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Characterization of the Rolipram-Sensitive, Cyclic AMP-Specific Phosphodiesterases: Identification and Differential Expression of Immunologically Distinct Forms in the Rat Brain

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ABSTRACT

To determine the properties of the cAMP-specific, rolipram-sensitive phosphodiesterases (cAMP-PDEs) that are expressed in different organs, monoclonal and polyclonal antibodies were raised against different epitopes present in the cAMP-PDE sequences. Of the several antibodies generated against peptides and fusion proteins, one monoclonal and four polyclonal antibodies recognized both the native cAMP-PDEs as well as the denatured proteins on Western immunoblot analysis. An immunoprecipitation assay demonstrated that these antibodies recognized the recombinant rat PDE4A, PDE4B, and PDE4D proteins with different avidity. The polyclonal antibody K118 and the monoclonal M3S1 were most specific for rat PDE4B and PDE4D forms, respectively, whereas the AC55 antiserum displayed the highest affinity for PDE4A forms. This selectivity was confirmed by Western blot analysis using recombinant rat PDE4A, PDE4B, and PDE4D proteins expressed in a heterologous system. These antibodies were used to characterize the cAMP-PDEs expressed in the rat brain. An immunoblot of extract of cortex and cerebellum demonstrated that at least seven different polypeptides specifically cross-reacted with the different antibodies, indicating that multiple cAMP-PDEs are expressed in this tissue. On the basis of cross-reactivity with PDE4D but not PDE4A or PDE4B antibodies, 93- and 105-kDa PDE4D species were detected in the cortex and cerebellum extract. These forms are different from the 68-kDa PDE4D form expressed in endocrine cells after hormonal stimulation. Although the 93-kDa form was recovered in both the soluble and particulate fractions, the 105-kDa polypeptide was mostly particulate in the cortex and cerebellum extracts. PDE4B forms of 90–87 kDa were recovered in both soluble and particulate compartments of the brain extract. These forms were different from the previously identified PDE4A variants of 110 and 75 kDa. These data demonstrate that the presence of multiple cAMP-PDE genes is translated into cAMP-PDE proteins of different sizes and distinct immunological properties and that multiple variants derived from these cAMP-PDE genes are expressed in different regions of the brain and different subcellular compartments. These immunological tools will be useful to identify different cAMP-PDE forms expressed in organs targeted for pharmacological intervention with PDE4 inhibitors.

The high affinity, cAMP-specific phosphodiesterases [type 4 according to the nomenclature proposed by Beavo *et al.* (1994)] are a class of enzymes with similar kinetic properties that are inhibited by the antidepressant rolipram and structurally related compounds. Although the presence of these forms has long been recognized, their distinctive properties are becoming evident only recently (Conti *et al.*, 1995b). Early attempts to purify these forms have been hampered by their low abundance and instability (Conti and Swinnen, 1990). The molecular mass attributed to this group of enzymes ranges between 29 and 89 kDa (Conti and Swinnen, 1990). The definition of the exact properties and site of expression of these forms is made difficult by the presence of nonlinear kinetics and by contaminating cGMP hydrolytic activity (Strada *et al.*, 1989).

Cloning of the rat cDNAs that encode cAMP-PDEs (Colicelli *et al.*, 1989; Davis *et al.*, 1989; Swinnen *et al.*, 1989) has provided a first indication for the presence of at least four different *PDE4* genes in the rat. Despite an early report indicating the presence of only one cAMP-PDE gene in the humans (Livi *et al.*, 1990), more recent findings (Bolger *et al.*, 1993; Obernolte *et al.*, 1993) point to the conclusion that four genes are present in this species and therefore is not a pecu-

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ABBREVIATIONS: PDE, phosphodiesterase; GST, glutathione-*S*-transferase; ELISA, enzyme-linked immunosorbent assay; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TBS-T, Tris-buffered saline-Tween 20.

liarity of rodents. The partial structure of two of the rat genes has been characterized recently (Monaco *et al.*, 1994). These findings provide an explanation of the wide variety of physicochemical properties attributed to these cAMP-PDEs. Northern blot analysis or reverse transcription-polymerase chain reaction of different tissues has established that not all genes are expressed at all times and that different cells express a different set of cAMP-PDE forms. In the rat testis, for instance, somatic cells express predominantly PDE4D and PDE4B mRNAs (Swinnen *et al.*, 1989) and germ cells express preferentially PDE4C and PDE4A mRNAs (Welch *et al.*, 1992). In the rat brain, transcripts have been detected corresponding to PDE4A, PDE4B, and PDE4D but not to PDE4C (Bolger *et al.*, 1994; Davis *et al.*, 1989; Engels *et al.*, 1995; Iwahashi *et al.*, 1996; Swinnen *et al.*, 1989). Despite the established presence of multiple genes and of cognate mRNAs, it remains unclear whether different cAMP-PDE proteins are in fact expressed in a cell. An example would be our current understanding of PDE4 expression in inflammatory cells in which PDE4A, PDE4B, and PDE4D mRNAs have been detected (Engels *et al.*, 1994; Torphy *et al.*, 1992; Verghese *et al.*, 1995), but little information is available on the cAMP-PDE proteins expressed. This occurs because no clear-cut biochemical criteria are available to identify and classify the cAMP-PDE variant proteins expressed in the different organs.

In view of the difficulty of using a biochemical approach to separate and characterize the different cAMP-PDE forms, we developed an immunological strategy to identify the cAMP-PDE forms expressed in any given tissue. Using a panel of nonselective and form-selective antibodies, we demonstrate that apparently homogeneous cAMP-PDE preparations are a mixture of forms derived from different genes and that different variants are derived from each gene.

Experimental Procedures

Materials. Waymouth 752/1 medium, gentamycin, and horse serum were purchased from GIBCO (Grand Island, NY). *Crotalus atrox* snake venom was purchased from Sigma Chemical (St. Louis, MO). Pansorbin cells were purchased from Calbiochem (San Diego, CA). Immobilon was from Millipore (Bedford, MA). [2,8-^{3}H]cAMP (20–50 Ci/mmol) and ^{125}I/protein A were purchased from DuPont-New England Nuclear (Boston, MA). AG 1-X8 resin was purchased from BioRad (Richmond, CA). ECL Western blot detection kit was purchased from Amersham (Arlington Heights, IL). Rolipram (4-[3-(butoxy-4-methoxybenzyl)-2-imidazolidone]) was provided by Syntex (Palo Alto, CA). Except where otherwise designated, all other chemicals were the purest grade available and were provided by Sigma.

