Articles

Characterization of a Hormone-Inducible, High Affinity Adenosine 3′−5′-Cyclic Monophosphate Phosphodiesterase from the Rat Sertoli Cell†

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ABSTRACT: In previous reports we have shown that FSH and β-adrenergic agonists regulate the levels of mRNA and increase the activity of a high affinity cAMP phosphodiesterase (cAMP-PDE) in the immature rat Sertoli cell in culture. To identify and characterize the hormone-inducible form(s), the cAMP-PDE activity of the Sertoli cell was partially purified and its properties were determined using biochemical and immunological tools. The cAMP-PDE activity present in the 100000*g* supernatant of Sertoli cell extracts was purified more than 2000-fold by four HPLC chromatographic steps. The major purified form of cAMP-PDE had a specific activity of 1−2 μmol/(min·mg of protein). Polyacrylamide gel electrophoresis and silver staining analysis showed that a 67−68 kDa polypeptide comigrated with the major peak of cAMP hydrolytic activity. The molecular weight of the crude or purified enzyme determined by gel filtration and sucrose density gradients was 150000, suggesting that the native enzyme is an oligomeric structure. This PDE hydrolyzed cAMP with a K_m of 1.97 ± 0.26 μM. The hydrolysis of cAMP was neither inhibited nor stimulated by cGMP concentrations lower than 50 μM. Cyclic nucleotide catalysis required Mg^{2+}, but was insensitive to Ca^{2+}. The activity of this form was competitively inhibited by several inhibitors with the following potency: rolipram > RO 20-1724 > methylisobutylxanthine > cilostamide = milrinone. Because mRNAs derived from two distinct PDE4B and PDE4D genes are present in the Sertoli cell, selective and nonselective PDE antibodies were used to determine the origin of the inducible PDE protein. The partially purified PDE was quantitatively precipitated with nonselective or PDE4D-specific antibodies. Conversely, only negligible activities were immunoprecipitated with the PDE4B-selective antibody. Immunoblot analyses using crude preparations or preparations at different stages of purification demonstrate the presence of a predominant 67 kDa band. The nonselective and the PDE4D-selective antibody, but not the PDE4B-selective antibody, recognized this polypeptide. The accumulation of the 67 kDa polypeptide was induced by FSH, confirming that this is the major hormone-regulated form. This 67 kDa PDE recovered from the Sertoli cell had a molecular weight different from the form with similar immunological properties expressed in brain. These data indicate that the major PDE form regulated by hormones and cAMP is a high affinity, cGMP insensitive 67 kDa cAMP-PDE protein variant product of the PDE4D gene.

It is now clear that a large number of cyclic nucleotide phosphodiesterases are expressed in mammalian cells. It has been established that at least 7 different families of PDEs are present, and several forms have been extensively characterized (Beavo & Reifsnyder, 1990, Beavo et al., 1994). The physiological significance of this heterogeneity is unclear, but it is generally agreed that differences in specificity, catalytic activity, and regulation provide the cells with a complex means to dispose of cyclic nucleotides (Beavo, 1988).

Characterization of members of the 4th family of PDEs, termed cAMP-PDEs, has been hampered by the low abundance and instability of these proteins. Discrepancies have been reported on the kinetic characteristics as well as sizes and specific activities of these forms (Thompson et al., 1979; Marchmont et al., 1981). In several instances (Thompson et al., 1979; Marchmont et al., 1981; Moore & Schroedter, 1982; Nemoz et al., 1989; DeMazancourt & Guidicelli, 1988) attempts to purify cAMP-PDEs have yielded specific activities 1 order of magnitude lower than that shown for the Ca^{2+}, calmodulin-dependent cGMP PDE (CaM PDE) (Sharma et al., 1980, 1984), the cGMP-stimulated PDE (Martins et al., 1982; Yamamoto et al., 1983), or the cGMP-inhibited PDE (Grant & Colman, 1984; Degerman et al., 1987; Harrison et al., 1987). To date, purification of a native cAMP-PDE and

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the clear association of the activity with a nonproteolyzed polypeptide are still lacking.

In the Sertoli cell of the rat testis, a cAMP-PDE is under the control of the gonadotropin FSH (Conti et al., 1982). Increase in intracellular cAMP by several means mimics the hormone effect (Conti et al., 1982). In addition, at least two different cDNAs (PDE4B and PDE4D) that code for cAMP-PDEs have been isolated from Sertoli cell libraries prepared from cells treated with dibutyryl cAMP (Swinnen et al., 1989b). These cDNAs are derived from the PDE4D and PDE4B genes. Using these cDNAs as a probe, we have demonstrated that hormones and cAMP regulate mRNA levels coding for these PDEs (Swinnen et al., 1989b). Here, we report the partial purification and characterization of a cAMP-PDE protein from the rat Sertoli cell and the association of the catalytic activity of this form with a 67 kDa polypeptide derived from the PDE4D gene.

EXPERIMENTAL PROCEDURES

Antibody Generation. The peptide CVSEYISNTFLD-KQHEVEIPSPT (peptide no. 2224) was designed on the basis of a region of the amino terminus domain of the deduced amino acid sequence of ratPDE3, a PDE cDNA isolated from an immature rat Sertoli cell library (Swinnen et al., 1989b). The characteristics of the PDE encoded by this clone have been reported (Swinnen et al., 1989b). Two antisera (K111 and K116) generated by injecting this peptide were used because they had different affinity for the PDE4A and PDE4B protein. While K116 has an affinity for recombinant PDE4D 100-fold higher than for PDE4A and PDE4B, antibody K111 recognized PDE4D, PDE4A, and PDE4B with affinities in the same range (less than 10-fold differences; Iona et al., manuscript submitted). To generate antibodies that would discriminate between ratPDE3/PDE4D and ratPDE4/PDE4B, the carboxyl terminus region of these proteins was used. *Bam*HI−*Eco*RI fragments were prepared from the 3′ end of ratPDE3.1/PDE4D.1 and ratPDE4.2/PDE4B.2 cDNAs and were subcloned in the bacterial expression vector pGEX (Smith & Johnson, 1988). Expression of these constructs in *Escherichia coli* produces a protein which is the result of the fusion of the coding region of the glutathione *S*-transferase (GST) and the carboxyl terminal portion of ratPDE3/PDE4D and ratPDE4/PDE4B. These fusion proteins were isolated on a single step affinity chromatography on glutathione−Sepharose according to the manufacturer's recommendations. The GST-PDE4B protein was used to generate polyclonal antibodies in rabbits (K118), while the GST-PDE4D protein was used to generate monoclonal antibodies (M3S1). These antibodies had affinities for their respective antigen that was at least 3 orders of magnitude higher than for other PDE4. A thorough characterization of the monoclonal antibody M3S1 against PDE4D and polyclonal K118 against PDE4B is detailed elsewhere (Iona et al., manuscript submitted).

