PDE4 splice variants are classified into long and short forms depending on the presence or absence of two unique N-terminal domains termed upstream conserved regions 1 and 2 (UCR1 and -2). We have shown previously that the UCR module mediates dimerization of PDE4 long forms, whereas short forms, which lack UCR1, behave as monomers. In the present study, we demonstrate that dimerization is an essential structural element that determines the regulatory properties and inhibitor sensitivities of PDE4 enzymes. Comparing the properties of the dimeric wild type PDE4D3 with several monomeric mutant PDE4D3 constructs revealed that disruption of dimerization ablates the activation of PDE4 long forms by either protein kinase A phosphorylation or phosphatidic acid binding. Moreover, the analysis of mutants consisting of a catalytically inactive PDE4D3 subunit indicates that protein kinase A phosphorylation of both subunits is essential for fully active PDE4 enzymes. In addition to affecting enzyme regulation, disruption of dimerization reduces the sensitivity of the enzymes toward the prototypical PDE4 inhibitor rolipram. Parallel binding assays indicated that this shift in rolipram sensitivity is likely mediated by a decrease in the number of inhibitor binding sites in the high affinity rolipram binding state. Thus, although dimerization is not a requirement for high affinity rolipram binding, it functions to stabilize PDE4 long forms in their high affinity rolipram binding conformation. Taken together, our data indicate that dimerization defines the properties of PDE4 enzymes and suggest a common structural and functional organization for all PDEs.

The mammalian cyclic nucleotide phosphodiesterases (PDEs) comprise a large group of over 20 genes that are subdivided into 11 PDE families on the basis of their sequence homology (1). PDE families can also be distinguished on the basis of inhibitor selectivity and by their catalytic properties as some specifically hydrolyze cAMP or cGMP or show dual specificity. In addition, most PDE families possess unique regulatory domains such as Ca\(^{2+}\)/calmodulin binding sites (PDE1), GAF domains (where the GAF domain is named for its presence in GTP-binding proteins, adenyl cyclases, and the Escherichia coli Ehp1A protein; PDE2, -5, -6, -9, and -10), PAS domains (where PAS domain is named for its presence in the Per, ARNT, and Sim proteins; PDE8), and UCR domains (PDE4; see Fig. 1). Nearly all PDE genes are expressed as several splice variants resulting in a total of ~50 PDE isoenzymes that show differences in their tissue distribution, subcellular localization, and post-translational modifications.

Despite this functional and structural diversity, some properties are common to all PDE isoenzymes. First, PDEs show a multidomain structure with a C-terminal catalytic domain of about 270 amino acids that is conserved between 25 and 45% (1). Second, N-terminal domains of most PDE families contain unique regulatory domains harboring binding sites for small messenger molecules such as Ca\(^{2+}\)/calmodulin, cGMP (GAF domains), and phosphatidic acid (UCR domains) and/or recognition sites for protein kinases including the Ca\(^{2+}\)/calmodulin-dependent kinase and the protein kinases A, B, and G (Table II). Although these domains are structurally diverse, they exhibit a functional conservation in that PDEs are generally activated upon occupancy of their respective binding sites or phosphorylation at the N terminus (1–9). Some differences are present, however, in the mechanism of enzyme activation. Most PDEs are activated through an increase in \(V_{\text{max}}\) (PDE1 and PDE3–6), whereas PDE2 is thought to be activated through a decrease of the \(k_m\) value (1, 4). Third, most PDEs exist as dimers or oligomers, and dimerization is usually mediated by the N-terminal sequences (3, 10–17). The dimerization domains have been mapped in greater detail for PDE2, -5, -6 (10, 15, 16), and PDE4 (14) and were found to lie within the highly conserved N-terminal GAF and UCR domains, respectively (Fig. 1 and Table II). The finding that most PDEs are dimers and that dimerization is mediated by highly conserved sequences indicates that oligomerization itself is an important structural property of a PDE conserved through evolution. It is known, however, that neither dimerization nor the N-terminal regulatory domains are essential for PDE activity as a single subunit of a catalytic domain is sufficient for cyclic nucleotide hydrolysis.

In a previous report (14), we showed that dimerization of PDE4 isoenzymes is mediated by a domain encompassing the C-terminal half of UCR1 (UCR1C) and the N-terminal half of UCR2 (UCR2N) and that nested deletion of either subdomain ablates enzyme dimerization. The four genes that make up the PDE4 family are all expressed as several splice variants that differ with regard to the presence or absence of the UCR domains and, therefore, their subunit structure. Only the so-called long PDE4 forms, which have a complete UCR1/UCR2 module, are dimers, whereas the so-called short forms, which
only partially contain the UCR1/2 module, are monomers (Fig. 1).

It is generally accepted that PDE4 proteins exist in vivo in several different conformations that are distinguishable by their inhibitor sensitivity, efficiency of catalysis, and metal ion sensitivity. The structural basis for this variety of conformers is largely unknown. It is well established, however, that PKA-mediated phosphorylation (a critical event in the short term feedback regulation of cAMP signals) or binding of phosphatidic acid to the N terminus of PDE4 long forms activates the enzymes, possibly by stabilizing one conformation (6–8). Another example is the existence of PDE4 in several distinct conformations that bind the PDE4 inhibitor rolipram with different affinities (high and low affinity rolipram binding sites) (20–24).