Selection of the epitopes and preparation of the antigens. Comparison of the deduced sequences of the four cAMP-PDEs indicated that the sequences are similar except for the amino- and carboxyl-terminal regions (Conti *et al.*, 1991; Conti and Swinnen, 1990). Several peptides were synthesized on the basis of the rat PDE4D1 sequences residue (Conti *et al.*, 1991). Peptide 2224 corresponds to residue 105–126 of PDE4D1 (accession number U09455), a region that is homologous in the four different cAMP-PDEs. Rat PDE4A differs in one residue (Asn57 of RD1; accession number M26715), and rat PDE4B differs in two residues (Asn102 and Asp103 of rat PDE4; accession number M25347). The same epitope is present in the human cAMP-PDEs (Bolger *et al.*, 1993; Obernolte *et al.*, 1993). This peptide was injected into several rabbits (Conti *et al.*, 1995a). The antisera from rabbits K111 and K116 were used for the current study. To generate antibodies that would discriminate among PDE4A, PDE4B, and PDE4D, the carboxyl-terminal region was used (Fig. 1). The *Bam*HI/*Eco*RI fragments were prepared from the 3′ end of rat PDE4D1, PDE4B, and PDE4A1 cDNAs and were subcloned in the bacterial expression vector pGEX-3X in-frame with the GST coding sequence (Crowl *et al.*, 1985). Expression of these constructs in *E. coli* produces a protein that is the result of fusion of the coding region of the GST and the carboxyl-terminal portion of rat PDE4D, rat PDE4B, and rat PDE4A. These fusion proteins were isolated on a single-step affinity chromatography on glutathione-Sepharose according to the manufacturer's recommendations (Pharmacia). The GST-rat PDE4B and GST-rat PDE4A proteins were used to generate polyclonal antibodies in rabbits, whereas the GST-rat PDE4D protein was used to generate monoclonal antibodies. Eight-week-old female BALB/c mice were immunized by an intraperitoneal injection of 20 μg of GST-PDE4D in Freund's adjuvant followed by three injections at 4-week intervals with the same dose of antigen. Three days after the fourth injection, the splenocytes were fused with P3X63Ag8NS1 murine myeloma cells according to standard procedures; 1.0×10^{10} splenocytes were mixed with 2×10^{10} myeloma cells in 50% polyethylene glycol (PEG 1500; Boehringer-Mannheim) in RPMI 1640 medium. After fusion, cells were seeded onto 96-well microtiter plates (model 3598; Costar, Cambridge, MA). A first screening for the presence of antibodies reacting with the immunizing antigen was performed with an ELISA using GST-PDE fusion protein. Hybridoma-secreting antibodies specific for rat PDE4D were cloned by limiting dilution and injected intraperitoneally into 8-week-old female BALB/c mice primed with Pristane (Aldrich Europe, Berse, Belgium) to produce ascitic fluid. The hybridoma isotype was determined by ELISA (Boehringer-Mannheim). The M3S1 antibody used in the current report was an IgG1 isotype. The titers of the antisera and monoclonal antibodies were determined by ELISA using the fusion proteins, purified GST, or partially purified recombinant PDEs as antigens.

Cell culture. MA-10 cells, a cell line derived from a Leydig cell tumor, were generously provided by Dr. Mario Ascoli (see Ascoli, 1981). Cells were routinely cultured in Waymouth medium supplemented with 20 mM HEPES and 15% horse serum as reported previously (Conti *et al.*, 1995a). Cells were cultured in 75-cm flasks (Corning Glassworks, Corning, NY) at 37° in an atmosphere of 95% air/5% CO_2 in a humidified incubator. MA-10 cells were seeded onto 90-mm dishes (Corning) in Waymouth medium supplemented with 15% serum. After 24 hr, cells were transfected with 10–20 μg of

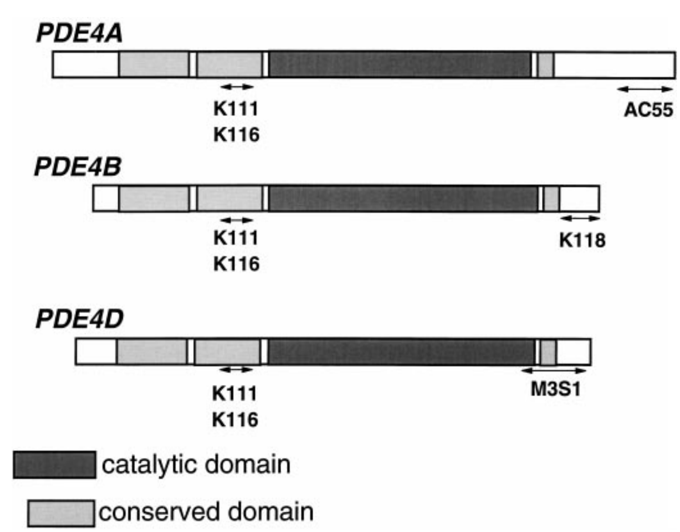

Fig. 1. Primary structure of rat PDE4A, PDE4B, and PDE4D and location of the epitopes used to generate monoclonal and polyclonal antibodies. □, primary structure of the proteins; ■, highly conserved catalytic domain; ▨, domains outside the catalytic domain conserved in the three sequences. *Double-headed arrows*, region corresponding to peptides or fusion proteins used to generate the antibodies.

pCMV5-rat PDE4D1, pCMV5-rat PDE4B2, or pCMV5-rat PDE4A1 plasmids as described in detail previously (Swinnen *et al.*, 1991) using the $CaPO_4$ method (Graham and van der Eb, 1973). Primary Sertoli cell cultures were prepared and maintained as reported previously (Conti *et al.*, 1982).

Preparation of cell extracts. At 24 hr after transfection, cells were harvested in homogenization buffer consisting of 20 mM Tris·HCl, pH 8.0, 1 mM EDTA, 0.2 mM EGTA, 50 mM NaF, 10 mM sodium pyrophosphate, 50 mM benzamidine, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 4 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, and 2 mM phenylmethylsulfonyl fluoride. Cells were homogenized and centrifuged for 10 min at 14,000 × *g*. Both the homogenates and soluble extracts were used for PDE assay. In some experiments, soluble extracts were subjected to immunoprecipitation with the different antibodies. PDE4 proteins were expressed in Sf9 insect cells using the baculovirus expression system, and cell extracts were prepared as described previously (Sette and Conti, 1996).

Preparation of brain and heart extracts. The brain was removed rapidly from cervically transected adult rats, and the cerebellum and cortex were isolated, weighed, rinsed, and then homogenized at 4° in a buffer containing 250 mM sucrose, 20 mM Tris·HCl, pH 7.8, 1 mM EGTA, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 μM microcystin, 50 mM benzamidine, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 4 μg/ml aprotinin, 10 mg/ml soybean trypsin inhibitor, and 2 mM phenylmethylsulfonyl fluoride. After centrifugation at 20,000 × *g* for 30 min, the supernatant was set aside as the soluble fraction. The pellet was washed twice and then extracted according to the procedure of Penman (He *et al.*, 1990) as modified by Ndubuka *et al.* (1993) with a solution containing 250 mM sucrose, 10 mM piperazine-*N,N′*-bis(2-ethanesulfonic acid), pH 6.8, 0.1 M NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 2 mg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 1% Triton X-100. After incubation for 10 min at 4° and centrifugation at 20,000 × *g*, a Triton-extracted fraction was obtained. The pellet was washed twice and then resuspended in a solution containing 10 mM Tris·HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1% Tween-20, and 0.5% sodium deoxycholate. After this step, a deoxycholate-extracted fraction (mean ± standard deviation) was obtained. Finally, the pellet was washed twice and then resuspended in a RIPA buffer without SDS (consisting of 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM Tris·HCl, pH 7.5, 25 mM benzamidine, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 2 μg/ml aprotinin, 5 mg/ml trypsin soybean inhibitor, 2 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 1 μM microcystin). An aliquot was removed for the assay of the PDE activity, and SDS was added to the remaining extract to a final concentration of 0.1%, yielding an RIPA-extracted fraction. Heart extracts were prepared in a similar manner.