Sertoli Cell Cultures. Sertoli cell cultures were prepared from 15−25-day-old Sprague-Dawley rats. In a typical preparation, between 60 and 100 rats were used. The seminiferous tubule preparation by enzymatic treatment followed the procedure described by Dorrington et al. (1975) with minor modifications (Conti et al., 1982). Cells were plated in 75 or 150 cm^2 flasks in minimum essential medium with Earle's salts supplemented with 20 mM glutamine, 1 mM pyruvate, and 5% inactivated fetal calf serum. After 24 h, medium was removed, cells were rinsed once with Hank's balanced salt solution (HBSS), and fresh medium without serum was added. On the fourth day of culture, fresh medium containing 1 mM dibutyryl cAMP was added and the cells were maintained in the presence of the cAMP analog for 24−36 h.

Cell Harvesting and Preparation of Soluble Extracts. Each flask was rinsed once with 10 mL of HBSS, and the medium was aspirated. Flasks were placed on ice, and all the following manipulations were conducted at 4 °C. Approximately 2 mL/flask of homogenization buffer (HB) containing 20 mM Tris-HCl, pH 8.0, 10 mM NaF, 1 mM EDTA,[1] 0.2 mM EGTA, 50 mM benzamidine, 0.5 mg/L leupeptin, and 0.7 mg/L pepstatin was added, and the cells were scraped with a rubber policeman. The cell suspension was transferred to an all-glass homogenizer (Dounce), and the flask was rinsed with 1 mL of homogenization buffer. The procedure was repeated for each flask (50−60 flasks in a typical experiment). Prior to homogenization, the suspension was adjusted to 2 mM phenylmethanesulfonyl fluoride (PMSF) by adding the appropriate volume of a concentrated solution of this protease inhibitor. Cells were disrupted by 30 strokes of a tight pestle at 4 °C. The homogenate was transferred to centrifuge tubes placed on ice and then centrifuged at 20000*g* for 30 min in a refrigerated Sorvall centrifuge. The pellet was discarded and the supernatant centrifuged at 100000*g* in a Beckman ultracentrifuge for 1 h at 4 °C. The floating lipid layer was carefully removed and the cytosol collected. These extracts were used for either chromatography or immunoprecipitation.

Ion Exchange Chromatography of Crude Extracts. Extracts prepared from Sertoli cells incubated in the absence or presence of FSH or dibutyryl cAMP were applied to an HPLC DEAE 5PW (Waters Associates, Milford, MA) ion exchange column (size 75 mm × 7.5 cm) preequilibrated with 70 mM sodium acetate, pH 6.5, containing 5 mM 2-mercaptoethanol, 10 mM NaF, 1 mM EDTA, 0.2 mM EGTA, 0.5 mg/L leupeptin, 0.7 mg/L pepstatin, and 0.2 mM PMSF (starting buffer). The filtered and degassed cytosol was applied to the column at a flow rate of 1 mL/min through an auxiliary valve on the low pressure side of the HPLC system. After application of the sample, the column was washed with starting buffer until absorbance returned to basal levels. Bound protein was eluted with a 75−750 mM sodium acetate gradient. The gradient was applied at a flow rate of 1 mL/min (30 min). Fractions were collected in siliconized tubes containing ethylene glycol (final concentration 33% v/v), aprotinin (0.12 IU/tube), soybean trypsin inhibitor (10 μg/mL), and freshly added PMSF (0.2 mM). The PDE

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; rolipram, 4-(3-(cyclopentyloxy)-4-methoxyphenyl)-2-pyrrolidone; RO 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidone; cilostamide, *N*-cyclohexyl-*N*-methyl-4-((1,2-dihydro-2-oxo-6-quinoyl)-oxy)butyramide; HPLC, high pressure liquid chromatography. The nomenclature used for the phosphodiesterases described herein follows the recent recommendations of Beavo et al. (1994). In previous reports the PDEs expressed in the Sertoli cells were named ratPDE3 (PDE4D) and ratPDE4 (PDE4B) while the PDE4D variants had been called ratPDE3.1 (PDE4D1), ratPDE3.2 (PDE4D2), and ratPDE3.3 (PDE4D3).

activity of the fractions was assayed using 1 μM cAMP as substrate.

PDE Assay. Phosphodiesterase activity was measured following the method of Thompson and Appleman (1971) with minor modifications (Conti et al., 1982). Products of the reaction were separated by ion exchange chromatography as described (Thompson & Appleman, 1971). Adenosine recovery from the columns was 70−80%. No correction for this recovery was introduced in the calculation of the activity. To determine the kinetic characteristics of the purified enzyme, rates of cAMP hydrolysis were determined with increasing concentration of substrate and data were plotted according to Lineweaver and Burk (1934). Protein concentration of the samples was measured by the method of Bensadoun and Weinstein (1976) or by polyacrylamide gel electrophoresis and silver staining (Degerman et al., 1987) using bovine serum albumin as standard.

Immunological Procedures. The antisera were tested by solid phase immunoprecipitation of purified cAMP-PDE, following the procedure of Harrison et al. (1986) with minor modifications. Briefly, appropriate dilution of the antiserum in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) was preincubated with Staph A suspension (Pansorbin, Calbiochem) or protein G−Sepharose. Protein G−Sepharose was used for the monoclonal antibody M3S1 because of the higher affinity of this protein for the mouse IgG. After incubation for 1 h at 4 °C, the immobilized antibody was washed twice with PBS-BSA containing 0.5 M NaCl. The enzyme solution was mixed with the immobilized antibody and incubated for 1.5 h at 4 °C. After sedimentation at 15000g for 5 min, the supernatant was saved and the pellet washed twice with PBS-BSA. Aliquots of the supernatant and of the resuspended pellet were assayed for PDE activity using 1 μM cAMP as substrate. To control for the nonspecific adsorption of the PDE to the immobilizing support, samples without the antisera were included in every immunoprecipitation and processed in a manner identical to the samples containing the antisera.

