or toxic effect.

For this purpose, theophylline was first added to the lung conditioned media and at different time intervals the final supernatants were extracted as described in the "Methods" section and loaded onto a DEAE-cellulose column. The

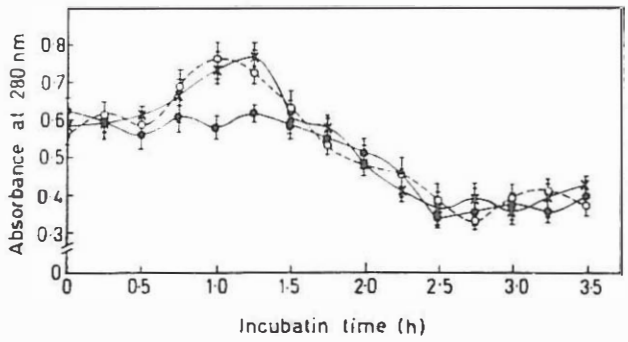


Fig. 5: Change in inhibitor protein content in lung tissue at different time intervals in the presence and absence of theophylline. (×----×) Control, (●----●) 100 µg/mL, (○----○) 10 µg/mL. Mean ±SD of four experiments.

maximum points of the elution profiles of C-subunits were drawn against the incubation time. The results from the control and samples treated with 10 and 100 µg/mL theophylline are shown in Fig 1. It is seen that with progression along the incubation time interval axis, the concentration of C subunit in the normal state (control) remains at about baseline value until 2h of incubation but reaches a maximum value at 3h and then declines. In comparison, in drug-treated samples, this value is shifted to 2.5h and 1.5h with 10µg/mL and 100 µg/mL theophylline, respectively. According to these results, when theophylline exists at 100 µg/mL in the media, C subunit would have more time to affect protein synthesis in comparison to the control in the lung tissue.

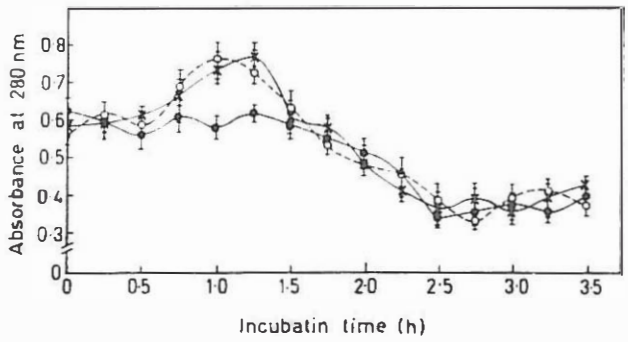
To see whether the peaks eluted from the column corresponded to the catalytic subunit of the protein kinase, the phosphorylation of casein was carried out in the presence and absence of C subunit. Fig. 2 demonstrates these results. It is shown that when C subunit is incubated with casein for 20 or 35 min, a new band running slightly faster than casein appears on the gel. This band corresponds to the phosphorylated form of casein with its higher negative charge causing higher mobility on the gel.

Fig. 3 shows that phosphorylation of casein by the catalytic subunit can be suppressed in the presence of a heat stable inhibitor protein. Inhibitor protein extracted from the lung tissue as described in the "Methods" section shows a single band with a low mobility on the SDS-gel as compared to casein and C subunit. On the other hand, addition of cAMP to the mixture of C and R subunits released the subunits from each other which could be clearly seen on the SDS-gel electrophoresis pattern (data not shown). These results indicate that the eluted peaks correspond to the catalytic subunit of protein kinase.

Fig. 4 illustrates the cAMP concentration of the lung tissue conditioned media in the presence and absence of theophylline as a function of incubation time. As is observed, the maximum concentration of cAMP for the control is 2.5h, but for 10 and 100µg/mL drug-treated samples it is reduced to 2.15 h and 1.15 h, respectively. Calculation of the area under the peaks reveals that the areas of catalytic subunit in the drug-treated samples are greatly increased from 8.17 cm^2 to 10.65 cm^2 for 100µg/mL theophylline which is equivalent to 1.31 fold of the normal. Fig. 5 shows the changes of protein inhibitor content of the conditioned medium in the presence and absence of drug. As indicated, theophylline also affects the value of protein inhibitor so that its content is considerably decreased when 100µg/mL theophylline is used as compared to the control and 10µg/mL of the drug.

DISCUSSION

Cyclic AMP, an intracellular regulatory agent, has long been considered to play a role in the control of cell proliferation and differentiation in various cell types[15] and exerts its effect on eukaryotes by binding to regulatory subunits of cAMP-dependent protein kinases.[16,17] A number of results clearly show that theophylline exerts its biological effect on many metabolic pathways through cAMP and recently many reports have indicated its involvement in the control of cellular protein synthesis and genome activity.[8,18] The identification of specific regulatory proteins and the modification of the activity of cAMP-dependent protein kinase at the cellular level contribute to the understanding of the mechanisms for these effects. According to all experiments performed, different processes may exist by which genes are regulated by AMP since different regulatory agents have been described and some cAMP-induced genes are under the control of more than one external stimulus.[19] Cyclic AMP responds to various haemopoietic regulators implying its participation in the regulation of CFU-S proliferation.[20] It appears that cAMP activation of transcription occurs through the regulation of the phosphorylation activity of C subunit.[21] In this report, we have investigated the kinetic role of the three most important elements in the pathway that lead to the regulation of protein synthesis. Preliminary results suggest that in the lung tissue, the level of cAMP is increased as a function of theophylline concentration. As is seen, at high concentrations of drug (100 µg/mL), this increment is more significant and the peak appears earlier than 10 µg/mL and normal profiles. The measurement of C subunit in the presence and absence of theophylline shows that its elevation corresponds to cAMP elevation in the lung in both drug-treated and control groups. This is desirable since it confirms the role of cAMP in the release of C subunit from the protein kinase complex. When 100 µg/mL and 10 µg/mL theophylline are used, a broad range of time is provided, a time in which the free subunit will be able to elevate the basal synthesis of certain proteins, depending however on feasibility of conditions. On the other hand, the effect of partially purified inhibitor protein which blocks the



