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Characterization of the Structure of a Low K_m, Rolipram-sensitive cAMP Phosphodiesterase

MAPPING OF THE CATALYTIC DOMAIN*

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Considerable structural similarities are present in a region of approximately 270 amino acids in most known cyclic nucleotide phosphodiesterase (PDE) sequences, opening the possibility that this region encodes the catalytic domain of the enzyme. To test this hypothesis, the structure of a high affinity cAMP PDE (cAMP-PDE) was analyzed by deletion mutations and site-directed mutagenesis. A ratPDE3 cDNA was mutated using a strategy based on fragment amplification by polymerase chain reaction. The effect of the introduced mutations was determined by expressing wild type and mutated proteins in prokaryotic and eukaryotic cells. The level of expression of the PDE protein was monitored by immunoblot analysis using two specific cAMP-PDE polyclonal antibodies and by measuring the PDE activity. After removal of a 99-amino acid region at the carboxyl terminus flanking the conserved domain, the protein retains its catalytic activity even though its K_m and velocity were changed. Internal deletions at the amino terminus of this PDE showed that the enzyme activity was increased when a 97-amino acid fragment (from Tyr^{49} to Lys^{145}) was removed. Further deletions within the amino terminus produced inactive proteins. Within the domain that appears essential for catalysis, 1 threonine and 2 serine residues are conserved in all PDEs. Substitutions of the invariant threonine (Thr^{349}) present in the most conserved region with alanine, proline, or serine yielded proteins of the correct size and a level of expression comparable to the wild type PDE. However, in both expression systems used, proteins were completely devoid of the ability to hydrolyze cyclic nucleotides, except when the threonine was substituted with a serine. Conversely, mutations of 2 other conserved serine residues (Ser^{305} and Ser^{398}) present in the catalytic domain either had no effect or produced changes only in K_m and V_{max}, but did not abolish catalytic activity. In addition, 2 histidine residues (His^{278} and His^{311}) present in proximity to Thr^{349} appeared to be essential for the structure of the catalytic domain, since any substitution performed in these residues yielded an inactive enzyme. Mutations of a serine residue (Ser^{295}) in the region homologous to the cAMP binding site of the regulatory subunit of the cAMP-dependent protein kinase demonstrated that this region does not have the same function in the two proteins. These data provide direct evidence that a 37-kDa domain, which in part corresponds to the region of conservation in all PDEs, contains the catalytic domain, and that threonine and histidine residues are probably involved in catalysis and/or are essential for the conformation of an active enzyme.

Intracellular inactivation of the second messengers cAMP and cGMP is dependent on cleavage of the phosphodiester bond of the cyclic phosphate ring by specific cyclic nucleotide phosphodiesterases (PDEs).[1] These enzymes constitute a complex family of forms that can be distinguished on the basis of their catalytic properties and regulation (1, 2). The recent acquisition of the primary sequences of a number of PDEs has provided new insight into the structure of these enzymes. Knowledge of the structure of the catalytic and allosteric sites is needed especially in view of the fact that these enzymes are targets for a large number of drugs. Moreover, pharmacological modulation of the PDE activity is a primary tool for manipulating intracellular cyclic nucleotide levels.

With the exception of the *Dyctiostelium discoideum* PDE (3) and the *Saccharomyces cerevisiae* PDE1 (4), all other PDEs possess a region of approximately 270 amino acids in which homology is present. Sequence alignment of this conserved region of these proteins reveals that 45 residues (61 if the yeast low K_m PDE (5) is not considered due to its smaller conserved region) are identical or chemically conserved among 15 different sequences available (6, 7). This observation suggests that the 270-residue region of the enzyme has a common function, possibly that of catalysis. Controlled proteolysis studies on the calmodulin-dependent PDE (CaM-PDE[1]) (8, 9) and the cGMP-stimulated PDE (cGS-PDE[1]) (10–12) have revealed that the functional domain of these proteins can be separated from regulatory domains. In both cases limited

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[1] The abbreviations used are: PDE, cyclic nucleotide phosphodiesterase; cAMP-PDE, cAMP-specific phosphodiesterase; CaM-PDE, calmodulin-dependent phosphodiesterase; cGS-PDE, cGMP-stimulated phosphodiesterase; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RO 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; rolipram, 4-{3-(cyclopentyloxy)-4-methoxyphenyl}-2-pyrrolidone; milrinone, 1,6-dihydro-2-methyl-6-oxo-(3,4′-bipyridine)-5-carbonitrile; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s); HPLC, high-performance liquid chromatography; aa, amino acid.

proteolysis produces 30–40-kDa fragments that are catalytically active and are no longer activated by calmodulin and cGMP, respectively. Furthermore, these fragments are known to include the conserved region of PDEs. The presence of separate regulatory and catalytic domains, however, has not been explored for the cAMP-specific PDE (cAMP-PDE[1]), even though this group of PDEs has been shown to be regulated by hormones and neurotransmitters (see Ref. 13 for review).

Within the conserved region, the low K_m, cAMP-specific PDEs including the *Drosophila melanogaster* dunce PDE (14) and the four rat cAMP-PDEs (15) contain a short sequence (ELALMYN) also present in the RII_a regulatory subunit of cAMP-dependent protein kinase. It is intriguing that this sequence in the RII_a subunit is thought to be involved in cAMP binding by forming a hydrogen bond between the conserved glutamate residue (E) and the 2′-hydroxyl group of the ribose ring of cAMP (16). Cyclic AMP binding is abolished when the enzyme conformation is changed following the substitution of the conserved glycine, the residue adjacent to the glutamate, with glutamic or aspartic acid residue (17–19). Whether this homology in the sequence of the cAMP-PDEs has a similar role is not known. Here we report experiments examining possible implications of this 7-amino acid sequence homology in cAMP-PDEs.

Sequence analyses also have shown that out of the 45 conserved residues 5 positions are occupied by histidine (6, 7), suggesting the importance of these residues in the enzyme function. Epstein *et al.* (20) have investigated the kinetic properties of a cAMP-PDE from dog kidney and reported data consistent with the presence of a histidine and a sulfhydryl group in the active site of the enzyme. Studies on substrate specificity of the beef heart cAMP-PDE have led to the conclusion that hydrolysis of cAMP probably takes place by two nucleophilic substitution reactions initiated by a nucleophilic group of the enzyme (21). This mechanism recently has been agreed upon by Van Lookeren Campagne *et al.* (22) who studied the yeast low K_m cAMP-PDE. Thus, amino acid residues that are conserved in all known PDEs and also possess a nucleophilic group such as serine, threonine, and histidine are thought to be directly involved in catalysis. Furthermore, studies on the bovine intestinal 5′-nucleotide phosphodiesterase have shown that a threonine residue is the active site of the enzyme. This residue forms a covalent phosphothreonine intermediate in the catalytic mechanism (23).

The purpose of this study was to test the following hypotheses: 1) to determine if the conserved domain corresponds to the catalytic domain of the cAMP-PDE; 2) to test if the region of homology with the RII_a regulatory subunit behaves as in the cAMP-dependent protein kinase; 3) to examine which residue(s) among the conserved threonine, serine, and histidine are important for the enzyme activity. For this purpose deletion and site-directed mutations of a low K_m cAMP-specific PDE, ratPDE3 (24), were performed using the polymerase chain reaction technique. The rationale for choosing ratPDE3 protein for this study is that the corresponding cDNA contains the entire open reading frame which encodes a cAMP-PDE of 584 amino acids (24). This protein has been successfully expressed in prokaryotic and eukaryotic cells, and this is the form that we have characterized extensively and found to be indistinguishable from the cAMP-PDE endogenous in the Sertoli cell (24, 25).

EXPERIMENTAL PROCEDURES

Materials—Reagents were purchased from the following companies: Taq DNA polymerase, Perkin-Elmer/Cetus, Norwalk, CT; dNTP and the large fragment of *Escherichia coli* polymerase I, Bethesda Research Laboratories (BRL); T4 DNA ligase, New England Biolabs; *Crotalus atrox* snake venom, lysozyme, and cAMP, Sigma; RO 20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone), Hoffmann-LaRoche; rolipram (4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidone), Berlex, NJ; [2,8-^{3}H]cAMP (20–50 Ci/mmol), ^{125}I-protein A (2–10 μCi/μg), and [^{35}S]dATP (1000 Ci/mmol), Du Pont-New England Nuclear; Immobilon, Millipore; pZ523 column, 5prime-3prime Inc.; Pansorbin cells, Calbiochem Corp.; AG 1-X8 resin, Bio-Rad. Restriction enzymes were from either Bethesda Research Laboratories or United States Biochemical Corp. Milrinone (1,6-dihydro-2-methyl-6-oxo-(3,4′-bipyridine)-5-carbonitrile) was provided by Glaxo Inc., Research Triangle Park, NC.

