Phosphodiesterase 4D and Protein Kinase A Type II Constitute a Signaling Unit in the Centrosomal Area*

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The mediation of cAMP effects by specific pools of protein kinase A (PKA) targeted to distinct subcellular domains raises the question of how inactivation of the cAMP signal is achieved locally and whether similar targeting of phosphodiesterases (PDEs) to sites of cAMP/PKA action could be observed. Here, we demonstrate that Sertoli cells of the testis contain an insoluble PDE4D3 isoform, which is shown by immunofluorescence to target to centrosomes. Staining of PDE4D and PKA shows co-localization of PDE4D with PKA-RIIα and RIIβ in the centrosomal region. Co-precipitation of RII subunits and PDE4D3 from cytoskeletal extracts indicates a physical association of the two proteins. Distribution of PDE4D overlapped with that of the centrosomal PKA-anchoring protein, AKAP450, and AKAP450, PDE4D3, and PKA-RIIα co-immunoprecipitated. Finally, both PDE4D3 and PKA co-precipitate with a soluble fragment of AKAP450 encompassing amino acids 1710 to 2872 when co-expressed in 293T cells. Thus, a centrosomal complex that includes PDE4D and PKA constitutes a novel signaling unit that may provide accurate spatio-temporal modulation of cAMP signals.

The broad specificity protein kinase A (PKA)1 and phosphodiesterases (PDEs) have complementary roles in cAMP signaling. Whereas PKA phosphorylation is activated by cAMP (1), PDEs degrade cAMP and inactivate the cAMP signal (2). Subcellular targeting of components in the cAMP signaling pathway is essential to elicit discrete cellular effects. Compartmentalization of PKA is mediated by interaction with a number of protein kinase A-anchoring proteins (AKAPs) (3), whereas little is known about subcellular targeting of PDEs even though examples of interactions with putative anchor proteins is now emerging as shown, e.g., for PDE4D3 (4) and PDE4D5 (5). Here we have investigated the colocalization of PDE4D and PKA regulatory subunits in an endocrine cell and provided evidence that PDE4D3 and PKA are in a functional complex coordinated by AKAP450.

EXPERIMENTAL PROCEDURES

Cell Culture—Sertoli cell primary cultures were prepared from testes of 19-day-old Harlan Sprague Dawley rats (B&K Universal AS, Nittedal, Norway) according to the method of Dorrington et al. (34) with some modifications (6). The cells were plated in 10-cm culture dishes (Nunc, Copenhagen, Denmark) for protein analysis and cultured in Eagle’s minimum essential medium (Life Technologies, Inc.; Grand Island, NY) with addition of streptomycin (100 mg/liter), penicillin (105 IU/liter), fungizone (0.25 mg/liter), L-glutamine (2 mM), and fetal calf serum (10% Life Technologies, Inc.) at 32 °C in a humidified atmosphere with 5% CO2. After 3 days, the cells were incubated further in serum-free modified minimum essential medium. For immunocytochemistry, Sertoli cells were plated densely to avoid overgrowth of peritubular cells, cultured for 4 days to allow release of germinall cell, and then trypsinized and spread on glass coverslips at lower density to allow examination of single cells.

Cell Fractionation—Sertoli cells (10 culture dishes; 10 cm; 12 × 10⁶ cells/dish) were washed in cold phosphate-buffered saline and then scraped in isonic buffer (250 mM sucrose, 20 mM Tris-Cl, pH 7.8, 1 mM EDTA, 10 mM MgCl2, 50 mM NaF, 10 mM β-mercaptoethanol, 50 mM benzamidine, 2 mM PMSF, and Complete™ antiprotease mix; Roche Molecular Biochemicals), homogenized (Dounce homogenizer, 10 strokes), and centrifuged at 200,000 × g. Supernatants (S200) and subsequent extracts of the pellet in isonic buffer with 1% Triton X-100 (15,000 × g supernatant, TX-100) and then with RIPA buffer (15,000 × g supernatant, RIPA) were homogenized in pH 8.0, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) were prepared.

Immunological Procedures and Antibodies—Immunoblotting, immunoprecipitation, and immunofluorescence analyses were performed as described elsewhere (7, 8). For immunofluorescence, observations were made with an Olympus BX60 microscope, and photographs were taken with a JVC camera and AnalySIS software (Soft Imaging Systems). Monoclonal Ab M3S1 directed against PDE4D (9), mAbs directed against RIIα, RIIβ, and RIβ (cat. nos. P53620, P55120, and 54720, respectively; developed by K. Tasken in collaboration with Transduction Laboratories, Lexington, KY) (10, 11), and affinity-purified rabbit polyclonal antibodies against RIα and RIβ (12) were used for Western blotting, immunoprecipitation, and immunofluorescence as indicated in the figure legends. The centrosomal marker mAb CTR453 (13) was kindly supplied by Dr. Michel Bornens, Curie Institute, Paris, France. A24 antibody was raised against a purified recombinant fragment of the chicken AKAP450, affinity purified and characterized and shown to specifically interact with human AKAP450.7 The anti-GFP antibody was from CLONTECH (Palo Alto, CA).