Immunoprecipitation. The soluble or solubulized extracts from MA-10 cells or brain tissue were immunoprecipitated using antibodies immobilized on fixed *Staphylococcus aureus* cells (Pansorbin) or Protein G-Sepharose. Pansorbin was used for the polyclonal anti-cAMP-PDE antiserum K116, AC55, or K118, and Protein G-Sepharose was used for the monoclonal anti-cAMP-PDE3 M3S1. When not specified, the polyclonal and monoclonal antibodies were used at a 1:30 or 1:100 dilution. Adsorption of the antibody to the insoluble substrate followed the procedure described by MacPhee *et al.* (1988) with minor modifications (Conti *et al.*, 1995a). Extracts were incubated with antibodies immobilized to Pansorbin and Protein G-Sepharose for 1.5 hr at 4° by gentle mixing. At the end of the incubation, the samples were centrifuged at 14,000 × *g* in an Eppendorf centrifuge for 5 min. The supernatants containing nonadsorbed PDE were removed and saved for the PDE assay. The pellets were washed three times with phosphate-buffered saline and 0.1% BSA. After the last centrifugation, pellets were resuspended in the same buffer, and an aliquot was used for the PDE assay. Adsorbed proteins were then eluted with 1% SDS in phosphate-buffered saline and diluted to a final concentration of 1× sample buffer (see below) for Western blot analyses. To determine the specificity of the immunoprecipitation, several controls were performed. Nonspecific adsorption was monitored by incubating identical amounts of extracts with the immobilized immune or preimmune serum or with a comparable concentration of BSA (1 mg/ml). Additional control experiments were performed by using Pansorbin preadsorbed to K116 antiserum preincubated with the immunogenic peptide 2224 (5 μg/ml). These different controls gave comparable results. For the monoclonal antibody, Protein G-Sepharose preincubated with 0.1% BSA was used as a control. The PDE activity was measured in both the resuspended pellets and the supernatants of the immunoprecipitation.

Western blot analysis. Immunoprecipitated samples were prepared in 1× sample buffer [62.5 mM Tris-Cl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 0.7 M 2-mercaptoethanol, and 0.0025% (w/v) bromphenol blue]. The samples were boiled for 5 min and subjected to electrophoresis on an 8% SDS-polyacrylamide gel. The proteins were then blotted onto an Immobilon membrane followed by blocking of the membrane in TBS solution (20 mM Tris·HCl, pH 7.6, 14 mM NaCl) containing 5% BSA and 0.1% Tween 20. After several washes, the membrane was incubated with the primary antibody in TBS-T (20 mM Tris·HCl, pH 7.6, and 14 mM NaCl with 0.1% Tween 20). Because of the high background obtained with the K118 antiserum, this was used routinely in the presence of 1 μg/ml recombinant GST. After a 90-min incubation, the membrane was washed in TBS-T followed by a 1-hr incubation with either ^{125}I-protein A (Amersham) or peroxidase-linked anti-rabbit or anti-mouse IgG. After several washes with TBS-T, the membrane was exposed to XAR-5 X-ray film (Kodak, Rochester, NY) or incubated for 1 min with the ECL detection reagents (Amersham) and exposed to XAR-5 X-ray film for 5–60 sec (Kodak) to detect the peroxidase-conjugated secondary antibodies. To control for the specificity of the immunoreactive bands, the antibodies were preadsorbed with the corresponding peptide or fusion protein.

PDE assay. PDE activity was measured using 1 μM cAMP as a substrate, according to the method of Thompson and Appleman (1971). Samples were assayed in a total volume of 200 μl of reaction mixture including 40 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 1.25 mM 2-mercaptoethanol, 0.1 mg/ml BSA, and 1 μM [^{3}H]cAMP (≈0.1 μCi/tube). In some experiments, 10 μM rolipram (final concentration) was added to the reaction mixture. After incubation at 34° for 5–15 min, the reaction was terminated by the addition of an equal volume of 40 mM Tris-Cl, pH 7.5, containing 10 mM EDTA, followed by heat denaturation for exactly 1 min at 100°. Fifty micrograms of *Crotalus atrox* snake venom was added to each reaction tube, and the incubation was continued at 34° for 20 min. The reaction products were separated by anion-exchange chromatography on AG1-X8 resin, and the amount of radiolabeled adenosine collected was quantified by scintillation counting. Protein concentrations of the samples were measured according to the method of Bradford (1976).

Ion exchange chromatography. The soluble fractions from brain extract, Sertoli cells, or MA-10 cells were prepared as described above and then applied to a high pressure liquid chromatography DEAE ion exchange column. The column was preequilibrated with 200 mM Na-acetate, pH 6.5, buffer containing 50 mM NaF, 1 mM EDTA, 0.2 mM EGTA, 5 mM β-mercaptoethanol, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 2 mM phenylmethylsulfonyl fluoride. After application of the sample and extensive washing with the starting buffer, bound protein was eluted with a linear gradient (200–750 mM acetate; running time, 30 min at 1 ml/min). Fractions of 1 ml were collected. Fractions containing the highest activity were stored at −20° after the addition of ethylene glycol to a final concentration of 33%.

Results

Characterization of the anti-cAMP-PDE polyclonal and monoclonal antibodies. Polyclonal and monoclonal antibodies against peptides and fusion proteins have been generated according to the strategies described. A summary of the epitopes used to generate the different antibodies is reported in Fig. 1.

To evaluate the properties and specificity of the antisera and monoclonal antibodies, recombinant PDE4 proteins were expressed in eukaryotic cells. Because no full-length PDE4C cDNAs were available, the cross-reactivity of the antisera against this PDE form could not be assessed; however, transcripts corresponding to this form have been detected in the rat kidney (Swinnen *et al.*, 1989), rat meiotic germ cells (Welch *et al.*, 1992), and a rat thyroid cell line (FRTL-5) but not in the rat brain (Bolger *et al.*, 1994; Engels *et al.*, 1995; Iwahashi *et al.*, 1996; Swinnen *et al.*, 1989), Sertoli cells (Swinnen *et al.*, 1991), or MA-10 cells (Swinnen JV and Conti M, unpublished observations). Therefore, interpretation of the current data would not be affected by cross-reactivity with PDE4C.

Transient transfection of these constructs produced a large increase in the PDE activity present in the homogenate (Table 1). The increase in activity was inhibited 95–98% by 10 μM rolipram (data not shown). This indicated that the recombinant PDE activity represented >90% of the PDE activity of the unfractionated soluble extracts. For this reason, the recombinant PDE proteins were not purified further, and crude soluble extracts were used directly for the immunoprecipitation assay. A representative immunoprecipitation with the K116 antiserum is reported in Fig. 2. The amount of PDE activity recovered in the pellet of the immunoprecipitation was proportional to the amount of antiserum used, and a commensurate decrease was observed in activity recovered in the supernatant of the immunoprecipitation. Furthermore, the immunoprecipitation of the cAMP-PDE activity was blocked by preadsorption of the antiserum to the appropriate immunogen, and background PDE activity could be immunoprecipitated when a preimmune serum (Fig. 2) or BSA (data not shown) was used. The antibodies tested did not recognize a recombinant or native CaM-PDE, a cGMP-stimulated PDE from rat brain, or a cGI-PDE partially purified from HL60 cells (data not shown). The ED_{50} value of an antibody measured with this immunoprecipitation was dependent on the amount of Pansorbin used (data not shown) but independent of the amount of PDE antigen added to the assay (data not shown). All of the following experiments were performed using similar concentrations of antigen and fixed concentrations of Pansorbin. In several instances, it was noticed that the activity recovered in the immunoprecipitated pellet exceeded that measured in the extract before precipitation. Although activation of the cAMP-PDE on binding to the immunoglobulin cannot be excluded, it is possible that the binding of the immunoglobulin to the PDE molecule causes a stabilization of the PDE activity by rendering the PDE protein inaccessible to proteases or phosphatases.