PCR Primers—For the sake of simplicity, the oligonucleotides used for PCR amplification are identified by letters as listed below. The positions of the oligonucleotides used for deletion mutation are reported in Fig. 1 together with the complete nucleotide and deduced amino acid sequences of ratPDE3.

A = 5′ GACGGAATTCAATATGACTCTAGAAGACCATTA 3′; where the *Eco*RI site, the translation initiation sequence, and the *Xba*I site are *underlined* in order. The 21 nucleotides at the 3′ end including the ATG codon and the *Xba*I site correspond to the ratPDE3-specific sequence from nucleotide 822 to nucleotide 842 (see Fig. 1). The *Eco*RI site was added to correspond to the *Eco*RI site present in the expression vector, and to allow the creation of the deletion from the 5′ end to base 822 of ratPDE3 cDNA (see Fig. 2). The native ATG codon preceding the *Xba*I site was used to initiate the translation of the amino-terminal truncated proteins thus generated. For the purpose of prokaryotic expression, the sequence AAT located between the *Eco*RI site and the ATG codon was added to correspond to the sequence in the wild type construct (detailed below).

B = 5′ AGGCAAGCTTACTGGTACCACTCACGATTGT 3′ ; where the *Hin*dIII site and the *Kpn*I site are *underlined* in order. The 20 nucleotides at the 3′ end including the *Kpn*I site correspond to the antisense sequence of the ratPDE3 from nucleotide 1605 to nucleotide 1586 (see Fig. 1). The *Hin*dIII site was added to correspond to the *Hin*dIII site in the expression vector and to allow the generation of the deletion from the 3′ end to the *Kpn*I site of ratPDE3 cDNA (see Fig. 2). For these carboxyl-terminal truncated proteins, the introduced stop codon (TAA), which overlaps the *Hin*dIII site, was used to terminate the translation.

C = 5′ ACGAGGCCTTGATGCACAGCTCCAGC 3′; where the *Stu*I site is *underlined* and is followed by the ratPDE3-specific sequence from nucleotide 587 to 603.

D = 5′ CCAGCAGATTCATGTGC 3′, which corresponds to the antisense sequence of the ratPDE3 cDNA from nucleotide 1225 to 1209.

E = 5′ ACGAGGCCTGGGGCCTCCACGTTTTCC 3′; where the *Stu*I site is *underlined* and is followed by the ratPDE3-specific sequence from nucleotide 689 to 706.

F = 5′ ACGAGGCCTTTAAAATCCCAGTGGACAC 3′; where the *Stu*I site is *underlined* and is followed by the ratPDE3-specific sequence from nucleotide 788 to 806.

Oligonucleotides A through F were used to create deletion mutations of the ratPDE3 cDNA using PCR technique. PCR fragments of 807, 648, 546, and 838 bp were amplified by using paired primers, A and B, C and D, E and D, and F and B, respectively, in which primers C, E, and F were designed to contain a *Stu*I site at their 5′ ends in order to generate internal deletions (see Figs. 2 and 3).

The following primers were synthesized to produce site-directed mutations of the ratPDE3.

G = 5′ GAGAATCAT(A/G/T)ATTTGGCTGT 3′; where the single mutated base is *underlined*. These three mixed oligonucleotides correspond to the ratPDE3 cDNA from nucleotide 1072 to 1091.

H = 5′ ACAGCCAAAT(A/C/T)ATGATTCTC 3′; where the mutated base is *underlined*. These oligomers correspond to the antisense sequences of oligomers G.

Oligomers A, B, G, and H were used as primers in PCRs in order to obtain mutations of His^{311} to Asn, Asp, and Tyr (His^{311}/Asn, His^{311}/Asp, and His^{311}/Tyr).

I = 5′ GTACTAGCG(T/G/C)CAGACATGTC 3′; where the mutated base is *underlined*. These oligomers correspond to the ratPDE3 cDNA sequence from base 1186 to 1205.

J = 5′ GACATGTCTG(G/C/A)CGCTAGTAC 3′; where the mutated base is *underlined*. These oligomers correspond to the antisense sequences of oligomers I.

The use of oligomers A, B, I, and J in PCRs was able to generate

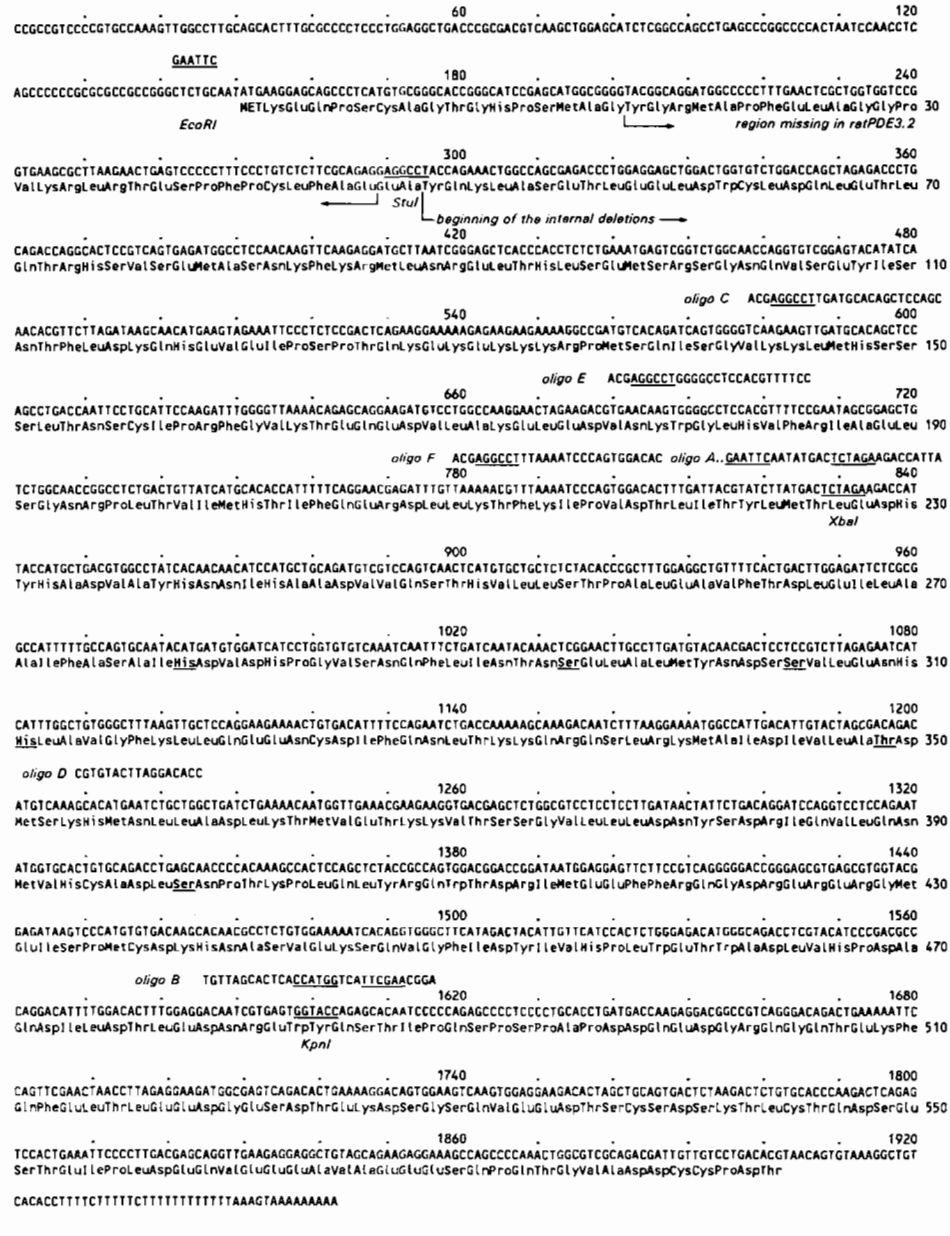

FIG. 1. **Nucleotide and deduced amino acid sequences of ratPDE3 cDNA.** Nucleotide numbers are reported above the sequence. Deduced amino acid residue numbers are reported to the right of the amino acid sequence. Nucleotides *underlined* in the sequence correspond to restriction sites used for the various constructs detailed under "Experimental Procedures." The name of the corresponding restriction enzyme is reported below the sequence. The sequences of the oligonucleotides used to construct deleted proteins (see "Experimental Procedures" for details) are reported above the cDNA sequence. Restriction sites present in the oligonucleotides are *underlined*. The *arrow* next to the *Stu*I restriction site marks the beginning of the internal deletion. Amino acid residues *underlined* are targets for site-directed mutagenesis. *Small arrows* mark sequence missing in the ratPDE3.2 cDNA. A nucleotide sequence corresponding to an *Eco*RI restriction site is reported above the cDNA between bases 142 and 147. This sequence was introduced in the native ratPDE3 cDNA by PCR mutagenesis. Digestion of the mutated cDNA with *Eco*RI causes the removal of 147 bases of the 5′ untranslated sequence.

mutations of Thr^{349} to Ser, Ala, and Pro (Thr^{349}/Ser, Thr^{349}/Ala, and Thr^{349}/Pro).