The present study was aimed at elucidating the role of dimerization in enzyme kinetics, inhibitor sensitivity, and the mechanisms regulating PDE4.

EXPERIMENTAL PROCEDURES

PCR Primers—The oligonucleotides used for PCR amplifications are numbered consecutively; restriction enzyme sites and exchanged nucleotides are underlined, and start and termination codons are shown in italics within the nucleotide sequences as follows: P1, 5′-GCTGAAATCATCATGCA/CTGAGATCTAA-3′; EcoRI; P2, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; CTGAGATCTAA-3′; P3, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; HincII; P4, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; HincII; P5, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; HincII; P6, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; HincII; P7, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P8, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P9, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P10, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P11, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P12, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P13, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P14, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P15, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P16, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P17, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P18, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P19, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P20, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P21, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P22, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P23, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P24, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI; P25, 5′-GGGGGCTGCTAGCCCCAGCTTCTGACGAGCTTTC-3′; XbaI.

Design of Expression Vectors, Cloning, and Mutagenesis—PCR was routinely performed using Pwo DNA polymerase (Roche Diagnostics) according to the manufacturer’s protocol. The generation of vectors for the expression of wild type human PDE4D3 and PDE4D2 in mammalian cell lines was reported by us previously (14). In brief, a cDNA encoding the entire open reading frame of PDE4D3 had been ampliﬁed from a PDE4D3 plasmid available in our lab using primers P1/P2 and cloned into restriction enzyme sites EcoRI/SalI of the pCMV5 vector. The construct was completely sequenced to exclude random mutations and was used as template for all other cloning strategies. The vector encoding PDE4D2 was constructed by ligating the EcoRI/BstEI fragment obtained from the digest of the PCR product P3/P4 with the BstEI/EcoRI vector fragment excised from the PDE4D3-pCMV5 vector. To generate constructs carrying small nested deletions within the UCR1C and UCR2N-subdomains, the EcoRI/BstEI fragment within the PDE4D3-pCMV5 construct was replaced by ligating the rest of the vector with each of two small fragments that had been generated by PCR and digested with the respective restriction enzymes EcoRI/XbaI and XbaI/BstEI, respectively. The PCR fragments were amplified with primer pairs P1/P5 and P6/P4, and P1/P7 and P8/P4 to obtain 4D3-D1 (197–194) and 4D3-D2 (147–155), respectively. Single point mutations were inserted into the PDE4D3-pCMV5 vector using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). First, mutations V93A/L97A, V100A/F104A, L145A/L148A, L152A/L155A, R98A/R101A, and E147A/D149A were introduced using primers P9/P10, P11/P12, P13/P14, P15/P16, P17/P18 and P19/P20, respectively. Some of the resulting constructs were then used as PCR templates for a second round of mutagenesis to generate the following constructs, each carrying four mutations: V100A/F104A/L145A/L155A (4D3-M1), V100A/F104A/L145A/L148A (4D3-M2), L145A/L148A/L152A/L155A (4D3-M3), and R98A/R101A/E147A/D149A (4D3-K1). A PCR product amplified from either wild type or mutant PDE4D3-pCMV5 constructs using primers P21/P22 was cloned into the HindIII/XbaI/BstEII fragment obtained from the digest of the PCR product P3/P4 with the BstEI/EcoRI vector fragment excised from the PDE4D3-pCMV5 vector. To obtain the catalytically inactive PDE4D3-dead-V5/His constructs, the mutagenesis H326A was introduced into the PDE4D3-pCMV5/V5/His vector with primers P23/P24 using the QuikChange site-directed mutagenesis kit (Stratagene). The PDE4D3 construct carrying the S54A mutation was generated by replacing the EcoRI/ClaI fragment within the PDE4D3-pCMV5 vector with a PCR product amplified using primer pair P1/P2 from the wild type PDE4D3 template and digested with the same restriction enzymes. A similar strategy was used to generate the PDE4D3-dead-V5/His/S54A construct, except that the PCR product was generated with primers P21/P25 and inserted into the HindIII/ClaI restriction enzyme sites of the PDE4D3-dead-V5/His vector. All plasmids containing PCR-generated DNA fragments were sequenced to exclude random mutations.

Cell Culture and Transfection—COS7 cells (African green monkey kidney cells) were cultured at 37 °C and under a 5% CO2 atmosphere in Dulbecco’s modiﬁed Eagle’s medium supplemented with 10% fetal calf serum, 30 µg/ml penicillin, and 100 µg/ml streptomycin. For recombinant expression of PDE constructs in COS7, cell cultures were grown on 100-mm dishes to 50% conﬂuency and were then transfected with recombinant vector DNA using the Effectene transfection system (Invitrogen) according to the manufacturer’s protocol. To obtain similar expression levels for all constructs, 1.5 µg of plasmid DNA of the dimeric wild type PDE4D3 and 4 µg of plasmid DNA for all monomeric constructs were transfected for the experiments reported in Figs. 6–10. Three days after transfection, cells were routinely harvested in 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 0.2 mM EGTA, 150 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, a protease inhibitor mixture.

Significance of PDE4 Dimerization
from Roche Diagnostics, and 1 mM 4-(2-aminoethoxy)-benzenesulfonyl fluoride hydrochloride (Roche Diagnostics).

