Selective inhibition of purified human phosphodiesterase 4A expressed in yeast cell GL62 by ciclamilast, piclamilast, and rolipram[1]

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KEY WORDS phosphodiesterase inhibitors; ciclamilast; 3-(cyclopentyloxy)-*N*-(3,5-dichloro-4-pyridyl)-4-methoxybenzamide; rolipram; zaprinast; 3',5'-cyclic-nucleotide phosphodiesterase; *Saccharomyces cerevisiae*

ABSTRACT

AIM: To improve the specific activity of human phosphodiesterase 4A (PDE4A) expressed in yeast cell GL62 and investigate the effects of selective phosphodiesterase 4 (PDE4) inhibitors (ciclamilast, piclamilast, and rolipram), selective phosphodiesterase 5 (PDE5) inhibitor zaprinast, and cyclooxygenase (COX) inhibitors (aspirin, indomethacin) on human PDE4A activity expressed in yeast cell GL62. **METHODS:** Human PDE4A was expressed in yeast cell GL62 after $CuSO_4$ induction and the specific activity of human PDE4A was improved by ammonium sulfate fractionation, DEAE Sephadex A-50 chromatography, and Sephadex G-100 chromatography. The activity of PDE4A was measured by high performance liquid chromatography (HPLC). **RESULTS:** Induced PDE4A activity expressed in crude yeast cell GL62 supernatant and pellet was (340±21) nmol·g^{-1}·min^{-1} and (250±25) nmol·g^{-1}·min^{-1} respectively. The specific activity of recombinant PDE4A in supernatant was improved 6.4 fold. Ciclamilast, piclamilast, and rolipram could inhibit PDE4A activity. The IC_{50} values (95 % confidence limits) of ciclamilast, piclamilast, and rolipram were 1.27 (0.84-1.91), 66.4 (33.3-132.2), and 3.73 (2.51-5.53) μmol/L respectively. Zaprinast, aspirin, and indomethacin had no obvious inhibitory effect on PDE4A activity. **CONCLUSION:** The specific activity of PDE4A expressed in yeast cell GL62 can be improved by ammonium sulfate fractionation, DEAE Sephadex A-50 chromatography, and Sephadex G-100 chromatography. Ciclamilast, piclamilast, and rolipram can inhibit PDE4A activity while zaprinast, aspirin, and indomethacin have no obvious inhibitory effect on PDE4A activity. Human PDE4A expressed in GL62 might be useful in the research and screening of new selective PDE4 inhibitors.

INTRODUCTION

The cyclic nucleotides cAMP and cGMP are key regulators of diverse cellular processes. The levels of the cyclic nucleotides are regulated by the rates of the related synthesis and degradation: the synthesis is generated by adenylate and guanylate cyclases and the degradation is linked to phosphodiesterase activity. Phosphodiesterase which is a large superfamily of proteins can be classified into 11 families in their substrate specificity, kinetic properties, responsiveness to endogenous regulators, and susceptibility to inhibition by vari-

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ous compound[1,2]. Among those family members, cAMP specific phosphodiesterase 4 (PDE4), which is composed of four subtypes (PDE4A to PDE4D), has emerged as the predominant isoenzyme in inflammatory cells, pulmonary smooth muscle cells, heart cells, and brain cells. Numerous studies have demonstrated the modulation of inflammatory and immunocompetent cell activation by selective PDE4 inhibitors[3,4]. The basis for the application of the selective PDE4 inhibitors in the treatment of many inflammatory and allergic diseases, such as asthma and chronic obstructive pulmonary disease, remains unclear. We need to learn more about this important enzyme family, but the paucity and lability of PDE4, especially from human sources, have severely constrained the development of our understanding of this important enzyme family. A better understanding of the properties of these enzymes has come with the cloning of the genes and cDNAs corresponding to PDE4 subtypes. Since the first report on the cloning of PDE4, PDE4 activity has been recovered by expression in *Escherichia coli*, *Saccharomyces cerevisiae*, and some mammalian cell strains[5-7].