K = 5′ CAACGACTCC(A/C)CCGTCTTAGAG 3′; where the mutated base was *underlined*. These two oligomers correspond to the ratPDE3 cDNA sequence from base 1053 to 1074.

L = 5′ CTCTAAGACGG(G/T)GGAGTCGTTG 3′; where the mutated base is *underlined*. These oligomers correspond to the antisense sequences of oligomers K.

Oligomers A, B, K, and L were employed in PCRs in order to produce mutations of Ser^{305} to Thr and Pro (Ser^{305}/Thr and Ser^{305}/Pro).

M = 5′ AATACAAACG(A/G)GGAACTTGC 3′; where the two mutated bases are *underlined*. These oligomers correspond to the ratPDE3 cDNA sequence from base 1024 to 1043.

N = 5′ GCAAGTTCC(C/T)CGTTTGTATT 3′; where the two mutated bases are *underlined*. These oligomers correspond to the antisense sequences of oligomers M.

Oligomers A, B, M, and N were used to obtain mutations of Ser^{295} to Gly and Glu (Ser^{295}/Gly and Ser^{295}/Glu).

O = 5′ CCACATCAGCTATTGCACTG 3′; where the two mutated bases are *underlined*. This oligomer corresponds to the antisense sequence of the ratPDE3 cDNA from base 991 to 972.

Using oligomers A and O in a PCR, a 183-bp fragment was amplified, in which the His^{278} codon was mutated to encode alanine (His^{278}/Ala). This PCR fragment (also called megaprimer (26)) and oligomer B were then employed as primers in the second round of PCR to produce a fragment that contained the base substitution.

P = 5′ GCAGACCTGGCCAACCCCAC 3′; where the two mutated bases are *underlined*. This oligomer corresponds to the ratPDE3 cDNA sequence from base 1333 to 1352.

As oligomer O, oligomer P also was designed to generate a megaprimer. In the presence of oligomers B and P, a PCR fragment of 284 bp was amplified, in which the Ser^{398} codon was substituted with an Ala codon (Ser^{398}/Ala).

PCR Amplification and Product Purification—PCR was performed using Taq polymerase and a DNA thermal cycler (Perkin-Elmer/Cetus). Amplification was achieved using conditions provided by Perkin-Elmer/Cetus. Briefly, in a final volume of 100 μl, the reaction mixture contained 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM each of dNTP, 1 μM of each primer, 5–10 ng of template (ratPDE3 cDNA for both deletion and site-directed mutations), and 2.5 units of Taq polymerase. Reactants were overlaid with 50 μl of mineral oil and subjected to 10–15 cycles of denaturation (1 min, 94 °C), annealing (2 min, 55 °C), and extension (3 min, 72 °C). The PCR products were electrophoresed on a 1% agarose gel (1.5% for the products shorter than 300 bp), followed by staining with ethidium bromide and visualizing with UV light. DNA bands of correct sizes were cut out and removed from the gel by electroelution (unidirectional electroelutor, International Biotechnologies Inc., New Haven, CT).

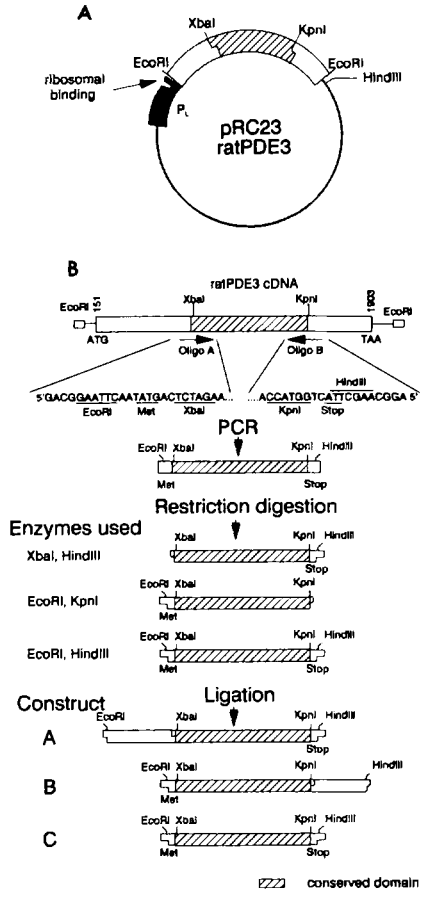

FIG. 2. **Schematic representation of the strategy used for generation of terminal deletions of ratPDE3.** *A*, structure of prokaryotic expression vector, pRC23, bearing the ratPDE3 cDNA inserted at the *Eco*RI site. The ratPDE3 cDNA has been modified to shorten its 5′-untranslated sequence as described under "Experimental Procedures." *B*, to determine if the conserved domain (*hatched bar*) corresponds to the catalytic domain of ratPDE3, mutants with deletions of 5′ and/or 3′ domain were generated using PCR technique. A pair of oligonucleotide primers (oligoA and oligoB) were designed to produce a PCR fragment of 807 bp using the ratPDE3 cDNA as template. The complete sequences of oligoA and oligoB are reported under "Experimental Procedures" and in Fig. 1. The PCR product contains the *Eco*RI site, translation initiation codon (ATG), and *Xba*I site at its 5′ end and the *Kpn*I site, stop codon (TAA), and *Hin*dIII site at the 3′ end. The PCR fragment was digested with *Xba*I and *Hin*dIII enzymes (for construct A) or *Eco*RI and *Hin*dIII enzymes (for construct C), and then inserted into the expression vector, pRC23-ratPDE3, to replace the corresponding wild type fragment. To prepare construct B, the PCR fragment was digested with *Eco*RI and *Kpn*I enzymes. A *Kpn*I-*Hin*dIII fragment (approximately 390 bp) encoding the carboxyl terminus of the protein was also excised from the wild type pRC23-ratPDE3 and was purified from an agarose gel. These two restriction fragments were then ligated to the vector that has been digested with *Eco*RI and *Hin*dIII followed by dephosphorylation. The assembly of this construct was verified by restriction mapping and DNA sequencing of the ligation sites. Plasmids thus constructed have deletions of ratPDE3 cDNA at the 3′ end (construct A, corresponding to carboxyl-terminal 99-amino acid deletion), at the 5′ end (construct B, corresponding to amino-terminal 224 amino acid deletion), or at both 5′ and 3′ ends (construct C).

To create deletion mutations of the ratPDE3 cDNA, paired primers (oligomers A and B; C and D; E and D; and F and B) were used in PCR (Figs. 1 and 3). For site-directed mutations, either three primers (megaprimer method, (26)) or four primers (overlap method, (27)) and two rounds of PCR were employed. Details of this PCR-based mutagenesis strategy have been described elsewhere (26, 27).

Prokaryotic Expression Vector and Mutation Constructs—To express wild-type and mutated ratPDE3 in bacteria, the prokaryotic expression vector pRC23 (28) was used. The plasmid pRC23 contains a synthetic Shine-Dalgarno ribosomal binding sequence, which is located downstream from the tightly regulated phage P_L promoter (Fig. 2*A*). The SD sequence is immediately followed by the *Eco*RI site where the ratPDE3 cDNA has been inserted (Fig. 2*A*). Since the distance between the ribosomal binding site and the AUG codon is critical for transcription efficiency (29), the ratPDE3 cDNA has been modified by removing the untranslated sequence of 147 bases from the 5′ end of the cDNA using PCR technique (detailed in Ref. 25). The resulting 1.8-kb ratPDE3 cDNA encoding the wild-type ratPDE3 enzyme has its AUG codon only 3 bases (AAT, from base 148 to 150) 3′ to the *Eco*RI site (see Fig. 1). This ratPDE3-bearing plasmid, designated pRC23-ratPDE3 (Fig. 2*A*), was used in the present study to create the mutation constructs.

To prepare mutation constructs, PCR fragments were digested with appropriate restriction enzymes (as shown in Figs. 2 and 3), analyzed by agarose gel electrophoresis, and eluted from the gel. These purified fragments were inserted into pRC23-ratPDE3 that has been digested with the same restriction enzymes followed by dephosphorylation. One exception was the construct B shown in Fig. 2*A* where the restriction enzymes for the pRC23-ratPDE3 were different from those for the PCR fragment (see Fig. 2*A* legend).

