Expression and mutagenesis of the catalytic domain of cGMP-inhibited phosphodiesterase (PDE3) cloned from human platelets

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We have used reverse transcriptase PCR, platelet mRNA and degenerate primers based on platelet peptide sequences, to amplify a fragment of platelet cGMP-inhibited phosphodiesterase (cGI-PDE; PDE3). Sequence analysis of this clone established that both the platelet and the cardiac forms of PDE3 were derived from the same gene (PDE3A). A RT-PCR product representing the C-terminal half of platelet PDE3 cDNA and corresponding to amino acid residues 560–1141 of the cardiac enzyme, was cloned and expressed in *Escherichia coli* cGI-PDEΔ1. Further deletion mutants were constructed by removing either an additional 100 amino acids from the N-terminus (cGI-PDEΔ2) or the 44-amino-acid insert characteristic of the PDE3 family, from the catalytic domain (cGI-PDEΔ1Δi). In addition, site-directed mutagenesis was performed to explore the function of the 44-amino-acid insert. All mutants were evaluated for their ability to hydrolyse cAMP and cGMP, their ability to be photolabelled by [³²P]cGMP and for the effects of PDE3 inhibitors. The K_m values for hydrolysis of cAMP and cGMP by immunoprecipitates of cGI-PDEΔ1 (182 ± 12 nM and 153 ± 12 nM respectively) and cGI-PDEΔ2 (131 ± 17 nM and

99 ± 1 nM respectively) were significantly lower than those for immunoprecipitates of intact platelet PDE3 (398 ± 50 nM and 252 ± 16 nM respectively). Moreover, N-terminal truncations of platelet enzyme increased the ratio of V_{max} for cGMP/ V_{max} for cAMP from 0.16 ± 0.01 in intact platelet enzyme, to 0.37 ± 0.05 in cGI-PDEΔ1 and to 0.49 ± 0.04 in cGI-PDEΔ2. Thus deletion of the N-terminus enhanced hydrolysis of cGMP relative to cAMP, suggesting that N-terminal sequences may exert selective effects on enzyme activity. Removal of the 44-amino-acid insert generated a mutant with a catalytic domain closely resembling those of other PDE gene families but despite a limited ability to be photolabelled by [³²P]cGMP, no cyclic nucleotide hydrolytic activities of the mutant were detectable. Mutation of amino acid residues in putative β -turns at the beginning and end of the 44-amino-acid insert to alanine residues markedly reduced the ability of the enzyme to hydrolyse cyclic nucleotides. The PDE3 inhibitor, lixazinone, retained the ability to inhibit cAMP hydrolysis and [³²P]cGMP binding by the N-terminal deletion mutants and the site-directed mutants, suggesting that PDE3 inhibitors may interact exclusively with the catalytic domain of the enzyme.

INTRODUCTION

In human platelets, increases in cAMP or cGMP inhibit agonist-induced platelet aggregation [1–4]. Intracellular concentrations of cyclic nucleotides are tightly controlled by the activities of adenylate and guanylate cyclases and various phosphodiesterases (PDEs). Three distinct forms of PDEs were initially isolated by DEAE-cellulose chromatography of platelet extracts [5]. Further studies in platelets led to the identification and purification of a low- K_m cAMP PDE that was competitively inhibited by cGMP [6,7], a cGMP-binding cGMP PDE [8,9] and a cGMP-stimulated PDE that hydrolyses both cAMP and cGMP [10]. Seven mammalian PDE gene families have now been recognized and classified according to substrate affinities, inhibitor sensitivities, modes of regulation and primary sequence homology, and a standardized nomenclature for these enzymes has now been adopted [11]. According to this classification, platelet cGMP-inhibited phosphodiesterase (cGI-PDE), cGMP-stimulated phosphodiesterase and cGMP-binding phosphodiesterase are members of the PDE3, PDE2 and PDE5 gene families respectively. Hydrolysis of cGMP results predominantly from PDE5, an enzyme possessing a high K_m for cAMP and a low K_m for cGMP. Although PDE2 and PDE3 enzymes both hydrolyse cAMP, they are differentially regulated by cGMP. PDE2, for instance, is stimulated by cGMP through an allosteric binding site, whereas PDE3 is competitively inhibited by cGMP, which is hydrolysed very slowly. In platelets, the PDE3 enzyme is the

major cAMP PDE and is thought to account for 80% of the hydrolysis at sub-micromolar concentrations of cAMP [12]. Increases in platelet cAMP attributed to the inhibition of PDE3 by cGMP, have been shown to account for the synergistic inhibition of platelet aggregation by activators of guanylate and adenylate cyclases [13].

All PDEs contain a conserved 270-amino-acid segment located in the carboxy terminus of the molecule which encompasses the catalytic domain of the enzyme [14,15]. The highly divergent amino-terminal regions of the PDE molecules are likely to confer some of the distinctive regulatory properties characteristic of the individual PDE gene families. cDNAs encoding two distinct cGMP-inhibited PDEs have been previously cloned from a human cardiac cDNA library (PDE3A) [16] and a rat adipocyte cDNA library (PDE3B) [17]. Both PDE3A and PDE3B possess a common domain organization [18]. The deduced amino acid sequences of these two subfamily members indicate a high degree of homology (88%) at the C-terminal catalytic region and a significant amount of divergence at the N-terminus. Located within the catalytic domain is a 44-amino-acid insert which is characteristic of the PDE3 gene family. Although the function of this insert is not known, it could play a role in substrate binding or interaction with PDE3-specific inhibitors. In this study, we have used reverse transcriptase PCR (RT-PCR) to clone the C-terminal half of human platelet cGI-PDE. The resulting truncation mutant, cGI-PDEΔ1 was expressed in *Escherichia coli*, purified for kinetic and pharmacological analysis and compared

Abbreviations used: RT, reverse transcriptase; PDE, phosphodiesterase; TBS, Tris-buffered saline; cGI-PDE, cGMP-inhibited phosphodiesterase; HEL, human erythroleukaemia; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine.