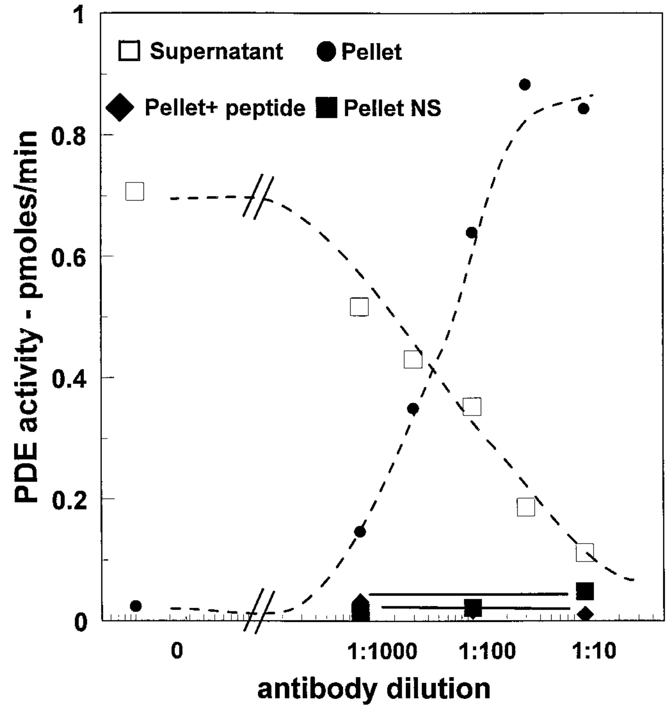

Fig. 2. Immunoprecipitation of the recombinant rat PDE3 by the K116 antibody: specificity of the immunoprecipitation. Aliquots of MA-10 cells expressing recombinant PDE4D3 were incubated with Pansorbin alone, Pansorbin preadsorbed with increasing concentrations of the K116 antibody, increasing concentration of the K116 antibody preadsorbed to peptide 2224 (5 μg/ml), or 1:10–1000 dilutions of preimmune serum. After immunoadsorption, pellets and supernatant were separated according to the procedure described in Experimental Procedures, and PDE activity recovered was measured. *Points,* mean of three observations.

Comparison of the efficiency of the five antibodies in immunoprecipitating the three recombinant proteins demonstrates that these antibodies recognized the cAMP-PDEs to different extents (Fig. 3). Antisera K111 and K116 raised against peptide recognized all three recombinant proteins, albeit with different avidity. On the other hand, antiserum K118 preferentially immunoprecipitated the recombinant PDE4B and could not immunoprecipitate significant activity of PDE4A or PDE4D at a 1:10 dilution. The AC55 antiserum generated against the PDE4A carboxyl-terminal sequence preferentially immunoprecipitated PDE4A but not PDE4B or PDE4D. One of the several monoclonal antibodies generated, M3S1, efficiently immunoprecipitated PDE4D but not PDE4A or PDE4B (Fig. 3).

The cross-reactivity of the five antibodies with the PDE4 proteins was tested further by Western blot analysis with the recombinant proteins (Fig. 4). Soluble extracts from MA-10 cells transfected with the three PDE4 cDNAs were immunoprecipitated with K116 antibody as described above (see Experimental Procedures). Comparable immunoprecipitated activities were analyzed by Western blot analysis. The K116 and K111 antisera recognized all three recombinant proteins (Fig. 4). K116 cross-reacted with the recombinant PDE4B only when present in large amounts, and K111 reacted weakly with the PDE4A protein. As expected from the im-

TABLE 1
Expression of rat PDE2, rat PDE3, and rat PDE4 in MA-10 cells
MA-10 cells were transfected with 10–15 μg of plasmid according to the $CaPO_4$ method. Mock-transfected cells received no plasmid. Experiments done using the vector only gave results identical to those with mock transfection. Cells were harvested 24 hr after transfection, and PDE activity in the homogenate was measured. Each value is the mean ± standard error of at least three transfections performed on different days.

Form transfected	PDE activity
	pmol/min/mg of protein
Mock-transfected cells	23.2 ± 1.2
Rat PDE4A	529.01 ± 249
Rat PDE4B	719.62 ± 40
Rat PDE4D	1549.0 ± 572

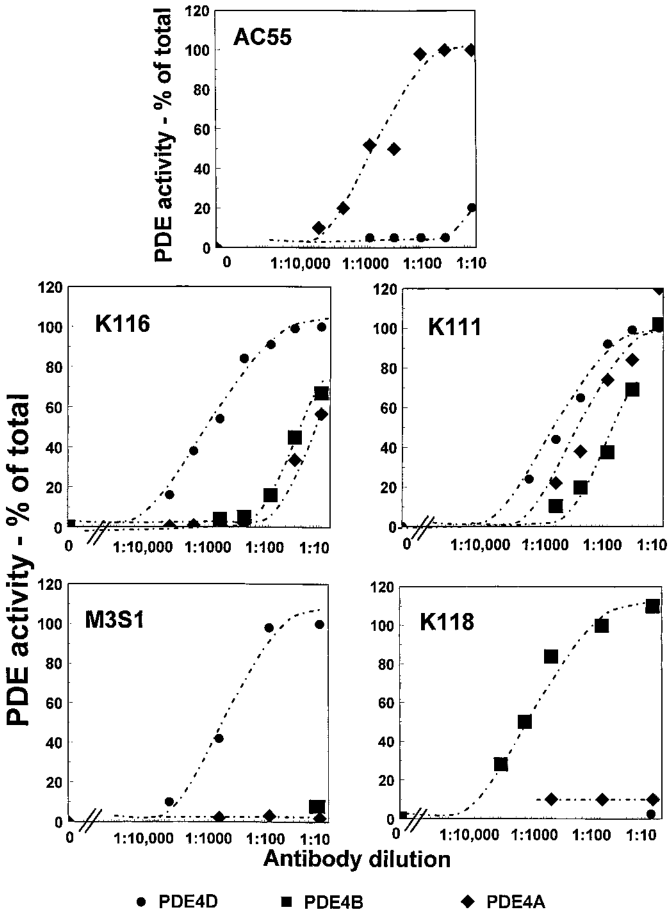

Fig. 3. Comparison of the potency of the K111, K116, AC55, and K118 polyclonal and monoclonal M3S1 antibodies in immunoprecipitating different recombinant cAMP-PDEs. The recombinant rat PDE4A, PDE4B, and PDE4D cAMP-PDEs were partially purified by DEAE-ion exchange chromatography. Comparable amounts of recombinant PDE activity were incubated in the absence or presence of increasing antibody concentration. Bound and free enzymes were separated and assayed as described in Experimental Procedures. Data are expressed as a percentage of the activity added in the immunoprecipitation assay.

munoprecipitation data, M3S1 recognized PDE4D but not PDE4A or PDE4B (Fig. 4). AC55 recognized PDE4A but not PDE4B or PDE4C. Conversely, K118 recognized the recombinant PDE4B but not rat PDE4D or rat PDE4A. Under these experimental conditions, only K111 recognized the MA-10 cell endogenous protein of 108–110 kDa. On the basis of these data, it was concluded that different antibodies had different properties, with AC55, K118, and M3S1 being sufficiently selective for PDE4A, PDE4B, and PDE4D, respectively.