Transformation and Recombinant PDE Expression—E. coli strain DH5α and its plasmid-bearing derivative DH5α(pRK248cIts) were used for transformation. The hybrid plasmid pRK248cIts is compatible with the expression vectors derived from pBR322 (30). It contains the cIts gene encoding a temperature-sensitive repressor (cIts) which acts in *trans* on the phage P_L promoter present in the pRC23 expression vector to prevent expression from the promoter at the nonpermissive temperature of 30 °C. DH5α(pRK248cIts) cells were transformed with the wild type (pRC23-ratPDE3) and mutant constructs using the procedure described by Maniatis *et al.* (31). Cells were grown at 30 °C at which the ratPDE3 enzyme was not expressed due to the presence in the cell of the cIts repressor that controls the P_L promoter. Transformants were selected on ampicillin-containing medium.

Individual colonies (6 to 29) were picked from each transformation. Cells were grown at 30 °C and then stored as frozen stocks at −80 °C. Each bacterial clone was screened for PDE expression by PDE assay and by Western blot analysis. Expression of ratPDE3 in bacteria was achieved by shifting incubation temperature from 30 to 42 °C. After 3 h at 42 °C, the cells were pelleted and lysed in 1X sample buffer for Western blot analysis (detailed below). For PDE assay, the bacterial pellet was resuspended and lysed for 15 min in a buffer containing 20 mM Tris-Cl, pH 8.0, 1 mM EDTA, and 2.2 mg/ml lysozyme. The cell lysate was then adjusted to also contain 0.2 mM EGTA, 10 mM NaF, 50 mM benzamidine, 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin.

Isolation of Plasmid and DNA Sequencing Analysis—Plasmid DNAs were isolated from mutant clones using the alkaline lysis method provided by the manufacturer (5prime-3prime, Inc.), and were mapped using restriction enzyme analysis to confirm that the correct DNA fragment had been inserted. For site-directed mutation, subsequent double-stranded DNA sequencing was performed by the dideoxy termination method (32) to confirm that nucleotide substitution(s) took place in the expected position(s). The entire 807-bp PCR fragment of a representative clone(s) of each amino acid mutation was also sequenced by subcloning the fragment into M13 bacteriophage to determine whether random mutation(s) were introduced by Taq polymerase. The clones analyzed in this study either were free of random mutations or contained random mutations that did not alter the amino acid sequence.

Eukaryotic Expression—The wild type and mutated ratPDE3 cDNAs were inserted into the *Eco*RI site of pCMV5, a eukaryotic expression vector (33). After transformation of DH5α bacteria, ampicillin-resistant cells were examined for the presence and the orientation of the ratPDE3 cDNAs. Plasmids were prepared from 1-liter bacterial cultures as described above. Mouse Leydig tumor cells (MA-10) were grown in the medium as described previously (34). When MA-10 cells in 10-cm dishes reached a density of approximately 40% of confluency, the cells were transfected with 10 μg of plasmid DNA using a calcium phosphate procedure as described previously (35). After an overnight incubation with the plasmid/$CaPO_4$ mixture, the cells were shocked with 10% glycerol in medium for 1.5 min at room temperature, washed with PBS, and then incubated in the growth medium overnight. To harvest, the cells were washed with PBS, scraped with a rubber policeman, and homogenized in an all-glass Dounce homogenizer (30 strokes) in a buffer containing 20 mM Tris-Cl, pH 8.0, 10 mM NaF, 1 mM EDTA, 0.2 mM EGTA, 50 mM benzamidine, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 2 mM

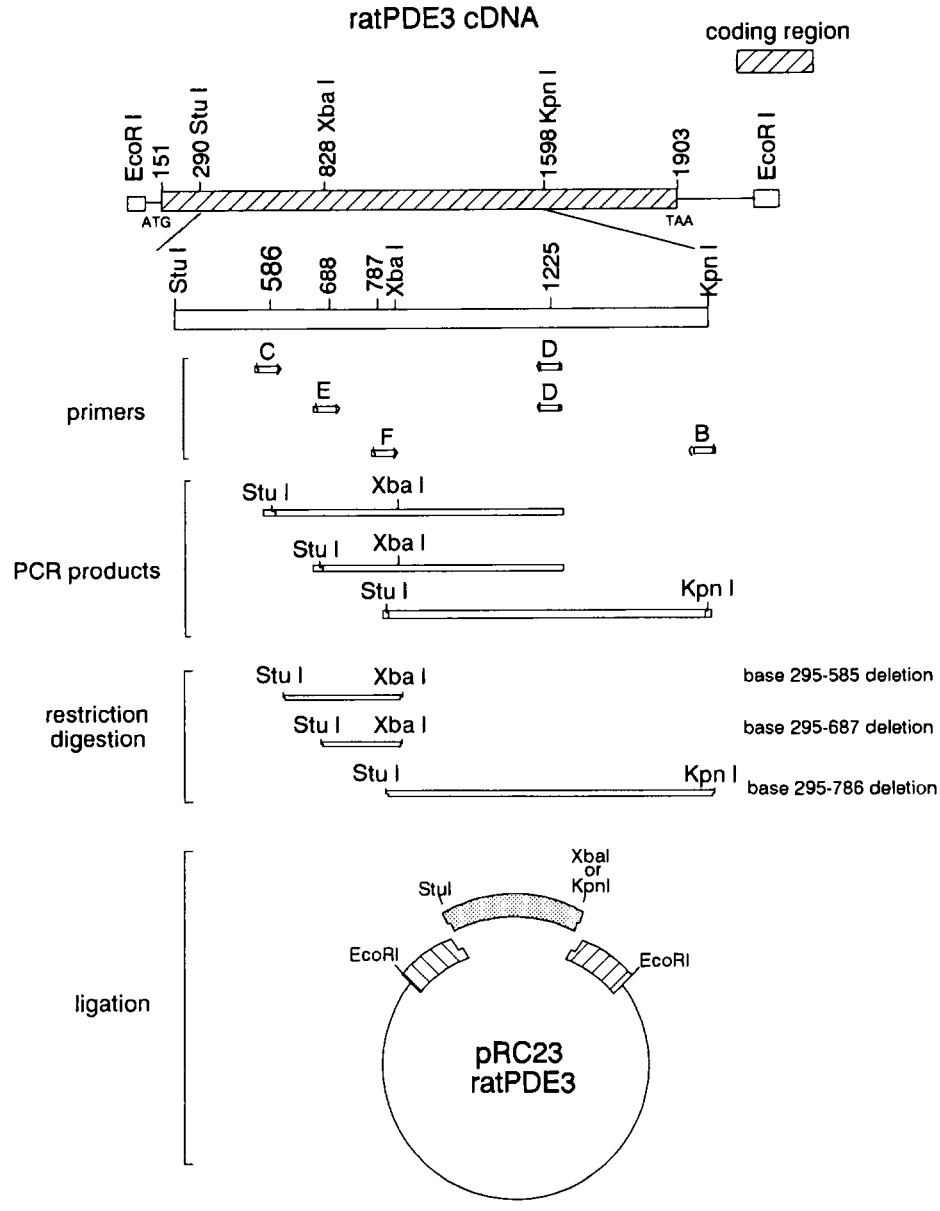

FIG. 3. **Schematic representation of the strategy used for internal deletions within the amino-terminal region of ratPDE3.** Internal deletions of different lengths (291, 393, and 492 bp) were generated using PCR technique. Fragments of the ratPDE3 cDNA were amplified using the ratPDE3 cDNA as template and paired oligonucleotides, C and D, E and D, and F and B, as primers (also see Fig. 1). To create deletions starting at the *StuI* site of the cDNA, the primers C, E, and F were designed to contain a *StuI* sequence at their 5′ ends. The complete sequences and base locations of these primers are reported under "Experimental Procedures" and in Fig. 1. PCR products were digested with either *StuI* and *XbaI* or *StuI* and *KpnI* restriction enzymes to generate the *StuI-XbaI* or *StuI-KpnI* fragment, followed by ligation to the expression vector to replace the wild-type fragment. Plasmids thus constructed have deletions within the 5′ region of the ratPDE3 cDNA starting at the base position 295 to the positions 585, 687, and 786, which correspond to the locations of primers C, E, and F, respectively (see Fig. 1). The *StuI-KpnI* fragment was used to create the 295–786-bp deletion instead of the more obvious *StuI-XbaI* fragment, since the small size of the *StuI-XbaI* fragment (44 bp) made it difficult to purify and clone. The proteins thus produced have deletions of 97 amino acids (from Tyr^{49} to Lys^{145}), 131 amino acids (from Tyr^{49} to Lys^{179}), and 164 amino acids (from Tyr^{49} to Thr^{212}). Since the deletions were produced internally, the translation start site (at base 151) of these mutants was unchanged.

phenylmethylsulfonyl fluoride. The cell homogenates were centrifuged for 15 min at 14,000 × *g*, and the soluble fraction was subjected to PDE assay, immunoprecipitation, or ion-exchange chromatography purification. "Mock"-transfected cells were treated exactly the same way but did not receive plasmid DNA.

