Identification, characterization and regional distribution in brain of RPDE-6 (RNPDE4A5), a novel splice variant of the PDE4A cyclic AMP phosphodiesterase family

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COS-7 cells were transfected with a plasmid encoding a putative splice variant of PDE4A cyclic AMP-specific phosphodiesterase, RPDE-6 (RNPDE4A5). This led to the expression of a novel, cyclic AMP-specific, rolipram-inhibited phosphodiesterase activity. In such transfected cells a novel ~ 109 kDa species was recognized by anti-peptide sera raised against a dodecapeptide whose sequence is found at the extreme C-terminus of both RPDE-6 and another PDE4A splice variant, RD1 (RNPDE4A1A). RPDE-6 activity and immunoreactivity was found distributed between both pellet (~ 25%) and cytosol (~ 75%) fractions of transfected COS-7 cells. Soluble and pellet RPDE-6 activities exhibited similar low K_m values for cyclic AMP (~ 2.4 μM) and were both inhibited by low concentrations of rolipram, with IC_{50} values for the soluble activity being lower (~ 0.16 μM) than for the pellet activity (~ 1.2 μM). Pellet RPDE-6 was resistant to release by either high NaCl concentrations or the detergent Triton X-100. Probing brain homogenates with the anti-(C-terminal peptide) sera identified two immunoreactive species, namely an ~ 79 kDa species reflecting RD1 and an ~ 109 kDa species that co-migrated with the immunoreactive species seen in COS cells transfected to express RPDE-6. The ~ 109 kDa species was found distributed between both the low-speed (P1) and high-speed (P2) pellet fractions as well as the cytosol fractions derived from both brain and RPDE-6-transfected COS cells. In contrast, RD1 was found exclusively in the P2 fraction. Phosphodiesterase (PDE) activity immunoprecipitated by these antisera from brain cytosol had the characteristics of COS cell-expressed RPDE-6 with $K_m^{cyclicAMP}$ ~ 3.7 μM and $IC_{50}^{rolipram}$ ~ 0.12 μM. The distribution of PDE activity immunoprecipitated from the cytosol of various brain regions paralleled that seen for the distribution of the ~ 109 kDa immunoreactive species. It is suggested that the 109 kDa species identified in brain cytosol and pellet fractions is the native form of RPDE-6. The PDE4A splice variants, RD1 and RPDE-6, were shown to have distinct patterns of expression among various brain regions. PDE4A and PDE4B activities appear to provide the major source of PDE4 activity in brain membranes, whereas the cytosolic PDE4 activity is suggested to reflect predominantly the activity of the PDE4D family. Alternative splicing of the PDE4A gene confers distinct N-terminal domains on RPDE-6 and RD1, which attenuates the $V_{max.}$ of these enzymes and defines their distinct subcellular distribution pattern.

INTRODUCTION

Cyclic AMP phosphodiesterase (PDE) activity provides the sole route for inactivation of the key second messenger cyclic AMP. This activity is provided for by a complex gene family whose members show distinct kinetic and regulatory properties [1–8]. Within the PDE family is a distinct class of enzymes known as the PDE4, rolipram-inhibited cyclic AMP-specific phosphodiesterase family [7,8]. The cloning of a cyclic AMP-specific phosphodiesterase from *Drosophila* [5] has allowed for the isolation of cognate cDNAs from both rat [4,5,7,9–11] and human [9–16] sources. This has allowed for the identification of four distinct genes (PDE4A, PDE4B, PDE4C and PDE4D), distributed on three different chromosomes [10,11], which encode members of this enzyme family. Such enzymes can be specifically and selectively inhibited by the antidepressant drug 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidone (rolipram) and related compounds [4,5,8,13,17–20].

There is now, however, an increasing body of molecular genetical evidence [5,7,10–12,21] for the occurrence of splice variants within the PDE4 family in both human and rat. This notion of potential multiplicity of protein products from genes encoding PDE4 species has recently been consolidated through immunological analyses [22]. These have not only identified multiple protein products derived from a PDE4 gene but have also shown, in the case of the PDE4B isoforms in rat, both a selective association with membranes of a particular isoform and the differential expression of splice variants in various brain regions [22].

The first mammalian PDE4 to be cloned [5,23] was a species called RD1 (RNPDE4A1A), which provides the first member of the PDE4A family. Using an antiserum generated against a dodecapeptide that reflects the C-terminus of RD1 we were able to immunoprecipitate selectively a PDE4 activity from cerebellum whose kinetic properties and size reflected that of RD1 when it was expressed transiently in COS cells. Native RD1, from cerebellum, and RD1 expressed transiently in COS cells are both membrane-bound species requiring detergent for solubilization [24,25]. Such membrane association is apparently conferred upon RD1 because of a membrane targeting domain found

Abbreviations used: PDE, cyclic AMP phosphodiesterase (the PDE4 enzymes are rolipram-inhibited cyclic AMP-specific phosphodiesterases for which there are four known genes, giving rise to PDE4A, PDE4B, PDE4C and PDE4D); RD1, rat '*dunc*-like' PDE also referred to as either RNPDE4A1A or rPDE-IV_{A1} representing rat PDE4, isoform subfamily A splice variant 1 (GenBank accession number M26715); RPDE-6, a splice variant of the PDE4A gene referred to formally as RNPDE4A5 (GenBank accession number L27057); CaM, calmodulin; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; rolipram, 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidone.