**High Pressure Liquid Size Exclusion Chromatography**—For gel filtration experiments, cells were harvested in a 10 mM sodium acetate buffer (pH 6.5) containing 1 mM EDTA, 0.2 mM EGTA, 5 mM β-mercaptoethanol, 150 mM NaCl, 20% ethylene glycol, 1 mM 4-(2-aminoethoxy)-benzenesulfonyl fluoride hydrochloride, and a protease inhibitor mixture from Roche Diagnostics. The cell homogenates were centrifuged first at 14,000 × g for 20 min followed by a 30-min centrifugation at 100,000 × g, and 500 μl of the high speed supernatants were then loaded on a TSK-3000 analytical gel filtration column (Toosoh Bioscience LLC, Montgomeryville, PA). The samples were eluted with a 10 mM sodium acetate buffer (pH 6.5) containing 1 mM EDTA, 0.2 mM EGTA, 5 mM β-mercaptoethanol, 150 mM NaCl, and 20% ethylene glycol in a flow rate of 0.5 ml/min. Fractions of 500 μl were collected and assayed for PDE activity. Bovine thyroglobulin (670 kDa; 85 Å), horse ferritin (440 kDa; 61 Å), bovine γ-globulin (158 kDa), rabbit aldolase (158 kDa; 48.1 Å), bovine serum albumin (monomer = 67 kDa; 35.5 Å), chicken ovalbumin (44 kDa; 30.5 Å), horse myoglobin (17 kDa), and coelobamin (1.35 kDa) were used as molecular weight markers.

**Density Gradient Centrifugation**—Density gradients from 5 to 33% sucrose in 50 mM Tris-HCl (pH 8.0) containing 0.2 mM EDTA, 150 mM NaCl, and 5 mM β-mercaptoethanol were prepared in 14 × 89-mm centrifugation tubes (Beckman Instruments, Fullerton, CA) using a gradient former (Jule Inc., New Haven, CT). The gradients were stored overnight at 4 °C before they were loaded with 100 μl of the soluble cell extracts (100,000 × g supernatant) or marker proteins and centrifuged for 24 h at 36,000 rpm in a Beckman SW41 rotor (Beckman Instruments). Fractions of ~220 μl were then collected starting from the bottom of the tube using a peristaltic pump and analyzed for PDE activity or protein concentration (molecular weight markers). The following proteins were used as molecular weight markers: bovine thyroglobulin (670 kDa; 19 S), bovine liver catalase (250 kDa; 11.3 S), rabbit aldolase (158 kDa; 7.3 S), bovine serum albumin (67 kDa; 4.6 S), and chicken ovalbumin (44 kDa; 3.5 S).

**Calculation of Molecular Weights**—The molecular weights of PDE4D constructs were calculated by the method of Siegel and Monty (25) using sedimentation coefficients obtained from gel filtrations and density gradient centrifugations, respectively, using Equation 1,  

\[ M = (s_{0.5} \cdot N_A \cdot 6 \cdot \pi \cdot v \cdot R_g(1 - v) \cdot \rho) \]  

(Eq. 1)

where \( s_{0.5} \) indicates sedimentation coefficient in Svedberg units; \( N_A \) indicates Avogadro’s constant (6.022 × 10²³ mol⁻¹); \( \gamma \) indicates viscosity of medium (0.01 cm³ s⁻¹); \( R_g \) indicates Stokes radius in nm; \( \varepsilon \) indicates partial specific volume of a protein (0.73 g cm⁻³); and \( \rho \) indicates density of medium (1.0 g cm⁻³).

**Immunoprecipitation (IP)**—Protein-G-Sepharose beads were washed three times with PBS and then loaded with the α-V5 antibody (Invitrogen) in a reaction mixture containing 20 μl of protein G-Sepharose, 100 μl of PBS, and 1 μl of the affinity-purified antibody. The samples were incubated in a rotating mixer for 60 min at 4 °C and then centrifuged for 2 min at 1,000 × g. The pellet beads were washed twice with PBS to remove unbound antibodies and were then incubated with the enzyme extract for 2 h at 4 °C in a rotating mixer. The reactions were then centrifuged for 2 min at 1,000 × g to pellet the immunocomplex. The beads were washed three times by resuspending in cell lysis buffer and subsequent re-centrifugation. After the last centrifugation step, the beads were resuspended in cell lysis buffer and subjected to SDS-PAGE or the immunoprecipitate was eluted from the Sepharose beads for further analysis by SDS-PAGE and Western blotting.

**In Vitro Phosphorylation by PKA**—For in vitro phosphorylation experiments, cytosolic supernatants of recombinant PDE constructs expressed in COS7 cells were incubated for 15 min at 30 °C in a reaction mixture containing 20 mM MgCl₂, 5 mM β-mercaptoethanol, 1 μM CAMP, 0.1 mM EGTA, 0.1 mM GDP, and 0.1 μM of ATP. ATP was purified from bovine muscle ATPase (Boehringer Mannheim) and added to 1 μM MgCl₂ and 2 mm 2-mercaptoethanol. ATP was added to a final concentration of 10 μM. The reaction product 5'-AMP was then hydrolyzed by incubating the assay mixture with 50 μg of Crotalus atrox snake venom (Sigma) for 20 min at 37 °C, and the resulting adenosine was separated by anion exchange chromatography on 1 ml of AG1-X8 resin (Bio-Rad) and quantitated by scintillation counting.