RESULTS AND DISCUSSION

We investigated localization of PDEs in Sertoli cell primary cultures that are highly hormonally responsive and known to contain isoforms of the cAMP-specific PDE4D (14). Subcellular fractionation of Sertoli cells from 19-day-old rats revealed the presence of a soluble PDE4D1/2 (Fig. 1a, S200), which was highly inducible by cAMP (compare basal B versus cultured

7 W. A. Kemmner, unpublished observations.

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Figs. 1 and 2. Immunolocalization of PDE4D and PKA in Sertoli cells. Sertoli cells were stimulated with 300 μM 8-CPT-cAMP for 48 h (a, right panels, b and c) or left untreated (a, left panels). Cells were fixed with 3% paraformaldehyde and permeabilized with 0.5% Triton X-100 (a, b, left panel, and c) or simultaneously permeabilized, extracted, and fixed with −10 °C methanol for 5 min to eliminate soluble cytoplasmic epitopes (b, right panel). Immunofluorescence analysis was performed using mAb M3S1 directed against PDE4D (20 μg/ml), affinity-purified rabbit polyclonal antibodies against RIα (1 μg/ml) or RIβ (5 μg/ml, mAb CTR453) (13) (140 ng/ml), and fluorescein isothiocyanate-labeled anti-mouse IgGs (green) and TRITC-labeled anti-rabbit IgGs (red) in the second layer (1:100 dilutions). Dual image overlays of PDE4D (green) and RIα (a, upper row, red) or RIβ (a, lower row and b, red) are shown. c, separate and merged images of centrosome (CTR453, green) and RIβ (red) labeling. DNA was stained with 0.1 μg/ml Hoechst 33342. Bars, 10 μm. Representative observations from examination of many fields from three or more experiments are presented.

Fig. 1. Subcellular localization of PDE4D isoforms in Sertoli cells. a, Sertoli cell primary cultures from 19-day-old rats were incubated with a permeable cAMP analog, 8-CPT-cAMP (100 μM), for 24 h (S) or left untreated (B) and subsequently homogenized in isotonic buffer. Supernatants (S200) and subsequent extracts of the pellet in isotonic buffer with 1% Triton X-100 (15,000 × g supernatant, Ts-100) and then with RIPA buffer (15,000 × g supernatant, RIPA) were subjected to SDS-PAGE (40 μg of each fraction) and immunoblot analysis for the presence of PDE4D (upper panel, M3S1 mAb that recognizes all PDE4D isoforms; similar data were obtained with mAb F34–8F4 from Pfizer), RIα (upper middle panel), RIβ (lower middle panel), and R1α (lower panel). The mobility of molecular size markers (in kDa) are indicated. b, cells were fractionated to yield a RIPA extract from the detergent-insoluble fraction as in a and subjected to immunoblot analysis for PDE4D. Recombinant PDE4D2 and PDE4D3 were used as standards (Std). A representative of three experiments is shown. (S)). Furthermore, an insoluble PDE4D3 isoform was observed in the pellet from the 200,000 × g fractionation, a majority of which could be solubilized by non-ionic detergent (Fig. 1a, Ts-100). In addition, a minor pool of PDE4D3 that remained in the pellet after repeated detergent extraction could be extracted by a RIPA buffer containing 0.1% SDS (Fig. 1, a and b, RIPA). In agreement with observations made in thyroid cells, the level of PDE4D3 was not regulated by cAMP (15). Examination of PKA distribution revealed RIα associated with soluble and particulate fractions, whereas RIβ was present at lower levels but was induced by cAMP in all cellular compartments (as described previously in Refs. 16 and 17). Notably, induction of RIβ by cAMP seemed to displace RIα from the particulate fraction. RIα was mainly present in the soluble fraction but appeared also in particulate fractions after cAMP treatment.