Because most of the epitopes recognized are present in the carboxyl-terminal domain of the different PDEs, the antibodies described cross-reacted equally well with the long PDE variants (Conti *et al.*, 1995b) derived from the three genes (Fig. 5). Furthermore, no appreciable difference was observed in the cross-reactivity with cAMP-PDEs from rat and human species (data not shown).

Immunoprecipitation and characterization of the cAMP-PDEs from brain soluble extracts

Crude soluble extracts were prepared from rat brain, and the cAMP-PDE polypeptides were identified directly by immunoprecipitation and Western blot analysis using the above-described antibodies (Fig. 6A). In some instances, the rat Sertoli cell extracts containing a 68-kDa PDE4D (Conti *et al.*, 1995a) were used as a control. Three immunoreactive polypeptides of 110, 98, and 93 kDa were observed from the immunoprecipitation and immunoblots of soluble rat brain extracts using a K116-nonselective antibody (Fig. 6A). The 110-kDa polypeptide cross-reacted with the AC55 antibody, whereas the 92-kDa polypeptide cross-reacted with the M3S1 antibody (Fig. 6A). The identity of the third band is less certain even though in several experiments it cross-reacted with the PDE4A-antibody. When the PDE4A-selective antibody was used for the Western blot, immunoreactivity of a 75-kDa polypeptide was observed in some but not all soluble preparations of brain (Fig. 6A). Similarly, a 105-kDa immunoreactive peptide was observed with M3S1 monoclonal antibody (Fig. 6A). An 83–92-kDa doublet could be immunoprecipitated from rat brain extracts with either K116 or K118 antibodies, but it cross-reacted only with K118 in the Western blot analysis (Fig. 6A). Confirming our previous observation, a polypeptide of 67–68 kDa was detected from the immunoprecipitation of the Sertoli cell extracts (data not shown). No signal in this molecular weight range was observed in the rat brain extracts (Fig. 6A) (Conti *et al.*, 1995a).

Although immunoprecipitated by the nonselective PDE4 antibody (K116), the observed 83–92-kDa doublet cross-reacted in a Western blot analysis only with the PDE4B-selective antibody. Two additional experiments were performed with proteins derived from the *PDE4B* gene to confirm the identity of these polypeptides. The above-characterized antibodies were used to determine whether the immunoreactive doublet coeluted with the cAMP-PDE after high performance liquid chromatography/DEAE ion exchange chromatography (Fig. 6B). When the fractions of rolipram-sensitive PDE activity were analyzed by Western blotting, the doublet of 83–92 kDa as well as a faint 64-kDa band was present with the PDE4B-selective antibody, K118. The immunoreactivity of the 64-kDa polypeptide could not be blocked by preadsorption of the antibody, suggesting it most likely is a nonspecific band. When different fractions of the DEAE chromatography were tested in a Western blot, it was found to be a good correlation between the intensity of the immunoreactive bands and PDE activity (data not shown).

Because several different polypeptides migrated in the 83–92-kDa region of the gel with brain extract, we tested heart extract to determine whether the identity of the PDE4B polypeptide could be distinguished clearly in a tissue in which other PDE4 proteins are expressed at low levels. Immunoblot analysis of heart soluble extracts with K118 demonstrated the presence of only the 90–92-kDa polypeptide, not the 83-kDa polypeptide. Although efficiently immunoprecipitated by K116, this polypeptide cross-reacted very weakly with the K116 antibody in Western blot analysis (Fig. 6C). More importantly, no additional polypeptides cross-reacted with K116 in that region of the gel (Fig. 6C). Finally, the polypeptides immunoprecipitated by the PDE4B-specific K118 antibody did not cross-react with the PDE4D-specific M3S1 antibody (data not shown). Thus, the 90–92-kDa polypeptide is the product of the *PDE4B* gene and not the result of cross-reactivity of the antibody with PDE4A and PDE4D proteins.

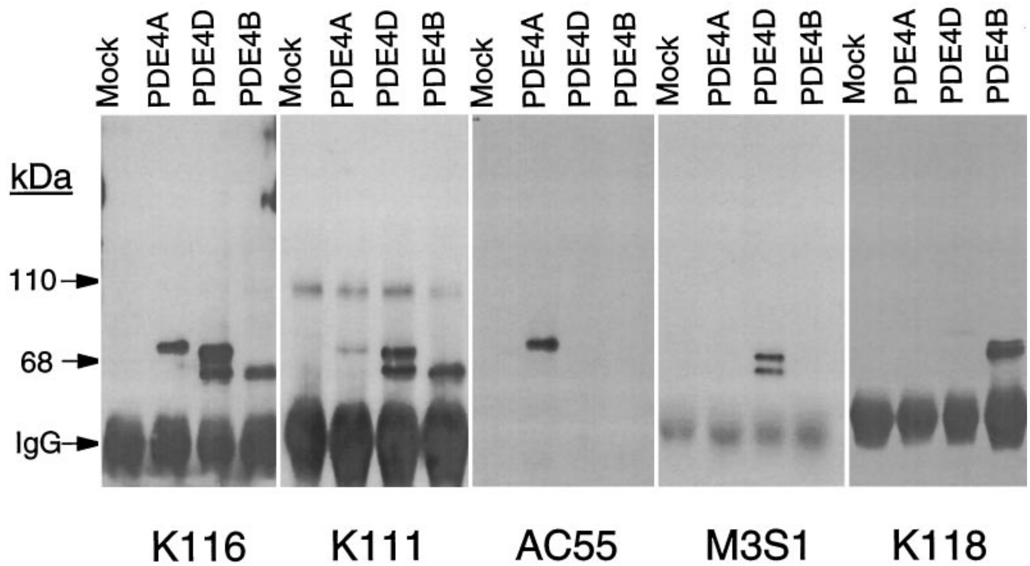

Fig. 4. Western blot analysis of the recombinant cAMP-PDEs expressed in MA-10 cells using K111, K116, K118, AC55, and monoclonal M3S1 antibodies. The cDNAs coding for the short forms of PDE4A, PDE4D, and PDE4B were subcloned into pCMV5 vectors and transfected by the $CaPO_4$ method into MA-10 cells. Cytosol from mock-transfected cells or cells transfected with rat PDE4A1, PDE4D1/2, and PDE4B2 expression vectors was immunoprecipitated with antibody K116 (1:30 dilution) and separated by SDS-PAGE. Replicate blots were incubated with antibody K116, K111, AC55, M3S1, and K118 at a dilution of 1:100. The primary antibody was visualized using peroxidase-linked secondary antibodies, and enzyme activity was visualized by chemiluminescence. Exposure time was adjusted to the intensity of the signal and varied between 5 and 30 sec.