**Calculations**—The GraphPad Prism program (GraphPad Software Inc., San Diego) was used to calculate \( R_0 \), \( k_o \), \( IC_{50} \), and \( K_r \) values using linear and nonlinear, Michaelis-Menten, sigmoid dose response, and one-site binding curve fits and equations, respectively.
We have demonstrated previously that the nested deletion of the C-terminal part of UCR1 (UCR1C) and/or the N-terminal part of UCR2 (UCR2N) ablates PDE4D3 dimerization. The constructs PDE4D3-H9004UCR1C and PDE4D3-H9004UCR2N used in these studies carried the deletions of 37 and 34 amino acid residues, respectively (14). Here we identified mutations that disrupt enzyme dimerization with only minor changes in the PDE4D3 amino acid sequence. As shown in Fig. 2A, both UCR1C and UCR2N are predicted to contain long -helices as a characteristic secondary structural element. By using gel filtration to analyze a set of mutants carrying nested deletions of about 10 amino acid residues, we further mapped the regions mediating dimerization within the UCR1C and UCR2N domains to the center of both helices (data not shown). Depicting these regions as helical wheels (Fig. 2B) revealed that the surface of both helices can be subdivided into a hydrophobic surface shown in blue, a charged cluster of amino acids shown in red, and a segment containing mostly uncharged hydrophilic residues shown in green, and all other amino acids in black. Name, amino acid substitutions, and subunit structure of PDE4D3 constructs generated and utilized in this study.

**RESULTS**

**Design and Expression of Monomeric PDE4D3—**We have demonstrated previously that the nested deletion of the C-terminal part of UCR1 (UCR1C) and/or the N-terminal part of UCR2 (UCR2N) ablates PDE4D3 dimerization. The constructs PDE4D3-UCR1C and PDE4D3-ΔUCR2N used in these studies carried the deletions of 37 and 34 amino acid residues, respectively (14). Here we identified mutations that disrupt enzyme dimerization with only minor changes in the PDE4D3 amino acid sequence. As shown in Fig. 2A, both UCR1C and UCR2N are predicted to contain long -helices as a characteristic secondary structural element. By using gel filtration to analyze a set of mutants carrying nested deletions of about 10 amino acid residues, we further mapped the regions mediating dimerization within the UCR1C and UCR2N domains to the center of both helices (data not shown). Depicting these regions as helical wheels (Fig. 2B) revealed that the surface of both helices can be subdivided into a hydrophobic surface shown in blue, a charged cluster of amino acids shown in red, and a segment containing mostly uncharged hydrophilic residues shown in green. A construct carrying the mutation of several charged residues in UCR1C and UCR2N had been generated previously (mutant 4D3-K1; exchanged residues are highlighted in red in Fig. 2A). The mutation of these residues has been reported to disrupt an intramolecular interaction between UCR1 and UCR2 thought to be crucial for PKA activation (18). They did not, however, disrupt PDE4D3 dimerization (14). Given the above results, we directed our mutagenesis to the

**Fig. 2. Generation of monomeric PDE4D3 constructs.** A, the scheme shows the domain structure (top), the amino acid sequence (middle), and a secondary structure prediction (bottom) for the human PDE4D3 encompassing the region between the C-terminal part of UCR1 (UCR1C) and the N-terminal part of UCR2 (UCR2N). Predicted -helical regions are shown as yellow barrels connected by wires representing predicted unstructured sequence or loops. Indicated with small vertical arrows and their amino acid position are the first and the last amino acids of the sequences shown in B and C in their helical wheel presentations. The regions deleted in the 4D3-D1 and 4D3-D2 mutants are indicated by horizontal arrows. Hydrophobic and charged amino acid residues that were mutated in this study are shown in blue and red, respectively. B and C, helical wheel presentation of UCR1C and UCR2N of the wild type enzyme (B) and the 4D3-D1 and -D2 deletion mutants (C). Charged amino acid residues are shown in red; uncharged but polar residues are shown in green; hydrophobic residues are shown in blue, and all other amino acids in black. Name, amino acid substitutions, and subunit structure of PDE4D3 constructs generated and utilized in this study.

