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ACCELERATED COMMUNICATION

Expression of Human Recombinant cAMP Phosphodiesterase Isozyme IV Reverses Growth Arrest Phenotypes in Phosphodiesterase-Deficient Yeast

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SUMMARY

The low-K_m cAMP-specific phosphodiesterases (PDEases) are of great pharmacological significance because of their involvement in regulating cAMP concentrations, which, in turn, are responsible for mediating the cellular response to extracellular signals such as hormones and neurotransmitters. We recently reported the isolation of a cDNA clone that encodes a human monocyte low-K_m, rolipram-sensitive, cAMP PDEase (isozyme IV). We have engineered the inducible expression of this human PDEase in yeast. Cells of *Saccharomyces cerevisiae* contain two genes that encode cAMP PDEases. PDEase-deficient mutants are viable but exhibit specific growth arrest phenotypes associated with elevated intracellular cAMP content; these phenotypes include heat shock sensitivity and the inability to grow on acetate as a carbon source. We show that functional expression of our human cAMP PDEase in a genetically engineered PDEase-deficient strain of *S. cerevisiae* reverses these aberrant phenotypes. Furthermore, under conditions for growth arrest, rolipram is cytotoxic to PDEase-deficient mutants expressing the human cAMP PDEase, indicating that it is capable of inhibiting the human recombinant enzyme *in vivo*. This system can be used in the development of a yeast cell-based assay for isozyme-selective inhibitors of the human recombinant cAMP PDEase.

The hydrolysis of 3′,5′-cyclic nucleotides into 5′-nucleoside monophosphates is catalyzed by a large and heterogeneous family of cyclic nucleotide PDEases (1, 2). The mammalian PDEases have been classified into five isozyme families, based on differences in substrate specificity, cofactor requirements, and selective inhibition by various compounds (1, 2).

One isozyme family consists of the low-K_m (high affinity) cAMP-specific PDEases [also referred to as PDEase isozyme IV (2)]. Members of the PDEase isozyme IV family have been of particular pharmacological interest because of their possible involvement in neurobiochemical processes (3). Moreover, cAMP PDEase induction has been observed in a number of different cell types following increases in cellular cAMP levels induced by hormones and neurotransmitters (4–8); this regulation of PDEase expression occurs at the level of transcription (8). Thus, these enzymes appear to play a central role in signal transduction, mediating the cellular response to external stimuli. Additional evidence for a neurobiochemical function comes from the fact that mutations in the *dunce*$^+$ locus of *Drosophila melanogaster*, which encodes a low-K_m cAMP PDEase, cause learning and memory dysfunction in flies (9, 10). Furthermore, the pharmacological inhibition of the mammalian cAMP PDEases clearly affects mood. In fact, the type IV PDEase isozymes have been defined by their specific inhibition by neurostimulators such as the antidepressant drugs rolipram and RO 20-1724 (1–3, 11). Inhibitors of the cAMP PDEases may also be useful in the therapy of asthma and certain types of inflammation (12–16).

We recently reported the isolation of a cDNA that encodes a low-K_m, rolipram-sensitive, cAMP PDEase expressed in human monocytes (17). The human recombinant PDEase was found to exhibit all of the biochemical characteristics associated with enzymes of the isozyme IV class (2, 17). The predicted protein sequence contains significant homology to several rat cAMP PDEases (8, 9, 18–20), as well as to the *Drosophila dunce*$^+$ gene product (21). In an effort to generate human recombinant protein for biochemical studies and to develop a rapid, high throughput screen for human cAMP PDEase isozyme IV-selective inhibitors, we have engineered the expression of this human PDEase in yeast. Cells of *Saccharomyces cerevisiae* contain two genes for cAMP PDEases, *PDE1*, which encodes a low affinity cAMP PDEase of ~42,000 Da (22, 23), and *PDE2*, which encodes a high affinity enzyme of ~61,000 Da (24–26). Cells containing null mutations in both *PDE1* and *PDE2* are