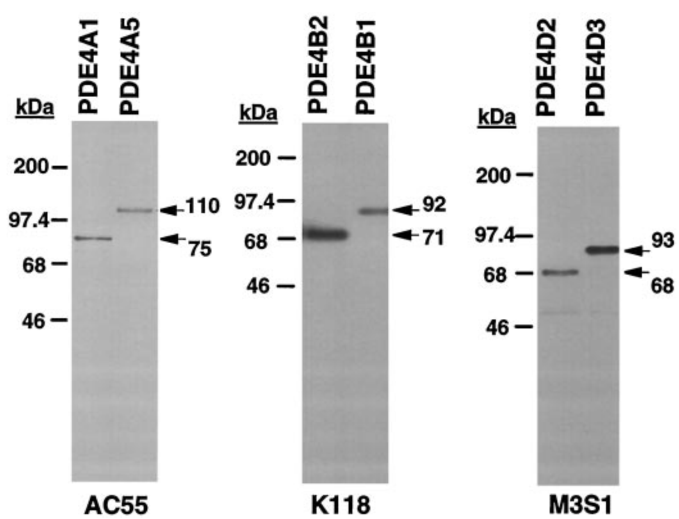

Fig. 5. Western blot analysis of the recombinant short and long forms derived from the *PDE4A*, *PDE4B*, and *PDE4D* genes. Long and short forms of PDE4A, PDE4B, and PDE4D were generated by expression in MA-10 or insect cells. Approximately 5–10 ng of recombinant PDE protein was loaded onto each lane. The recombinant proteins were immunodetected by Western blot using the primary antibody (*bottom*). The molecular mass of the immunoreactive proteins was calculated by comparing their mobility with the following protein standards: myosin heavy chain (250 kDa), phosphorylase B (97.5 kDa), BSA (68 kDa), and ovalbumin (45 kDa). Shown is an experiment representative of the five performed.

Distribution of the cAMP-PDEs in the soluble and particulate compartments

As mentioned above, two additional immunoreactive polypeptides of 75 and 105 kDa were sometimes observed in the brain soluble extracts with PDE4A- and PDE4D-selective antibodies, respectively. A possible explanation for this variability is that the 75- and 105-kDa polypeptides are particulate proteins that are sometimes recovered in the soluble extracts. It has been reported that a substantial amount of cAMP-PDE activity is present in the particulate fraction of the brain (Thompson and Appleman, 1971). This was confirmed by measuring the rolipram-sensitive PDE activity in soluble and particulate fractions of cortex and cerebellum homogenates (data not shown). To further test this hypothesis, the subcellular localization of the different PDE4 forms was determined. The particulate fraction from cortex and cerebellum were extracted sequentially according to the method of Penman (He *et al.*, 1990), and the fractions obtained were analyzed by immunoprecipitation and immunoblot with the different available antibodies. These studies are reported in Fig. 7. In confirmation of previous reports (McPhee *et al.*, 1995; Shakur *et al.*, 1995), the PDE4A-selective antibody identified a 110-kDa polypeptide in the soluble and particulate fractions of the cortex, and a 75-kDa polypeptide was detected almost exclusively in the particulate fraction in both the cortex and cerebellum extracts. Two PDE4B species of 90–92 and 83 kDa were present in the particulate fraction, but in the cerebellum only the 90–92-kDa polypeptide could be identified. The 93-kDa polypeptide cross-reacting with the PDE4D antibody was recovered more in the particulate than in the soluble fraction. More importantly, the 105-kDa polypeptide cross-reacting with the PDE4D antibody was recovered almost exclusively in the particulate fraction of the homogenate of both the cortex and cerebellum extracts (Fig. 7). That this polypeptide is a PDE4 was supported by the observation that this form is immunoprecipitated with either nonselective or PDE4D-selective antibodies and cross-reacted with the nonselective PDE4D (K116) antibody (data not shown). The recovery of the polypeptides in the particulate fraction is not an artifact of the homogenization because the cytosolic LDH enzyme was recovered predominantly in the soluble fraction (data not shown).

Discussion

The cloning of cDNAs for the cAMP-PDEs from a different species has demonstrated that four genes, encoding closely related proteins, are present in mammals (Conti *et al.*, 1995b). Despite many reports identifying different mRNAs expressed in any given tissue (Bolger *et al.*, 1994; Engels *et al.*, 1994, 1995; Iwahashi *et al.*, 1996; Swinnen *et al.*, 1989; Torphy *et al.*, 1992; Verghese *et al.*, 1995), little information is available on whether this multiplicity of genes is translated in the presence of multiple proteins with similar catalytic properties. The data reported herein demonstrate the presence of several PDE4 proteins of different molecular

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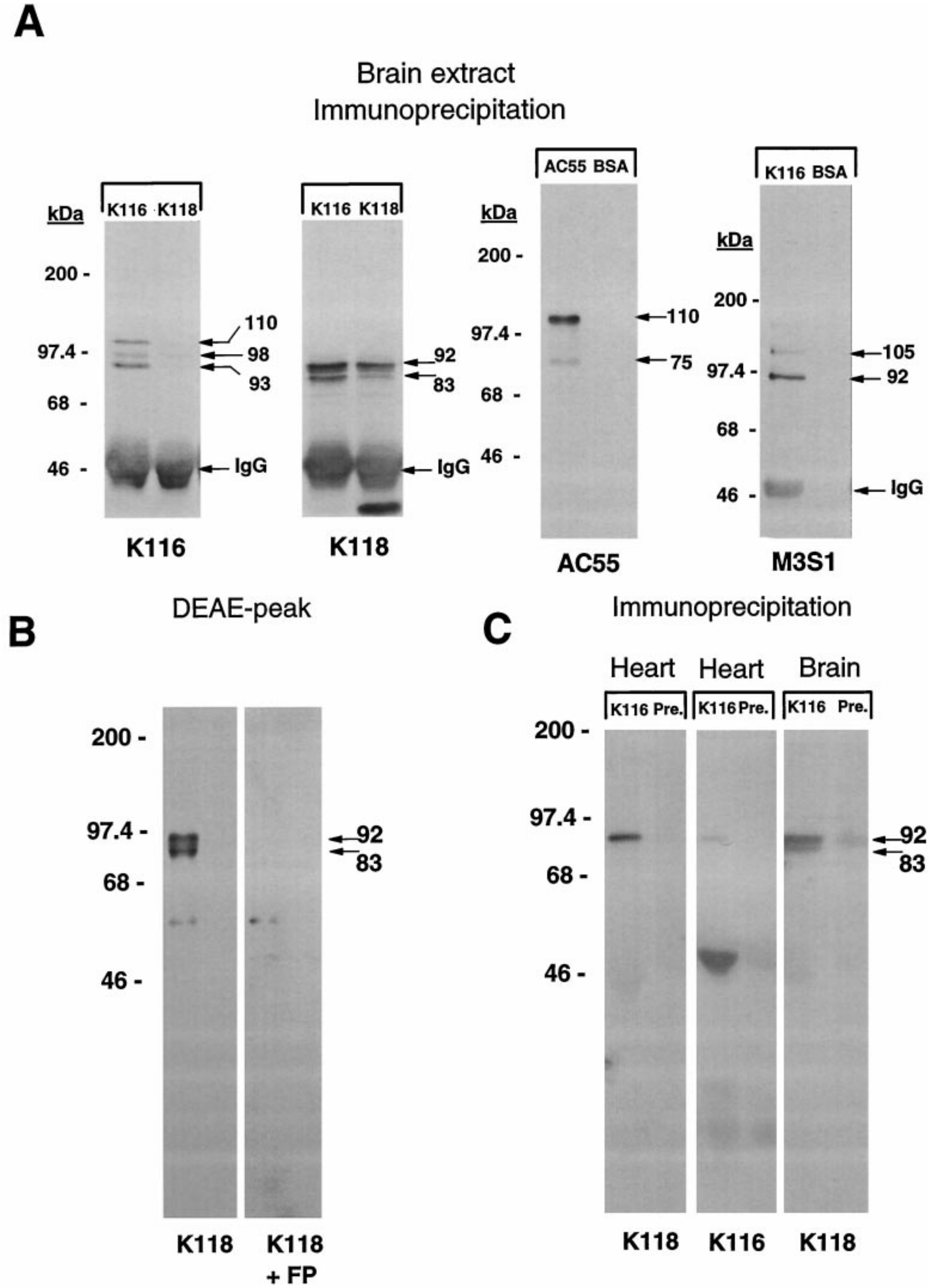