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with the intact platelet enzyme. The effects of deletion and site-directed mutagenesis of the 44-amino-acid insert were also studied.

EXPERIMENTAL

Materials

[^3H]cAMP (27 Ci/mmol), [^3H]cGMP (9.3 Ci/mmol), [^{14}C]AMP (590 mCi/mmol) and [^{14}C]GMP (50 mCi/mmol) were obtained from Du Pont (Mississauga, ON, Canada). [^{32}P]cGMP (3000 Ci/mmol) was supplied by ICN Pharmaceuticals Inc. (Costa Mesa, CA, U.S.A.). Leupeptin, heparin, Protein A-Sepharose, cAMP, cGMP, Nonidet P40, PMSF and protein standards (myosin heavy chain, 205 kDa; β -galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa) were purchased from Sigma (St. Louis, MO, U.S.A.). Immobilion P was obtained from Millipore (Canada) Ltd. (Mississauga, ON, Canada), *Taq* polymerase from Gibco/BRL (Burlington, ON, Canada) and all restriction enzymes from New England BioLabs (Beverly, MA, U.S.A.). The following compounds were gifts: zaprinast (May & Baker, Dagenham, Essex, U.K.), lixazinone (Syntex Corporation, Palo Alto, CA, U.S.A.), milrinone (Sterling Drug, Rensselaer, NY, U.S.A.) and rolipram (Schering, Berlin, Germany). *Erythro*-9-(2-hydroxy-3-nonyl)-adenine (EHNA) was generously provided by Dr. T. Podzuweit (Max Planck Institute, Bad Nauheim, Germany). All oligonucleotide primers were synthesized by the Institute for Molecular Biology and Biotechnology (MOBIX) at McMaster University.

Preparation of platelet soluble proteins

Human platelets were first isolated and washed in Ca^{2+} -free Tyrode's solution supplemented with 5 mM Pipes, pH 6.5, 50 units of heparin/ml, 75 μg of apyrase/ml and 3.5 mg of BSA/ml as described by Mustard et al. [19]. After centrifugation at 1000 g for 15 min, the platelets were finally resuspended at 2.5×10^9 /ml in Buffer A (100 mM KCl, 2.5 mM EGTA, 25 mM Hepes, pH 7.4, and 0.2 mM leupeptin), frozen in 1 ml aliquots using a solid- CO_2 /methanol bath and stored at -70°C . When required, the platelets were thawed and sonicated on ice for 4×30 s; soluble proteins were obtained by centrifugation at 100000 g for 1 h. These proteins were used either for photolabelling or for immunoprecipitation (see below).

Reverse transcriptase PCR

Human platelet mRNA was isolated using the FastTrack mRNA Isolation kit (Invitrogen, San Diego, CA, U.S.A.) according to the manufacturer's instructions. Platelet cDNA was then prepared using Superscript reverse transcriptase (Gibco/BRL, Burlington, ON, Canada) and an oligo(dT) primer (1 h at 37°C), after which the sample was heated to 99°C for 5 min to inactivate the enzyme. Degenerate primers, each with two 5' restriction sites (in bold) were designed based on the sequences of peptides isolated from platelet cGI-PDE, and also found in the cloned human cardiac enzyme [16] (forward primer: 5'-GGTC-TAGAGGATCC(C/A/T/G)GA(G/A)AC(A/T/C/G)ATGA-TGTT-3', corresponding to amino acid residues 661–666 of the cardiac PDE3; reverse primer: 5'-GGGAATTCGTCGAC(G/A)-AA(G/A)TC(G/A)AA(G/A)TG(C/T)TT-3', corresponding to amino acid residues 911–917). An 800 bp PCR product generated using these primers and platelet cDNA as a template was purified by low-melting-point agarose-gel electrophoresis (FMC BioProducts, Rockland, ME, U.S.A.), cloned into pBluescript II

Figure 1 Cloning strategy for the generation of platelet cGI-PDE deletion mutants and the specific removal of the 44-amino-acid insert from the catalytic domain

(a) A 1.8 kb region of platelet cGI-PDE was amplified using RT-PCR and platelet mRNA. The PCR product (see photograph of agarose gel), which coded for the complete catalytic domain of cGI-PDE, was cloned into pBluescript II SK⁺ for sequencing and then subcloned into the bacterial expression vector pQE-30. The His₆-tagged protein, representing a truncated form of the human platelet cGI-PDE (cGI-PDEΔ1), was expressed to high levels in *E. coli*. (b) The 44 amino acid insert characteristic of the PDE3 family was specifically removed from cGI-PDEΔ1 using a PCR approach, to give cGI-PDEΔ1Δi. Primers 1 and 3 were used to amplify the region upstream of the insert and primers 2 and 4 to amplify the downstream region. By incorporating an *Afl*II restriction site into primers 3 and 4, the boundary formed by the ligation of these two regions produced the required PILS amino acid sequence. Diagonal shading, catalytic domain; solid area, 44-amino-acid insert; horizontal shading, His₆ sequence from vector. Ab, antibody binding site.

SK⁺ (Stratagene, La Jolla, CA, U.S.A.) and sequenced in both directions using Sequenase Version 2 (Amersham, Oakville, ON, Canada). PCR reaction conditions using *Taq* DNA polymerase were: first five cycles, denaturation for 1 min at 95°C , annealing for 2 min at 45°C and extension for 1.5 min at 65°C , followed by 25 cycles of 1 min at 95°C , 2 min at 65°C , and 1.5 min at 72°C , with a final extension step of 10 min at 72°C .

