ISSN 1671-4083
Shanghai Institute of Materia Medica
Chinese Academy of Sciences
http://www.ChinaPhar.com

Inhibition of human phosphodiesterase 4A expressed in yeast cell GL62 by theophylline, rolipram, and acetamide-45[1]

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KEY WORDS 3',5'-cyclic-nucleotide-phosphodiesterase; *Saccharomyces cerevisiae*; cyclic AMP; cyclic GMP; theophylline; rolipram; acetamide-45

ABSTRACT

AIM: To study the inductive expression of human phosphodiesterase 4A (hPDE4A) in yeast cell GL62 and investigate the inhibitory effects of theophylline, rolipram, and acetamide-45 on PDE4A activity of the expressed product in yeast cell GL62. **METHODS:** Yeast cell GL62 were transfected with human PDE4A gene cloned in the expression plasmid p138NB. Expression was induced by adding $CuSO_4$ to a final concentration of 150 μmol/L, and the expressed product was extracted. The activity of PDE4A was detected by HPLC. **RESULTS:** Yeast cell GL62 expressed a certain protein at $CuSO_4$ 150 μmol/L, the size of the expressed product was between 62 kDa and 83 kDa, the activity of PDE4A of the expressed product at 3 h was in maximum (188±23) $\mu mol \cdot g^{-1} \cdot min^{-1}$, and the K_m was (17.7±2.6) μmol/L. Theophylline, rolipram, and acetamide-45 could inhibit the activity of PDE4A extracted from yeast cell GL62. The IC_{50} (95 % confidence limits) of theophylline, rolipram, and acetamide-45 were 1642 (989–2727), 4.58 (3.45–6.08), and 275 (170–444) μmol/L respectively. **CONCLUSION:** PDE4A expressed in yeast cell GL62 is biologically active. Theophylline, rolipram, and acetamide-45 can inhibit the PDE4A activity. The expressed product in yeast cell GL62 may be used in the research work of PDE4 and its inhibitors.

INTRODUCTION

Phosphodiesterases (PDE) are a family of enzymes that play a critical regulatory role in cellular signal transmission by catalyzing the hydrolysis of 3',5'-cyclic nucleotide to the corresponding nucleotide 5'-monophosphate. PDE are classified into at least eleven isozymes on the basis of their functional characteristics such as substrate specificity and responsibility to selective PDE inhibitors[1]. A high-affinity cAMP-selective isozyme, known as PDE4, is the predominant form of PDE found in all inflammatory cells, which is divided into four subtypes, PDE4A, PDE4B, PDE4C, and PDE4D. PDE4A, PDE4B, and PDE4D are distributed mainly in inflammatory cells, while PDE4C is contained in brain cells[2].

cAMP has important regulatory roles in virtually all cell types in the pathophysiological processes of inflammation. Of paramount importance is the observation that, in general, cAMP broadly suppresses the

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Received 2001-11-26 Accepted 2002-08-03

activity of immune and inflammatory cells. PDE4 appears to be an attractive target for anti-inflammatory drugs. Inhibition of PDE4 results in increased levels of cAMP that leads to functional inhibition of eosinophils, macrophages, neutrophils, mast cells, basophils, monocytes, lymphocytes, and release of inflammatory mediators[3-6].

Recently, several PDE and PDE4 inhibitors have been developed, and clinical trials are already showing the potential use of these inhibitors in asthma and chronic obstructive pulmonary disease (COPD). Unfortunately, the anti-inflammatory effect of PDE4 inhibitors has been considered to be associated to some extent with nausea and vomiting as adverse effects. With respect to those adverse effects, it is concerned with inhibition of PDE4C. PDE4C is not present in inflammatory cells, but is abundantly expressed in brain cells[7]. For this reason, PDE4 subtype-selective inhibitors are eagerly explored. Now PDE4A gene has been identified from the human cDNA library and transfected in yeast cell GL62. The purpose of this study is to induce the yeast cells to express PDE4A and detect the PDE4A activity in the expressive product, and then determine the effects of theophylline, rolipram, and acetamide-45 on it to provide experimental references for the exploration of new PDE4 subtype-selective inhibitors.

MATERIALS AND METHODS

Strain of *Saccharomyces cerevisiae* The strain of *S cerevisiae* used in this study was GL62 *(MATa leu2 ura3-52 adel his3 trp1 lys2-208 pde1::LEU2 pde2::URA3 pep4::HIS3*), which was kindly provided by Prof Allan R SHATZMAN (SmithKline Beecham, USA). The yeast cell were PDE-deficient and harboring plasmid p138NB-hPDE4A, which was a transfecting plasmid containing full length of human PDE4A gene isolated from human cDNA library[8].