PDE Assay—PDE activity was measured according to the method of Thompson and Appleman (36) and as detailed previously (37). Briefly, bacterial or MA-10 samples were assayed in a total volume of 200 μl of reaction mixture including 40 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 1.25 mM 2-mercaptoethanol, 1 μM cAMP, 0.14 mg of bovine serum albumin, and 1 μM [^{3}H]cAMP (0.1 μCi). After incubation at 34 °C for 5 min, the reaction was terminated by adding an equal volume of 40 mM Tris-Cl, pH 7.5, solution containing 10 mM EDTA followed by heat denaturation for 1 min. To each reaction tube 50 μg of *C. atrox* snake venum was added, and the incubation was continued at 34 °C for 20 min. The reaction products were separated by anion-exchange chromatography on AG 1-X8 resin, and the amount of radiolabeled adenosine collected was quantitated by scintillation counting. Protein concentrations of the samples were measured according to Lowry *et al.* (38). To study the kinetic characteristics of the recombinant proteins, rates of cAMP hydrolysis were determined at the incubation times of 2, 4, 6, and 8 min with increasing concentrations of unlabeled cAMP (0.156–10 μM). Data were plotted according to Lineweaver and Burk (39). For inhibition studies, samples were assayed in the presence of 10^{-8} to 10^{-5} M rolipram, 10^{-8} to 3×10^{-5} M RO 20-1724, or 10^{-6} to 10^{-3} M milrinone which were dissolved in dimethyl sulfoxide. Control tubes contained equal amounts of dimethyl sulfoxide.

Immunoprecipitation—The soluble fraction of MA-10 cells transfected with plasmid DNAs was immunoprecipitated with the antiserum K111 using fixed *Staphylococcus aureus* cells (Pansorbin cells) by procedures described previously (25). The antigen-antibody complexes were prepared in a final concentration of 1 × sample buffer for Western blot analysis.

Western Blot Analysis—Bacterial lysates and immunoprecipitated MA-10 samples were prepared in 1 × sample buffer (62.5 mM Tris-Cl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 0.7 M 2-mercaptoethanol, and 0.0025% (w/v) bromphenol blue). The samples were boiled for 5 min and subjected to electrophoresis on a 10% SDS-polyacrylamide gel (SDS-PAGE). The proteins were then blotted onto Immobilon membrane followed by blocking the membrane in a PBS solution containing 5% (w/v) nonfat dry milk and 0.05% (w/v) Tween 20. After blocking, the membrane was incubated with primary antiserum, K110 or K111, at a dilution of 1:100 in blocking buffer. Antisera K110 and K111 were raised against synthetic peptides of ratPDE3 located at the amino acid positions 416 to 431 and 105 to 126, respectively. The antisera have been characterized previously.[2] After K110 anti-

[2] M. Conti, J. Odeh, J. V. Swinnen, and M. E. Svoboda, manuscript submitted.

serum incubation, the membrane was washed in PBS, followed by incubation with peroxidase-conjugated goat anti-rabbit antibody at a 1:1000 dilution in blocking buffer. After washing with PBS, peroxidase was visualized with the HRP color development reagent (Bio-Rad) in the presence of hydrogen peroxide. For K111 antiserum staining, the membrane was washed with PBS containing 1% (w/v) Nonidet P-40 and 0.2% (w/v) Tween 20, followed by incubation with ^{125}I-protein A (0.5 μCi/ml) in blocking buffer. After washing with PBS the membrane was exposed to XAR-5 x-ray film.

Ion-exchange Chromatography—The soluble fraction of transfected MA-10 cells was prepared as described above and then were applied to a high-performance liquid chromatography DEAE column for the partial purification of recombinant PDEs. The conditions and procedures used for the column is detailed in Ref. 34. The PDE activity of each fraction was measured and the activity recovered from the columns was corrected for the amount of protein loaded. The fractions containing the recombinant PDEs were subjected to kinetic and inhibition studies.

RESULTS

Expression of Wild Type ratPDE3 in Prokaryotic and Eukaryotic Cells—The complete nucleotide and deduced amino acid sequences of ratPDE3 are reported in Fig. 1. As previously reported, expression of this cDNA in *E. coli* leads to the appearance of cAMP hydrolytic activity with properties identical to those of a cAMP-PDE purified from rat Sertoli cell (24, 25). SDS-PAGE and Western analysis using K110 antibody of bacterial extract expressing recombinant ratPDE3 demonstrated the presence of a 70–71-kDa immunoreactive band (see Figs. 4 and 8). An additional doublet of 47–50 kDa was also present in all experiments performed (Figs. 4 and 8). This is probably derived from endogenous proteolysis of the amino terminus of the PDE protein, since this doublet could not be detected with the K111 antibody which recognizes an epitope located at amino acid position 105–126 of the protein (data not shown). This molecular weight species is most likely catalytically inactive (see below). Identical results were obtained with a modified cDNA lacking 147 bases of the 5′-untranslated region (see Fig. 1 for the location of the *Eco*RI site). This modified cDNA was used for all experiments reported below.

Mapping of the Catalytic Center of ratPDE3 Enzyme by Deletion Mutation—To determine if the conserved region corresponds to the catalytic domain of ratPDE3, constructs bearing deletions of 5′ and/or 3′ domain flanking the conserved region were generated based on the strategy outlined in Fig. 2*B*. Since the initiation codon of the wild type cDNA (at the base position 151) was removed from deletion of the 5′ domain (constructs B and C), an AUG codon present immediately 5′ to the *Xba*I site in the oligomer A was designed to initiate the translation of these amino-terminal truncated proteins. Similarly, the stop codon added immediately 3′ to the *Kpn*I site in the oligomer B was used to terminate the translation of those proteins truncated at the carboxyl terminus (constructs A and C). The three truncated ratPDE3 proteins thus generated are: one with a deletion of 99 amino acids at the carboxyl terminus (construct A), one with deletion of the entire 224 amino acids at the amino terminus (construct B), and one containing only the 261 amino acids of the conserved region of ratPDE3 (construct C). The three constructs were expressed in *E. coli*, and PDE activity was measured in bacterial lysates. Only expression of the construct bearing a 99-amino acid deletion at the carboxyl terminus led to the appearance of a major immunoreactive PDE protein of 53–55 kDa (Fig 4*A*). This truncated protein retained the ability to catalyze cAMP hydrolysis (PDE activity of bacterial lysate, pmoles/min/mg protein: antisense construct, 9.8 ± 2.1; wild type construct, 176.8 ± 4.9; carboxyl-deletion construct, 67.0 ± 15.0; also see Fig 5). This indicated that the carboxyl-terminal fragment of ratPDE3 is not necessary for catalysis. In contrast, the expression of the other two constructs did not result in any detectable PDE activity. These truncated proteins, however, were expressed at a low level in bacteria (data not shown), rendering the interpretation of the data difficult.

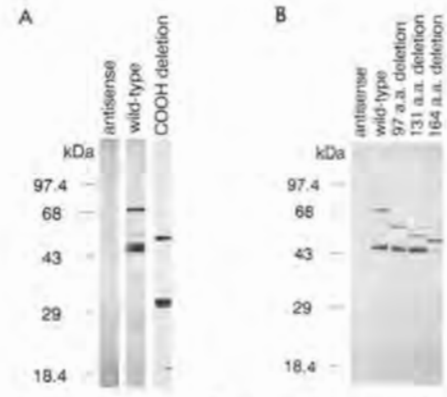

FIG. 4. **Expression of wild-type and truncated ratPDE3 in *E. coli*.** Bacteria were grown, pelleted, lysed in sample buffer, fractionated by SDS-PAGE, and immunostained with K110 antibody as described under "Experimental Procedures." Lysates were prepared from bacteria transformed with plasmids containing one of the following inserts: the full length ratPDE3 cDNA inserted in antisense orientation or in sense orientation (wild-type), the ratPDE3 cDNA deleted at the 3′ end (COOH deletion; *A*), or deleted by an internal fragment in the 5′ region (*B*). The sizes of internal deletions are as indicated. Eighty μg (in *A*) or 40 μg (in *B*) each of lysate protein was applied to the gel.