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within the N-terminal first 25 amino acids of RD1 [24,25]. Indeed, chimeric constructs between RD1 and chloramphenicol acetyltransferase (CAT) have been used to demonstrate that the N-terminal domain of RD1 can confer membrane association upon this normally soluble, cytosolic enzyme [26]. Thus the N-terminal domain of RD1 appears to provide a membrane anchor for an essentially soluble, 'core' PDE protein as well as conferring enhanced thermostability on this enzyme [25] and attenuating its activity through lowering the $V_{max.}$ of the reaction [24].

It has been suggested that splice variants of RD1 may be produced [5,23]. In this regard two cDNAs, called RD2 and RD3 [23], have been identified, which encode species that are smaller than RD1. However, these are considered [5,7,10,23] to be either incomplete clones (RD2) or to have arisen through cloning artefacts (RD3). More recently, however, a clone (pRPDE-6) encoding a putative PDE4A (RNPDE4A5) of greater size than RD1 has been identified [10]. Alignment [10] of the nucleotide sequence of this PDE4 species with that of RD1, whose sequence was independently confirmed in the study [10], showed that they shared, nucleotide for nucleotide, a large block of sequence within which was encoded the putative catalytic domain. Such data were consistent with RD1 and RPDE-6 forming distinct splice variants of the PDE4A gene [10]. Intriguingly, the cDNA for RPDE-6 would appear to encode a protein that shows identical amino acid sequence to RD1 over a region extending from the C-terminus of RD1 until 23 amino acids from the N-terminus of RD1. After this, the sequence of RPDE-6 changes dramatically and a novel N-terminal domain, formed by some 256 amino acids, is found. Thus, alternative splicing of the PDE4A gene would seem to delete from this variant the membrane targeting domain that is contained within the N-terminal 25 amino acids of RD1 [24–26].

In this study we show that when the RPDE-6 cDNA was expressed in COS cells then a dramatic increase in PDE activity occurred. This increase was due to the expression of a novel PDE4 activity. Use of antisera generated [24] against a dodecapeptide that reflects the sequence found at the extreme C-terminus of RPDE-6 enabled us to detect a novel immunoreactive species in transfected COS cells that co-migrated with a species found in brain homogenates and which we suggest may provide the native form of RPDE-6.

MATERIALS AND METHODS

The rat dunce cDNA for RD1 was kindly provided by Professor R. L. Davis, Baylor College of Medicine, Houston, TX, U.S.A. Restriction enzymes, Dulbecco's modified Eagle's medium (DMEM) and fetal-calf serum (FCS) were from Gibco/BRL (Paisley, U.K.). Tris, Hepes, DEAE-dextran (500 kDa), cytochalasin B, benzamidine hydrochloride, PMSF, aprotinin, pepstatin A, antipain, EDTA, EGTA, cyclic AMP, cyclic GMP, Dowex 1X8-400 (chloride form, 200–400 mesh), 3-isobutyl-1-methylxanthine, snake venom (*Ophiophagus hannah*), and bovine brain calmodulin (CaM) were from Sigma Chemical Co. (Poole, Dorset, U.K.). [^{3}H]Cyclic AMP and [^{3}H]cyclic GMP were from Amersham International (Amersham, Bucks., U.K.). Leupeptin was from Peptide Research Foundation (distributed by Scientific Marketing Associates, London, U.K.). Dithiothreitol, Triton X-100 and Triton X-114 were from Boehringer (UK), Ltd. (Lewes, U.K.). Triethanolamine was from BDH (Glasgow, U.K.). Glycerol was from Fisons (Loughborough, Leics., U.K.). Bradford reagent was from Bio-Rad (Herts., U.K.). DMSO was from Koch-Light Ltd (Haverhill, U.K.). Rolipram was a generous gift from Schering Aktiengesellschaft, Postfach 650311, D-1000 Berlin 65, Germany.

Generation of a COS cell expression vector for RPDE-6

The generation of the expression vector for RD1, namely pSVL-RD1, and for that encoding a truncated species lacking the first 25 amino acids of RD1, namely pSVL-Met^{26}RD1, has been described before by us [25]. Similar procedures were adopted to create a suitable expression system for RPDE-6 except that the plasmid pSV.SPORT was employed. Thus the pBluescript plasmid containing RPDE-6, as described before by one of us [10], was used as a source from which to subclone RPDE-6 into pSV.SPORT for expression in COS-7 cells. This was done by subcloning into the *Eco*R1 site of pSV.SPORT to generate the plasmid pSV.SPORT-RPDE-6 with restriction enzyme digests being used to check the orientation of the insert.