Chemicals Theophylline, rolipram, cAMP, cGMP, and dimethyl sulfoxide (Me_2SO) were purchased from Sigma Chemical Co. Acetamide-45 was kindly provided by Prof Guillaume Le BAUT (Nantes France). All drugs were first dissolved in Me_2SO, and the final concentration of Me_2SO was less than 1 %.

Expression and extraction of PDE4A Yeast cells were grown in YPD (Pierce Co) at 30 °C, to A_{540}=1.0. PDE4A expression was induced by adding $CuSO_4$ to a final concentration of 150 μmol/L. Cells were harvested at 3 h and centrifuged at 3000×*g* for 5 min at 4 °C, and then resuspended in 3 mL of Y-PER (Pierce Co) for 1 g of cell paste. The mixture was agitated gently at 20 °C for 20 min. The cell debris were collected by centrifugation at 13 000×*g* for 10 min, the soluble protein in cytoplasma was extracted. All steps were performanced as the direction of Y-PER. Sample of pellet was assayed for PDE4A activity immediately and the residual material was stored at -70 ℃ for later analysis. Protein was routinely measured by the method of Bradford[9].

PDE4A activity assay Extract 20 μL was added into the reaction system containing 20 μL substrate (cAMP) with a total volume of 200 μL in which the final concentration of substrate was 1 μmol/L. Theophylline, rolipram, or acetamide-45 was diluted into various concentrations when indicated and added into the reaction system. Another 20 μL extract that had been inactivated was added as control. Reactions were conducted at 37 ℃ for 30 min and terminated at 100 ℃, then the mixture was centrifuged at 12 000×*g* for 30 min at 4 ℃ and the supernatant was used to detect the remaining cAMP in HPLC (HP1100, HP Co). Enzyme activity of PDE4A was calculated by the following equation: Activity=[cAMP remaining (control)–cAMP remaining (reaction)]/(protein·reaction time) ($\mu mol \cdot g^{-1} \cdot min^{-1}$).

Method of statistics Data were expressed as mean±SD. Statistical significance of differences between means of groups was determined by *t* test. IC_{50} (95 % confidence limits) was calculated and compared by weighed probit analysis of Bliss method.

RESULTS

Analysis of PDE4A activity on cAMP in GL62 extract The analysis of SDS-PAGE showed that the product extracted at 3 h and 6 h appeared strong band between 62 kDa and 83 kDa, but the extract induced at 0 h and 1 h appeared only weak band in the corresponding site (Fig 1). The activity was related with the inducing time. From 0 h to 3 h, the activity increased gradually. At inducing time of 3 h, PDE4A activity on cAMP reached the maximal point. From 3 h to 9 h, the activity did not show obvious change (Tab 1).

Effect of cAMP concentration on velocity of enzyme reaction The 1/[cAMP] was designated as abscissa, 1/[velocity of enzyme reaction] (1/*v*) as ordinate and Lineweaver-Burk plot was made. According to Michaelis equation, the K_m was (17.7±2.6) μmol/L

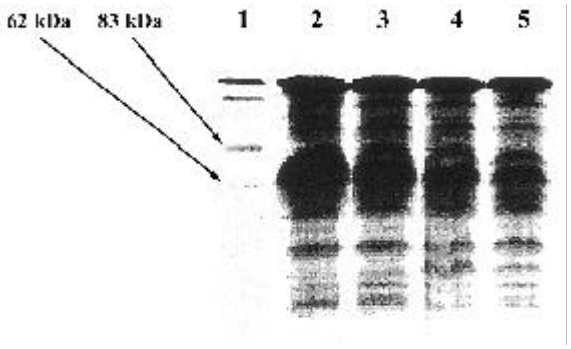

Fig 1. SDS-PAGE of GL62 extract at different inducing time. Lane 1: standard control; Lanes 2 to 5: different $CuSO_4$-induced time that are 6 h, 3 h, 1 h, and 0 h respectively.

Tab 1. PDE4A activity of GL62 extract on cAMP at different inducing time. *n*=6. Mean±SD.

Inducing time/h	Activity/μmol·g^{-1}·min^{-1}
0	16.3±0.7
1	16±3
1.5	56±8
2	66±11
2.5	92±12
3	188±23
6	173±55
9	183±18
12	169±24

(Fig 2).

Hydrolytic effect of GL62 extract on cGMP The hydrolyzing-cGMP activity of GL62 extract was (8.48±1.2) μmol·g^{-1}·min^{-1}, while the activity on cAMP was (188±23) μmol·g^{-1}·min^{-1}.