To overcome the potential disparity in the level of expression of the deleted proteins with different translation initiation sites, an alternative strategy of nested deletions was used to obtain deletions in the amino-terminal region. In addition to the ratPDE3 cDNA used in the present study, another clone, designated ratPDE3.2 (24), has previously been retrieved from a Sertoli cell library. The ratPDE3.2 cDNA contains an open reading frame coding for the ratPDE3 protein that lacks 78 amino acids at the amino terminus (Fig. 1). This truncated protein gave rise to an active enzyme when expressed in bacteria (24), indicating that this region is not necessary for catalysis. On the basis of this observation, a series of internal deletions starting at the *Stu*I site (corresponding to the amino acid position 49) within the 5′ region of the ratPDE3 cDNA were constructed using PCR technique. The method for generating different length deletions between *Stu*I and *Xba*I sites is detailed in Fig. 3. The three constructs with deletions from base 295 to 585, to 687 and to 786 were predicted to direct the translation of truncated proteins lacking amino acids 49 through 145 (97-aa deletion), amino acids 49 through 179 (131-aa deletion), and amino acids 49 through 212 (164-aa deletion), respectively. Western blot analyses of bacterial lysates expressing these truncated proteins showed immunoreactive bands of 60–61, 57–58, and 54–55 kDa, respectively (Fig. 4*B*). These molecular masses were comparable to those predicted by the size of the deletions. In addition, the efficiency of expression of these truncated proteins was similar to that of the wild type enzyme (Fig. 4*B*). When the internal 97-amino acid fragment was removed from the PDE, cyclic AMP-hydrolyzing activity observed was consistently 2–3-fold greater than the wild type enzyme (Fig. 5). Further deletions produced completely inactive proteins when expressed in bacteria (Fig. 5). A similar conclusion was reached when these truncated proteins were expressed in eukaryotic cells. Expression of the PDE with the 97-amino acid deletion in MA-10 cells produced a protein with approximately twice

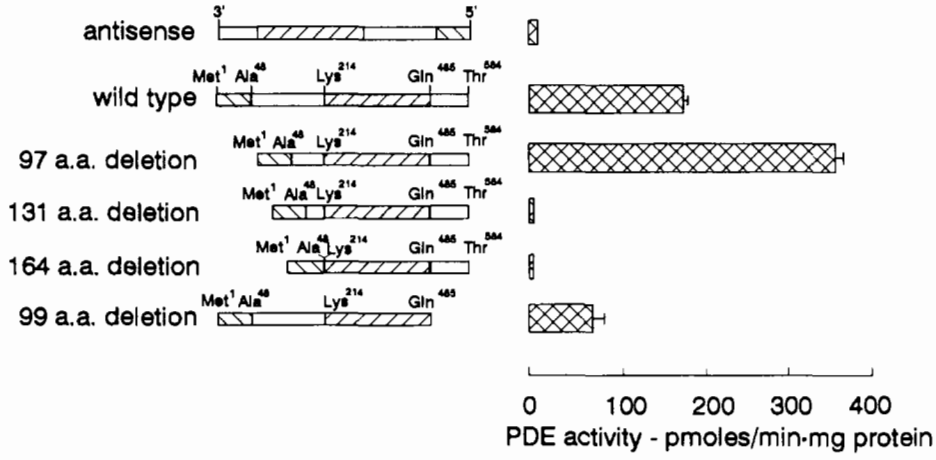

FIG. 5. **Comparison of the PDE activity of wild-type and truncated ratPDE3.** Bacteria were grown, pelleted, lysed in homogenization buffer, and assayed for PDE activity as described under "Experimental Procedures." Lysates were prepared from bacteria transformed with plasmids containing different DNA inserts. *Antisense*, the full length ratPDE3 cDNA inserted in antisense orientation, representing the basal level of PDE activity. *Wild type*, the ratPDE3 cDNA inserted in sense orientation, serving as a positive control. Deletions, DNA inserts encoding truncated ratPDE3 proteins including 97-, 131-, and 164-amino acid deletion (Tyr^{49}-Lys^{145}, Tyr^{49}-Lys^{179}, and Tyr^{49}-Thr^{212}, respectively) within the amino-terminal region and 99-amino acid deletion (Ser^{486}-Thr^{584}) at the carboxyl terminus. PDE activity represents the mean ± S.E. of at least three independent experiments (triplicate determinations of each experiment). Bacterial lysate was prepared independently in each experiment. The *left hatched bar* indicates the amino-terminal region of 48 amino acids unchanged in the deletion mutations.

TABLE I

Comparison of the properties of wild type and truncated ratPDE3 expressed in E. coli and MA-10 cells

Values of the apparent K_m (μM) and V_{max} (nmoles/min/mg protein) were determined for the wild type and truncated ratPDE3. Deletions introduced were the 97-amino acid internal deletion at the amino terminus (NH_2 97-aa deletion) and the 99-amino acid deletion at the carboxyl terminus (carboxyl-terminal deletion). Bacteria were lysed in homogenization buffer, and the lysate was directly used in the assay. MA-10 cells were harvested, and extracts were prepared as described under "Experimental Procedures." The recombinant PDE was partially purified by DEAE ion exchange chromatography, and the fractions containing the recombinant activity were used for the kinetic determinations. Each value is the mean ± S.E. of more than three independent experiments. The number of experiments is reported in parenthesis. The V_{max} values for the eukaryotic expression are the means of two independent experiments.

	Bacterial expression		Eukaryotic cell expression	
	K_m	V_{max}	K_m	V_{max}
Wild type	2.84 ± 0.62 (5)	0.36 ± 0.09 (5)	2.02 ± 0.30 (4)	9.90
Carboxyl-terminal deletion	3.73 ± 1.96 (5)	0.24 ± 0.10 (5)	-[a]	-[a]
NH_2 97-aa deletion	2.36 ± 0.25 (4)	1.04 ± 0.04 (4)	1.81 ± 0.25 (3)	20.26

[a] Expression in eukaryotic cells of ratPDE3 bearing a deletion at the carboxyl terminus did not produce a significant accumulation of the deleted protein.

the activity of the wild type (Table I). Conversely, in agreement with what has been shown in bacteria, the 131-amino acid truncated protein expressed in MA-10 cells was inactive (data not shown). The lack of PDE activity of the internal 131- and 164-amino acid deletions further indicated that the observed doublet of 47–50 kDa in the Western blots (Figs. 4 and 8) has no enzyme activity.

Catalytic Properties of the Truncated ratPDE3 Enzyme—Measurement of the apparent K_m of the crude or partially purified enzyme with 97 amino acids deleted within the amino terminus expressed in bacteria or eukaryotic cells showed no significant difference from that of the wild type (Table I). The deletion at the carboxyl terminus produced an enzyme with similar or slightly decreased affinity for the cAMP substrate. Maximal velocities of the enzymes, however, were affected (Table I). The kinetics of cAMP hydrolysis were linear in all cases indicating a homogeneous catalytic site (data not shown). In addition, deletions at the amino and the carboxyl termini did not abolish the PDE inhibition by rolipram, RO 20-1724, or milrinone (Fig 6). This indicated that the deleted regions are not involved in the interaction with these inhibitors. In the three experiments performed it was noticed that the PDE bearing the 99-amino acid deletion at the carboxyl terminus was more sensitive to milrinone than the wild type (ED_{50}: wild type, 7.73 ± 1.10 μM; carboxyl-terminal deletion, 3.10 ± 0.48 μM).

Site-directed Mutagenesis of ratPDE3 Enzyme—Several lines of evidence indicate that a Ser, Thr, or His residue plays an important role in PDE catalysis (20–23). To assess the role of these residues in the structure of ratPDE3 catalytic site, all the Ser and Thr residues present in the conserved domain and identical in all PDEs, and two of the conserved histidine residues were systematically mutated to Ala or other residues (Fig 7). None of the mutations obtained altered the level of the PDE protein expressed in bacteria (Fig 8). On the other hand, changes were observed in the activity of the mutated proteins (Table II). Substitution of the Thr present at the position 349 to Ala or Pro (Thr*349*/Ala or Thr*349*/Pro) produced a completely inactive PDE protein. Substitution of the same Thr residue with Ser (Thr*349*/Ser mutation) produced a protein still displaying catalytic activity, even though the velocity of the reaction was markedly reduced. The mutations of 2 Ser residues present at the positions 305 and 398 either did not affect catalysis or produced a decrease in the catalytic activity. Substitutions of His^{278} and His^{311} resulted in a complete inactivation of the enzyme.