Western immunoblotting using the polyclonal (K111, K116, K118) or monoclonal (M3S1) was carried out following established procedures (Towbin et al., 1978). The binding of the specific primary antibody was visualized by incubation with either secondary antibody coupled to peroxidase, ^{125}I-protein A, or ECL (Amersham) and autoradiography. These different detection procedures gave identical results.

RESULTS

Induction of a cAMP-PDE by FSH and Dibutyryl-cAMP. Cultured Sertoli cells were incubated for 24 h in the absence or presence of 100 ng/mL ovFSH-S16. At the end of the treatment, cells were harvested, and the soluble fraction was prepared as described. As previously reported (Conti et al., 1982), hormone treatment produced a 5−10 fold increase in PDE activity. Similar effects were observed with dibutyryl cAMP (data not shown). Homogenates from Sertoli cells treated with 1 mM dibutyryl cAMP had a specific activity of 0.71 ± 0.38 nmol of cAMP hydrolyzed/(min·mg of protein) (N = 13). This initial specific activity was 5−10 times higher than that of total testis extracts (data not shown). This FSH-dependent PDE activation was further analyzed by separating the PDE activity by HPLC DEAE ion exchange chromatography (Figure 1). The FSH treatment had no effect on the Ca^{2+}−calmodulin-dependent cGMP-hydrolyzing activity present in these cells. Conversely, FSH produced a large increase in the activity of a cAMP-PDE (Figure 1). Thus, the cultured Sertoli cell had a relatively simple pattern of PDE activity, with only one other PDE being present in the soluble fraction. This PDE was easily separated from the FSH-stimulated cAMP-PDE by ion exchange chromatography.

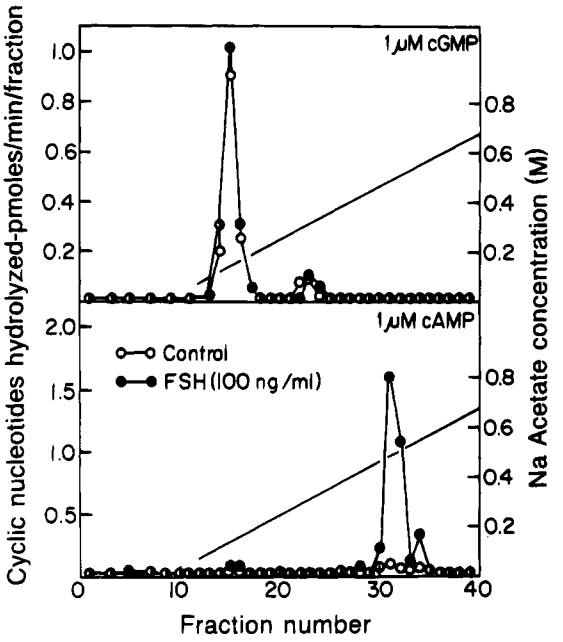

FIGURE 1: FSH stimulation of the cAMP-PDE of the Sertoli cell. After 4 days in culture in unsupplemented medium, Sertoli cells were incubated for 24 h in the absence or presence of 100 ng/mL FSH. At the end of the incubation, cells were harvested in homogenization buffer (see Experimental Procedures), and cytosol was prepared by centrifugation at 100000g. Supernatant was applied to an HPLC 5PW-DEAE ion exchange column equilibrated with 70 mM sodium acetate, pH 6.5, containing 5 mM 2-mercaptoethanol, 1 mM EDTA, 0.2 mM EGTA, and 10 mM NaF. After washing, adsorbed protein was eluted with a 30 min linear sodium acetate (75−750 mM) gradient. Fractions of 1 mL were collected, and PDE activity was measured using 1 μM cAMP or 1 μM cGMP as substrate. Activity recovered from control and FSH-treated cells was corrected for the amount of protein loaded on the column.

Purification of the Sertoli Cell cAMP-PDE. The major rolipram-sensitive, cAMP-specific PDE form present in the stimulated Sertoli cell was purified more than 2000-fold by four chromatographic steps. Two polypeptides of 67 and 86 kDa were present in the major peak of PDE activity (Figure 2). Details of the purification are reported in the supplementary material. In the four different experiments performed, the 86 kDa silver-stained band eluted with a pattern that does not coincide with the PDE activity. This lack of correlation was even more apparent during the gel filtration chromatography (data not shown). Conversely, the concentration of the 67 kDa band always followed closely the major peak of PDE activity.

Gel Filtration and SDS−PAGE. On the three different gel filtration matrices used (Sephacryl S-300, Sepharose AC34, HPLC-TSK), the Sertoli cell native cAMP-PDE behaved as a protein of approximate molecular weight 150 000. On the basis of the sedimentation coefficient and K_{av} derived from the TSK data, the molecular weight

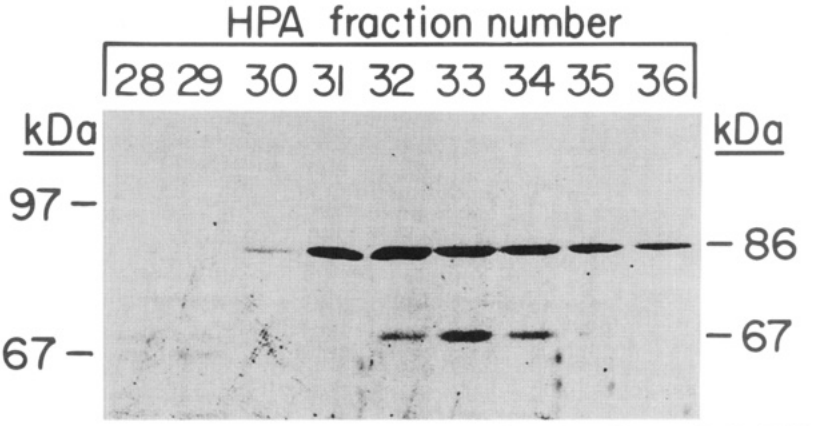

FIGURE 2: SDS–PAGE and silver staining of the fractions of the hydroxylapatite column, last step in the PDE purification. The fractions of the hydroxylapatite column (fractions 28–36, Figure 4, supplementary material) containing the major peak of activity were analyzed by electrophoresis in a 7.5% acrylamide gel according to Laemmli (1970). After electrophoresis, the proteins were visualized by silver staining (Wray et al., 1981). Molecular weight markers were phosphorylase B (MW 97 440) and bovine serum albumin (67 000).