Transfection of COS-7 cells

COS-7 cells were seeded at approx. one-third confluence on to 10 cm diam. plates, 18 h before the transfection. Immediately before transfection the culture medium was replaced with 5 ml of DMEM supplemented with 10% (v/v) Nuserum (Collaborative Biomedical Products) together with 0.1 mM chloroquine. This solution was prepared by diluting the DNA to 250 μl in TE buffer (10 mM Tris/HCl, 0.1 mM EDTA, pH 7.6) and adding 200 μl of a 10 mg/ml DEAE-dextran solution. The mixture was incubated at room temperature for 15 min before addition to the COS-7 cell culture. The cells were incubated for 3–4 h at 37 °C in a 5% CO_2 atmosphere before the medium was aspirated and the COS cell culture shocked for 2 min with a solution of 10% DMSO in PBS. The culture was then rinsed twice in PBS before DMEM containing 10% FCS was added and the cells incubated at 37 °C in a 5% CO_2 atmosphere for 72 h. Where indicated, experiments were also done to transfect cells with either native vectors (pSVL; pSV.SPORT) alone or with vectors, generated previously by one of us [24], which allowed for the expression of RD1 (pSVL-RD1) and Met^{26}RD1 (pSVL-Met^{26}RD1) or with pSV.SPORT-RPDE-6 to allow for the expression of RPDE-6. Such transfections of COS-7 cells were done in an identical fashion to that described above, with plates being transfected with a DEAE-dextran solution containing 10 μg of plasmid DNA.

Disruption of COS cells was done as described by us previously in some detail [24,25]. This was done in KHEM buffer (50 mM KCl, 50 mM Hepes/KOH, pH 7.2, 10 mM EGTA, 1.92 mM $MgCl_2$) containing 1 mM DTT and a mixture of protease inhibitors at a final concentration of 40 μg/ml PMSF, 156 μg/ml benzamidine and 1 μg/ml each of aprotinin, leupeptin, pepstatin A and antipain. Membrane pellets were resuspended in this mixture also. Homogenization was performed in the additional presence of 20 μM cytochalasin B [25].

Routinely a total pellet fraction was generated by centrifugation of homogenates for 60 min at 100000 g_{av}. However, in some instances fractionation into a P1 pellet (1000 g_{av} for 10 min) and a P2 pellet (60 min at 100000 g_{av}) leaving a high-speed supernatant (S) was done. The homogenization procedure was complete in that there was no detectable latent lactate dehydrogenase activity [26] present in the P1 pellet, indicating an absence of cytosol proteins.

Generation of anti-RD1/RPDE-6 sera

This was done as described previously [24]. Briefly, a rabbit polyclonal antiserum was generated against a peptide that corresponds to the 12 amino acids that characterize the C-terminus of both RD1 and RPDE-6, namely [(C)-T-P-G-R-W-G-S-G-G-D-P-A]. The peptide was synthesized with a cysteine at

the N-terminus to facilitate its conjugation to keyhole limpet haemocyanin. Antisera derived from immunization with such a peptide have been shown to recognize RD1 [24] and would thus be expected to recognize other PDE4A splice variants that possess common C-terminal regions, such as that predicted for RPDE-6. Similar results were obtained in this study using the antisera '1992', '270' and '271' that were raised in different rabbits.

Brain homogenates

This was done as described before by us in some detail elsewhere [22,24] but using whole brain rather than cerebellum as the source. Frozen aliquots were not subject to re-freezing and frozen samples were not stored beyond 7 days. Simple fractionation studies were also done with the homogenate being centrifuged at $1000\,g_{av}$ for 10 min to yield a 'low-speed' P1 pellet. The supernatant was decanted and re-centrifuged at $100000\,g_{av}$ for 1 h to yield a 'high-speed' P2 pellet together with a supernatant (S) fraction. The membrane pellets were each individually resuspended in ice-cold homogenization buffer [22,24] and then analysed.

Various brain regions were dissected and analysed as described before by us [22]. The 'membrane' fractions analysed were a pellet formed at $100000\,g$ for 60 min. This did not contain any latent lactate dehydrogenase activity, indicating that no cytosolic material was present [22,24].

Treatment with high salt concentrations

Membranes (0.2 mg) from rat brain were treated with KHEM buffer (50 mM KCl, 50 mM Hepes/KOH, 10 mM EGTA, 1.92 mM $MgCl_2$, pH 7.2) containing a range of NaCl concentrations (final pH 7.2). The membranes were left on ice for 30 min at 4 °C before centrifugation at $100000\,g$ for 1 h at 4 °C. The resulting pellet was resuspended in KHEM buffer containing the appropriate NaCl concentration and the pellet and supernatant fractions analysed by Western blotting [22,24].

Solubilization with Triton X-100

Membranes (0.2 mg) from rat brain were treated with KHEM buffer containing a range of Triton X-100 concentrations. The membranes were left on ice for 30 min at 4 °C before centrifugation at $100000\,g$ for 1 h at 4 °C. The resulting pellet was resuspended in KHEM buffer containing the appropriate Triton X-100 concentration and the pellet and supernatant fractions analysed by Western blotting.