Investigation of the PDE4D subcellular distribution by immunostaining demonstrated distinct perinuclear dots (Fig. 2a, upper and lower panels, green label) that could be identified as centrosomes by co-labeling with a centrosomal marker (compare Figs. 2e and 4a). In addition, some weaker and more disperse staining was observed in the Golgi region that could account for the detergent-soluble PDE4D3. In co-staining with RIα, PDE4D3 and RIα co-localized, but RIα had a wider distribution in the Golgi-centrosomal area (Fig. 2a, red label; co-localization appears yellow) as also described for a number of other cell types (12). In contrast, RIβ was only detected at low levels in the Golgi-centrosomal region of unstimulated cells and was distinctly co-localized with PDE4D in the centrosomal region of cAMP-treated cells (Fig. 2a, lower panels). Cyclic AMP treatment also induced strong labeling of PDE4D in the cytoplasm (Fig. 2a, right panels). This staining most likely represents the cAMP-induced soluble PDE4D1/2 described in Fig. 1, as fixation with cold methanol extracted the cytoplasmic PDE4D staining (Fig. 2b). Dual immunofluorescence labeling of cAMP-treated Sertoli cells for RIβ and a well-characterized centrosomal marker, CTR453 (13), revealed a full overlap in the merged image (Fig. 2c). This implies that RIβ and PDE4D (Fig. 2a, lower right panel) are targeted to the centrosomal region. Compared with the immunoreactive PKA and PDE4D in the soluble S200 fraction, there is an apparent lack of cytoplasmic PKA and PDE by immunostaining, presumably because such diffuse signals are below the detection level.

To further investigate the physical association of PDE4D and PKA type II in the centrosomal region, Triton X-100-insoluble fractions from cAMP-treated Sertoli cells were solubilized in RIPA buffer and photoaffinity-labeled by 8-azido-[32P]cAMP to visualize all R subunits. This approach demonstrated the presence of specifically labeled RI (α and β) as well as R1I (Fig. 3a, Input). Addition of excess unlabeled cAMP in the photoaffinity reaction showed specificity of labeling. Immunoprecipitation of photoaffinity-labeled extracts with PDE4D-specific antibody (mAb M3S1) showed co-precipitation of RIα and RIβ but not RI, whereas no precipitation was observed in controls with non-immune IgG (Fig. 3a, two right lanes). Conversely, immunoprecipitation of both RIα and RIβ co-precipitated PDE4D3 from RIPA extracts of cAMP-treated Sertoli cells (Fig. 3b and not shown). No co-precipitation was observed in soluble frac-
Cyclic AMP-binding proteins were photoaffinity-labeled by incubation with 1 μM 8-azido-[32P]cAMP in the absence (−) or presence (+) of 100-fold excess cold competitor for 1 h on ice followed by UV radiation at 254 nm for 10 min as described (32). Five percent of the labeling reaction was directly subjected to SDS-PAGE and autoradiography (Input). Immunoprecipitation was performed by incubation of labeled extract (500 μg protein, 2.5 μg/μl) with anti-PDE4D antibody (M3S1 mAb; 50 μg/ml) or non-immune mouse IgG (IgG) for 2 h at 4 °C followed by incubation with protein A/G beads (25 μl of 1:1 slush) for 16 h and four rounds of washing of the precipitates before analysis together with Input. b, immunoprecipitation was performed as above using RIPA extracts (500 μg of protein, 2.5 μg/μl) and anti-RIIα mAb (25 μg/ml) or irrelevant mouse IgG (IgG). Immunoblot analysis for PDE4D and RIIα was performed as in Fig. 1. Recombinant PDE4D3 was used as standard (Std.). Mobility of molecular size markers is indicated in kDa. c, immunoprecipitation was performed as in panel b but using the PDE4D2-containing S200 fraction from cAMP-treated cells (see Fig. 1) as source. Precipitates and supernatant after immunoprecipitation were analyzed for the content of PDE4D2. Recombinant PDE4D2 was used as standard (Std.). Mobility of molecular size markers is indicated in kDa. A representative of four (panels a and b) and two (panel c) experiments is shown.