Fig. 6. Comparison of the cAMP-PDEs expressed in the rat brain and heart extracts. A, Soluble extracts from freshly excised brain tissue were immunoprecipitated with nonselective (K116) or PDE4B-selective (K118) or PDE4A-selective (AC55) antibodies. The antibody used for the immunoprecipitation is reported (*top of each lane*). To control for the specificity of the immunoprecipitation, the primary antibody was substituted with BSA or a preimmune serum when available. The immunoprecipitated proteins were separated on SDS-PAGE. After transfer, immunoblots were performed using the primary antibody reported (bottom). B, The cAMP-PDE activity present in brain soluble extracts was purified partially by ion exchange chromatography. An aliquot of the fraction containing the highest rolipram-sensitive activity was fractionated by SDS-PAGE, followed by immunoblot with the K118 antibody. As a control, comparable blots were used for immunostaining with K118 preadsorbed to a GST-PDE4B fusion protein (1 μg/ml). C, Soluble extracts were prepared from brain and heart tissue as described in Experimental Procedures. PDE activity was immunoprecipitated with antibody K118 or K116 or an identical concentration of preimmune serum and separated on SDS-PAGE. Brain extract was used as a control. Western blot analysis was performed with antibody K118 or K116.

masses and distinct immunological properties expressed in the rat brain. Furthermore, our data indicate the presence of different variants derived from each gene.

Our immunoblotting studies have identified at least seven different polypeptides of 67–110 kDa that are recognized specifically by the different antibodies used. A summary of the different forms identified is given in Table 2. The conclusion that these polypeptides correspond to cAMP-PDEs is supported by several findings. These polypeptides are recognized specifically by two antibodies raised against different epitopes in an immunoprecipitation assay and coelute with the rolipram-sensitive cAMP-PDE activity on DEAE ion exchange chromatography. Most polypeptides also were recognized by two different antibodies in Western blot analysis, confirming that two cAMP-PDE epitopes are present in these proteins. In addition, in most instances, the migration of the immunoreactive polypeptide on SDS-PAGE was identical to the migration of a corresponding recombinant protein. Finally, several reports have shown that the *PDE4A*, *PDE4B*, and *PDE4D* genes are expressed to different extents in different brain regions (Bolger *et al.*, 1994; Engels *et al.*, 1995; Iwahashi *et al.*, 1996). The expression of any given protein form correlated well with the expression of the corresponding mRNA species.

In agreement with previous observations (Cherry and Davis, 1995; McPhee *et al.*, 1995; Shakur *et al.*, 1995), it was

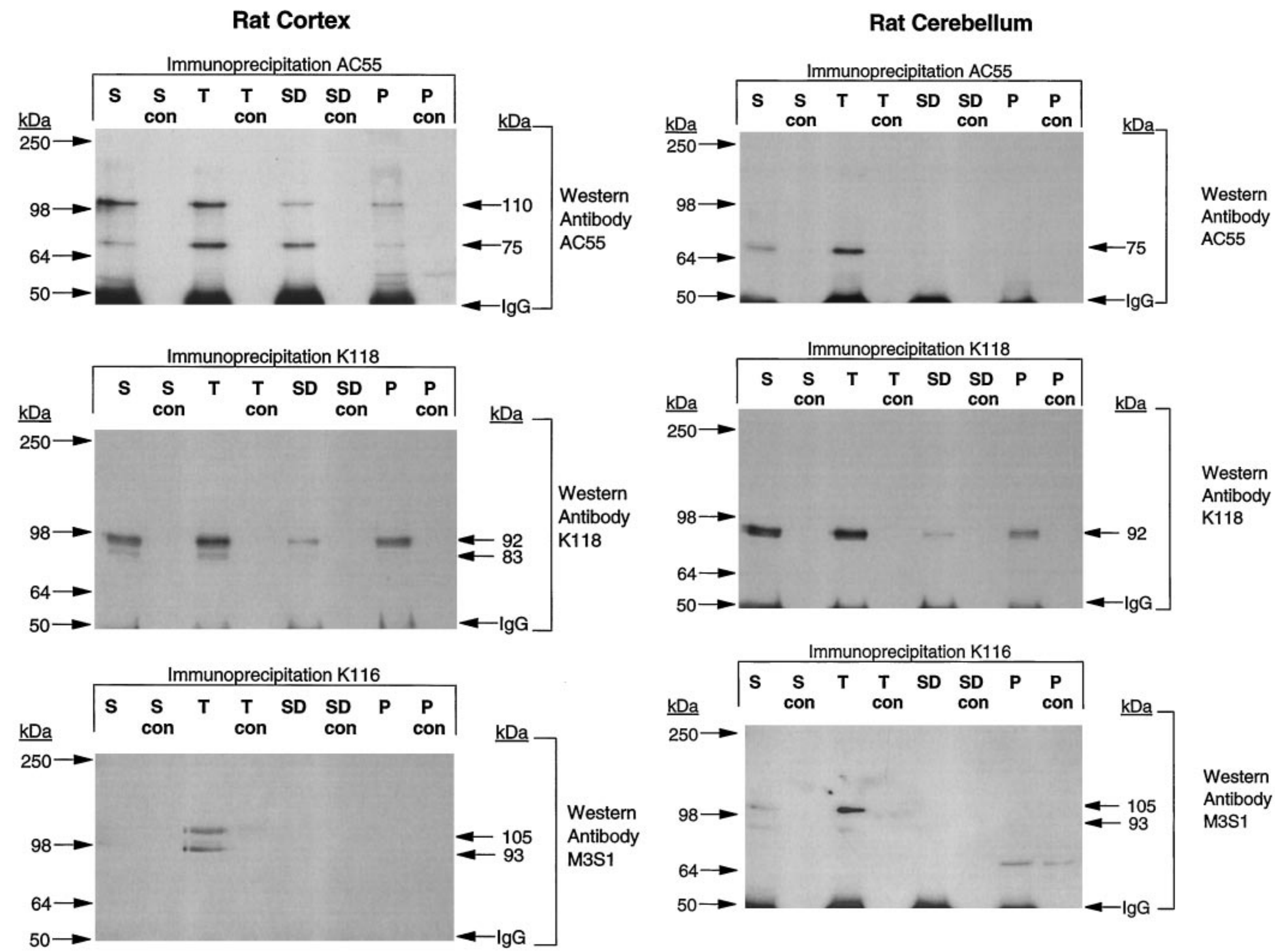

Fig. 7. Subcellular localization of cAMP-PDEs in the soluble and particulate fraction of cortex (*left*) and cerebellum (*right*). The soluble (*S*), Triton-extracted (*T*), deoxycholate-extracted (mean ± standard deviation), and RIPA-extracted (*P*) fractions were collected as described in Experimental Procedures. The samples were immunoprecipitated either with a PDE4-, PDE4A-, or PDE4B-specific antibody and were subsequently blotted with a PDE4D-, PDE4A-, or PDE4B-specific antibody, respectively. Immunoprecipitation with normal rabbit serum or BSA served as the control (*con*) lanes.