Immunoprecipitation

This was done as described before by us [22,24]. Briefly, membranes (1 mg of protein) were resuspended in 'immunoprecipitation buffer' (0.5% Triton X-100, 10 mM EDTA, 100 mM $NaH_2PO_4,2H_2O$, 50 mM Hepes, pH 7.2) containing protease inhibitors (as in KHEM buffer). Aliquots (25 μl) of antibody were added and the sample mixed overnight at 4 °C. An aliquot (100 μl) of 10% Pansorbin (Calbiochem) was added and the sample mixed at 4 °C for 2 h. Following this, the sample was centrifuged in a microfuge at $12000\,g_{av}$ for 2 min, the pellet washed twice in immunoprecipitation buffer and the pellet resuspended in KHEM buffer for PDE assay or Laemmli buffer [27] for SDS/PAGE. The addition of antiserum to solubilized membrane extracts from either brain or COS cells transfected with either pSVL-RD1 or pSV.SPORT-PDE-6 did not appear to alter (< 5%) the PDE activity.

Relative $V_{max.}$ determinations

This was done using the methodology described by us previously [24]. Briefly, increasing concentrations of membrane protein (25–200 μg) from either pSVL-RD1- or pSV.SPORT-PDE-6-transfected COS-7 cells were analysed by Western blotting. The resultant blots were scanned and the absorbance versus amount (μg) of sample protein plotted to gauge the relative concentrations of either RD1 or RPDE-6 in each of the preparations. For the $V_{max.}$ determinations, amounts of membrane protein from transfected COS-7 cells, which would provide equal amounts of these two PDE4 species, were taken for immunoprecipitation. They were then assayed for PDE activity over a range of cyclic AMP concentrations and the data plotted as a Lineweaver–Burke [28] plot to define the relative $V_{max.}$ values.

SDS/PAGE and Western blotting

Acrylamide (8% or 10%) gels were used and the samples boiled for 3 min after being resuspended in Laemmli [27] buffer. Gels were run at 8 mA/gel overnight or 50 mA/gel for 4–5 h with cooling. For detection of RD1 by Western blotting, 100 μg of protein samples was separated by SDS/PAGE and then transferred to nitrocellulose before being immunoblotted using RD1-specific antisera. Labelled bands were identified by using anti-(rabbit peroxidase-linked IgG) and the Amersham ECL Western blotting visualization protocol.

In a previous study [24] we had used a batch of Gibco BRL prestained molecular-mass markers to determine the size of RD1. In more recent batches, however, the manufacturers indicated revised apparent molecular-masses for their markers. Despite this, we found that RD1 migrated in an identical position, relative to the markers, to that we had noted before. The net effect of this was that the apparent molecular mass for RD1 that we now were able to calculate ($\sim$ 79 kDa) was somewhat larger than that reported previously by us as $\sim$ 73 kDa [25]. In order to establish whether this larger value ($\sim$ 79 kDa) was correct, we investigated this further in additional sets of experiments which employed different calibration standards. These were (i) unstained markers from GIBCO BRL (200 kDa, 97.4 kDa, 68 kDa, 43 kDa, 29 kDa, 18.4 kDa and 14.3 kDa), (ii) prestained markers from Sigma (β-galactosidase, 120 kDa; fructose-6-phosphate kinase, 84 kDa; pyruvate kinase, 75.2 kDa) and (iii) native protein markers from Sigma (myosin, 205 kDa; phosphorylase *b*, 97.4 kDa; carbonic anhydrase, 29 kDa). The results of such studies were consistent with a size of $\sim$ 79 kDa for RD1 on SDS/PAGE.

PDE assay

PDE activity using 1 μM cyclic AMP as substrate was assayed by a modification of the two-step procedure of Thompson and Appleman [29] and Rutten et al. [30] as described previously by Marchmont and Houslay [31]. All assays were conducted at 30 °C and in all experiments a freshly prepared slurry of Dowex/water/ethanol (1:1:1, by vol.) was used for determination of activities. In all the experiments described, initial rates were taken from linear time-courses of activity. Rolipram was dissolved in 100% DMSO as a 10 mM stock and diluted in 20 mM Tris/HCl/10 mM $MgCl_2$ buffer at a final pH of 7.4 to provide a range of concentrations for use in the assay. The residual levels of DMSO were shown not to affect PDE activity over the ranges used in this study. Double reciprocal plot data were analysed by computer fitting to the hyperbolic form of the Michaelis–Menten equation using an iterative least-squares procedure in order to obtain estimates of K_m and $V_{max.}$ values

together with associated errors (Ultrafit, Marquardt algorithm; Biosoft, Cambridge, U.K.).

Protein determination

Protein was routinely measured by the method of Bradford [32] using BSA as a standard.