Following demonstration that this PCR fragment was identical in sequence with human cardiac cGI-PDE (PDE3A) cDNA, sequence-specific forward and reverse primers were selected to amplify the C-terminal half of the platelet cDNA, including the whole of the catalytic domain (Figure 1a). This truncation mutant (cGI-PDEΔ1) was generated with primers 1 and 2, constructed with *Bam*HI and *Eco*RI restriction sites respectively (forward: 5'-TAGGATCCACAGGGCCTTAACCTTACACT-

CAG-3'; and reverse: 5'-TCGAATTCGGTACCATTGTCAC-TGGTCTGGCT-3'), yielding sequence corresponding to amino acids 560–1141 of the cardiac enzyme. In addition, a shorter truncation mutant (cGI-PDE Δ 2) was generated using the forward degenerate primer to yield sequence corresponding to amino acid residues 659–1141.

Removal of the 44-amino-acid insert characteristic of the PDE3 family (shown in black) from the catalytic domain of cGI-PDE Δ 1 was achieved using a PCR approach (Figure 1b). Regions upstream and downstream of this insert were selectively amplified, purified on a low-melting-point agarose gel, and ligated to generate a mutant, cGI-PDE Δ 1 Δ i. To conserve the amino acid sequence, PILS, at the site of ligation, *A*flIII restriction sites were incorporated into PCR primers 3 and 4 (Figure 1b). For construction of the above deletion mutants, the PCR profile consisted of 25 cycles containing denaturation at 95 °C for 1 min, primer annealing at 58 °C for 2 min and chain extension at 65 °C for 1.5 min. The extension step of the final cycle was increased to 10 min at 72 °C. All PCR products were purified, cloned and sequenced in pBluescript II SK⁺, as above.

Expression and purification of fusion proteins

cGI-PDE deletion mutants were subcloned into the *Bam*HI and *Kpn*I restriction sites of the bacterial expression vector pQE-30 (Qiagen, Chatsworth, CA, U.S.A.) to yield fusion proteins with an N-terminal MRGSH₆GS sequence. The *E. coli* M15 strain transformed with this plasmid were grown to an attenuation of 0.5–0.6 before being induced with 0.5 mM isopropyl thio-galactopyranoside. After growth at 30 °C for an additional 4 h, the cells were harvested and resuspended either in Buffer B (50 mM NaH₂PO₄, pH 7.8, 300 mM NaCl, 1 mM PMSF, 0.2 mM leupeptin) for isolation of His₆-tagged protein by nickel-resin chromatography, or in Buffer C (100 mM Hepes, pH 7.5, 2 mM EGTA, 0.2 mM leupeptin) for immunoprecipitation of protein. In both cases, bacterial suspension was sonicated on ice for 4 × 30 s and centrifuged at 100 000 *g* for 1 h. Supernatant proteins in Buffer B were loaded on to a nickel-resin column to bind the histidine-containing fusion protein. Contaminating proteins were removed by washing with Buffer B containing increasing concentrations of imidazole (5, 80 and 100 mM). Intact His₆-tagged protein was then eluted using Buffer B containing 500 mM imidazole. The eluant was dialysed overnight against Buffer C to remove imidazole before enzymic analysis. Alternatively, the expressed protein was immunoprecipitated from bacterial supernatant proteins in Buffer C using anti-PDE3A IgG, affinity-purified on Avid gel (BioProbe Inc., Tustin, CA, U.S.A.). Protein samples (100 μ l) were incubated for 90 min with 20–30 μ l of IgG at 0 °C, and were then shaken for 90 min at 4 °C with 50 μ l of 50% Protein A–Sepharose. The Protein A–Sepharose was isolated by centrifugation (12 000 *g*, 2 min), washed three times with Buffer C, and resuspended in an appropriate volume of Buffer C for assay of cAMP and cGMP PDE activities.

Site-directed mutagenesis of cGI-PDE Δ 1

Mutagenesis was performed according to the 2-step PCR protocol of Landt et al. [20] with minor modifications. Briefly, an intermediate PCR product was first generated using a 5' sequence-specific primer and a 3' mutagenic primer containing the modified deoxynucleotides. This fragment was purified on a low-melting-point agarose gel and used as the 5' mutagenic primer in a second PCR reaction with a 3' sequence-specific primer. The sequence-specific primers were 5' and 3' to unique restriction sites present in cGI-PDE Δ 1. The PCR products were digested

with appropriate restriction enzymes and subcloned into the corresponding regions of the pBluescript II SK⁺ containing cGI-PDE Δ 1. These mutant clones were sequenced and then further subcloned into pQE-30 for expression.

Western blotting

Bacterial proteins were separated by SDS/PAGE using 10% acrylamide and transferred electrophoretically (120 mA for 3 h) on to Immobilon P membrane. A solution of 5% (w/v) dried milk in Tris-buffered saline (TBS: 10 mM Tris/HCl, pH 8.0, 150 mM NaCl) was used to block the membrane. The primary anti-PDE3A antibody [21] was diluted 1:500 in TBS and incubated with membranes at room temperature for 1 h. Membranes were washed three times with TBS containing 0.05% (v/v) Nonidet P40 and once with TBS, before incubation with secondary antibody conjugated with alkaline phosphatase and detection of immune complexes (Promega, Unionville, ON, Canada).