However, the effects of prototype PDE4 inhibitors used in clinical trials, such as rolipram, have been compromised by side effects such as nausea and emesis[8]. It was reported that the four subtypes are found differentially expressed among tissues and cells[9-11]. PDE4A, PDE4B, and PDE4D gene products are found in most immune and inflammatory cells. PDE4A, PDE4C, and PDE4D gene are expressed in airway epithelial cells. The four genes are expressed in the brain showing a different distribution within the regions (PDE4D can be found in the area associated with nausea). The side effects may be the result of the inhibition of all PDE4 subtypes without discrimination, and so it is of great interest to develop new PDE4 subtype-selective inhibitors to inhibit the PDE4 subtypes with discrimination.

In the present study, we have further characterized the human PDE4A expressed in yeast cell GL62 after $CuSO_4$ induction and improved the specific activity of the recombinant human PDE4A by ammonium sulfate fractionation, DEAE Sephadex A-50 chromatography, and Sephadex G-100 chromatography. We also investigated the effects of ciclamilast, piclamilast, rolipram, zaprinast, aspirin, and indomethacin on it.

MATERIALS AND METHODS

Materials *Saccharomyces cerevisiae* strain GL62 *(MATa leu2 ura3-52 adel his3 trp1 lys2-208 pde1::LEU2 pde2::URA3 pep4::HIS3)* was a generous gift from Prof Allan R SHATZMAN (SmithKline Beecham, USA)[7]. Ciclamilast and piclamilast were kindly provided by Beijing Joinn Drug Research Center. Rolipram, pepstatin, phenylmethylsulfonyl fluoride (PMSF), 2-mercaptoethanol, zaprinast, aspirin, indomethacin, and dimethyl sulfoxide (Me_2SO) were purchased from Sigma Chemical Co. Yeast protein extraction reagent (Y-PER) was obtained from Pierce Chemical Co. DEAE Sephadex A-50 and Sephadex G-100 were purchased from Amersham Pharmacia Biotech. Other reagents used in experiments were of analytic grade or reagent grade as appropriate.

Expression and extraction of PDE4A GL62 was grown in YPD and PDE4A expression was induced with $CuSO_4$ essentially as described previously[7]. Cells were harvested by centrifugation and washed once with PBS, resuspended in yeast protein extraction reagent with 1 mmol/L PMSF following the manufacturer's instructions. Extracts were centrifuged at 13 000×*g* for 10 min at 4 ºC. PDE4A activity was determined both in the supernatant and pellet fractions.

Improvement of PDE4A specific activity Ammonium sulfate was added in the supernatant fraction to a final concentration of 33 %. After 30 min of equilibration, the precipitate was removed by centrifugation for 15 min at 12 000×*g*. Ammonium sulfate was further added in the supernatant fraction to a final concentration of 50 %. After 30 min of equilibration, the suspension was centrifuged again for 15 min at 12 000×*g*. The pellet was dissolved in Buffer A (sodium phosphate buffer 10 mmol/L, pH 7.4, $MgCl_2$ 1 mmol/L, pepstatin 1 mmol/L, edetic acid 0.1 mmol/L, 2-mercaptoethanol 5 mmol/L, PMSF 0.2 mmol/L) and dialysed against the same buffer, then applied to a DEAE Sephadex A-50 column equilibrated with Buffer A. After application of the sample, the column was washed with the same buffer until adsorbance at 280 nm had reached basal value. Proteins were eluted with a linear gradient of 30 mL of Buffer A and 30 mL of Buffer B (sodium phosphate buffer 200 mmol/L, pH 7.4, $MgCl_2$ 1 mmol/L, pepstatin 1 mmol/L, edetic acid 0.1 mmol/L, 2-mercaptoethanol 5 mmol/L, PMSF 0.2 mmol/L) at 30 mL/h and collected in 2 mL fractions. PDE4 activity was determined in each fraction. The more active fractions were pooled and concentrated by Ultrafree-4 (Millipore, USA) for ultrafiltration. The concentrated protein was subjected to Sephadex G-100 equilibrated

with Buffer A and eluted with the same buffer. The effluent was collected in 2 mL fractions at 10 mL/h. PDE4 activity was determined in each fraction. The more active fractions were pooled and concentrated for later analysis.