RESULTS AND DISCUSSION

Identification of RPDE-6 in brain and pSV.SPORT-RPDE-6-transfected COS-7 cells

We have previously demonstrated that the PDE4A species called RD1 is expressed as an ~ 79 kDa membrane-bound species in cerebellum [24]. Native RD1 was detected using specific antisera raised against a dodecapeptide that reflected the C-terminus of RD1. This species was shown to co-migrate on SDS/PAGE with RD1 found in membranes from transfected (pSVL-RD1) COS cells and to have identical kinetic properties to COS cell-expressed RD1 activity [24]. However, we show here that Western blot analyses of homogenates of whole brain with such antisera identified two immunoreactive species (Figure 1a). Recognition of both of these species was specific in that such a pattern was

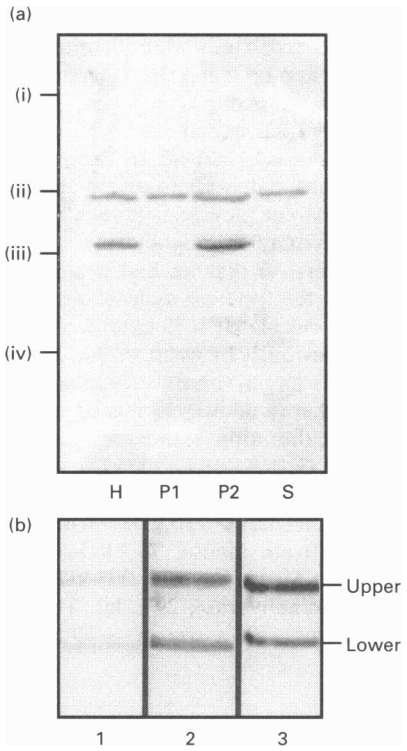

Figure 1 Immunoblotting of brain homogenates

This was done with an antiserum generated against a dodecapeptide [24] whose sequence is found at the extreme C-terminus of the PDE4A PDEs RD1 and RPDE-6 [5,7,10,23]. In (**a**) a whole brain homogenate (H) was resolved into low-speed pellet (P1), high-speed pellet (P2) and cytosol/supernatant (S) fractions, as described in the Materials and methods section. Samples were then subjected to SDS/PAGE followed by Western blotting with the antiserum. The lower band seen in the homogenate and P2 pellet migrated with a molecular mass of 79 ± 3 kDa and the upper band with a molecular mass of 109 ± 7 kDa. Results are from a typical experiment performed three times. Similar data were obtained with antisera raised in two different animals. The molecular-mass markers used in this study and those described in other Figures were prestained species of mass: (i) 214.2 kDa, (ii) 111.4 kDa, (iii) 74.25 kDa and (iv) 45.5 kDa. (**b**) Immunoblots demonstrating the upper (RPDE-6) and lower (RD1) bands detected by our antiserum (track 2) and the ability of the dodecapeptide used to generate the antiserum (C)-T-P-G-R-W-G-S-G-G-D-P-A (0.5 mg/ml) to block (track 1) their detection. An unrelated peptide (C)-A-T-E-D-K-S-L-I-D-T (0.5 mg/ml), in this case one used by us previously [22] to generate an antiserum to the PDE4B family, did not attenuate the signal (track 3).

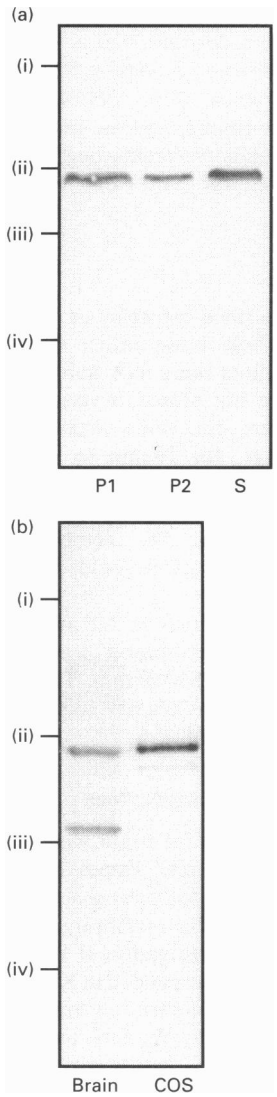

Figure 2 Immunoblotting of COS cells transfected with the cDNA for RPDE-6

As described in the Materials and methods section, COS-7 cells were transfected with pSV.SPORT-RPDE-6 DNA, harvested, disrupted and then subjected to differential centrifugation in order to give low-speed pellet (P1), high-speed pellet (P2) and cytosol/supernatant (S) fractions. Samples were then subjected to SDS/PAGE followed by Western blotting with the antiserum generated against a dodecapeptide whose sequence is found at the extreme C-terminus of the PDE4A PDEs RD1 and RPDE-6 [5,7,10,23]. As described before, no immunoreactive species was detected using COS-7 cells which had either not been transfected at all or had been 'mock-transfected' with the parent vector (pSV.SPORT) only (results not shown). (**a**) The single co-migrating band seen in all of these fractions [P1 pellet, P2 pellet and cytosol (S)] migrated with a molecular mass of 109 ± 4 kDa. Results are from a typical experiment which was performed at least three times. Similar data were obtained with antisera raised in two different animals. The molecular-mass markers used in this study were as described in the legend to Figure 1. (**b**) This shows that immunoblots of an homogenate made from pSV.SPORT-RPDE-6-transfected COS-7 cells (COS) yielded a single band which co-migrates with the upper band found in homogenates of whole brain (Brain). These data show typical single experiments which were performed at least three times. Similar data were obtained with antisera raised in two different animals. The molecular-mass markers used in this study were as described in the legend to Figure 1.