Measurement of PDE activity

PDE assays (0.1 ml) were performed in the presence of 50 mM Hepes (pH 7.5)/1.0 mM EGTA/5 mM MgCl₂/0.1 mM leupeptin. Nickel-purified or immunoprecipitated enzyme was incubated with [³H]cAMP or [³H]cGMP for 5 or 10 min at 30 °C. Reactions were terminated by adding 0.1 ml of 10 mM EDTA and boiling for 2 min. Approximately 2000 d.p.m. of [¹⁴C]AMP or 4000 d.p.m. of [¹⁴C]GMP was added to each sample to monitor recovery of [³H]AMP or [³H]GMP, which were isolated on Affi-Gel 601 Boronate affinity columns (Bio-Rad, Mississauga, ON, Canada) by a modification of the method of Davis and Daly [22]. Each column (0.8 ml bed volume) was washed with 16 ml of buffer containing 50 mM Hepes, pH 8.5, and 100 mM MgCl₂. [³H]AMP or [³H]GMP was then eluted with 2 ml of 250 mM acetic acid and counted for ³H and ¹⁴C in 10 ml of ACS scintillation fluid, using a Beckman LS5000TA counter. All assays were performed in triplicate or quadruplicate. Contaminating [³H]AMP or [³H]GMP were removed from the tritiated substrates ([³H]cAMP and [³H]cGMP) before their use in the assay by passing solutions of these substrates through Affi-Gel 601 Boronate columns. All IC₅₀ values and enzyme kinetic data (*K_m* and *V_{max}*) were determined using Graft version 3.0 (Erithacus Software Ltd., Staines, Middx., U.K.).

Photoaffinity labelling of proteins by [³²P]cGMP

The method used has been described previously [21]. Briefly, incubation mixtures (0.1 ml) containing 80–100 μ g of total bacterial supernatant protein in Buffer A and 5 μ Ci of [³²P]cGMP (33 nM after dilution) were stirred at 0 °C for 15 min in the dark and then for a further 15 min under UV light. The proteins were precipitated with trichloroacetic acid and analysed by SDS/PAGE (10% acrylamide). Autoradiography was performed using Kodak X-OMAT AR film.

RESULTS

Cloning, bacterial expression and purification of the human platelet cGI-PDE

Degenerate primers, based on platelet peptide sequences, and purified platelet mRNA were used in an RT-PCR reaction to determine whether the platelet PDE3 enzyme was distinct from the previously cloned human cardiac enzyme [16]. An 800 bp PCR product was generated and cloned into pBluescript II SK⁺. Nine independent clones were sequenced in both directions, and

Figure 2 Purification of cGI-PDE deletion mutants by nickel-resin column chromatography or immunoprecipitation

E. coli expressing cGI-PDE deletion mutants were harvested and lysed by sonication. After centrifugation at 100 000 *g*, the His₆-tagged proteins were purified either by nickel-resin column chromatography or by immunoprecipitation. (a) Soluble protein from *E. coli* expressing cGI-PDEΔ1 (lane 1) was loaded on to a nickel-resin column to bind histidine-containing proteins. After removal of unbound protein (lane 2) and a series of increasingly stringent washes (lanes 3–5), purified protein was eluted from the column (lanes 6 and 7) (see the Experimental section). Protein was analysed by SDS/PAGE; a Coomassie Blue-stained gel is shown. (b) Immunoblots of supernatants from bacteria expressing cGI-PDE deletion mutants are shown (lanes 1–3). Purification of the expressed proteins from the supernatants was achieved by immunoprecipitation and the immunoprecipitates were then analysed by immunoblotting (lanes 4–6). Lanes 1 and 4, cGI-PDEΔ1 (Δ1); lanes 2 and 5, cGI-PDEΔ1Δi (Δ1Δi); lanes 3 and 6, cGI-PDEΔ2 (Δ2). The positions of protein standards are shown on the left (see the Experimental section).

shown to be identical with nucleotide residues 1982–2752 of the cardiac enzyme (PDE3A). This established that both the platelet and cardiac forms of PDE3 are derived from the same gene. However, this result does not exclude the possibility of alternate splicing of the platelet enzyme at the N-terminus.

Sequence-specific primers were selected for RT-PCR amplification of the C-terminal half of the platelet enzyme, including the whole of the catalytic domain (Figure 1a). The 1769 bp PCR product (see Figure 1a, photograph insert), which represents amino acids 560–1141 of the cardiac enzyme, was cloned into the pBluescript II SK⁺ vector for sequencing. A *Bam*HI/*Kpn*I cDNA fragment was inserted into the corresponding restriction sites of the bacterial expression vector pQE-30. A His₆-tagged protein representing a truncated form of platelet cGI-PDE (cGI-PDEΔ1) was expressed to high levels in *E. coli*. A further deletion mutant, cGI-PDEΔ2, containing amino acid residues 659–1141, was constructed by replacing the forward PCR primer (see the Experimental section). In addition, the deletion of the 44-amino-acid insert from the catalytic domain was achieved by selective PCR amplification of upstream and downstream regions (cGI-PDEΔ1Δi). Amino acid residues surrounding the insert

Figure 3 Photoaffinity labelling of extracts of *E. coli* expressing cGI-PDE deletion mutants

(a) Bacterial supernatants were photolabelled with [³²P]cGMP as described in the Experimental section. Lane 1, control (*E. coli* transformed with vector alone); lane 2, cGI-PDEΔ1; lane 3, cGI-PDEΔ1Δi; lane 4, cGI-PDEΔ2. The positions of protein standards are shown on the left (see Experimental section). (b) Effects of PDE3 inhibitors on the photoaffinity labelling of bacterial supernatants containing cGI-PDEΔ1 (Δ1) and cGI-PDEΔ2 (Δ2). Lane 1, no inhibitor; lane 2, 10 μM lixazinone; lane 3, 40 μM milrinone; lane 4, 40 μM cGMP.