Protein determination and gel electrophoresis Protein concentration was routinely measured by the method of Bradford[12] with bovine serum albumin as a standard. The samples were subjected to SDS-PAGE through the 5 % stacking gel and 12 % separating gel as described by Laemmli with bromophenol blue as reference dye. The molecular mass marker used were calmodulin binding protein (130 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), and actin (43 kDa). Gels were fixed and stained with Coomassie blue.

PDE4A activity assay and PDE4A inhibition PDE4A activity was assayed and calculated as described previously[7]. For PDE4A inhibition experiments, ciclamilast, piclamilast, rolipram, and zaprinast were dissolved in 100 % dimethyl sulfoxide as a 10 mmol/L stock. Aspirin and indomethacin were dissolved in 100 % ethanol as a 10 mmol/L stock. Drugs were then diluted with PBS to provide a range of concentrations for use in the assay. The residual levels of dimethyl sulfoxide or ethanol in each assay mixture were less than 1 %. Remaining enzyme activity of PDE4A was calculated by the following equation: Remaining activity=[cAMP remaining (control) – cAMP remaining (reaction with drug)]/[cAMP remaining (control) –cAMP remaining (reaction with solvent)].

Data analysis Data were presented as mean± SD. Statistical analysis was performed with *t*-test. A probability level of $P<0.05$ was considered significant. IC_{50} (95 % confidence limits) was calculated and compared by weighted probit analysis of Bliss method.

RESULTS

Analysis of expressed PDE4A activity PDE4A activity was observed both in the supernatant fraction and pellet fraction with different proportion. The specific activity of PDE4A in supernatant was higher than that in pellet (Tab 1).

Improvement of PDE4A specific activity After ammonium sulfate fractionation, DEAE Sephadex A-50 chromatography, and Sephadex G-100 chromatography (Fig 1), PDE4A in the supernatant fraction was purified to apparent homogeneity by virtue of it giving a single band of 76 kDa on SDS-PAGE (Fig 2). The specific activity of PDE4A in supernatant was improved 6.4 fold (Tab 2).

Tab 1. Distribution of PDE4A activity in crude extract from GL62 yeast cells. *n*=6. Mean±SD. $^cP<0.01$ *vs* supernatant.

Part	Specific activity/ nmol·g^{-1} protein·min^{-1}	Percentage of total activity/%
Supernatant	340±21	34±5
Pellet	250±25^c	66±5^c