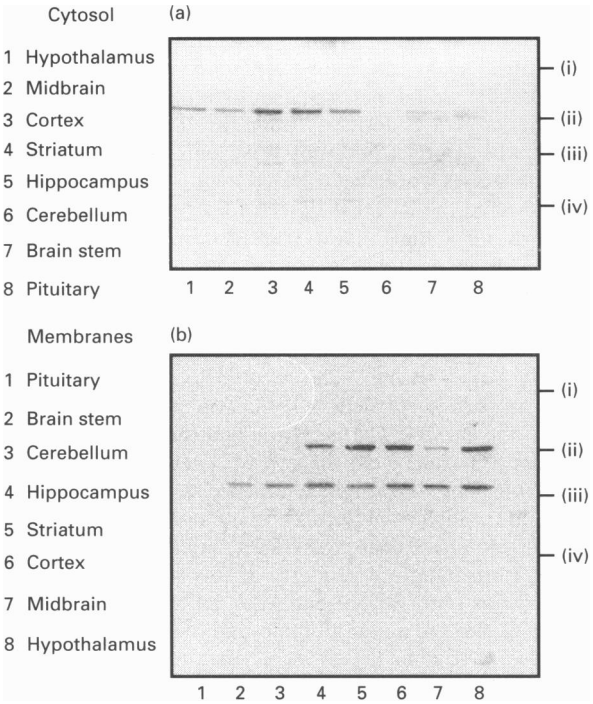

Figure 3 Distribution of immunoreactive RD1 and RPDE-6 in membrane and cytosol fractions from various brain regions

(**a**) Western blots of cytosol from various brain regions (100 μg of protein/lane) using PDE4A C-terminal antiserum. (**b**) Western blots of membranes from various brain regions (100 μg of protein/lane) using PDE4A C-terminal antiserum. These data show typical experiments of those performed at least three times. The molecular-mass markers used in this study were as described in the legend to Figure 1.

observed using three different antisera (results not shown) and recognition by these antisera could be competed out specifically using the C-terminal dodecapeptide used to raise these antisera but not with an unrelated peptide (Figure 1b). The lower band recognized by these antisera was a ~ 79 kDa species that, as noted before by us using cerebellum [24], co-migrated with RD1 expressed in membranes of COS-cells transfected with RD1 cDNA. The upper band, however, migrated at ~ 109 kDa (Figure 1a). Fractionation of this homogenate into a low-speed (P1) and a high-speed (P2) pellet and a high-speed (cytosol) supernatant (S) fraction showed that while the ~ 79 kDa band of RD1 was found exclusively in the P2 pellet, the ~ 109 kDa immunoreactive species was evident in all fractions (Figure 1a).

For a number of reasons we consider it to be unlikely that the ~ 79 kDa immunoreactive species found in cerebellum, which we have previously attributed to be RD1 [24], might be a proteolytic degradation product of the larger immunoreactive species identified here. Certainly, under the experimental conditions employed with rapid tissue processing in the presence of a range of protease inhibitions at ice-cold temperatures, we routinely obtained similar results with no evidence of a range of lower-molecular-mass immunoreactive species that might be expected if proteolysis was occurring. Indeed, immediate homogenization of brain, boiling in SDS sample buffer and analysis consistently identified these two immunoreactive species (Figure 1a). Furthermore, an ~ 109 kDa species was found associated with both membrane and cytosol fractions in brain with, in both instances, such species co-migrating on SDS/PAGE. Thus an identical protein species would appear to be able to occur not only in a soluble state but also immobilized, presumably by targeted interaction with another protein. This implies a fundamental difference between the ~ 109 kDa species and the ~ 79 kDa species which was observed solely in the P2 fraction. Furthermore, the association properties of the ~ 79 kDa species with membranes and its kinetic characteristics have been shown to be identical to those of the enzyme that was transiently expressed in COS cells transfected with RD1 cDNA [24,25], indicating that it is a native species.

In this regard, it has been demonstrated from both isolation of cDNAs [4,7,10,12,21,33] and genomic sequencing [11,34] that splice variants of PDE4 species, including PDE4A forms [7,10–12,33], occur in both humans and rat. One of us has recently isolated a rat cDNA (RPDE-6) [10] which would appear

Table 1 Distribution of RD1 and RPDE-6 in brain regions

Relative distribution (percentage), in these various brain regions, was determined for these two PDE isoforms. Namely RPDE-6 in the soluble, cytosolic fractions and both RD1 and PDE-6 in the membrane fractions. This was done by (i) subjecting 100 μg of protein from each of the fractions to SDS/PAGE, immunoblotting with designated antisera and then quantifying the amount of immunoreactivity (arbitrary units) as described in the Materials and methods section. The amount in each fraction was then expressed as a percentage of the total in order to determine the relative specific distribution between each of the fractions. A similar strategy was then adopted to determine the relative cyclic AMP phosphodiesterase activity using 1 μM cyclic AMP as substrate. Activity which was specifically immunoprecipitated from the cytosol fractions was attributed to RPDE-6 solely as no immunoreactivity representing RD1 was detected in Western blots and RD1 remains solely membrane-associated by virtue of its membrane targeting domain.