TABLE 2
Summary of the properties of the recombinant and native cAMP-PDEs derived from rat brain and Sertoli cell

PDE form	Recombinant	Brain		AB	Sertoli cell	
		mRNA	Protein		mRNA	Protein
	kDa					
PDE4A1	75	+	75 ± 0.8	K116/AC55	–	–
PDE4A5	110	+	110 ± 1.1	K116/AC55	–	–
PDE4A?	?	?	99 ± 2.3	K116/AC55	?	?
PDE4B1	92	+	91 ± 2.8	K116/K118	–	–
PDE4B2	71	+/–	?	K118	+	ND
PDE4B?	?	?	83 ± 1.2	K118	?	?
PDE4D1	73	–	–	K116/M3S1	+	?
PDE4D2	67	–	–	K116/M3S1	+	68 ± 0.5
PDE4D3	93	+	93 ± 1.5	K116/M3S1	–	–
PDE4D4	105	N.D.	105 ± 0.8	K116/M3S1	N.D.	–

N.D., not determined.

found that two predominant PDE4A forms are expressed in the rat brain. These correspond to the 75-kDa PDE4A1 and the 110-kDa PDE4A5 variants derived from the *PDE4A* gene. It was also confirmed that the 75-kDa PDE4A1 protein is recovered mostly in the particulate fraction of brain extracts (Shakur *et al.*, 1995), whereas the 110-kDa PDE4A5 protein is recovered in both the soluble and particulate fraction (McPhee *et al.*, 1995). Several observations indicated that the unique amino terminus of PDE4A1 might contain a signal for membrane compartmentalization (Houslay, 1996; Shakur *et al.*, 1993).

Our data show that proteins of 93 and 105 kDa from brain

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extracts are recognized by the nonselective and the PDE4D-selective antibodies, whereas the polypeptide with similar immunological properties derived from the Sertoli cell has a molecular mass of 67–68 kDa (Conti *et al.*, 1995a). This finding confirms that at least three proteins of different sizes are derived from the *PDE4D* gene. The conclusion is supported by the finding that the 5′ end of the Sertoli cell and brain PDE4D mRNAs are different (Monaco *et al.*, 1994). The 5′ end of the PDE4D3 RNA expressed in rat brain has been isolated and sequenced, confirming our hypothesis (Bolger *et al.*, 1994; Sette *et al.*, 1994). Transfection of a PDE4D3 cDNA produces the appearance of a band of 93 kDa that migrates in a manner identical to that of the brain cAMP-PDE (Sette *et al.*, 1994). Several cDNAs with distinct 5′ sequences have been isolated from human brain (Bolger *et al.*, 1993) and rat and mouse libraries (Jin SLC and Conti M, manuscript in preparation); this PDE4D4 mRNA variant likely encodes the 105-kDa polypeptide identified in the particulate fraction of cortex and cerebellum extracts. Therefore, we can conclude that at least three cAMP-PDE proteins are derived from the rat *PDE4D* gene: one protein is expressed in the Sertoli cell with a molecular mass of 67–68 kDa (PDE4D2), and two proteins of 93 kDa (PDE4D3) and 105 kDa (PDE4D4) are expressed in the brain. The production of proteins of different sizes is due to the presence of different promoters: one active in the Sertoli cell and the others active in the brain (Conti *et al.*, 1995b). Interestingly, both the 93- and 105-kDa proteins were recovered mostly or exclusively in the particulate fraction of the cortex and cerebellum homogenates, suggesting that these forms may be targeted to insoluble subcellular structures. Because substantial amounts of PDE4D3, but only traces of PDE4D4, were recovered in the soluble fraction, it is possible that the two proteins are present in two distinct compartments or the physical interaction with these structures is different. Regardless of the exact location and mechanism of the targeting of PDED3 and PDE4D4, our finding is at odds with a recent report (McPhee *et al.*, 1995) indicating that PDE4D products are exclusively soluble proteins. At present, the reason for these different conclusions is unknown.

Puzzling findings were obtained with the rat PDE4B-selective antibodies. These antibodies recognize recombinant PDE4B of 72 and 90–92 kDa and efficiently immunoprecipitate them. We documented the presence of two immunoreactive species of 90–92 and 83 kDa in the soluble and particulate fractions of cortex and cerebellum. These species can be immunoprecipitated from crude extracts with two antibodies against two different epitopes of the PDE4 molecule. Because they share two epitopes with the PDE4, it is highly unlikely that these polypeptides are proteins other than cAMP-PDE. Although the PDE4B-selective antibody recognized both polypeptides with high affinity, only the polypeptide of 90–92 kDa was weakly recognized by the K116 antibody in Western blot analysis. The 90–92-kDa species was the only polypeptide recognized by the two antibodies in rat heart extracts. Furthermore, the 90–92-kDa polypeptide had the same mobility of the recombinant PDE4B1 protein and coeluted with the rolipram-sensitive PDE activity. On the basis of these findings, we hypothesize that the 90–92-kDa polypeptide corresponds to the PDE4B1 variant. Although further experiments are necessary to exclude the possibility that the 83-kDa polypeptide is a product of degradation of the 90–92-kDa polypeptides, our findings suggest that this protein is a novel splicing variant derived from the *PDE4B* gene. Under our experimental conditions, only trace amounts of the 71-kDa PDE4B2 were observed in the brain suggesting that this “short” variant is not expressed widely in neuronal cells. This would be consistent with our reverse transcription-polymerase chain reaction data indicating that no PDE4B2 mRNA can be detected in rat brain (Monaco *et al.*, 1994). However, RNase protection analysis with a PDE4B2-specific probe detected expression of this form in several brain regions (Bolger *et al.*, 1994). The reason for these conflicting results is unclear. Our antibodies did not detect the PDE4B 64-kDa species described by Lobban *et al.* (1994), which according to the authors corresponds to the DPD form (Colicelli *et al.*, 1989). It should be pointed out that DPD is a truncated cDNA, encoding only a portion of PDE4B1 open reading frame.

In conclusion, the immunological data reported demonstrate that different cAMP-PDE proteins are expressed in different cells or tissues (as summarized in Table 2). Furthermore, a cAMP-PDE gene can produce at least two isoforms with distinct immunological and physicochemical properties, as demonstrated for the *PDE4A*, *PDE4B*, and *PDE4D* genes. Because heterogeneity of the 5′ end of the corresponding mRNA has been demonstrated for the four cAMP-PDEs, we must conclude that these mRNA are translated in distinct cAMP-PDEs. This conclusion is also supported by the comparison of the molecular masses of recombinant and native cAMP-PDEs (Table 2). The antibodies that we generated can be used to determine the repertoire of cAMP-PDEs expressed in each individual cell. These tools will be useful to design pharmacological approaches to manipulate cAMP levels through the inhibition of a specific cAMP-PDE. They also will be useful in determining the physiological significance of the large number of cAMP-PDE forms expressed in mammals. Together with the multiplicity of regulation (Conti *et al.*, 1995b), the distinct subcellular localization of different PDE4 forms may explain the existence of different variants.

Acknowledgments

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