ABBREVIATIONS: PDEase, phosphodiesterase; SC−Trp, synthetic complete medium minus tryptophan; kb, kilobase pairs.

viable but exhibit specific growth arrest phenotypes, such as heat shock sensitivity, the inability to grow under starvation conditions, or the inability to survive prolonged nitrogen starvation (18, 23, 25, 26); these phenotypes are a specific consequence of elevated cAMP levels in the cell. Starvation triggers meiosis (G1 arrest) in *S. cerevisiae* by apparently inducing the adenylate cyclase/cAMP-dependent protein kinase cascade, thereby increasing the cellular accumulation of cAMP (27). Colicelli *et al.* (18) have exploited this system to clone a sequence that encodes a high affinity cAMP PDEase from a rat brain cDNA expression library, constructed in a yeast expression plasmid, simply by its ability to suppress these phenotypic abnormalities.

In this report, we demonstrate (a) that our human cAMP PDEase can be functionally expressed in yeast, (b) that stable protein expression requires the absence of a major vacuolar protease, (c) that expression in PDEase-deficient cells reverses both the heat shock and starvation-sensitive phenotypes associated with elevated cAMP content, and (d) that the human recombinant cAMP PDEase is rolipram sensitive *in vivo*. These data are discussed with regard to their use in development of a yeast cell-based assay for human PDEase isozyme IV inhibitors.

Materials and Methods

Strains and growth conditions. The genotypes of the *S. cerevisiae* strains relevant to this study are shown in Table 1. A strain carrying a genomic disruption of *PDE1* was constructed as follows. (a) A C-terminal fragment of *PDE1* was generated by polymerase chain reaction, using oligonucleotide primers homologous to sequences within the coding region (25, 28). (b) This resulted in an 853-base pair *Bam*HI fragment, which was subcloned into the unique *Bam*HI site of pUC19. (c) The resulting plasmid (pUC19-yPDE1) was linearized with *Bal*I and ligated to a 2.2-kb *Xho*I-*Sal*I fragment carrying the *S. cerevisiae LEU2* gene, which was blunt-ended with Klenow polymerase, creating pUC19-yPDE1::LEU2. (d) An ~3.0-kb *Bss*HI (internal coding region site)-*Bam*HI fragment was purified from pUC19-yPDE1::LEU2 and used to transform strain SJ21R (*MATa ura3-52 leu2-3,2-112 ade1 MEL1*) (29), selecting for Leu$^+$ prototrophs. (e) Transformants were screened for those containing a genomic disruption of the cloned sequence by genomic Southern blotting, using ^{32}P-labeled (30) *PDE1* polymerase chain reaction fragment as a probe. One strain (MM1) was chosen for further study.

All other strains were obtained via standard yeast genetic crosses, sporulation, and tetrad disection, as previously described (31). Strain GL54 was obtained as a segregant from a diploid formed between RW134-2C and MM1. Strain GL61 was subsequently generated from a cross between GL54 and BJ3505 (*MATa pep4::HIS3 prb1-Δ1.6R his3-Δ200 lys2-208 trp1-Δ101 ura3-52 gal2 can1*) (Yeast Genetic Stock Center, Berkeley, CA). All media for growth [Yeast Extract Peptone Dextrose and SC−Trp], as well as for sporulation, were prepared as previously described (32). Mating type determinations were made by standard techniques (33). Yeast transformations were performed using the lithium acetate method (34).

Expression plasmid construction. The human cAMP PDEase yeast expression plasmid was constructed as follows. A 2.38-kb *Sal*I-*Xba*I fragment containing human monocyle cAMP PDEase isozyme IV was isolated from phPDE-1U [a modified version of the original λZAP (Stratagene Inc., La Jolla, CA) clone] (17) and subcloned into the unique *Xho*I and *Nhe*I polylinker sites of the yeast expression vector p138NB, creating p138NB-hPDE1. The parent vector (p138NB) was derived from p138 (35). Briefly, it contains the *TRP1* selectable marker and partial 2μ sequences for maintenance at high copy number, with expression driven by the copper-inducible *CUP1* gene promoter. A multiple cloning (polylinker) site is present downstream of the promoter, followed by the yeast *CYC1* transcriptional terminator.