calculated according to the method described by Siegel and Monty (1966) is 133 996. This value is twice as large as the values derived from SDS–PAGE and silver staining (67 kDa) (Figure 2), raising the possibility that the native cAMP-PDE is a oligomeric structure. The native PDE behaved as an asymmetric protein with an f/f_o of 1.74. The enzyme eluted from a chromatofocusing column at a pH of 5.55 (data not shown).

Kinetics of Cyclic Nucleotide Hydrolysis. The purified enzyme hydrolyzed cAMP with Michaelis–Menten kinetics. The calculated K_m for cAMP was 1.971 ± 0.26 μM (mean ± SE; $N = 3$), in close agreement with the K_m (1.65 μM) derived from crude PDE preparations (Conti et al., 1982). cGMP competed with cAMP hydrolysis only at concentrations higher than 50 μM. At concentrations of 1–10 μM, no inhibitory or stimulatory effect of cGMP was ever observed. The V_{max} calculated on the basis of protein content estimated by silver staining was 2.0-3.0 μmol/(min·mg of protein). If, instead, data were corrected for only the 67 kDa protein content of the major peak, the calculated V_{max} was 9–10 μmol/(min·mg of protein). These figures are in the same range as the specific activities obtained for purification of the Ca^{2+}, calmodulin-dependent PDE (Sharma et al., 1984), the cGMP-stimulated PDE (Martins et al., 1982; Yamamoto et al., 1983), and the cGMP-inhibited PDE (Grant & Colman, 1984; Degerman et al., 1987; Harrison et al., 1986). The hydrolysis of cAMP required the presence of Mg^{2+} ions (data not shown). Ca^{2+} ions at a concentration of 1 mM had no effect on the activity of the enzyme (data not shown).

The cAMP-PDE thus purified was most sensitive to RO 20-1724 (IC_{50} = 1.5 μM) and rolipram (IC_{50} = 0.9 μM). Methylisobutylxanthine (IC_{50} = 15 μM), cilostamide (IC_{50} >50 μM), and milrinone (IC_{50} >50 μM) were less effective. Both rolipram and RO 20-1724 inhibition appeared to be competitive (data not shown). The minor peak of PDE activity resolved by the hydroxylapatite column was also inhibited with the same order of potency (data not shown).

Immunoprecipitation of the Sertoli Cell PDE. Four different antibodies were used for the immunological characterization of the cAMP-PDE induced by FSH in the Sertoli cell. Two antipeptide polyclonal antibodies K111 and K116 are nonselective since they recognize different PDE4 although with different affinities. These were used together

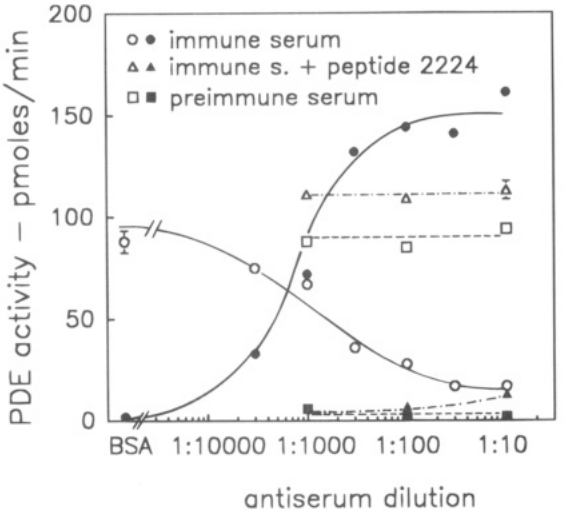

FIGURE 3: Immunoprecipitation of the Sertoli cell cAMP-PDE by a type IV PDE-specific antiserum. The polyclonal antiserum (K111) was used in a solid phase immunoprecipitation assay with the Sertoli cell cAMP-PDE partially purified by DEAE-HPLC ion exchange chromatography. After immunoprecipitation, the PDE activity in the pellet (filled symbols) and in the supernatant (empty symbols) was assayed as described in the Experimental Procedures. These activities are plotted against the dilution of the antiserum. Similar immunoprecipitations were carried out with preimmune serum (squares) and with immune serum in the presence of excess peptide 2224 (triangles), the peptide used as immunogen. A representative experiment of the three performed is presented.

with a monoclonal antibody specific for PDE4D and an antibody specific for PDE4B. Three antibodies recognized the cAMP-PDE from the Sertoli cell in a solid phase immunoprecipitation assay (Figures 3 and 4). Partially purified cAMP-PDE prepared from Sertoli cell extracts (specific activity = 200 nmol/(min·mg of protein)) was immunoprecipitated with K111 antibody dilution ranging between 1:5000 and 1:100 (ED_{50} 1:1000). Immunoprecipitation was completely suppressed by including the peptide used for immunization, while preimmume serum from the same animal did not display any precipitating activity (Figure 3). Similar results were obtained with antibody K116 (data not shown). The affinities of these antibodies were identical to the affinity determined with a recombinant PDE4D protein (data not shown). The PDE4D-selective M3S1 antibody immunoprecipitated most of the activity present in the peak fraction of the DEAE (Figure 4). Conversely, the PDE4B-