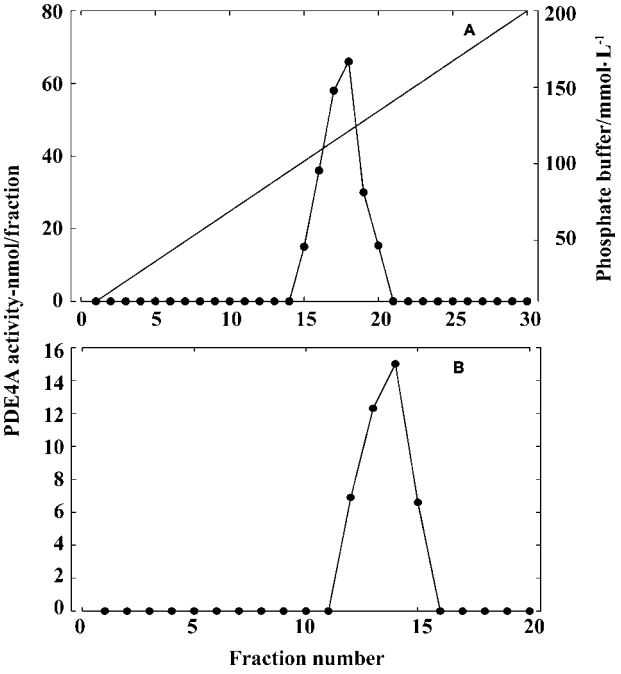

Fig 1. Purification of PDE4A from GL62 yeast cells after ammonium sulfate fractionation by DEAE Sephadex A-50 and Sephadex G-100. Fractionation on the two columns was performed as described in the text. The PDE4A activity eluted was measured with 1 μmol/L cAMP as substrate. The total PDE4A activity per fraction was reported. (A) DEAE Sephadex A-50. (B) Sephadex G-100.

Effects of selective PDE4 inhibitors, selective PDE5 inhibitor, and cyclooxygenase (COX) inhibitors on purified PDE4A activity PDE4A activity was potently inhibited by ciclamilast and rolipram. It was also inhibited by piclamilast at much higher concentration (Fig 3). The IC_{50} values (95 % CL) of ciclamilast, piclamilast, and rolipram were 1.27 (0.84-1.91), 66.4 (33.3-132.2), and 3.73 (2.51-5.53) μmol/L, respectively (Tab 3). On the other hand, selective PDE5 inhibitor zaprinast and COX inhibitor aspirin, indomethacin had

Tab 2. Purification of PDE4A from GL62 yeast cells.

Step	Total protein /mg	Total activity /nmol·min^{-1}	Specific activity /nmol·g^{-1} protein·min^{-1}	Purification /fold	Yield /%
Crude supernatant	130	45.5	0.35	1	100
Ammonium sulfate	60	29.4	0.49	1.4	65
DEAE Sephadex A-50	7	7.21	1.03	2.9	16
Sephadex G-100	0.6	1.36	2.27	6.4	3

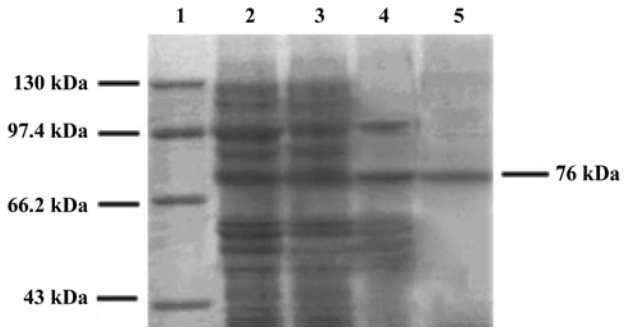

Fig 2. Samples were electrophoresed on a 12 % polyacrylamide gel under denaturing and reducing conditions. Protein bands were visualized by staining with Coomassie blue. Identities of protein loaded were: lane 1, molecular weight markers; lane 2, crude extract; lane 3, ammonium sulfate precipitated; lane 4, DEAE Sephadex A-50 eluate; lane 5, Sephadex G-100 eluate.

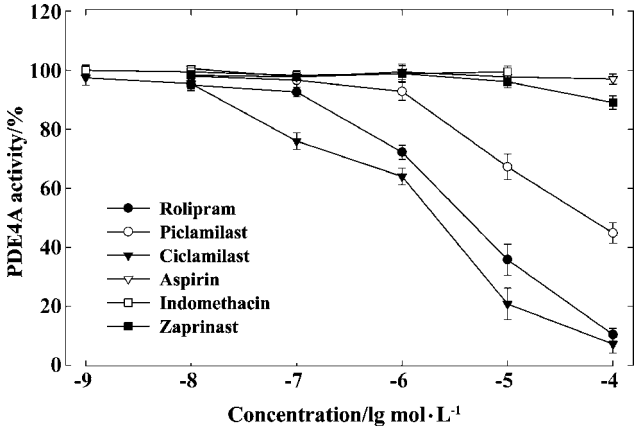

Fig 3. Effect of ciclamilast, piclamilast, rolipram, zaprinast, aspirin, and indomethacin on the purified PDE4A activity from GL62 yeast cells. Ciclamilast, piclamilast, and rolipram. *n*=5. Zaprinast, aspirin, and indomethacin. *n*=3. Mean±SD.