Immunoblot analysis. Yeast cells harboring plasmids were grown aerobically, in SC−Trp at 30°, to A_{540} = 1.0. PDEase expression was induced by the addition of 150 μM $CuSO_4$. Cells were harvested at various times and either incubated for 5 min at 100° in Laemmli sample buffer (36) (whole-cell extracts) or subjected to fractionation protocols. For the latter, harvested cells were washed once with distilled H_2O, resuspended in breaking buffer (50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride), and disrupted by vortexing a 4° in the presence of 0.45-mm glass beads. Extracts were centrifuged at 1,500 × *g* for 5 min at 4°. The soluble lysate fraction was then centrifuged at 100,000 × *g* for 30 min at 4°, the resulting supernatant was removed, and the pellet was resuspended in an equal volume of breaking buffer. One-fifth final volume of 5× sample buffer was added, and protein samples were separated on sodium dodecyl sulfate-8% polyacrylamide gels and electrophoretically transferred to nitrocellulose paper, using a Janssen semidry electroblotter. Blots were probed using antisera raised against a GalK-hPDE1 fusion protein expressed in *Escherichia coli*; protocols for immunoblotting and antiserum production have been described elsewhere (17).

PDEase assays and cAMP determination. Cyclic AMP PDEase activity was determined in the 100,000 × *g* supernatant fractions. Briefly, the reaction was initiated by addition of an aliquot of the yeast supernatant fraction to 0.1 ml (final volume) of a reaction mixture containing (final concentrations) 50 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 50 μM 5′-[^{14}C]AMP (400 dpm/nmol), and 1 μM [^{3}H]cAMP (200 dpm/pmol). Reactions were conducted at 30° for 30 min and terminated, substrate and product were separated, and PDEase activity was determined as previously described (17).

Yeast cells were prepared for cAMP determinations as described by Nikawa *et al.* (25). Cells were grown to A_{540} = 1.0, harvested, washed once with water, and resuspended in 5% trichloroacetic acid. Cells were disrupted as described above, and the resulting lysates were centrifuged at 100,000 × *g* for 30 min. Trichloroacetic acid was removed by five successive extractions with water-saturated ether, and cAMP concentrations were measured, following acetylation, via radioimmunoassay (37). Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce Chemical Co.), after cells were boiled for 5 min in 1 N NaOH.

TABLE 1

S. cerevisiae strains and cAMP levels

Genetic techniques used to construct strains for this study and protocols for determining intracellular cAMP content are described in Materials and Methods.

Strain	Genotype	cAMP	Source of strain
		pmol/mg of total cell protein	
DC6	*MATα leu2 his3 can1 gal2*	7.7	Livi *et al.* (42)
RW134-2C	*MATα leu2 his3 his4 trp1 ura3 pde2::URA3*	13.5	Wilson and Tatchell (26)
MM1	*MATa leu2 ura3-52 ade1 MEL1 pde1::LEU2*	7.6	This study
GL54	*MATα leu2 ura3-52 ade1 his3 trp1 pde1::LEU2 pde2::URA3*	12.6	This study
GL61	*MATα leu2 ura3-52 ade1 his3 trp1 lys2-208 pde1::LEU2 pde2::URA3 pep4::HIS3*	14.5	This study

Plate assay for functional PDEase expression. Cells were tested for growth arrest phenotypes using modifications of reported protocols (18, 23, 25, 26). Briefly, cells were grown in SC−Trp liquid medium for 2 days at 30°, spotted onto SC−Trp agar medium containing 5 mM cAMP and 150 μM $CuSO_4$, and incubated for 2 days at 30°. For heat shock, cells were replicated from the master plate onto two plates containing the same medium, one of which had been preheated to 55° for 1 hr. Cells transferred to the preheated plate were incubated at 55° for 5 min and then shifted to 30°. Growth was scored following 3 days of growth at 30°. For monitoring growth on acetate, the master plate was replicated to a plate containing the same medium that contained 2% potassium acetate instead of glucose. Growth was scored after 5 days at 30°.