Several lines of evidence indicate that the Glu residue at position 204 of the RII_α regulatory subunit of cAMP-depend-

ent protein kinase participates in the cAMP binding, probably forming an hydrogen bond with the 2′-hydroxyl group of the ribose of cAMP (16). Naturally occurring mutation of the conserved Gly residue adjacent to this Glu disrupts the cAMP binding (17–19). An identical stretch of 7 amino acids, ELALMYN, surrounding this Glu in RII_a is present in all cAMP-PDEs thus far sequenced. To test whether this region has a similar function in the cAMP-PDE, the residue (Ser^{295}) adjacent to the ELALMYN was mutated to Gly or Glu (Fig. 7), the wild-type or mutated residue observed in RII_a. Substitution of the Ser^{295} with Gly led to some reduction in catalytic activity of the PDE. Unlike what was observed for RII_a, mutation to Glu in ratPDE3 did not abolish enzyme activity (Table II). It indicates that this region of the protein can accept modifications which in RII_a disrupts the structure of the binding site.

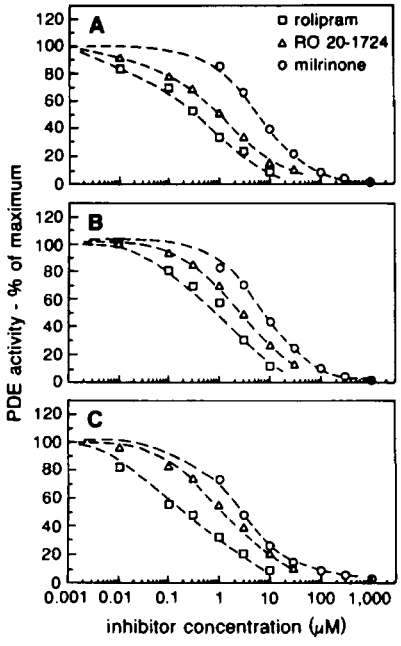

FIG. 6. **Effects of PDE inhibitors on the activity of wild-type and truncated ratPDE3.** Bacteria were lysed in homogenization buffer and assayed for PDE activity as described under "Experimental Procedures." Lysates were prepared from bacteria that expressed the wild-type ratPDE3 (*A*) or truncated proteins: 97-amino acid deletion within the amino-terminal region (*B*) and 99-amino acid deletion at the carboxyl terminus (*C*). PDE activity was assayed in the absence of the PDE inhibitors which represents the maximum activity (pmol/min/μl lysate: wild type, 0.94; NH_2 deletion, 2.37; carboxyl deletion, 0.32) and in the presence of 10^{-8} to 10^{-5} M rolipram, 10^{-8} to 3×10^{-5} M RO 20-1724, or 10^{-6} to 10^{-3} M milrinone. Each point represents the mean of triplicate determinations of a representative experiment of three performed. ED_{50} values in μM for the three inhibitors are: rolipram: wild type, 0.36 ± 0.08; NH_2 deletion, 0.94 ± 0.16; carboxyl-terminal deletion, 0.50 ± 0.10; RO 20-1724: wild type, 1.89 ± 0.37; NH_2 deletion, 3.13 ± 0.52; carboxyl-terminal deletion, 2.34 ± 0.53; milrinone: wild type, 7.73 ± 1.10; NH_2 deletion, 10.9 ± 1.51; carboxyl-terminal deletion, 3.10 ± 0.48 (mean ± S.E. of three independent experiments).

Characterization of the Thr349/Ser Mutation—To further analyze the effect of Thr*349*/Ser mutation, the cAMP-PDE carrying this substitution was inserted into a mammalian expression vector and expressed in COS and MA-10 cells. Data obtained in MA-10 cells will be reported here, although COS cell expression produced similar results. Immunoprecipitation of the soluble fraction of mock-transfected cells and cells transiently transfected with the wild type or Thr*349*/Ser mutant construct was followed by SDS-PAGE and immunoblot analysis. The expression of the Thr*349*/Ser mutation was comparable to that of the wild type (Fig 9). On the contrary, the amount of PDE activity recovered after transfection of the Thr*349*/Ser mutation was only a small fraction of the activity recovered with wild type PDE (PDE activity, pmol/min/mg protein: mock, 21.1; wild type, 646.8; Thr*349*/Ser, 58.5). To further characterize the kinetic properties of the Thr*349*/Ser PDE, the wild type, and the Thr*349*/Ser-mutated PDEs were partially purified on HPLC ion-exchange column, a method that allows separation of the recombinant PDE activity from the endogenous PDE (Fig 10). A major difference in the activity was retained after this chromatography. Comparison of the kinetics of these partially purified PDEs showed a significant difference in the apparent K_m (wild type, 2.06 μM; Thr*349*/Ser mutant, 3.55 μM) of the enzyme. Furthermore, the apparent V_{max} of the Thr*349*/Ser protein was approximately 10% of that of the wild type protein. The inhibition of the catalytic activity of the Thr*349*/Ser mutant by different PDE inhibitors was comparable to that of the wild type protein (data not shown).

DISCUSSION

Although information is available about the presence of different domains of the CaM-PDE (8, 9), cGS-PDE (10–12), and photoreceptor PDE (12), little is known about the structure of the cAMP-PDE family of enzymes. This is a group of PDEs which catalyzes cAMP degradation with a low K_m and whose activity is under hormonal control (8). Data reported show that these PDEs may play an important role in the mechanism of regulation of cell responses to hormones (25, 34, 42, 43). In the present study we have provided direct evidence that the catalytic domain of the cAMP-PDE is located in a central region of the protein which contains the domain conserved in most PDEs. We have also identified a Thr and 2 His residues located within the catalytic domain

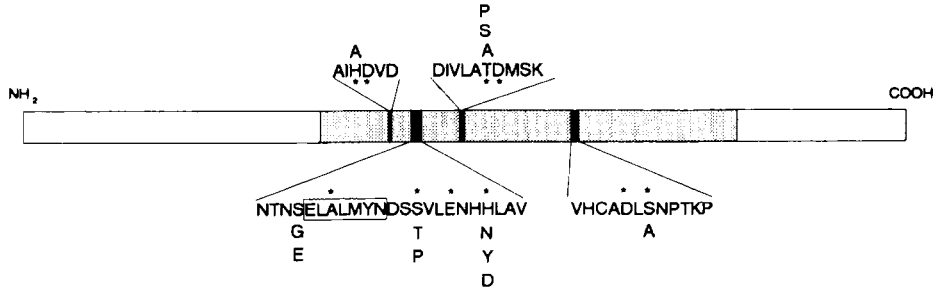

FIG. 7. **Schematic diagram to show the location of point mutations introduced in ratPDE3.** Amino acid sequences of the *filled bar* regions are shown by *one-letter codes*. Residues including His^{278}, Ser^{295}, Ser^{305}, His^{311}, Thr^{349}, and Ser^{398} were mutated to other residues as indicated on the top or the bottom of the wild-type residues. The *boxed* amino acid sequence corresponds to a sequence also present in the cAMP binding region of the RII_a regulatory subunit of the cAMP-dependent protein kinase (40, 41).

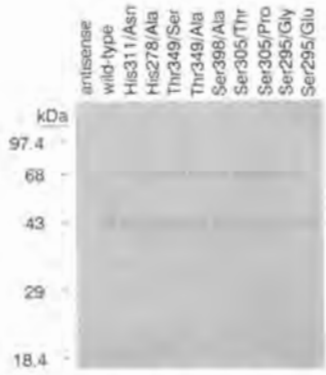

FIG. 8. **Expression of wild type and mutated ratPDE3 in *E. coli*.** Bacteria were transformed with plasmids containing either the full length ratPDE3 cDNA inserted in antisense orientation or in sense orientation (wild-type) or mutated ratPDE3 cDNA encoding proteins with a single amino acid substitution. The blot was immunostained with K110 antiserum. Forty-five μg each of lysate protein was applied to the gel.

TABLE II

Phosphodiesterase activities of wild type and mutated ratPDE3 expressed in E. coli

Bacteria were transformed with plasmids containing either the wild type ratPDE3 cDNA inserted in antisense or sense orientation or mutated ratPDE3 cDNA encoding proteins with a single amino acid substitution. Cells were lysed and assayed for activity as described under "Experimental Procedures." Results are the means ± S.E.M. of at least three independent experiments assayed in triplicate.

ratPDE3 construct	PDE activity
	pmol/min/mg protein
Wild type	
Antisense orient.	9.8 ± 2.0[a]
Sense orientation	176.8 ± 4.9
Mutations	
Ser^{295}/Gly^{295}	47.8 ± 4.2
Ser^{295}/Glu^{295}	74.7 ± 6.1
Ser^{305}/Thr^{305}	74.5 ± 1.5
Ser^{305}/Pro^{305}	80.8 ± 0.3
Ser^{398}/Ala^{398}	152.3 ± 9.5
Thr^{349}/Ser^{349}	20.8 ± 2.0
Thr^{349}/Ala^{349}	2.5 ± 0.5
Thr^{349}/Pro^{349}	1.8 ± 0.3
His^{278}/Ala^{278}	1.4 ± 0.3
His^{311}/Asn^{311}	6.7 ± 0.6
His^{311}/Tyr^{311}	2.1 ± 0.3
His^{311}/Asp^{311}	3.1 ± 1.0

[a] Mean ± S.E.M.

that play an important role in the structure and function of the catalytic site.