Effect of cGMP on PDE4A hydrolyzing-cAMP activity of GL62 extract cGMP had no effect on PDE4A hydrolyzing-cAMP activity of GL62 extract at a low concentration. As the concentration of cGMP were 0, 10, and 100 μmol/L, the activity of PDE4A on cAMP were (188±23), (163±8), and (167±15) μmol·g^{-1}·min^{-1}, respectively. There were no significant differences between experimental and control group (Fig 3).

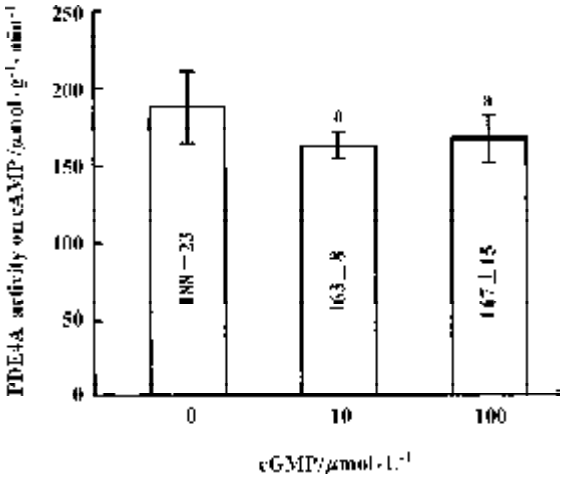

Fig 3. Effect of cGMP on PDE4A hydrolyzing cAMP activity of GL62 extract. *n*=6. Mean±SD. $^{a}P>0.05$ *vs* cGMP 0 mmol/L.

Effect of theophylline, rolipram, and acetamide-45 on PDE4A activity of GL62 extract As shown in Fig 4, theophylline, rolipram, and acetamide-45 gradually decreased the hydrolysis of cAMP by

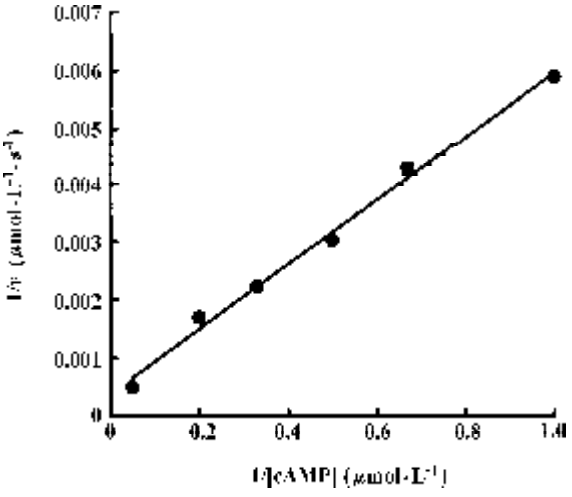

Fig 2. Effect of cAMP concentration on velocity of PDE4A enzyme reaction.

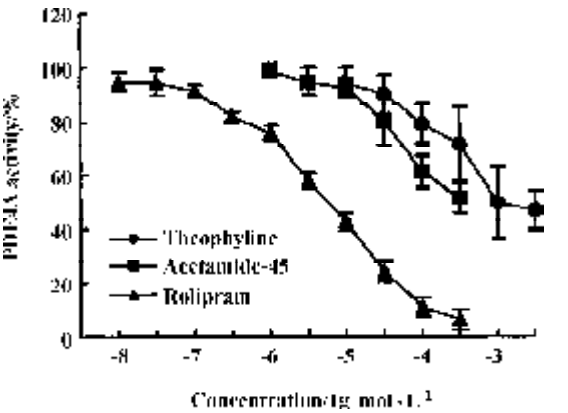

Fig 4. Effect of theophylline, rolipram, and acetamide-45 on PDE4A activity of GL62 extract. *n*=6. Mean±SD.

PDE4A extracted from yeast cell GL62 from 1×10^{-5} mol/L to 3×10^{-4} mol/L. At concentration of 3×10^{-4} mol/L, the remaining activity were 72 %±14 %, 7 %±5 %, and 52 %±5 %, respectively. IC_{50} (95 % CL) of theophylline, rolipram, and acetamide-45 were 1642 (989–2727), 4.58 (3.45–6.08), and 275 (170–444) μmol/L, respectively (Tab 2).

Tab 2. Inhibition of theophylline, rolipram, and acetamide-45 on activity of human PDE4A expressed in GL62 yeast cells. n=6. $^bP<0.05$ *vs* rolipram. $^eP<0.05$ *vs* theophylline.