(–PI[insert]LS–) were conserved by incorporating an *Afl*II restriction site into primers 3 and 4 (Figure 1b).

The His₆-tagged proteins were purified from bacterial supernatants using nickel-resin column chromatography (Figure 2a) or were selectively immunoprecipitated using anti-PDE3A antibody that binds to the C-terminal 14 amino acids of the enzyme [21]. An immunoblot of the immunoprecipitates (Figure 2b) indicated that the apparent molecular masses of the cGI-PDEΔ1, cGI-PDEΔ1Δi and cGI-PDEΔ2 mutants were higher (80, 75 and 66 kDa respectively) than their calculated molecular masses (67, 62 and 56 kDa respectively). The decrease in electrophoretic mobility of these fusion proteins probably results from the presence of the N-terminal His₆ tag.

Photoaffinity labelling of bacterial extracts expressing platelet cGI-PDE deletion mutants

The cGI-PDEΔ1 and cGI-PDEΔ2 mutants were readily photolabelled by [³²P]cGMP in proportion to the amounts of expressed protein present (compare Figure 2b, lanes 1 and 3, with Figure 3, lanes 2 and 4). Despite the fact that the cGI-PDEΔ1Δi mutant protein was expressed to the same level as cGI-PDEΔ1, photolabelling of the former by [³²P]cGMP was much weaker than the latter (Figure 3, lane 3). All three photolabelled species were absent from bacterial lysate transformed with the control pQE-30 vector alone (Figure 3, lane 1). These results indicate that

Table 1 Comparison of the kinetic properties of expressed cGI-PDE deletion mutants with those of the intact enzyme

Intact platelet PDE3 was immunoprecipitated from platelet supernatant and mutant PDEs from bacterial supernatants, as described in the Experimental section. K_m and V_{max} values were calculated by non-linear regression analysis using explicit weighting based on results obtained in triplicate assays. IC_{50} values were determined using $0.5 \mu M$ cAMP as substrate. To eliminate small amounts of PDE5 contaminating platelet cGI-PDE immunoprecipitates, $10 \mu M$ zaprinast was added to assays of this enzyme. Results are means $\pm$ S.E.M. from the numbers of separate enzyme preparations shown in parentheses.

Enzyme	K_m (cAMP) (nM)	K_m (cGMP) (nM)	V_{max} (cGMP)/ V_{max} (cAMP)	IC_{50} for lixazinone (nM)	IC_{50} for milrinone (nM)
cGI-PDE Δ 1	$182 \pm 12(3)$	$153 \pm 12(3)$	$0.37 \pm 0.05(3)$	$186 \pm 26(4)$	$1148 \pm 73(3)$
cGI-PDE Δ 2	$131 \pm 17(3)$	$99 \pm 1(3)$	$0.49 \pm 0.04(3)$	$165 \pm 41(1)$	$1207 \pm 151(1)$
Platelet cGI-PDE	$398 \pm 50(3)$	$252 \pm 16(3)$	$0.16 \pm 0.01(3)$	$53 \pm 10(4)$	$474 \pm 92(3)$

Table 2 Specificity of the effects of PDE inhibitors on cGI-PDE Δ 1, cGI-PDE Δ 2 and platelet cGI-PDE

Immunoprecipitates of cGI-PDE from platelets and *E. coli* expressing cGI-PDE deletion mutants were incubated with $0.5 \mu M$ cAMP and the indicated PDE inhibitors for 5 min at $30^\circ C$. Results are means $\pm$ S.E. from assays carried out in triplicate.

Inhibitor	Inhibition of cAMP hydrolysis (%)		
	cGI-PDE Δ 1	cGI-PDE Δ 2	Platelet cGI-PDE
Milrinone ($10 \mu M$)	84 ± 1	82 ± 1	86 ± 1
Zaprinast ($10 \mu M$)	1 ± 6	6 ± 5	0 ± 3
EHNA ($20 \mu M$)	-4 ± 6	-2 ± 5	6 ± 3
Rolipram ($10 \mu M$)	-5 ± 5	-1 ± 6	5 ± 6

Figure 4 Phosphodiesterase activities of expressed cGI-PDE Δ 1 and cGI-PDE Δ 1 Δ i

The mutant enzymes were immunoprecipitated from bacterial extracts, as described in the Experimental section and assayed for PDE activities. (a) Quantification of immunoprecipitated enzymes by immunoblotting. Lanes 1–3, 25, 50 and $100 \mu l$ of cGI-PDE Δ 1; lane 4, prestained 80 kDa marker; lanes 5–7, 25, 50 and $100 \mu l$ of cGI-PDE Δ 1 Δ i. (b) cAMP PDE activities. Immunoprecipitated enzymes ($50 \mu l$ aliquots) were incubated with $0.5 \mu M$ cAMP for 5 min at $30^\circ C$. (c) cGMP PDE activities. Immunoprecipitated enzymes ($50 \mu l$ aliquots) were incubated with $0.5 \mu M$ cGMP for 10 min at $30^\circ C$. The results are the means $\pm$ S.E. from triplicate assays.

although the expressed N-terminal deletion mutants retained the ability to bind cGMP, removal of the 44 amino-acid-insert from the catalytic domain greatly reduced cGMP binding. Thus cGMP binds to the 62 kDa C-terminal part of PDE3 and, for structural or other reasons, the 44-amino-acid insert facilitates this interaction.

The effects of PDE3 inhibitors on the photolabelling of cGI-PDE Δ 1 and cGI-PDE Δ 2 were studied. Lixazinone, milrinone and cGMP, all of which inhibit the activity of intact cGI-PDE, suppressed photolabelling of these deletion mutants by [^{32}P]cGMP (Figure 3b). These results confirm that all three compounds interact with the C-terminal part of the enzyme containing the catalytic domain.