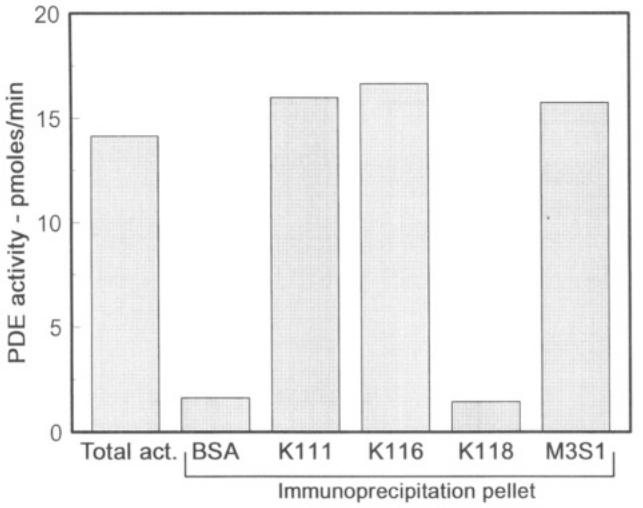

FIGURE 4: Immunoprecipitation of the partially purified cAMP-PDE with nonselective and PDE4B- and PDE4D-selective antibodies. The cAMP-PDE was partially purified by DEAE-HPLC ion exchange chromatography as described in the Experimental Procedures. Aliquots of the fractions containing the highest activity were incubated with the nonselective K111 and K116 antibodies and the PDE4D-selective (M3S1) and PDE4B-selective (K118) antibodies preadsorbed to protein G−Sepharose. The antisera were used at concentrations in excess of the antigen (1−10 dilution). To control for the nonspecific adsorption of the PDE to the protein G−Sepharose, a sample in which the antiserum was replaced with BSA was included in the experiment. After 1.5 h, the antibody antigen complexes were recovered by centrifugation, and the PDE activity recovered after two washings of the immunoprecipitation pellets was measured.

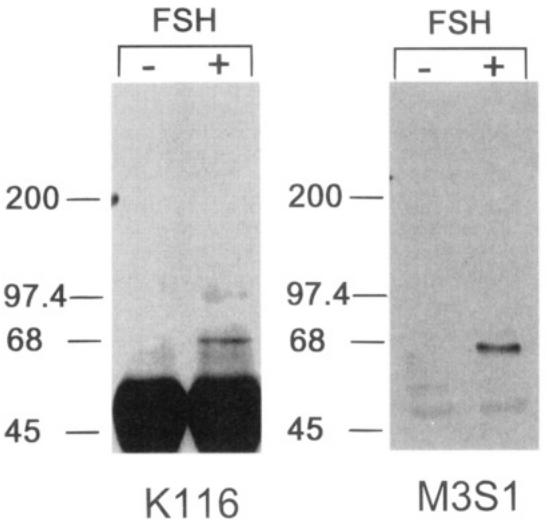

FIGURE 5: Immunoprecipitation and immunoblot analysis of the Sertoli cell cAMP-PDE induced by FSH. After 4 days in culture, Sertoli cells were incubated for 24 h in the absence or presence of 1 μg/mL FSH. At the end of the hormonal stimulation, cell monolayers were rinsed twice with Hank's balanced salt solution and harvested in homogenization buffer. An aliquot of the homogenate was used for protein and PDE assays. The soluble extract was subjected to solid phase immunoprecipitation using antibody K116 (dilution 1:10) immobilized to Pansorbin and monoclonal antibody M3S1 (1:10 dilution) immobilized to protein G−Sepharose. After centrifugation, the immunoprecipitation pellet was washed several times and the immunoadsorbed protein solubilized with SDS as described in the Experimental Procedures. An aliquot of the solubilized fraction was used for SDS−PAGE and immunoblot analysis. Comparable amounts of protein were loaded on each lane. The FSH stimulation produced an increase in soluble PDE activity from 22 to 103 pmol/(min·mg of protein). The molecular mass markers used are myosin (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), and ovalbumin (45 kDa). The predominant immunoglobulin band has a molecular mass of 52 kDa.

specific antibody immunoprecipitated minimal amounts of the partially purified cAMP-PDE from the Sertoli cell. Similarly, K118 antibody did not precipitate appreciable activity from crude Sertoli cell extracts (data not shown). This is not due to the inability of the K118 antibody to recognize the native PDE4B because this antibody efficiently immunoprecipitated PDE activity from crude brain extracts or recombinant PDE4B protein (data not shown). The cAMP-PDE activity could not be immunoprecipitated with a CaM-PDE-specific antibody (ACC-1; data not shown).

Western Blot Analysis of the cAMP-PDE Induced by FSH. To further characterize the cAMP-PDE induced by FSH, available anti-PDE antibodies were tested in Western blot. Immunoprecipitation followed by immunoblot analysis of the extracts from FSH-treated cells showed an enrichment of an immunoreactive polypeptide of 67 kDa when antibodies K116 and M3S1 (Figure 5) were used. This immunoreactive polypeptide was not present in extracts from control cells (Figure 5).

Western analysis of the rat cAMP-PDE after the first or second purification step showed multiple bands in the 67−68 kDa region of the gel (Figure 6). While the highest molecular weight band of 67−68 kDa was always present, the other two bands could not be always resolved. The lowest molecular weight band was recognized by K111 but not by the M3S1 monoclonal antibody or the polyclonal K116 antibody (Figure 6). The K118 antibody did not specifically recognize any polypeptide present in these fractions (Figure 6).

When the HPA column fractions were analyzed by Western blot, the antibody K111 recognized a single 67 kDa polypeptide that corresponds to the silver-stained band (Figure 7). An additional band with slightly lower molecular weight (65−66 kDa) coincided with the second peak of activity (fraction 23, Figure 7). The same polypeptides were recognized by the monoclonal antibody M3S1 (data not shown). Thus, two antibodies against two distinct epitopes of the PDE4D recognized the 67 kDa band resulting from the PDE purification.

On the contrary, none of the PDE antibodies available reacted with the 83−86 kDa band. That the larger polypeptide does not possess phosphodiesterase activity is also suggested by the fact that a mouse polyclonal antibody, which recognizes the 86 kDa band on a immunoblot, does not recognize the 67 kDa polypeptide and does not immunoprecipitate any PDE activity in a solid phase immunoprecipitation (data not shown).