Drugs	IC_{50} (95 % CL)/μmol·L^{-1}
Theophylline	1642 (989-2727)
Rolipram	4.58 (3.45-6.08)
Acetamide-45	275 (170-444)^{be}

DISCUSSION

The present study showed that the extract of yeast cell GL62 which was transfected by human PDE4A gene and inductively expressed by $CuSO_4$ possessed the PDE4A character, cAMP was a specific hydrolyzing substrate of the extract, cGMP had no influence on hydrolyzing-cAMP, and PDE4 inhibitors could inhibit the hydrolyzing-cAMP activity.

Yeast cell GL62 expressed certain protein after CuSO4 induction. The size of the product was between 62 kDa and 83 kDa, which was in accordance with the previous report[2]. The amount of the expressed product increased with the inducing time. The activity of PDE4A varied with inducing time. At 0 h, 1 h, 1.5 h, 2 h, 2.5 h, and 3 h, the activity was (16.3±0.7), (16±3), (56±8), (66±11), (92±12), and (188±23) μmol·g^{-1}·min^{-1}, respectively, increasing gradually with the inducing time. However, at 6 h, 9 h, and 12 h, the activity did not alter obviously. The K_m of this extract was (17.7±2.6) μmol/L, however the K_m of the PDE4A was reported as 4–20 μmol/L[2]. It is suggested that the amount of the expressed product induced by $CuSO_4$ increases with inducing time 0 h to 3 h and the product is maximally expressed at 3 h. The amount of product does not increase though the inducing time is further extended. cAMP is the specific substrate of the extract.

cGMP is another important messenger in cells. In PDE families, isozymes such as PDE3 and PDE5 have hydrolyzing effect on cGMP, which is not the specific substrate of PDE4. cGMP in low concentration does not affect the activity of PDE4 on hydrolyzing cAMP[10]. In this study, the extract of GL62 yeast cell had little hydrolyzing effect on cGMP. PDE4A activity on cAMP also was not influenced when cGMP existed in the reaction system. At the concentration of 10 μmol/L and 100 μmol/L, cGMP did not affect the PDE4A hydrolyzing-cAMP activity of the extract (Fig 3). These suggest that the extract selectively hydrolyze cAMP and that cGMP is not the specific substrate of the extract.

Theophylline is one of non-selective PDE inhibitors, which inhibits all members of the PDE family including PDE4. Rolipram is one of the second generation PDE inhibitors, which only inhibits PDE4, including PDE4A, PDE4B, PDE4C, and PDE4D[11]. In this study, the PDE4A activity was inhibited by theophylline and rolipram both in a concentration-dependent manner. Rolipram inhibited the PDE4A activity of the extract more strongly than theophylline. It is suggested that the expressed product of GL62 is PDE4A.

Acetamide-45, *N*-(Pyridin-4-yl)-[1-(4-fluorophenyl)indol-3-yl]acetamide, was reported as a new anti-inflammatory drug[12]. It has been introduced recently that acetamide-45 inhibited the ovalbumin-induced histamine release from peritoneal mast cells, the IL-4 and IL-5 production of Th2 lymphocyte, and the activation of eosinophil[12]. It was also reported that acetamide-45 inhibited the contraction of isolated guinea pig trachea induced by histamine and methacholine[13]. But the mechanism remains unknown. The present study showed that acetamide-45 had inhibitory effect on PDE4A activity of the expressed product. The nature of this inhibition varied with concentration. At a concentration of 1×10^{-5} mol/L, acetamide-45 caused feeble depression of hydrolysis of cAMP. However, when the concentration rose to 3×10^{-5} mol/L, acetamide-45 inhibited the PDE4A activity significantly. At a concentration of 3×10^{-4} mol/L, acetamide-45 achieved the maximal effect that the remaining activity was 52 %± 5 %. The study of higher concentrations was not carried out because of limited dissolution of the compound. Acetamide-45 inhibited the activity of PDE4A stronger than theophylline, but weaker than rolipram (Tab 2). So it is suggested that acetamide-45 may be an effective PDE4 inhibitor and the anti-inflammatory effect of the drug may depend on inhibition of PDE4.

In conclusion, the present studies indicate that PDE4A can be expressed in yeast cell GL62 induced by $CuSO_4$, and that acetamide-45, a kind of new anti-in-

flammatory drug, may inhibit the activity of PDE4A. These findings suggest that the product may be used to screen new PDE4 inhibitors and that inhibition of PDE4 may be one of the anti-inflammatory mechanisms of acetamide-45.

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