The centrosomal region harbors a number of large structural proteins that surround centrioles and participate in nucleation and stabilization of microtubules in interphase and regulation of spindle formation at mitosis (reviewed in Refs. 18, 19). Centrosomal proteins that have been shown to anchor PKA include AKAP450 (also called AKAP350, CG-NAP, Hyperion and yotiao), pericentrin, and in male germ cells hAKAP220 (20–24). To explore the possibility that PDE4D and PKA are tethered by a common anchoring protein, we examined whether PDE4D is associated with AKAP450. Dual immunostaining of PDE4D and AKAP450 showed overlap of the two proteins in the centrosomal region (Fig. 4a), but not in the Golgi region stained by several AKAP450 antibodies (20). As the epitope of the centrosomal marker used for dual staining with PKA in Fig. 2c also maps to AKAP450,2 PKA is also co-localized with the AKAP450-PDE4D complex in Sertoli cells. Furthermore, PDE4D3 and PKA-RIIα were detected in AKAP450 immune precipitates from extracts of partially purified centrosome-nuclei complexes (Fig. 4b, upper panels). Conversely, anti-PDE4D co-precipitated AKAP450 (lower panel). Finally, expression of a soluble fragment of AKAP450 (amino acids 1710–2572) fused to GFP-bound PDE4D3 as shown by co-immunoprecipitation of GFP-AKAP450 and PDE4D3 when co-transfected into 293T cells (Fig. 4c). In contrast, overlapping fragments spanning the other domains of AKAP450 did not associate with PDE4D3 when co-expressed (not shown). The interaction between AKAP450 and PDE4D is known to be mediated by the DXV domain (25–28) of the AKAP450 C terminus, which is essential for AKAP450 interaction with PKA (29). In contrast, the DXV domain is not essential for the interaction between AKAP450 and PDE4D3, as shown by the fact that the AKAP450 DXV mutant still interacts with PDE4D3 (not shown). Collectively, the data presented here indicate the presence of a common anchoring protein, we examined whether PDE4D is associated with AKAP450. Dual immunostaining of PDE4D and AKAP450 showed overlap of the two proteins in the centrosomal region (Fig. 4a), but not in the Golgi region stained by several AKAP450 antibodies (20). As the epitope of the centrosomal marker used for dual staining with PKA in Fig. 2c also maps to AKAP450,2 PKA is also co-localized with the AKAP450-PDE4D complex in Sertoli cells. Furthermore, PDE4D3 and PKA-RIIα were detected in AKAP450 immune precipitates from extracts of partially purified centrosome-nuclei complexes (Fig. 4b, upper panels). Conversely, anti-PDE4D co-precipitated AKAP450 (lower panel). Finally, expression of a soluble fragment of AKAP450 (amino acids 1710–2572) fused to GFP-bound PDE4D3 as shown by co-immunoprecipitation of GFP-AKAP450 and PDE4D3 when co-transfected into 293T cells (Fig. 4c). In contrast, overlapping fragments spanning the other domains of AKAP450 did not associate with PDE4D3 when co-expressed (not shown). The interaction between AKAP450 and PDE4D is known to be mediated by the DXV domain (25–28) of the AKAP450 C terminus, which is essential for AKAP450 interaction with PKA (29). In contrast, the DXV domain is not essential for the interaction between AKAP450 and PDE4D3, as shown by the fact that the AKAP450 DXV mutant still interacts with PDE4D3 (not shown). Collectively, the data presented here indicate the presence of...
a signaling complex consisting of PKA and PDE both assembled via a common anchoring protein. Because the components of this complex are present at low levels in the insoluble fraction, the stoichiometry of the complex is difficult to assess quantitatively. However, based on examination of input lysates and supernatants following immunoprecipitation, it appears that all the PDE4D3 and AKAP450 in the RIPa fraction goes into a PDE3-PKA-AKAP450 complex, whereas a part (5–20%) of PKA RIα and/or RIβ is in a complex with AKAP450 and PDE4D3. This is consistent with earlier observations of PKA associated with other cytoskeletal structures and other AKAPs in the insoluble material.

Targeting of PKA and PDE in close proximity to the PKA substrate will allow for a tight control of the phosphorylation state of proteins regulated by cAMP signaling. Spatial control is achieved by targeting of PKA. Temporal control and inactivation of the effect of cAMP on PKA is accomplished by complexing of PDE at the same site (Fig. 5). It should also be noted that the particular PDE4D3 variant that co-locates with PKA RIα can be phosphorylated and activated by PKA. Therefore, the physical association of PKA and PDE4D3 establishes a local feedback regulation whereby cAMP activates PKA by dissociation of C, which in turn phosphorylates and activates PDE4D3 (15). The resulting decrease in cAMP causes reassociation of C and inactivation of PKA, rather than diffusion of C into the cytoplasm. The proximity of PKA and PDE and this rapid feedback mechanism indicate that the cAMP response may occur only in a confined compartment of the cell. Several AKAPs have been shown to serve as anchoring proteins for multiple signal molecules to form signaling complexes where signals can be modulated by co-localization of PKA and protein phosphatases (25–27), or several signals can be integrated by co-localization of e.g. PKA and PKC (28, 29). The PKA-PDE complex facilitated by dual anchoring via AKAP450 adds to this list and provides a way of modulating signal intensity at the level of cAMP. Furthermore, the capacity of AKAP450 to anchor PDE4D3 extends the functions of this large protein that has already been shown to anchor PKA, protein kinase N, and PP1 (20, 22, 27). The fact that centrosomal PKA, at least in some cells, have a co-targeted PDE suggests the importance of a tight regulation of the phosphorylation state of centrosomal proteins, possibly implicated in regulation of microtubule stability where cAMP has a distinct regulatory effect (19, 30, 31). It will be interesting to determine whether PKA and PDEs also co-localize at other sites of PKA action where a tightly regulated mechanism of termination of the cAMP signal is important.

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