Region	Brain stem	Cerebellum	Cortex	Hippocampus	Hypothalamus	Mid-brain	Pituitary	Striatum
Cytosol								
RPDE-6 blot	0	0	35±5	10±3	20±3	5±2	0	30±4
RPDE-6 activity	0±2	0±3	37±5	9±3	27±4	2±3	0	24±4
Membrane								
RPDE-6 blot*	0	0	27±4	14±3	25±4	6±2	0	28±4
RD1 blot**	4±2	10±1	25±3	17±2	8±1	16±2	0	20±3
RPDE-6 + RD1 activity***	6±4	7±2	21±5	20±4	13±4	12±4	0	21±2

*** In contrast, activity which was immunoprecipitated from solubilized membranes represents, in those regions where both isoforms are expressed, the combined activities of both RD1 and RPDE-6. Such data were obtained with immunoprecipiatation done using the various anti-(C-terminal peptide) antisera able to recognize both RD1 and RPDE-6. * The magnitude of this was gauged from the upper band migrating at ~ 109 kDa; ** this was gauged from the lower band migrating at ~ 79 kDa. Errors are shown as S.D. on the mean for determinations done in three separate experiments using different samples ($n = 3$), with determinations done in triplicate in each instance. Determinations shown as zero indicate failure to observe significant activity or immunoreactivity above background in all three separate experiments.

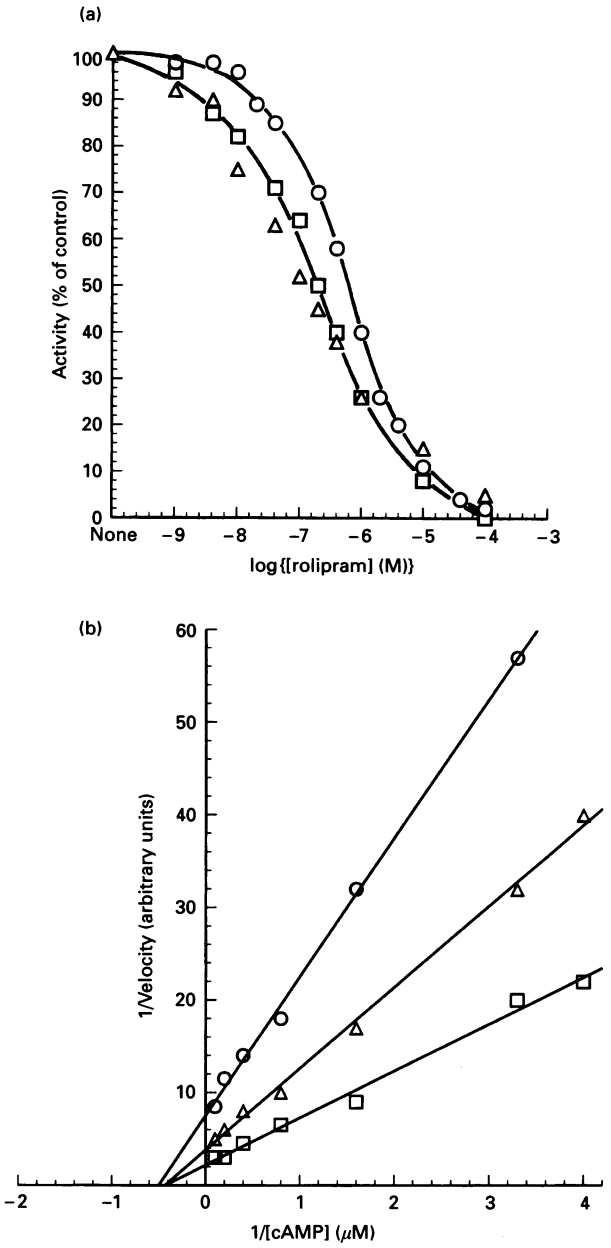

Figure 4 RPDE-6 from brain and pSV.SPORT-RPDE-6-transfected COS-7 cells is a rolipram-inhibited cyclic AMP phosphodiesterase

