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RETINAL CYCLIC-GMP PHOSPHODIESTERASE γ-SUBUNIT: USE OF MUTANT SYNTHETIC PEPTIDES TO DEFINE FUNCTION

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Previously, we have domain-mapped the 87 amino acid PDEγ inhibitory subunit of the retinal phosphodiesterase (PDE) $\alpha\beta\gamma_2$ complex using synthetic peptides (1). The PDEγ subunit has a binding domain for transducin-α (Tα) and for PDEα/β within residues #24-45. An inhibitory region for PDEα/β is within residues #80-87. In order to establish the role of individual amino acids in the function of the PDEγ inhibitory subunit, mutants were synthesized and utilized in PDE inhibition assays. The following mutants exhibited a decreased ability to inhibit PDEα/β: Tyr_{84}→Gly; Arg_{24}→Gly; and Arg_{33}→Pro. Sequence comparisons with cone PDEγ indicate that there is identity within these functional regions.

In the retinal rod outer segment, light activation of rhodopsin leads to the production of an activated state of the G-protein transducin-α subunit (Tα) (1-7). The resulting Tα-GTP binds to and relieves the inhibitory constraint of the phosphodiesterase-γ subunit (PDEγ) (8). As a result of lower cGMP levels, the cell membrane cGMP-binding channel protein closes, leading to membrane hyperpolarization (9, 10). The 87 amino acid PDEγ inhibitory protein sequence is highly conserved between human, mouse, and bovine species (13-15), as well as the cone outer segment (COS) PDEγ (16). The bovine rod outer segment (ROS) PDEγ inhibitory subunit contains a binding region for both Tα-GTP and for PDEα/β within residues #24-45 (11, 12) and an inhibitory binding region for PDEα/β within the C-terminus residues #80-87 (11, 12). Recombinant and synthetic PDEγ both have functional activity similar to native PDEγ (17, 18). In order to identify functionally significant amino acids within the binding and inhibitory regions, mutants of PDEγ were synthesized. Evidence is presented here in support of previous reports suggesting that Arg_{24} and Tyr_{84} are required for functional activity (18, 19). In addition, the functional requirement of Arg_{33} is also

Abbreviations: cGMP-guanosine 3', 5'-cyclic monophosphate; COS-cone outer segment; PDE-phosphodiesterase; PMSF-phenylmethyl-sulfonyl fluoride; ROS-rod outer segment; SDS-PAGE-sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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suggested. Sequence comparisons between ROS and COS PDEγs show similarity in these regions.

MATERIALS AND METHODS

Bovine eyes were obtained from Iowa Beef Packers (Emporia, KS). Amino acids and chemicals for peptide synthesis were from Vega Biochemicals, from Sigma, or from Pierce. [8-^{3}H]cGMP (15 Ci/mmol) was obtained from ICN Radiochemicals, Aquacide III from Behring Diagnostics, HPLC columns from Phenomenex, nitrocellulose from Schliecher and Schuell, X-ray film from DuPont, and developing solutions from Kodak. Trypsin (bovine pancreas, E. C. 3.4.21.4, 12,500 units/mg)and trypsin inhibitor (Soybean, Type 1-S) were from Sigma.

Rod outer segments (ROS) discs were prepared by the method of Papermaster and Dreyer (20). Soluble PDE$\alpha/\beta\gamma_2$ was eluted in room light from ROS discs in a buffer containing 10 mM Tris·HCl (pH 7.4), 0.1 mM dithioerythritol, 0.2 mM leupeptin, and 1 μM pepstatin. HPLC purification of proteins was as previously described (21).

The PDE assay was as described (22) using 400 μl final volume, 0.025 μg PDEα/β per 400 μl, in a buffer containing 50 mM Tris·Cl (pH 7.4), 5 mM $MgCl_2$, 40 μM cGMP, and [^{3}H] cGMP at 40,000 cpm/assay (15 Ci/mmol). Reaction was for 5 min at 30 °C. PDE was trypsin-activated to remove PDEγ by incubation for 1 min on ice using a stock solution of 20 μg of purified PDE$\alpha/\beta\gamma_2$,40 μg trypsin in 400 μl of the PDE assay buffer. The reaction was stopped by the addition of 2 x excess trypsin inhibitor.

PDEγ or mutants were synthesized, using the bovine sequence (13), by the method of Merrifield (23) as modified by Gorman (24). Cleavage was with anhydrous HF (25). Peptides were quantitated and checked for amino acid composition as previously reported (12, 21). Proteins were purified on HPLC TSK G200 columns and fractions monitored by absorbance at 220 nm, Western blots, and PDE inhibition assays, as previously detailed (18). Antiserum used for Western blots was directed against residues #1-49 of bovine PDEγ (18).