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activity of free C subunit was measured in the presence and absence of drug. According to the results, it is indicated that at low concentrations of theophylline (10 µg/mL) the change in inhibitor protein almost overlaps with control peak with approximately the same magnitude, but by using 100 µg/mL of drug, a considerable change occurs, producing no peak in this position. The results suggest that at higher concentrations of drug, a great deal of the inhibitor protein binds to the C subunit and prevents subunit activity completely, in contrast to low drug concentrations.

The results of this study are consistent with the proposed role of cAMP-dependent protein kinase as the intracellular mediator of the mechanical response to theophylline. Further investigations are needed to elucidate the actual role of cAMP-dependent protein kinase in protein synthesis in the presence of methylxanthines such as theophylline.

REFERENCES

1. Redpath NI, Proud CG: Cyclic AMP-dependent protein kinase phosphorylates rabbit reticulocyte elongation factor-2 kinase and induces calcium-independent activity. Biochem J 239: 31-34, 1993.
2. Taylor SS, Zheng JH, Radziandzelm E, Knighton DR, Teneyck LF, Yonemoto WM: Cyclic AMP-dependent protein kinase defines a family of enzymes. Biol Sci 340: 315-324, 1993.
3. Simo MN: Cyclic AMP-dependent protein kinases: Structure and role in the control of gene expression. Bul Inst Pasteur 89: 97-107, 1991.
4. Gonzalez GA, Montminy MR: Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB of serine 133. Cell 59: 675-680, 1989.
5. Ward A, McKenniff M, Evan J, Page C, Costello J: Theophylline-an immunomodulatory role in asthma. Am Rev Respir Dis 47: 3 Mar, 1993.
6. Tomes C, Rossi S, Moreno S: Isobutylmethylxanthine and other classical cyclic nucleotide phosphodiesterase inhibitors affect cAMP-dependent protein kinase activity. Cellular Signalling 5: 615-621, 1993.
7. Rabbani A, Boojar M, Goliaei B: The effect of theophylline on the production of colony stimulating factor (CSF) by the lung. Med J Islam Rep Iran 5: 148-155, 1991.
8. Moens U, Subramaniam N, Johansen B, Aarbakke J: The c-fos cAMP-response element: regulation of gene expression by a β_2-adrenergic agonist, serum and DNA methylation. Biochim Biophys Acta 1173: 63-70, 1993.
9. Schwoch G: Differential activation of type-I and type-II adenosine 3′,5′-cyclic monophosphate-dependent protein kinases in liver of glucagon-treated rats. Biochem J 170: 469-477, 1978.
10. Torphy TT, Freese WB, Rinard GA, Brunton LL, Mayer ES: Cyclic nucleotide-dependent protein kinases in air smooth muscle. J Biol Chem 257: 11609-11616, 1982.
11. Hofmann F: Apparent constants for the interaction of regulatory and catalytic subunits of cAMP-dependent protein kinase I and II. J Biol Chem 255: 1559-1564, 1980.
12. Walsh AD, Ashby CD, Gonzales C, Calkins D, Krebs EG: Purification and characterization of a protein inhibitor of adenosine 3′,5′-monophosphate- dependent protein kinases. J Biol Chem 246: 1977-1985, 1971.
13. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970.
14. Schultz G, Bohme E, Hardman JC: Separation and purification of cyclic nucleotides by ion-exchange resin column chromatography. In: Methods in Enzymology. Hardman JG (ed), Vol. 38, pp. 9-40, 1974.
15. Gonsalkorale WM, Dascombe MJ, Hutchinson IV: Cyclic AMP inhibits macrophage suppressor function and enhances lymphocyte proliferation. Immunology 80: 611-616, 1993.
16. Park K, Kim K: The site of cAMP action in the insulin induction of gene expression of acetyl-CoA carboxylase is AP-2. J Biol Chem 268: 17811-17819, 1993.
17. Sweet MT, Allis CD: Phosphorylation of linker histones by cAMP-dependent protein kinase in mitotic micronuclei of tetrahymena. Chromosoma 102: 637-647, 1993.
18. Yeh C, Chinchetru MA, Kousvelari E: Characteristics of c-fos and jun B gene expression in A5 cells after β-adrenoreceptor stimulation and during cell cycle. Biochim Biophys Acta 1090: 173-180, 1991.
19. Brindle P, Link S, Montminy M: Protein-kinase-A-dependent activator in transcription factor CREB reveals new role for CREM repressor. Nature 364: 821-824, 1993.
20. Bakala JW, Pines M, Guigon M, Lenfant M: Cyclic AMP response to various haemopoietic regulators. Cell Tissue Kinet 18: 297-306, 1985.
21. Thivierge M, Alami N, Muller E, Debrumfernandes A, Rolapleszczynski M: Transcriptional modulation of platelet-activating factor receptor gene expression by cyclic AMP. J Biol Chem 268: 17457-17462, 1993.