Materials. Racemic rolipram was synthesized by S. Christensen and colleagues, Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals (King of Prussia, PA). Cyclic AMP radioimmunoassay kits and [^{3}H]cAMP were obtained from New England Nuclear (Boston, MA).

Results and Discussion

Expression of bioactive human cAMP PDEase isozyme IV in PDEase-deficient yeast. A yeast expression plasmid was constructed in which cDNA encoding a human monocyte cAMP PDEase (isozyme IV) (17) was placed under the control of the copper-inducible *CUP1* gene promoter (see Materials and Methods). This plasmid (p138NB-hPDE1) was introduced into cells containing genomic disruptions of one or both of the two yeast cAMP PDEase genes (strains RW134-2C and GL54) (see Table 1 for complete genotypes). Although copper-inducible PDEase mRNA expression occurred in these transformed strains (as judged by Northern blotting), no recombinant protein was detected by immunoblotting (data not shown). One possible explanation for the lack of protein expression is that the recombinant protein is subject to rapid protease degradation.

To address this possibility, we constructed a PDEase-deficient *PEP4*-deleted yeast strain (GL61), using both classical and reverse genetics (see Materials and Methods). *PEP4* is a gene that codes for a major vacuolar protease (38, 39). Cells of strain GL61 (and GL54) exhibited an expected increase in intracellular cAMP content, as compared with that present in wild-type cells (strain DC6) and in cells containing a deletion of only *PDE1* (strain MM1) (Table 1). These data are consistent with previous reports (18, 23, 25). As shown in Fig. 1A, significant amounts of human cAMP PDEase protein could be expressed in GL61 (as well as in all other *PEP4*-deleted strains tested) following a 1-hr induction. Thus, stable protein expression was specifically dependent upon the absence of the *PEP4* aspartyl protease. Although the molecular mass of the protein predicted from the cDNA sequence is approximately 77,000 daltons (17), the protein expressed in yeast appeared larger, suggesting the presence of posttranslational modifications. We also observed that a significant proportion of the recombinant protein existed in a soluble cell fraction (Fig. 1B); the percentage of soluble cAMP PDEase protein appeared to be inversely related to the level of expression (data not shown).

The fact that the expressed human recombinant protein possesses cAMP PDEase activity was determined by comparing the level of cAMP-hydrolyzing activity in cytosolic fractions of GL61 harboring either p138NB-hPDE1 or the parent plasmid (p138NB) without insert. Table 2 shows that, whereas GL61 cells with p138NB contained no detectable cAMP PDEase activity, the same cells with p138NB-hPDE1 exhibited significant levels of enzyme activity, which were 8-fold higher than the endogenous activity present in wild-type cells. Furthermore, the recombinant enzyme was sensitive to rolipram (98% inhibition at 30 μM), whereas the endogenous yeast PDEase activity remained rolipram insensitive. These data are in agreement with our earlier observations of the recombinant protein transiently expressed in COS-1 cells (17).