Since a PDE4D is also expressed in brain and differences in mRNA species have been observed (Monaco et al. 1994), the properties of the cAMP-PDE induced by FSH in the Sertoli cell were compared to the corresponding activity expressed in brain. The PDE activities present in the soluble extracts of the Sertoli cell and brain were separated by HPLC DEAE chromatography, and the peak of rolipram-sensitive activity was characterized by SDS−PAGE and Western blot analysis (Figure 8). In the brain in addition to other immunoreactive bands, a 93 kDa polypeptide cross-reacted with K116 and M3S1 but not with K118 (Figure 8). The Sertoli cell band (67 kDa) was considerably smaller than the corresponding immunoreactive band expressed in brain (93−97 kDa). This is an indication that the Sertoli cell PDE

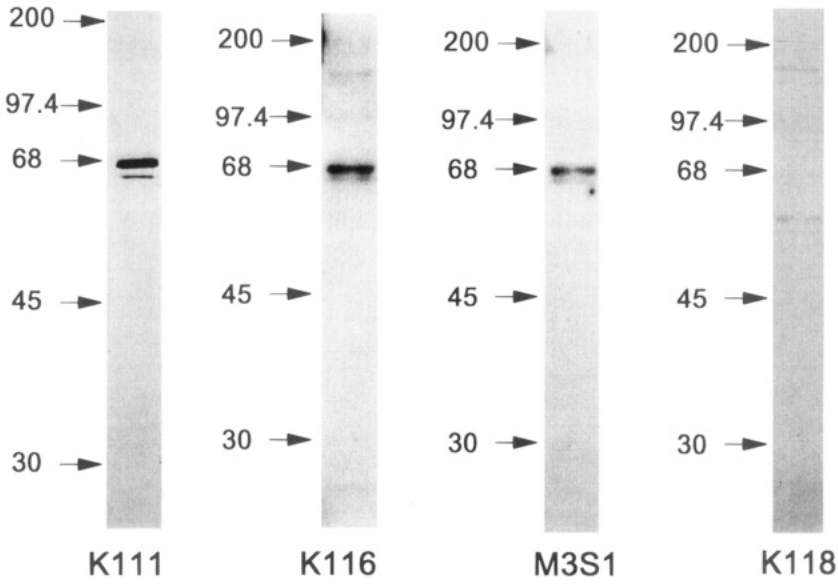

FIGURE 6: Immunoblot analysis of the cAMP-PDE from the Sertoli cell partially purified on DEAE ion exchange chromatography. Sertoli cell were treated for 24 h with 1 mM dibutyryl cAMP. At the end of the treatment, cells were harvested, and the cytosol was applied to a DEAE-HPLC column (see Experimental Procedures). An aliquot (10 μL) of the fractions containing the peak of cAMP-PDE activity was separated on a 10% gel. After transfer, the nylon membrane was incubated with antibody K111, K116, M3S1, or K118. Molecular weight markers were phosphorylase B (97 400), bovine serum albumin (68 000), and ovalbumin (45 000). The detection method used was peroxidase-conjugated secondary antibody for K111 or ^{125}I-protein A for the other antibodies. A representative experiment of the four performed is reported.

is a variant distinct from he major PDE4D form expressed in the central nervous system. The brain 93 kDa band had electrophoretic mobility identical to the recombinant PDE protein derived from mRNA variant PDE4D3 (data not shown). Furthermore, the K118 antibody, which does not recognize any polypeptide in the partially purified preparations from the Sertoli cells, recognized polypeptides of 97 and 85 kDa in the partially purified brain preparations (Figure 8).

DISCUSSION

With the present report we have described a high affinity cAMP-specific phosphodiesterase that, in the Sertoli cell, is under the control of FSH and cAMP. On the basis of the immunological data and the properties of the protein, we can conclude that hormone stimulation of the PDE activity observed in these cells is mostly due to the synthesis and accumulation of a 67 kDa protein which is derived from the PDE4D gene. Because activation of similar PDEs has been shown for several cell types (D'Armiento et al., 1972; Manganiello & Vaughan, 1972; Bourne et al., 1973, Uzunov et al., 1973), we believe that induction of a 67 kDa PDE4D is a ubiquitous regulation to adjust the cell responsiveness to hormones and neurotransmitters.

The following findings support the conclusion that a cAMP-PDE of 67 kDa derived from the PDE4D gene is the major PDE form induced in the Sertoli cell. The major peak of phosphodiesterase activity isolated from the Sertoli cell after hormonal stimulation was associated with two polypeptides of 86 and 67 kDa. However, only the 67 kDa was the form whose concentration closely followed the PDE activity in the chromatography steps used. Second, the 67 kDa polypeptide from crude or purified preparations is recognized by two types of antibodies cross-reacting with the cAMP-PDEs and a monoclonal antibody that is more selective for the PDE4D gene product. It does not cross-react, instead, with antibodies raised against the PDE4B cAMP-PDE. Finally, the electrophoretic properties of the 67 kDa cAMP PDE are identical to the properties of a recombinant PDE4D form expressed in prokaryotic or eukaryotic expression systems (Sette et al., 1994). More than one mRNA and PDE protein is encoded in the PDE4D gene. Our data show that the Sertoli cell PDE is smaller than the PDE with identical immunological properties derived from soluble brain extracts. This is consistent with the finding that two different mRNA variants were detected in immature Sertoli cells and in brain (Sette et al. 1994). On the basis of these findings, we propose that the FSH-induced cAMP-PDE of the Sertoli cell is derived from the PDE4D2 mRNA variant (Sette et al., 1994) while the major brain PDE4D is derived from the PDE4D3 variant mRNA.

The nature of the 86 kDa band copurifying with the catalytic activity, instead, is at present unknown. Because large quantities of an 86 kDa protein were present in the chromatographic steps preceding the hydroxylapatite column, this is probably a contaminating protein. Further experiments are, however, required to unequivocally exclude that this protein is interacting with the 67 kDa PDE.