Comparison of the enzymic properties of cGI-PDE deletion mutants with that of the intact platelet enzyme

Nickel-resin-purified cGI-PDE Δ 1 was assayed for enzyme activity. Kinetic analysis indicated that the K_m values for cAMP and cGMP hydrolysis were 153 nM and 175 nM respectively, and that the ratio of V_{max} for cGMP/ V_{max} for cAMP was 0.36 . On the basis of the ratios of V_{max}/K_m , the inhibition of cGI-PDE Δ 1 by lixazinone was mixed, with approximately equal competitive and uncompetitive components. Although good kinetic plots were obtained, the enzyme activity of the nickel-resin-purified preparation was unstable and was lost within 2–3 days when the enzyme was stored at $4^\circ C$. The sensitivity of the nickel-resin to strong reducing agents, chelating agents and ionic detergents restricted the steps that could be taken to stabilize the enzyme during purification, and dialysis to remove the high concentration of imidazole used to elute the enzyme may have increased the likelihood of inactivation. For these reasons, this method was not routinely used and measurement of enzyme activity in immunoprecipitates was adopted for comparison of the expressed mutant enzymes with the platelet enzyme. The immunoprecipitation procedure provided a more rapid means of enzyme purification that minimized the risk of proteolysis.

The kinetic properties of the immunoprecipitated cGI-PDE Δ 1 mutant were very similar to those of the nickel-resin-purified enzyme. The K_m values for hydrolysis of cAMP and cGMP were $182 \pm 12 \text{ nM}$ and $153 \pm 12 \text{ nM}$ respectively, whereas, the ratio of V_{max} for cGMP/ V_{max} for cAMP was 0.37 ± 0.05 (Table 1). Since the latter value was very different from published results with the intact cGI-PDE [23], we directly compared the K_m values and V_{max} ratios of our expressed truncation mutants with that of the immunoprecipitated intact platelet enzyme (Table 1). A trend towards progressively lower K_m values accompanied the deletion of N-terminal sequences from cGI-PDE. Although both cGI-PDE Δ 1 and cGI-PDE Δ 2 showed significantly lower K_m values for cAMP and cGMP than intact enzyme, only the K_m of cGI-PDE Δ 2 for cGMP was significantly lower than that of cGI-PDE Δ 1 ($P < 0.05$, Student's *t* test). Moreover, successive deletions of the N-terminus led to an increase in the ratio V_{max} for cGMP/ V_{max} for cAMP from 0.16 ± 0.01 in intact cGI-PDE, to 0.37 ± 0.05 in cGI-PDE Δ 1 and to 0.49 ± 0.04 in cGI-PDE Δ 2. Thus our results indicate that loss of the N-terminus enhanced the hydrolysis of cGMP relative to that of cAMP, implying that N-terminal sequences may exert a selective effect on the activity of the catalytic domain.

The effects of PDE3 inhibitors on the immunoprecipitated enzymes were studied. Lixazinone showed the same pattern of mixed inhibition of immunoprecipitated cGI-PDE Δ 1 as it did with the nickel-resin-purified enzyme (not shown). However, in the case of the immunoprecipitated platelet enzyme, inhibition

Figure 5 Site-directed mutagenesis of the 44-amino-acid insert within the catalytic domain of cGI-PDE Δ 1

(a) The 44 amino acid inserts from rat adipocyte PDE3B (RA cGI-PDE) and human cardiac PDE3A (HC cGI-PDE) were aligned to identify conserved amino acid residues. Selected residues were replaced with alanine by site-directed mutagenesis of cGI-PDE Δ 1. The mutants shown were cloned and expressed in *E. coli* (see Experimental section). (b) Bacterial supernatants containing expressed mutants of cGI-PDE Δ 1 were photolabelled with [32 P]cGMP and the protein was analysed by SDS/PAGE. An autoradiograph is shown. (c) Immunoprecipitates from the same supernatants were analysed by immunoblotting. The supernatants used in (b) and (c) were from the following sources: lane 1, *E. coli* transformed with vector alone; lane 2, *E. coli* expressing cGI-PDE Δ 1; lanes 3–7, *E. coli* expressing the following cGI-PDE Δ 1 insert mutants: 3, D17A; 4, D17A D19A; 5, P1A G2A; 6, G25A; 7, Y42A G43A. Mutated residues are numbered according to their positions in the 44 amino acid insert (see a).

by lixazinone could be characterized as competitive or predominantly competitive. This kinetic profile was distinct from that of the expressed cGI-PDE Δ 1. Measurements of IC_{50} values for inhibition of cAMP hydrolysis by lixazinone and milrinone (Table 1) indicated three-fold increases for the deletion mutants cGI-PDE Δ 1 and cGI-PDE Δ 2, relative to the intact platelet enzyme. This must largely reflect decreases in the K_m for cAMP rather than reduced affinities for the inhibitors. Comparison of the effects of selective inhibitors of PDEs 2, 3, 4 and 5 indicated that the specificity for PDE3 inhibitors resides in the expressed C-terminal fragment of the enzyme and is not significantly affected by removal of the N-terminus (Table 2).

Immunoprecipitates of cGI-PDE Δ 1 and cGI-PDE Δ 1 Δ i containing similar amounts of immunoreactive protein (Figure 4a) were compared with respect to their abilities to hydrolyse cAMP and cGMP (Figures 4b and 4c). Despite its limited capacity for binding [32 P]cGMP, no enzyme activity was detected in cGI-PDE Δ 1 Δ i.