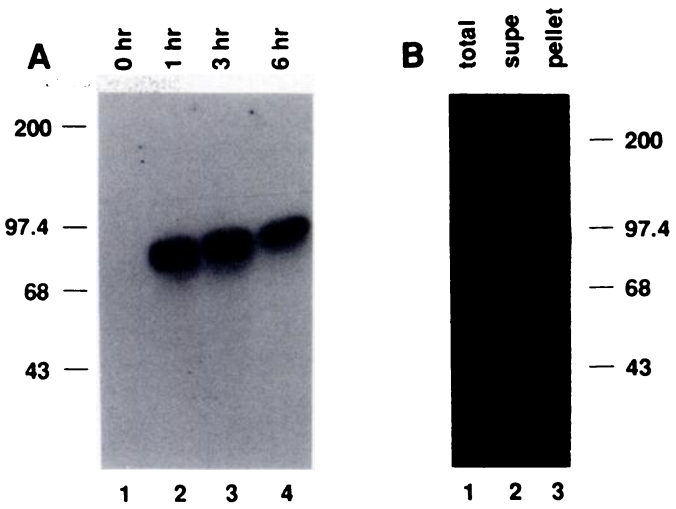

Fig. 1. Expression of human cAMP PDEase in yeast. Cells of strain GL61 containing p138NB-hPDE1 (see Materials and Methods) were grown to mid-logarithmic phase (A_{540} = 1.0), and expression of human cAMP PDEase was induced with the addition of 150 μM $CuSO_4$. Cell lysates were subjected to 0.1% sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with rabbit anti-human cAMP PDEase isozyme IV (17). A, Induction profile (5 × 10^6 cells/lane). *Lane 1*, time zero (before addition of $CuSO_4$); *lane 2*, 1 hr after induction; *lane 3*, 3 hr after induction; *lane 4*, 6 hr after induction. B, Subcellular fractionation analysis of human recombinant cAMP PDEase. Cells were induced for 5 hr, harvested, and broken as described in Materials and Methods. Each lane contains the equivalent material from 2 × 10^7 cells. *Lane 1*, total cell lysate; *lane 2*, 100,000 × *g* supernatant; *lane 3*, 100,000 × *g* pellet. Protein size markers are in kDa.

TABLE 2

Expression of human cAMP PDEase activity in PDEase-deficient yeast

cAMP-hydrolyzing activity was determined using 1 μM [^{3}H]cAMP as the substrate (see Materials and Methods).

Strain	Plasmid	cAMP hydrolysis[a]	
		−Rolipram	+Rolipram (30 μM)
		pmol/min/mg of protein	
DC6	None	49	46
GL61	p138NB	0	0
GL61	p138NB-hPDE1	399	8

[a] With or without racemic rolipram.

Yeast cell-based assay for functional human cAMP PDEase expression. Previous workers have determined that PDEase-deficient yeast cells exhibit specific growth arrest phenotypes that are attributed to increased levels of intracellular cAMP (18, 23, 25, 26). Likewise, in the presence of exogenous cAMP, our PDEase-deficient strain GL61 exhibited similar growth defects, such as heat shock sensitivity and the inability to utilize acetate as a carbon source (Fig. 2). As clearly shown in Fig. 2, functional expression of human cAMP PDEase in GL61 cells rescued them from these lethal effects, presumably by reducing the intracellular content of cAMP. Furthermore,