The following lines of evidence identify the catalytic domain of this cAMP-PDE (ratPDE3). An initial observation made in our laboratory has shown that a complementary DNA encoding a cAMP-PDE missing the first 78 amino acids of ratPDE3 enzyme when expressed in bacteria leads to the appearance of an active enzyme, suggesting that the amino-terminal region is not part of the catalytic domain (24). The deletion mutations described herein have allowed the identification of a region that contains all the information necessary for catalysis. Since the enzyme with the 97-amino acid deletion (Tyr^{49}-Lys^{145}) is active and that with the 131-amino acid deletion (Tyr^{49}-Lys^{179}) is inactive, the beginning of the catalytic domain should be located between Leu^{146} and Lys^{179}. At the carboxyl terminus, Gln^{485} is the tentative end of the catalytic domain, because further deletions from Gln^{485} toward the amino terminus were not performed. This roughly coincides with the conserved domain of approximately 35–37 kDa.

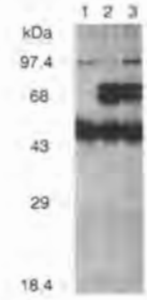

FIG. 9. **Immunoblot of wild type and Thr*349*/Ser ratPDE3 expressed in MA-10 cells.** Cells were either mock-transfected (*lane 1*) or transiently transfected with the wild type construct (*lane 2*) or the Thr*349*/Ser mutant construct (*lane 3*). Cells were harvested, and the supernatants were prepared and immunoprecipitated with the antiserum K111 as described under "Experimental Procedures." The resulting antigen-antibody complexes were solubilized and fractionated by SDS-PAGE followed by immunostaining with the same antiserum. The 53-kDa band corresponds to the immunoglobulin used for the immunoprecipitation, since it was also present in the immunoprecipitated sample without cell extracts. The 97-kDa band probably corresponds to the endogenous cAMP-PDE, since this band could not be detected in the recombinant PDE samples that have been partially purified through a DEAE column (data not shown). The wild type and mutated PDEs migrated as a doublet of 68–72 kDa.

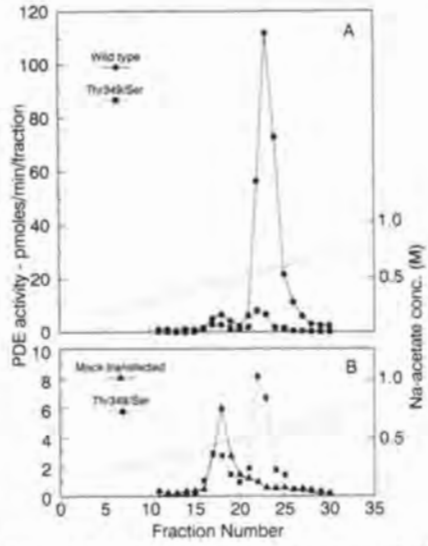

FIG. 10. **Ion-exchange chromatography of wild type and Thr*349*/Ser ratPDE3 in MA-10 cells.** Mock- or ratPDE3-transfected cells were harvested, and extracts were prepared and fractionated on a DEAE column, as described under "Experimental Procedures." The PDE activity present in each fraction was measured and was corrected for the amount of protein loaded on the column. The column profiles of wild type and Thr*349*/Ser ratPDE3 (*A*) and mock transfection and Thr*349*/Ser ratPDE3 (*B*) are reported. The *broken line* shows the sodium acetate gradient applied to the column. The results of a representative experiment of the two performed are shown.

Interestingly, this is similar to the size of the active fragment obtained by controlled proteolysis of a CaM-PDE (9), a cGS-PDE (10), and a photoreceptor PDE (12). The 35–37-kDa fragment includes the region (Lys^{214}-Asn^{480}) conserved in most PDEs, but it is not limited to this domain. In fact, an additional region of 68 amino acids flanking the conserved domain toward the amino terminus appears to be necessary for catalysis. DNA sequence analysis indicates that this fragment is conserved among the different cAMP-PDE forms, suggesting again an important function for this region. However, no homology was found with the PDEs of other families.

The data showed that the ratPDE3 protein bearing a deletion of amino acids 49–145 (NH_2 97-aa deletion) not only retains catalytic activity but also that the velocity of the reaction catalyzed by this truncated protein is approximately 2–3-fold higher than that of the wild type protein. Since the activities have been measured in crude or partially purified PDE preparations, it cannot be excluded that differences in activity are due to differences in the level of expression or in protein stability. If our preliminary data are confirmed with a purified enzyme it would be an indication that within this 97-amino acid region there is a domain that inhibits the catalytic activity. This would be reminiscent of what is observed for other PDEs (8). It is accepted that regulatory domains present in a CaM-PDE exert an inhibitory constraint on the catalytic domain. Proteolytic removal of these domains causes an irreversible activation of the CaM-PDE together with loss of calmodulin regulation (8, 9). Thus, it can be speculated that cAMP-PDEs also have regulatory domain(s) similar to the CaM-PDE. The identity of the regulatory signal that modulates the function of this domain in the cAMP-PDE is not known.

The location of the catalytic domain within the fragment included between Leu^{146} and Ser^{486} is also supported by site-directed mutagenesis of residues within this region. Of the 6 residue substitutions performed, only one (Ser^{398}) had no effect on catalysis. All the other mutations decreased or abolished catalytic activity of the PDE. This is a further indication that these residues are located in the catalytic center of the enzyme.

Substitution of His^{278}, His^{311}, and Thr^{349} abolished the catalytic activity of the enzyme. At present we cannot distinguish whether these residues participate in catalysis or are necessary for the conformation of the catalytic center. The finding that Thr^{349} could be substituted by a Ser with retention of some catalytic activity, while substitution with an alanine gave rise to an inactive enzyme is an indication that the hydroxyl group present in Thr^{349} and Ser^{349} may play an important role in enzyme function. It is to be emphasized that Thr^{349} is present in the most conserved region of all PDE sequences available (6). In this region 7 residues are identical or conserved. Interestingly, it has been found that in 5′-nucleotide phosphodiesterase a Thr residue plays an important role in catalysis, because it forms an intermediate bond with phosphate (23). Similarly, data thus far accumulated using cAMP analogs with different group substitutions have suggested that an electron donor residue such as Thr is required in the PDE for the nucleophilic attack of the phosphodiester bond (21, 22). It is, therefore, tempting to speculate that this Thr has a crucial function in PDE catalysis.

Chen *et al.* (14) first observed that in *Drosophila* dunce PDE there is a short segment (ELALMYN) of identity with the RII_a subunit of cAMP-dependent protein kinase. In the regulatory subunit of the kinase the Glu residue present in this region is thought to play a crucial role in stabilizing the binding of cAMP to the protein by forming a hydrogen bond with the 2′-hydroxyl group of the ribose moiety of cAMP (16). However, our mutation data provide evidence that the ELALMYN region behaves differently in the cAMP-PDE. In RII_a, substitution of the Gly residue adjacent to this region with Glu disrupts cAMP binding. Conversely, an identical substitution in the cAMP-PDE has only marginal effects on catalysis. This is in line with the observation that 2′-deoxy cAMP lacking the hydroxyl group at the position 2′ competes efficiently for the catalytic domain of several PDEs (21, 22, and our laboratory). This suggests that, unlike RII_a, there is probably no hydrogen bond formed between 2′-OH of cAMP and the Glu residue of ELALMYN in the cAMP-PDE. Therefore, even though this sequence is present in the catalytic center of the cAMP-PDE, it probably has a different function.

In summary, these data demonstrate that a 37-kDa fragment of a cAMP-PDE contains the catalytic domain of the protein. It also contains the site of interaction with the selective inhibitors RO 20-1724 and rolipram. Within this region the Thr^{349} residue conserved in the cAMP-PDEs and in other PDEs plays an important role in the catalytic center. Conversely, the role of the amino and carboxyl terminus domains remains largely unknown. Preliminary evidence reported suggests the presence of a regulatory domain located at the amino terminus of the protein.

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