**Fig. 3. Definition of the state of oligomerization of PDE4D3 constructs using gel filtration and density gradient centrifugation.** A, COS7 cells were transfected with vectors encoding wild type 4D3 (○) or the 4D3-M1 mutant (○). After cell harvest and lysis, the corresponding high speed supernatants were applied to size exclusion chromatography. The elution of 4D3-M1 is representative also for the 4D3-M2, -M3, -D1, and -D2 constructs. The molecular weight standard proteins reported were separated on the gel filtration column under identical conditions. B, recombinant wild type 4D3 (○) and the 4D3-M1 mutant (○), both expressed in COS7 cells, were applied to density gradient centrifugation using 5–33% linear sucrose gradients and centrifuged for 24 h at 36,000 rpm. After centrifugation, fractions of ~220 μl were collected and analyzed for PDE activity. Several marker proteins were separated on parallel gradients at the same time. The graph for 4D3-M1 is representative also for the 4D3-M2, -M3, -D1, and -D2 constructs that migrated similarly. BSA, bovine serum albumin.
modifications of the hydrophobic surfaces of both helices. To avoid disturbing the integrity of the UCR1/2 domains, selected hydrophobic residues were not substituted by charged or hydrophilic amino acids. Instead, only the hydrophobic side chains were removed by mutating the selected amino acids to alanine. Sets of four hydrophobic residues were exchanged to obtain constructs 4D3-M1, -M2, and -M3 (Fig. 2D; residues are shown in blue in Fig. 2A). Secondary structure predictions indicate that both UCR1C and UCR2N would still form α-helices in the mutant proteins despite the amino acid exchanges. In addition, we designed constructs, 4D3-D1 and 4D3-D2, that carry the nested deletion of 8 and 9 residues, respectively. Both deletion mutants were designed with the intention of shortening the helices in UCR1C and UCR2N without causing torsion. It is apparent from the comparison of helices UCR1C and UCR2N (Fig. 2, B and C) that the residues following the deletion remain in their original orientation within the helix. All mutations of four hydrophobic residues as well as the two nested deletions disrupted PDE4D3 dimerization as determined by a combination of gel filtration (Fig. 3A and Table I) and sucrose density gradient centrifugation (Fig. 3B and Table I). All monomeric 4D3 constructs behaved similarly in gel filtration and in sucrose density gradient centrifugation to the 4D3-M1 construct shown in Fig. 3, A and B. Disruption of oligomerization in the 4D3 mutants was confirmed in pull-down experiments utilizing differentially tagged 4D3 constructs (Fig. 4). Two constructs C-terminally tagged with either a V5/His or a Myc/His tag were generated for each wild type and mutant PDE4D3. The Myc/His-tagged constructs were then overexpressed in COS7 cells either alone or cotransfected with the V5/His-tagged constructs. After harvesting the cells, the V5/His-tagged constructs were immunoprecipitated from the cell lysates by using an α-V5 antibody, and the IP pellets were probed for the presence of the Myc/His-tagged proteins. As shown in Fig. 4, the α-V5 antibody specifically immunoprecipitated the V5/His-tagged constructs (lanes 2, 4, 6, and 8; immunoblot with α-V5), whereas Myc/His-tagged constructs are not recognized by the antibody (lanes 1, 3, 5, and 7; immunoblot with α-Myc). Therefore, the pull down of the Myc/His-tagged wild type PDE4D3, when co-transfected with the corresponding PDE4D3-V5/His construct (Fig. 4, lane 2), is indirect and indicates an intermolecular interaction between the two differentially tagged proteins. This interaction, however, is ablated in the mutant proteins.

**Specific Activity and Enzyme Kinetics of Monomeric PDE4D Constructs**—Having generated a set of five PDE4D3 constructs carrying minimal amino acid sequence modifications that ablate enzyme dimerization, we determined the functional consequences of dimer disruption. First, we examined if the subunit structure has an impact on the specific activity or the substrate affinity of the enzyme. When transiently expressed in COS7 cells, the wild type PDE4D3 construct was expressed at ~4 pmol/min/μg. The PDE activity expressed for all monomeric PDE4D3 mutants as well as the short splice form PDE4D2 was found to be significantly lower, ranging from 25 to 50%, in comparison to that of the PDE4D3 wild type enzyme. The expression level of all constructs was monitored by Western blotting by using three different PDE antibodies. As shown in Fig. 5A, the intensity of the K116 immunoblot signal of the mutant proteins in comparison to the wild type enzyme corresponded to the level of PDE activity relative to the wild type control. This indicates that the differences in activity recovered between the mutant and wild type proteins are due to different expression levels and not to different specific activities of the constructs. Consequently, similar specific enzyme activities are obtained for all constructs when the PDE activity measured was corrected for the Western signal intensity (Fig. 5A, black column). The data shown in Fig. 5A also were reproduced using two other PDE4D antibodies (M3S1 and α-4D3; see Fig. 1). To minimize artifacts caused by different expression levels, plasmid concentrations were adjusted to yield comparable expression levels for experiments shown in Figs. 6–10.

In addition to comparable specific activities, the PDE4D dimer (wild type PDE4D3) and monomers (PDE4D2, PDE4D3-M1) exhibit similar substrate affinities of ~2.5 μM cAMP (Fig. 5B).

### Table I

| Construct | M<sub>theor</sub> | Stokes radius (R<sub>Stokes</sub>) | Sedimentation coefficient (s<sub>v</sub>) | M<sub>calc</sub> | Co-immunoprecipitation |
|-----------|------------------|-------------------------------|---------------------------------|--------------|---------------------|
|           | ×1000            | A                             | s                               | ×1000        |                     |
| 4D3       | 76               | 62 ± 1.5                      | 5.9 ± 0.2                       | 154          | Oligomer            |
| 4D3-M1    | 76               | 49 ± 1.1                      | 3.7 ± 0.2                       | 76           | Monomer             |
Requirement of Dimerization for PKA-mediated Activation of PDE4D3

We then investigated if the mechanisms controlling PDE4 activity require enzyme dimerization. As shown in Fig. 6A, wild type PDE4D3 is activated by PKA phosphorylation by about 2.5-fold, whereas this activation is absent in all monomeric PDE4D constructs. It was shown previously that the mutation of several charged residues within UCR1C and UCR2N blocks an intramolecular interaction between these subdomains thought to be important for PKA-mediated enzyme activation (18). These mutations do not disrupt dimerization, and we have used this construct, termed 4D3-K1, as a control to distinguish between effects caused by ablation of an intramolecular interaction and disruption of dimerization. As shown in Fig. 6A, the exchange of these residues reduces but does not abolish PKA-mediated PDE4 activation. Thus, the complete loss of PKA activation in the monomeric PDE4D3 constructs is most likely caused by disruption of dimerization and not by disruption of the intramolecular UCR1/UCR2 interaction.