Tab 3. Inhibition of ciclamilast, piclamilast, and rolipram on the purified PDE4A activitiy from GL62 yeast cells. *n*=5. $^{b}P<0.05$ *vs* rolipram. $^{c}P<0.01$ *vs* piclamilast.

Drugs	pIC_{50}	IC_{50} (95 % CL)/μmol·L^{-1}
Rolipram	5.45±0.11	3.73 (2.51-5.53)
Piclamilast	4.23±0.21	66.4 (33.3-132.2)
Ciclamilast	5.91±0.32^{bc}	1.27 (0.84-1.91)

no obvious inhibitory effect on purified PDE4A activity expressed in yeast cell GL62 (Fig 3).

DISCUSSION

In the present study, yeast strain GL62 lacking endogeneous PDE4 activity is available, providing a system with the undisputed advantage that the recombinant protein produced is virtually free of contamination by other endogenous PDE to the expression system. Once a suitable expression system has been identified, the improvement of specific activity can be performed by chromatographic step. However, some steps often suffer from two related problems: low yield and difficulty in controlling inactivation of enzyme. It also happened in this experiment (Tab 2). Several laboratories have used His-tag or GST system for expression and improvement of specific activity with affinity chromatography to avoid above mentioned problems, while the addition of a tag may have unknown effects on the structure and properties of the recombinant PDE4 proteins[6,13].

The strain GL62 expressed soluble PDE4A activity in supernatant, together with an insoluble activity in pellet. PDE4 activity in both soluble and pellet forms in GL62, as well as the purified form, showed similar IC_{50} values for inhibition by ciclamilast, piclamilast, and rolipram (unpublished data made by our lab). This indicates that, whatever gives rise to the aggregated form of PDE4A in GL62, the catalytic/inhibitor-binding site

would appear to be relatively unaffected.

The present study showed that selective PDE5 inhibitor zaprinast and COX inhibitors aspirin, indomethacin did not inhibit the purified PDE4A activity expressed in yeast cell GL62 obviously. Rolipram is known as a standard PDE4 inhibitor[14] and most of the other inhibitors have been based on rolipram[3]. Piclamilast [3-cyclopentyloxy-*N*-(3,5-dichloro-4- pyridyl)-4-methoxybenamide, Novartis Co] is an analogue of rolipram, and they both displayed anti-inflammatory effects *in vivo* and *in vitro*[3]. Ciclamilast is a newly developed "me-too" drug of piclamilast by Beijing Joinn Drug Research Center. It was found that both piclamilast and ciclamilast inhibited ovalbumin-induced bronchoconstriction and release of slow reactive substance-A (SRS-A) from lung tissue of sensitized guinea pig, with more potent action of ciclamilast[15]. The present study also showed that ciclamilast, piclamilast, and rolipram could inhibit the purified PDE4A activity expressed in yeast cell GL62 in a concentration-dependent manner. Ciclamilast showed higher potency against PDE4A than rolipram and piclamilast, with an IC_{50} value (95 % confidence limits) of 1.27 (0.84-1.91) μmol/L in comparison to 66.4 (33.3-132.2) and 3.73 (2.51-5.53) μmol/L of piclamilast and rolipram, respectively. The result indicates that these PDE4 inhibitors can discriminate between inhibitions of PDE4A subtype and the effect of inhibition of bronchoconstriction and release of SRS-A by piclamilast and ciclamilast may be partly based on the inhibition of PDE4A activity. Therefore, human PDE4A expressed in GL62 might be useful in the screening and verification of new selective PDE4 inhibitors.

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