Site-directed mutagenesis of the 44-amino-acid insert in cGI-PDE Δ 1

Alignment of the 44-amino-acid inserts of human cardiac PDE3A and rat adipocyte PDE3B showed that 15 amino acid residues

Figure 6 Enzymic properties of site-directed mutants of cGI-PDE Δ 1

The expressed mutant enzymes were immunoprecipitated from bacterial extracts. (a) The enzyme activities of the immunoprecipitates were determined in 5 min incubations at 30 °C with 0.5 μ M cAMP (solid bars) and 0.5 μ M cGMP (striped bars). The amounts of the different immunoprecipitated enzymes assayed were compared by immunoblotting (see insert). (b) Inhibition of cAMP hydrolysis by 0.1 μ M lixazinone was determined in 5 min incubations at 30 °C with 0.5 μ M cAMP. All results are the means $\pm$ S.E. of three identical assays.

were conserved (Figure 5a). Analysis of the sequences of PDE3A and PDE3B according to Garnier et al. [24] was used to identify conserved regions with potential structural roles. A probable β -turn was located at the C-terminus of the insert (YGC) and a possible β -turn at the N-terminus (PGL). Two residues in each of these sequences were replaced with alanine residues (Figure 5a). A region containing a cluster of negative charges (SDSDS), as well as a conserved downstream glycine residue were also mutated (Figure 5a). Photolabelling of these mutants with [32 P]cGMP was unaffected in four of the five examined (Figure 5b). In these experiments, Western blot analysis of the immunoprecipitates permitted correlation of the extent of photolabelling with the amount of expressed protein used in each sample (Figure 5c). Despite the presence of sufficient amounts of expressed protein, the P1A G2A mutant remained very poorly photolabelled relative to the cGI-PDE Δ 1 control (Figure 5b, compare lanes 2 and 5). Cyclic nucleotide hydrolytic activities of immunoprecipitates of these mutants were compared with those of immunoprecipitates of cGI-PDE Δ 1 (Figure 6). The results indicated that both cAMP and cGMP hydrolysis by the P1A G2A and Y42A G43A mutants were markedly reduced relative to cGI-PDE Δ 1 and the other mutants (Figure 6a). The ratio of cAMP to cGMP hydrolysis, at 0.5 μ M substrate concentrations, was unchanged by any of these alanine substitutions. Moreover, the inhibitory effect of 0.1 μ M lixazinone was very similar for all the mutant enzymes examined and was not diminished in the P1A G2A and Y42A G43A mutants despite their lower enzyme activities (Figure 6). These results suggest that the 44-amino-acid insert may not be critical for inhibition of the enzyme.

DISCUSSION

Purified platelet mRNA and degenerate PCR primers, based on published platelet peptide sequences [16], were used to clone a fragment of platelet PDE3. The nucleotide sequence of this partial clone was identical with a segment of the previously cloned human cardiac enzyme [16], indicating that both platelet and cardiac forms of PDE3 were derived from the same gene (PDE3A). Despite this common origin, fundamental differences exist between the platelet and cardiac enzymes. Platelet PDE3 is cytosolic with a subunit molecular mass of about 110 kDa [7,21], whereas the cloned cardiac enzyme is larger (126 kDa) and appears to be predominantly membrane-associated [25]. Moreover, the PDE3 inhibitor, vesnarinone, has been reported to distinguish between the PDE3 enzymes from human heart and platelets, preferentially inhibiting the former [26]. To date, two PDE3 genes have been identified [16,17] and shown to be expressed in a tissue-specific manner. As defined in the latest nomenclature [11], the PDE3A gene is expressed in vascular smooth muscle, platelets and cardiac muscle, whereas PDE3B is found predominantly in adipose and liver cells. Recently, a 74 kDa soluble human placental PDE was cloned [27] and found to be identical in sequence with the C-terminus of the human cardiac enzyme. Northern blots and RNase protection assays identified two transcripts (4.4 kb and 7.6 kb) in human placenta. The smaller transcript was generated from an alternate transcriptional start site and encoded the 74 kDa cytosolic enzyme, whereas the larger 7.6 kb transcript coded for a membrane-associated enzyme. A comparable relationship between the soluble platelet and particulate cardiac enzymes may exist, given that two transcripts have also been detected in human erythroleukaemia (HEL) cells, a megakaryocytic human cell line that expresses many platelet proteins [28]. Thus the presence of an additional transcriptional start site or alternate splicing at the N-terminus of the PDE3A gene could account for the differences observed between the platelet and cardiac isoforms of PDE3. Sequences corresponding to the N-terminus of the cloned membrane-bound cardiac enzyme have very recently been amplified from platelet mRNA [28], but this does not resolve the identity of the N-terminus of the smaller, soluble platelet enzyme.

We have cloned and expressed the C-terminal half of platelet PDE3A (cGI-PDE Δ 1) in the *E. coli* strain M15. Since the N-terminus of the platelet cGI-PDE remains to be defined and is unlikely to be identical with that of the cloned cardiac enzyme, N-terminal truncation mutants of platelet cGI-PDE were compared with the full-length native platelet enzyme. To facilitate rapid isolation of the undegraded enzymes, immunoprecipitates were isolated and compared with respect to enzyme kinetics and inhibitor sensitivity. Our results demonstrate that K_m values for the hydrolysis of cAMP and cGMP were significantly lower for the N-terminal deletion mutants than for the intact enzyme. Other groups have also reported on the effects of N-terminal deletions in the PDE3A gene. Pillai et al. [29] demonstrated that truncated forms of the cardiac enzyme expressed in yeast hydrolysed cAMP with K_m values higher than that of the expressed intact cardiac enzyme. Kasuya et al. [27] expressed the 74 kDa placental cGI-PDE in a baculovirus system and reported that, although the K_m value for cAMP was unchanged, that for cGMP was increased to $3.27 \pm 1.75 \mu\text{M}$. However, in a very recent study of enzymes expressed in Sf9 cells [30], kinetic analysis of two N-terminal deletion mutants of cardiac PDE3 did not demonstrate any significant difference in the K_m for cAMP for these mutants, relative to the intact expressed cardiac enzyme (0.15–0.22 μM). There are several possible explanations for the differences between our kinetic results and those reported by