PDE4D3 is phosphorylated by PKA primarily at two sites, serine 13 and serine 54 (Fig. 6B). The phosphorylation of Ser-54 mediates PKA activation (6), whereas phosphorylation of both Ser-13 and Ser-54 is necessary to cause a significant shift of the phosphorylated PDE4D3 as compared with the unphosphorylated enzyme in the migration in SDS-PAGE (7). As shown in Fig. 6C, both wild type and mutant proteins...
were subjected to then harvested, and cytosolic supernatants further cultured for 3 days. The cells were APDE4D3 constructs as shown in Fig. 7A. PKA-dependent activation of PDE4D3 may be mediated in sites (Ala-54). PKA-dependent activation of Ser-54 or Ala-54 are the wild type (Ser-54) sample 3, 4, 5, or phosphorylation of both subunits of the PDE4D3 dimer might be necessary for enzyme activation (sample 3). A V5-tagged catalytically active PDE4D3 was used as a positive control for PKA-dependent PDE activation in sample 7. B–E, COS7 cells were transfected with PDE4D3 constructs as shown in A, and further cultured for 3 days. The cells were then harvested, and cytosolic supernatants were subjected to in vitro PKA phosphorylation assays. Aliquots of the resulting reactions were immunoprecipitated using the α-V5 antibody. The PDE activity of the supernatants (B and C) and the IP pellets (D and E) was then determined at a substrate concentration of 1 μM cAMP. Shown is one representative experiment out of three performed. *** significantly different, p < 0.001 (paired t test); n.s. = not significantly different.

Mechanism of PKA-mediated PDE4 Activation—To gain further insight into the mechanism of PKA-mediated activation, we applied a co-transfection/immunoprecipitation assay that allowed us to analyze changes in the activity of a single subunit within the PDE4D3 dimer. Three different oligomers are obtained by co-transfection of an enzymatically active V5-tagged PDE4D3 construct in mammalian cells (Fig. 7, A and B). After immunoprecipitation of the V5-tagged constructs, the catalytically active subunit of the 4D3/4D3-dead-V5 heterodimer is the only subunit harboring enzyme activity in the IP pellet (compare activities of samples 1–3 in Fig. 7D). By using constructs that carry an additional mutation of the crucial PKA phosphorylation site Ser-54 (Fig. 7, A and C), we could show further that the phosphorylation of both dimer subunits is necessary for full PDE activation (sample 3, Fig. 7E). The phosphorylation of only a single subunit cannot fully activate the same subunit in cis (sample 4, Fig. 7E) or its counterpart in trans (sample 5, Fig. 7E).

Role of Dimerization for Activation of PDE4D3 by Phosphatidic Acid and the K116 Antibody—Another mechanism to increase the Vmax of the long PDE4 splice variants is the binding of phosphatidic acid to the N terminus of UCR1 (UCR1N) (8). Disruption of dimerization in the 4D3-M1, -M2, -M3, -D1, and -D2 constructs greatly reduced PA-mediated PDE4 activation, whereas the up-regulation was retained in the 4D3-K1 control (Fig. 8A).

We found previously that the physiologically relevant mechanisms of PDE4 activation by PKA and PA could be simulated by using the K116 antibody generated against the C-terminal part of UCR2 (UCR2C) (28). As shown in Fig. 8B, the disruption of dimerization in the mutant constructs once again greatly reduced the ability of this antibody to increase cAMP hydrolysis. The ~20% activation that remained in the monomeric PDE4D3 constructs was present also in the monomeric splice variant PDE4D2.

Inhibitor Sensitivity of Monomeric PDE4D Constructs—PDE4 isoenzymes exist in at least two different conformations termed HARBS and LARBS (20–22). In order to determine whether the oligomerization state of a PDE4 has an impact on
these conformational states, (R)-rolipram binding assays and inhibition studies were performed. As shown in Fig. 9, the binding affinity of HARBS was unchanged in all constructs with $K_d$ values $\leq 3 \text{ nM}$ (Fig. 9B), whereas the total binding in the high affinity binding state was reduced in the monomeric constructs to 25–45% compared with that of the wild type enzyme (Fig. 9, A and B). To further elucidate these differences, we measured the dose-dependent rolipram inhibition of the 4D3 constructs. As shown in Fig. 10, the dimeric wild type 4D3 displayed a significantly lower IC$_{50}$ for (R)-rolipram than the monomeric constructs, whereas the sensitivity toward RP73401, an inhibitor that binds to HARBS and LARBS with similar affinities, was identical between all constructs. The rolipram dose-response curves of dimers and monomers also differed significantly in their slopes.