On the basis of gel filtration and sucrose-density gradient analysis, the native Sertoli cell PDE has an estimated molecular mass of 130−150 kDa, approximately twice the molecular mass estimated by SDS−PAGE. This suggests that the native enzyme is an oligomer. Although the most likely possibility is that the native enzyme is a dimer of two identical 67 kDa subunits, we have no firm evidence to exclude that the cAMP-PDE purified is an heterodimer of two subunits derived from two different genes. Although two mRNAs are expressed in the Sertoli cell, it is unlikely that the PDE is a heterodimer of two subunits derived from the PDE4B and PDE4D genes, since the PDE4B antibody could not immunoprecipitate any activity. Similarly, immunoprecipitation data available do not support the hypothesis that the 86 kDa polypeptide is a subunit of the PDE, because, under the conditions used, it does not immunopre-

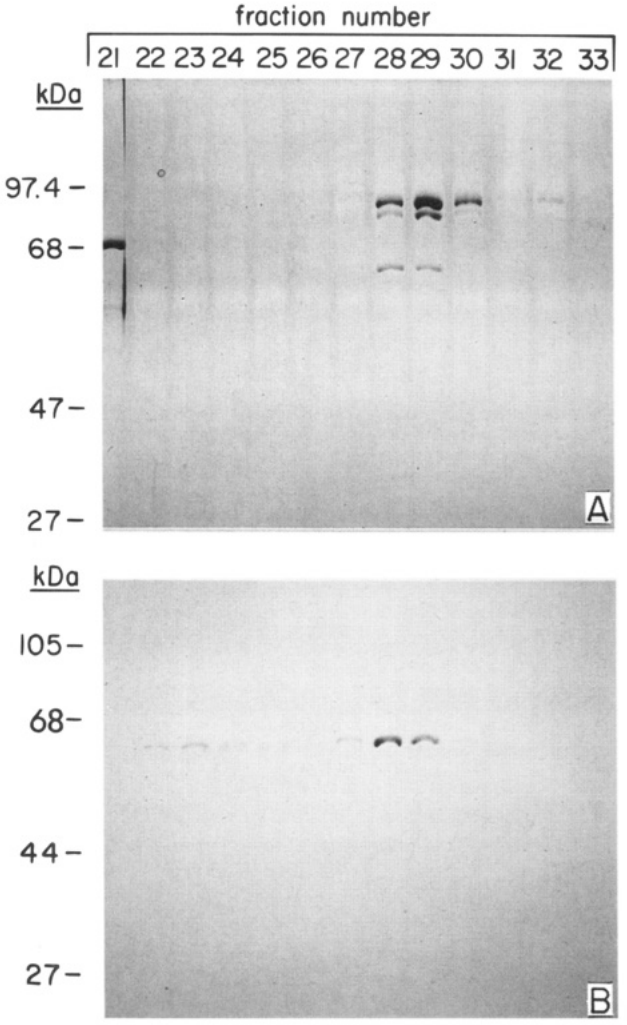

FIGURE 7: Immunoblot analysis of the purified cAMP-PDE from the Sertoli cell. Fractions of the hydroxylapatite column containing the PDE activity were divided into two aliquots and were loaded onto two 10% SDS−PAGE gels. After electrophoresis according to Laemmli (1970), one gel was stained with silver (A) and the other was transferred to a nylon membrane (B). The blot was incubated with K111 antibody, followed by peroxidase conjugated to the secondary antibody. Molecular weight markers were phosphorylase B (97 400), bovine serum albumin (68 000), egg albumin (45 000), and soybean trypsin inhibitor (21 500). Slight differences in the migration of phosphorylase B (105 000), bovine serum albumin (68 000), and ovalbumin (44 000) markers were observed after chromophore coupling to protein markers used for the lower panel. A representative experiment of the two performed is reported.

cipitate and could be dissociated from the catalytic activity.

The PDE purification yielded two peaks of activity with similar or identical kinetics properties. This is supported by the finding that the two species have comparable sensitivity to different inhibitors, and close MW on SDS−PAGE. It is likely that these PDE forms are the product of a single gene and that, during the purification, partial proteolysis yields the two peaks of activity. The minor peak was associated with a polypeptide of a molecular mass of approximately 66 kDa, only slightly smaller from that of the major form. If proteolysis has occurred, the fragment removed is then minimal, yet sufficient to allow separation on the HPA column. Other possible causes of the presence of two peaks of activity are (a) alternate splicing or different initiation methionine used from the same message; (b) differences in post-translational modification of the same protein; (c) different states of aggregation (monomeric−oligomeric) of the same protein. It is unlikely that these two closely related PDEs are derived from two distinct genes because the monoclonal antibody M3S1, which is selective for the product of the PDE4D gene, recognizes both bands. It is also worth noting that silver staining of fractions corresponding to the major peak of PDE clearly showed the presence of a 67 kDa band. On the contrary, in the several experiments performed, no band associated with the minor peak of activity could be detected by silver staining. Since the activity of the minor peak is about 50% of the major peak, it would be expected that polypeptide concentration would be well within the detection limits of silver staining. Also, the band that is associated with the minor peak of activity on immunoblot analysis is present in a ratio of 1:5 when compared to the major band. A possible explanation for this discrepancy is that the PDE eluting with the minor peak has a much higher specific activity than the major PDE peak. Further characterization of this second form is necessary to establish its exact properties, as controlled proteolysis may be a physiological mechanism of cAMP-PDE activation. This possibility would be in line with the finding that a truncated cAMP-PDE is more active than the full-length enzyme (Jin et al., 1992).

The kinetic characteristics of the enzyme activity that we have isolated are similar to the characteristics of other cAMP-PDEs. These PDE forms are probably ubiquitous and have been best studied from bovine lung (Thompson et al., 1979), dog kidney, human lymphoblastic cells (Onali et al., 1981), rat liver (Marchmont et al., 1981), and more recently rat brain (Nemoz et al., 1989). All these forms share, with the enzyme purified from the Sertoli cell, an affinity for cAMP of 1−2 μM, little or no inhibition by cGMP (EC_{50} higher than 100 μM), and high sensitivity to rolipram and RO 20-1724 but not to cilostamide or milrinone. The early reports of purification of these high affinity cAMP-PDEs from dog kidney (Thompson et al., 1979), lung, lymphoblasts, and, more recently, rat brain describe proteins with specific activities of less than 100 nmol/(min·mg of protein) and an overall purification of approximately 100-fold. This is more than 10-fold lower than the specific activity that we have reported for our enzyme and for the specific activities of the CaM PDEs or the cGMP stimulated or cGMP-inhibited PDEs. More recently, Thompson and co-workers (1988) have further purified the PDE form from dog kidney to specific activities in the micromolar range, which are similar to the specific activity of the Sertoli cell PDE and other low affinity PDEs. Recently, recombinant PDEs (PDE4A) have been purified from a yeast expression system as native or fusion proteins. These preparations again have specific activities similar to what we have observed (Torphy et al., 1993; Wilson et al., 1994). Therefore, from our present data and those reported by others it appears that cAMP-PDEs are not different from the other PDE families in terms of specific activity or turnover number.