others. First, our results were derived from enzymes purified by immunoprecipitation or nickel-resin chromatography and were not assayed in crude homogenates. Thus it is possible that extracts of yeast or Sf9 cells contain proteins or enzymes capable of modifying the activity of the expressed cGI-PDEs. In addition, the deletion mutants used in our studies are 27 to 77 residues shorter than those discussed above [29,30]. Finally, differences between investigators in terms of the PDE assay conditions used (Mg^{2+} versus Mn^{2+}) and the protein expression systems used (yeast versus baculovirus versus *E. coli*) may have contributed to differences in the kinetic results.

Shortly after completion of the present study, Cheung et al. [28] reported the isolation of cDNA clones expressing PDE3 sequences from a HEL cell library. The clones were 100% identical at the nucleotide level with the cardiac enzyme. These investigators contend that N-terminal truncated forms of these clones expressed enzymes with K_m values that were virtually identical with that of the purified platelet enzyme. Since it is well established that purification of platelet PDE3 results in the isolation of proteolysed forms [6,23], it is quite likely that the reported K_m values for 'intact' enzyme are representative of PDE3 fragments. Indeed, the K_m value of 0.2 μM [28] is consistent with our value of 0.18 μM for the truncated enzyme. As previously demonstrated [21], the immunoprecipitation procedure utilized in the present study permits the isolation of non-proteolysed platelet PDE3 enzyme, which can then be directly compared with various truncation mutants.

The K_m values obtained for hydrolysis of cAMP and cGMP by immunoprecipitates of intact platelet enzyme were $398 \pm 50 \text{ nM}$ and $252 \pm 16 \text{ nM}$ respectively. The respective values given in the literature for these two parameters using the platelet enzyme are quite variable, for example: 0.18 μM and 0.02 μM [6], 0.20 μM and 0.30 μM [23] and 1.0 μM for cAMP [31]. As discussed above, it is likely that some of these published values represent the enzyme activities of fragments rather than intact cGI-PDE. PDE3 is known not only to have a high affinity for cGMP, but also a low ratio of the V_{max} for cGMP to that for cAMP [11]. We therefore compared the cGMP hydrolytic abilities of our N-terminal deletion mutants with those of the intact platelet enzyme. Our results indicated that as N-terminal sequences were deleted, a progressively lower K_m for cGMP was generated and a concomitant three-fold increase in the ratio of V_{max} for cGMP/ V_{max} for cAMP was detected. Thus deletion of sequences from the N-terminus of platelet PDE3 increased the activity of the enzyme towards cGMP relative to cAMP. This implies that directly or indirectly, the N-terminal sequences in part determine the substrate specificity of the enzyme.

A distinguishing feature of the PDE3 gene family is the presence of a 44-amino-acid insertion located within the catalytic domain. The function of this insert, which bears no relationship to other sequences currently in the protein databases, is unknown, though it initially seemed reasonable to speculate that it had a role in the unique properties of the PDE3 family. Although removal of this insert destroyed enzyme activity, a limited ability to bind cGMP, as detected by photolabelling, was retained. Structural analysis of the 44-amino-acid inserts of human cardiac and rat adipocyte enzymes [24] identified a high potential for a β -turn at the C-terminus of the inserts and a possible β -turn at the N-terminus. Site-directed mutagenesis of pairs of amino acids in either of these putative β -turns markedly decreased enzyme activity, though cGMP binding, as determined by photoaffinity labelling, was inhibited only by mutagenesis of the N-terminal insert residues. Mutagenesis of a conserved cluster of negatively charged residues in the centre of the insert was without effect. Although our results did not identify any specific functional role

for amino acid residues in the insert, though it seems clear that the insert is required to preserve an effective catalytic domain structure.

The effects of the PDE3 inhibitor lixazinone, on the cAMP hydrolytic activities of intact platelet cGI-PDE and the N-terminal truncation mutants, were studied. Our results demonstrated that lixazinone increased the K_m of the intact platelet enzyme, but left the V_{max} largely unaffected, indicating that it is a competitive inhibitor. In contrast, mixed inhibition with roughly equal competitive and uncompetitive components was seen with the cGI-PDE Δ 1 deletion mutant. The previously reported inhibitory effect of lixazinone on platelet cGI-PDE purified by DEAE-cellulose chromatography [32] also indicated a mixed pattern of inhibition, similar to that obtained for the N-terminal deletion mutant, cGI-PDE Δ 1. Since purification of PDE3 by this method is known to generate proteolytic fragments, it is possible that the inhibitory profile reported in [32] is representative of a PDE3 fragment rather than the intact platelet enzyme. The high inhibitory potency of lixazinone is due to the molecular interaction between the compound and a bulk-tolerant, lipophilic region of the enzyme [33]. Since inhibition by this compound changes from predominantly competitive, for the intact enzyme, to mixed in the deletion mutant, it is possible that removal of the N-terminus provides the inhibitor with access to an alternate binding site.

Regardless of the mechanism, it is clear that the N-terminal deletion mutants retain the capacity of the intact enzyme to respond selectively to PDE3 inhibitors. Moreover, no changes in inhibition of the enzyme by lixazinone were seen in site-directed mutants of the 44-amino-acid insert. Thus the characteristic inhibitor profile of PDE3 probably reflects the structure of the catalytic domain, though C-terminal sequences, which were not deleted in this study, could also be involved.

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