**DISCUSSION**

The identification of $>50$ PDE isoenzymes, the comparison of their primary structures, and their subsequent classification into 11 PDE families have established that PDEs share a conserved domain organization consisting of a C-terminal catalytic domain and an N-terminal domain containing unique structural elements that function to regulate PDE activity (1). Furthermore, it is generally accepted that most PDEs exist as dimers. In the present study, we provide evidence that enzyme dimerization is a structural requirement for the activation of PDE4D mediated by PKA phosphorylation of, or phosphatidic acid binding to, its N-terminal UCR domains. The PDE4 dimerization domain was mapped previously within the region encompassing the C-terminal half of UCR1 (UCR1C) and the N-terminal half of UCR2 (UCR2N) (14). In addition, it has been reported that several charged residues...
located within the same subdomains mediate an intramolecular UCR1C/UCR2N interaction but are not involved in dimerization (18, 14). In the present study, we show that substitution of several of their neighboring hydrophobic amino acid residues with alanine disrupts dimerization. Based on the helical wheel presentations shown in Fig. 2, it is tempting to speculate that hydrophobic surfaces of their α-helices mediate dimerization, and the surfaces consisting of charged residues mediate an intramolecular interaction. This model of tertiary and quaternary structure of PDE4 is, however, hypothetical and can only be confirmed by three-dimensional structure analysis. In this context, it also should be noted that identification of several hydrophobic amino acids that disrupt dimerization cannot be taken as proof that these are indeed the very residues that mediate the dimer interaction of the PDE4 long forms. A crucial role of these residues for the assembly of single subunits into a dimer during the protein folding process might provide an alternative explanation. As an interesting analogy, however, the atomic structure of its GAF domains revealed that PDE2 dimerization is mediated by several hydrophobic residues located within α-helices (10).

Several lines of evidence suggest that the functional differences between the dimeric wild type enzyme and the monomeric mutants are indeed due to disruption of dimerization and are not the result of other effects caused by the amino acid exchanges. First, all five monomeric constructs behaved similarly with regard to all parameters tested. Considering the structural heterogeneity of the mutations and the minimal disruptions introduced, it is likely that the functional properties affected are only a consequence of the disruption of dimerization. Second, several enzyme properties such as specific activity, substrate affinity, and sensitivity toward the PDE4 inhibitor RP73401 are identical in both mutant and wild type enzymes indicating that the mutant proteins are properly folded and their overall structure and function are not affected. Third, the monomeric constructs behave similarly to the native monomeric PDE4 splice variant PDE4D2 but are functionally distinguishable from a mutant carrying mutations in UCR1/2 that does not disrupt dimerization (4D3-K1). Finally, a co-transfection/immunoprecipitation assay provided independent proof that a single PDE4D3 subunit cannot be fully activated in cis by PKA phosphorylation, confirming a critical role of dimerization in PDE4D.

Although the specific activity of all PDE4D constructs was found to be similar, we observed that monomeric constructs were expressed at a lower level than the dimeric wild type PDE4D3 when similar amounts of plasmid DNA were transfected into COS7 cells. These differences might be explained by a reduced transcription/translation level and/or a higher sensitivity toward protein degradation of PDE4 monomers in com-

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**Table II**

**Subunit structure and regulatory features of PDE families**

| PDE family | Subunit structure (interacting domain) | Ligand binding or phosphorylation site at the N terminus (effect on PDE activity) |
|------------|---------------------------------------|---------------------------------------------------------------------------------|
| PDE1       | Dimer (unknown)                       | CaM⁺ (Activation)                                                               |
| PDE2       | Dimer (N terminus, GAF-A)             | cGMP (Activation)                                                              |
| PDE3       | Dimer (unknown)                       | PKA, PRB (Activation)                                                          |
| PDE4       | Dimer (N terminus, UCR1/2)            | PKA, PA⁺ (Activation)                                                          |
| PDE5       | Dimer (N terminus)                    | cGMP/PKG (Activation)                                                          |
| PDE6       | Dimer (N terminus, GAF-A)             | Tdc/Pk⁺/γcGMP (Activation)                                                     |
| PDE7       | Unknown                               | Unknown                                                                         |
| PDE8       | Unknown                               | Unknown                                                                         |
| PDE9       | Unknown                               | Unknown                                                                         |
| PDE10      | Unknown                               | Unknown                                                                         |
| PDE11      | Unknown                               | Unknown                                                                         |

CaM indicates Ca²⁺-calmodulin; PA indicates phosphatidic acid; Tdc indicates transducin; Pk indicates PDE6-Pk subunit; PKB indicates protein kinase B; PKG indicates protein kinase G.
Significance of PDE4 Dimerization

W. Richter and M. Conti, unpublished observations.

parison to the dimeric enzyme. This assumption would be consistent with mechanisms regulating PDE activity often observed in vivo. The activity of PDE4 long forms is typically regulated by phosphorylation, ligand binding, and protein/protein interactions (8, 29, 30, 35, 36), whereas their protein level in the cell is relatively stable or varies only slightly (e.g. -3-fold inductions of protein were reported). In contrast, the activity of short splice forms in the cell is often controlled by major changes of transcription/translation levels (e.g. a 100-fold induction of mRNA and protein) in response to hormonal or neurotransmitter signals (31–33). Thus, although long PDE4 splice forms show a slow protein turnover and are regulated primarily in short term feedback loops by post-translational modifications, the short PDE4 splice forms likely exhibit a fast protein turnover, and changes in PDE activity are dependent on rapidly reversible accumulation of the protein in the cell. However, these possibilities were not investigated further in the present study.