Protein concentrations were determined by the method of Bradford (26) or by scanning-gel densitometry of Coomassie-blue-stained SDS-PAGE (27) using bovine serum albumin as a standard. Gels were scanned on a Gilford multimedia densitometer using a Shimadzu integrator.

RESULTS AND DISCUSSION

Amino acid analysis of synthetic PDEγ and mutants were as for native PDEγ (data not shown). A Western blot from a SDS-PAGE gel (Fig. 1) indicated single bands that migrated at the predicted region for PDEγ.

The results of PDE inhibition assays using synthetically produced PDEγ and the corresponding mutant synthetic peptides are shown in Table 1. Synthetic PDEγ

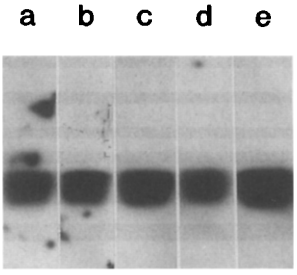

Fig. 1. SDS-PAGE of native purified PDEγ (lane a) and mutant PDEγs (lanes b-e) were blotted to nitrocellulose and immunoreacted with PDEγ 1-49 antiserum. Lane a: native PDEγ; lane b: $Tyr_{84}\rightarrow Gly$; lane c: $Arg_{24}\rightarrow Gly$; lane d: $Arg_{33}\rightarrow Pro$; lane e: -10 amino acids C-terminus.

Table 1. Trypsin-activated PDE (0.025 μg of purified native PDE digested for 2 min on ice with 1.0 unit of insoluble trypsin) was incubated with increasing amounts of synthetic PDEγ or with the following mutants: Tyr_{84}→Gly, Arg_{24}→Gly, Arg_{33}→Pro, and -10 aminio acid C-terminus. Data is the mean ± S.D. of n=3. Background (2,292 ± 90) has been subtracted from all samples. Maximum counts per min were 21,940 ± 800; counts per min with trypsin-activated PDE only were 11,114 ± 400.

PDEγ peptide	counts per min		
	10 μg	40 μg	100 μg
synthetic PDEγ	8,347 ± 916	3,810 ± 730	899 ± 38
Tyr_{84}→Gly	8,066 ± 345	8,209 ± 870	6,947 ± 1,083
Arg_{24}→Gly	10,154 ± 752	8,415 ± 905	2,993 ± 615
Arg_{33}→Pro	10,163 ± 587	10,558 ± 774	1,941 ± 254
-10 amino acids	12,563 ± 279	12,770 ± 910	7,837 ± 413

inhibited trypsin-activated PDEαβ with an I_{50} ≈ 40 μg. Mutants demonstrated reduced ability to inhibit PDEαβ, particularly at 40 μg of peptide. The C-terminal mutant, lacking the last ten amino acids, did not inhibit PDE. This agrees with previous reports (17, 19). In addition, the Tyr_{84}→Gly mutant failed to effectively inhibit PDEαβ activity. The two arginine mutants, Arg_{24}→Gly and Arg_{33}→Pro, had decreased inhibitory activity (I_{50} ≈ 100 μg).

Using the program SEQALIGN[1](Fig. 2), comparisons of rod and cone PDEγ (16) indicate homology at positions Arg_{24} and Arg_{33}. However, cone PDEγ has a phenylalanine at the corresponding rod PDEγ Tyr_{84} position. This suggests that functionally an aromatic amino acid rather than a tyrosine hydroxyl group is important for inhibitory activity.

The bovine gene for PDEγ has been sequenced and is reported to have three exons coding for residues #1-49, 50-62, and 63-87 (28). Using synthetic peptides, we have identified a binding region for TαGTP and PDEαβ within residues #24-45 (11, 12, 18) and an inhibitory region within residues #63-87 (11, 12, 18). This study more specifically reveals several key residues within the binding region (Arg_{24} and Arg_{33}) and within the inhibitory region (Tyr_{84}) that are required for PDEγ inhibitory activity.

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                                          ↓          ↓
1:    ----MNLEPP KAEIRSATRV MGGPVTPRKG PPKFKQRQTR QFKSKPPKKG
2:    MSDNTVLAPP TSN-------  -QGPTTPRKG PPKFKQRQTR QFKSKPPKKG
          * **                ** ***** ********** **********

                                                   ↓
1:    VQGFGDDIPG MEGLGTDITV ICPWEAFNHL ELHELAQYGI I
2:    VKGFGDDIPG MEGLGTDITV ICPWEAFSHL ELHELAQFGI I
      * ******** ********** ******* ** ******* ** *
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Fig. 2. Sequence comparison between PDEγs from bovine ROS [13] and COS [16] using the computer program SEQALIGN . Asterisks denote identity. Functionally important residues identified in this paper are notated by arrows (1: ROS PDEγ; 2: COS PDEγ).

[1]SEQALIGN is a program authored by Clark, K. L., Teller, D. C., and Reeck, G. R., copyright (c) 1989, Kansas State University Research Foundation.

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