Previous observations from our laboratory indicated that PDE4D and PDE4B mRNAs are expressed in the Sertoli cell (Swinnen et al., 1991). As previously discussed, the 67−68 kDa polypeptides are most likely the product of the PDE4D gene. On the other hand, it is not clear which polypeptides correspond to PDE4B protein. In the first step of purification, a third immunoreactive band was often observed in immunoblots of the DEAE ion exchange chromatography (see Figure 4) using K111 as antibody. This

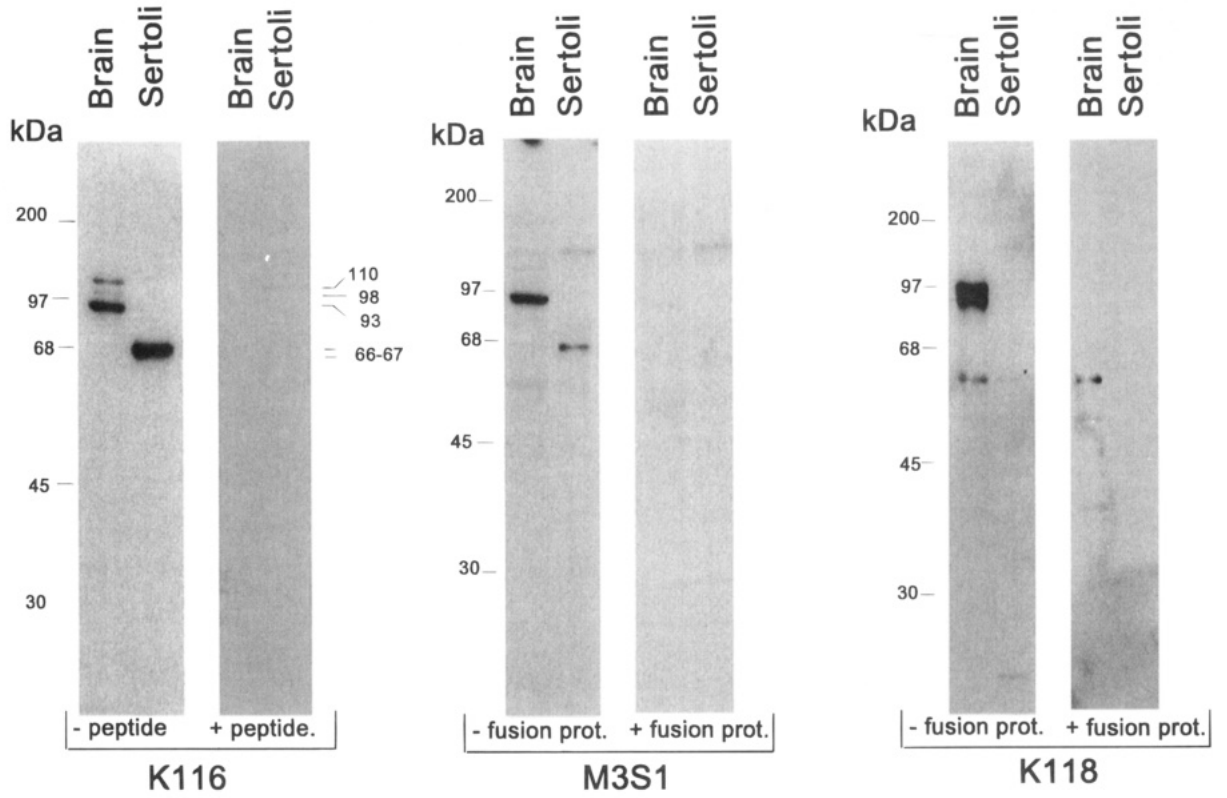

FIGURE 8: Comparison of the properties of the cAMP-PDEs expressed in the Sertoli cell and in brain. The cAMP-PDE activity was partially purified from Sertoli cell extracts and from brain soluble extracts by ion exchange chromatography. An aliquot of the fraction containing the highest activity was fractionated by SDS−PAGE, followed by immunoblot with the K116 antibody, the M3S1 antibody, and the K118 antibody. As a control, comparable blots were used for immunostaining using K116 preadsorbed to peptide 2224, M3S1 preadsorbed to GST-PDE4D fusion protein, and K118 to GST-PDE4B fusion protein. The molecular weight markers are as in Figure 5.

band was lost during purification, suggesting that the polypeptide is highly unstable. It is possible that this polypeptide corresponds to the product of ratPDE4, even though the cross-reactivity of this species with PDE4B-selective antibody could not be unequivocally determined. A possible explanation of the absence of a PDE4B polypeptide in the soluble extracts or partially purified preparation is that the product of the PDE4B gene is confined to the particulate fraction of the cell. This hypothesis is compatible with the finding that a recombinant PDE4B is largely particulate (Swinnen et al, 1991) and that a similar form is recovered in the particulate fraction from the rat brain (Lobban et al., 1994). Further experiments are necessary to clarify these points.

In conclusion, our data demonstrate that FSH induces a 67 kDa cAMP-PDE in the Sertoli cell, causing a major increase in PDE activity. This is a product of the PDE4D gene. Although a message for an additional cAMP-PDE (PDE4B) is expressed in these cells and regulated by hormones, the protein product of this gene could not be recovered in the soluble fraction of the Sertoli cell.

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SUPPLEMENTARY MATERIAL AVAILABLE

Details of the purification of the cAMP-PDE activity from the Sertoli cell, one table giving the purification data, and four figures showing purifications by nonpreparative HPLC-DEAE, by hydrophobic interaction chromatography, by TSK 3000 gel filtration chromatography, and on a hydroxylapatite column (10 pages). Ordering information is given on any current masthead page.

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