Comparing several monomeric PDE4D3 mutants with the dimeric wild type PDE4D3, we were able to show that disruption of dimerization ablates PDE activation mediated by either PKA phosphorylation, phosphatidic acid binding, or binding of the antibody K116 shown previously to mimic the PDE activation triggered by PKA or PA (28). To explain these findings, one could envisage that the wild type enzyme can exist in two states: a “high activity” and a “low activity” conformation. PKA phosphorylation or PA binding stabilizes the enzyme in its high activity conformation. As monomeric PDE4 constructs are not further activated by PKA phosphorylation or PA binding, the question remains whether they are confined in a low activity or a high activity conformation. Two findings suggest that monomeric constructs are in a low activity state and cannot switch to a high activity conformation. First, the specific activities of unphosphorylated monomers and dimers are very similar, indicating that the monomers are in a low activity state similar to the dimeric construct. Second, it is well established that PDE4D3 shows a shift toward a higher rolipram sensitivity upon phosphorylation by PKA. The monomeric PDE4D4 constructs, however, show a reduced rolipram sensitivity as compared with the dimer (Fig. 10A), and their rolipram sensitivity does not change upon PKA phosphorylation.2

It is noteworthy that disruption of dimerization does not only block the activation of PDE4 mediated by PA and PKA, which act N-terminally to the UCR1C/UCR2N dimerization module, but also the activation mediated by the K116 antibody, which binds C-terminally to the dimerization domain. This indicates that the conformational differences caused by the disruption of dimerization are not restricted to the UCR1C/UCR2N module but impact the overall conformation of the PDE4 protein.

Despite differences in the primary structure of their N-terminal domains, there is some structural and functional homology among the PDE families. Most PDEs exist as dimers (and dimerization is most likely mediated by their N-terminal domains), and they are all regulated (usually activated) by phosphorylation of, or ligand binding to, their N terminus (see Table II). Thus, the possibility should be entertained that similar molecular mechanisms regulate enzyme activity upon modulation of the PDE N termini. We show here that activation of PDE4 by PKA phosphorylation or PA binding depends on enzyme dimerization and, furthermore, that both dimer subunits must be PKA-phosphorylated to fully activate the enzyme. It will be interesting to determine whether other PDEs have similar structural requirements for modulation of their enzyme activity. At odds with our hypothesis, one report on the frog rod photoreceptor PDE suggested that a single subunit of the PDE6 α/β-heterodimer can be activated quite independently by releasing the inhibitory Pγ subunit bound to it (37). However, PDE6 is the only PDE that forms heterodimers instead of homodimers, and it is the only enzyme directly activated by a G-protein (transducin), and its activation mechanism involves two additional inhibitory Pγ subunits that bind to the catalytic α/β subunits. Differences in the affinity between α and Pγ and β and Pγ may explain the independent activation of the α and β subunits of PDE6.

Because of their role in regulating cell responses in immune cells, PDE4 isoenzymes are considered a promising target for the development of PDE4 inhibitors as anti-inflammatory drugs, and clinical trials are presently underway probing the use of PDE4 inhibitors as anti-asthmatics (20). It has been shown that PDE4 proteins exist in at least two conformations that exhibit different affinities toward the classic PDE4 inhibitor rolipram termed HARBS and LARBS (21, 22). It has been proposed that LARBS is predominant in inflammatory cells and correlates with several (although not all) anti-inflammatory effects of PDE4 inhibitors, whereas HARBS is predominant in brain tissue and has been associated with undesirable side effects of PDE4 inhibitors such as emesis. It has been suggested that HARBS is represented by a PDE4 with fully occupied metal ion binding sites, whereas LARBS represents the apoenzyme (23, 24). As the apoenzyme is enzymatically inactive, this hypothesis cannot fully explain the changes of inhibitor sensitivities observed upon post-translational modifications of PDE4 (6, 30, 34). Therefore, it is more likely that PDE4 exists in multiple conformations that can be distinguished according to their rolipram sensitivity.

In our present study, we show that although dimerization is not a requirement for high affinity rolipram binding, it functions to stabilize long PDE4 forms in their HARBS conformation. Conversely, the monomeric short splice forms exist to a higher proportion in their LARBS conformation. Our data show that PDE4 monomers can be recovered in HARBS conformation, although to a lesser extent than dimers. Therefore, the predominance of LARBS reported in immune cells and HARBS in neuronal cells cannot be explained solely by the predominance of monomeric PDE4 short forms or dimeric PDE4 long forms in these tissues. Besides post-translational modifications, protein/protein interactions, and tissue-specific expression of pde4 genes, the subunit structure of PDE4 splice forms may, however, contribute to some extent to the tissue-specific distribution of HARBS and LARBS.

In summary, we show that dimerization is a structural requirement for regulating PDE4 activity by post-translational modifications and the stabilization of PDE4 in its high affinity rolipram binding conformation. Based on the structural and functional homologies between different PDE families, we suggest that dimerization and the modification of both dimer subunits may be properties that are evolutionarily conserved among all PDEs as a requirement for enzyme activation.

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The Oligomerization State Determines Regulatory Properties and Inhibitor Sensitivity of Type 4 cAMP-specific Phosphodiesterases
Wito Richter and Marco Conti

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