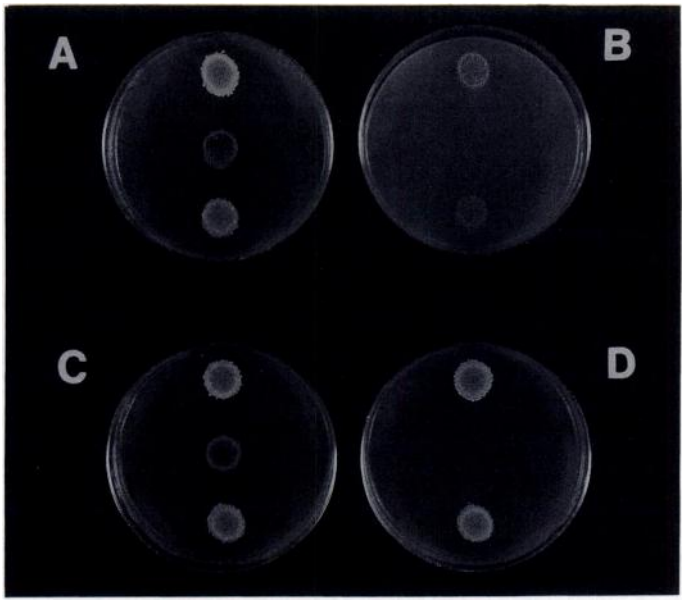

Fig. 2. Plate assay for functional human cAMP PDEase expression. A and C, Cells of three strains were plated on SC−Trp, 150 μM $CuSO_4$, 5 mM cAMP medium. *Top*, strain DC6 (wild-type); *middle*, strain GL61 (*pde1::LEU2 pde2::URA3 pep4::HIS3*) containing p138NB (no insert); *bottom*, strain GL61 containing p138NB-hPDE1. B, The master plate in A was replicated to SC−Trp/acetate medium, containing 150 μM $CuSO_4$ and 5 mM cAMP, and incubated for 5 days at 30°. D, The master plate in C was replicated to SC−Trp medium containing 150 μM $CuSO_4$ and 5 mM cAMP, which had been preheated to 55° for 1 hr; the replica was incubated at 55° for 5 min and then at 30° for 3 days.

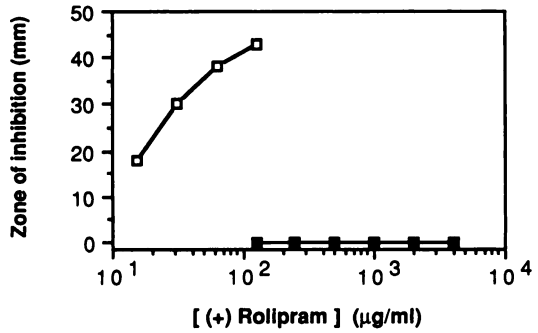

Fig. 3. Effect of (+)-rolipram on yeast strains. The zone of inhibition assay was performed as previously described (40). Cells of strain DC6 (■) or strain GL61/p138NB-hPDE1 (□) were spread on Sc−Trp/acetate agar medium containing 150 μM $CuSO_4$ and 3 mM cAMP. Wells were made in the agar and 100 μl of solution containing (+)-rolipram at the indicated concentrations were added. Zones of inhibition were measured after incubation at 30° for 6 days. The same level of resistance to (+)-rolipram seen in strain DC6 was observed with GL61 cells plated on Yeast Extract Peptone Dextrose and with cells of GL61/p138NB-hPDE1 plated on SC−Trp/glucose. Similar results were obtained with (−)-rolipram.

Fig. 3 shows that, under the conditions for growth arrest, rolipram was toxic to PDEase-deficient cells expressing the human cAMP PDEase but was not toxic to wild-type cells; in addition, rolipram had no effect on GL61 cells grown on glucose medium (see ref. 40 for zone of inhibition assay). Thus, rolipram is capable of entering yeast cells and inhibiting the activity of the human recombinant cAMP PDEase, thereby unmasking the endogenous growth arrest phenotype.

This system for rapidly determining functional enzyme expression offers a means for the development of a high throughput screen for human cAMP isozyme IV-selective inhibitors. New compounds found to specifically inhibit the recombinant enzyme *in vitro* can be compared with rolipram in their ability to unmask the inherent growth arrest phenotypes of host cells. Moreover, a screen could be configured to identify agents that kill cells under the prescribed conditions for growth arrest but do not kill cells under normal conditions of growth and that do not affect wild-type cells. Although yeast cells may possess certain barriers that make them less sensitive than mammalian cells to these types of inhibitors, there are ways of increasing yeast cell permeability via mutations that affect the cell membrane (41). This system also offers an excellent method for rapidly assaying genetically engineered site-specific mutants of our human recombinant cAMP PDEase for functionality (catalytic activity) *in vivo*. Further work in this area will advance our understanding of the structure-activity relationship of this pharmacologically significant enzyme.

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