(**a**) Ability of rolipram to effect the dose-dependent inhibition of PDE activity assayed in the presence of 2 μM cyclic AMP as substrate. This was done using PDE activity immunoprecipitated from brain cytosol (△) using PDE4A C-terminal antiserum and from membranes (○) and cytosol (□) of COS-7 cells which had been transfected with pSV.SPORT-RPDE-6 DNA. 100% activity is denoted as that observed in the absence of any inhibitor. In (**b**) is shown a double-reciprocal plot for cyclic AMP hydrolysis catalysed by PDE activity immunoprecipitated from brain cytosol (△) using the PDE4A C-terminal antiserum and from the membrane (○) and cytosol (□) fractions of COS-7 cells which had been transfected with pSV.SPORT-RPDE-6 DNA. Over the concentration range examined such plots were routinely linear with correlation coefficients > 0.997. PDE activity is shown in arbitrary units. These data show typical experiments with details of parameters and (n) values given in Table 1. We noted, however, that very similar data (< 5% difference) for these parameters (K_m, IC_{50}) were obtained for the RPDE-6 activity in pSV.SPORT-RPDE-6-transfected COS-7 cells whether or not the membrane and cytosolic activities had been immunoabsorbed.

to encode a novel PDE4A of greater size than RD1 and has shown [10], using RNase protection assays, that transcripts for this species occur in rat brain. The cDNA for RPDE-6 is predicted to encode a PDE4 species that is identical to RD1 from the C-terminus up until 23 amino acids from the N-terminus of RD1. Thus, such alternative splicing, to yield RPDE-6 (RNPDE4A5), would be expected to remove the N-terminal membrane targeting domain found in RD1 [24–26] and to replace it with a novel N-terminal domain of some 256 amino acids. This species would be expected to be ~ 94 kDa in size. We have engineered the RPDE-6 cDNA for expression in COS cells and shown here that, upon transfection, this leads to the expression of an ~ 109 kDa species that is recognized by our anti-(C-terminal peptide) sera (Figure 2a). This species, as seen with RD1 [24], appears to migrate on SDS/PAGE with less mobility than might be predicted from its sequence, yielding an apparently larger species. This may reflect particular conformational properties, covalent modification or anomalous binding of SDS to the enzyme. Certainly the fact that the 'core' soluble PDE4A species which lacks the spliced N-terminal domains, and is represented by the engineered species Met^{26}RD1 [24,25], migrates at a higher molecular mass (~ 76 kDa) than that predicted from its sequence (65.1 kDa) suggests that anomalous migration is an inherrent property of this core unit. Consistent with this is our observation that RD1, RPDE-6 and Met^{26}RD1 all, similarly, showed apparent molecular masses on SDS/PAGE which were ~ 10 kDa higher than those predicted from their amino acid sequence. It is possible that the binding of SDS to this protein is reduced, relative to other species, by the large content of acidic amino acids (15%), some of which form glutamate-rich regions, hence effecting a lower migration on SDS/PAGE. Nevertheless, COS cell-expressed RPDE-6 (Figure 2b; track 'cos') can be seen to co-migrate with the ~ 109 kDa immunoreactive species identified in rat brain homogenates (Figure 2b; track 'brain'). And, as transcripts for RPDE-6 have been found in brain [10] we thus consider it plausible that the ~ 109 kDa species that was specifically identified by our anti-(C-terminal peptide) sera is the endogenous form of RPDE-6.

In cerebellum, RD1 was the only species identified by these antisera and thus we were able to characterize this endogenous activity in immunoprecipitates made from solubilized membranes [24]. However, analysis of membranes from a number of different brain areas with our antisera (Figure 3b) showed that while RD1 was expressed in both cerebellum and brain stem, in no region did we find solely RPDE-6 expressed. This precluded our achieving any analysis of membrane-bound, endogenous RPDE-6 by selective immunoprecipitation with these anti-(C-terminal peptide) sera. However, unlike RD1, immunoreactive RPDE-6 was found in a soluble cytosolic form in both transfected COS cells and in various brain regions (Figure 3b). Thus we set out to determine whether PDE activity could indeed be immunoprecipitated from these cytosolic fractions with our antisera.

The addition of PDE4A anti-(C-terminal peptide) sera to brain cytosol did not alter the total PDE activity (< 3%), but it did allow for the immunoprecipitation of PDE activity from various brain regions (Table 1). This activity was insensitive (< 5% change) to either Ca^{2+}/CaM (100 μM; 20 ng/ml) or low concentrations of cyclic GMP (1 μM) but it was dose-dependently inhibited by low concentrations of rolipram and exhibited a low K_m value for the hydrolysis of cyclic AMP (Figure 4; Table 2); characteristics consistent with PDE4 activity.

Such properties coupled with our observations that the distribution, between various brain regions, of immunoprecipitated cytosolic PDE activity paralleled that seen for the ~ 109 kDa species observed upon immunoblotting (Table 1); that the only immunoreactive species observed in these various brain regions was the ~ 109 kDa species and that transfection of COS cells with RPDE-6 yielded both soluble and membrane-bound species

Table 2 K_m^{cAMP}, $IC_{50}^{rolipram}$ and relative $V_{max.}$ values for RD1 and RPDE-6

The properties of RPDE-6 were studied using enzyme derived from COS cells transfected with RPDE-6-pSVL and also from rat brain cytosol. Analyses of PDE activity were done over a substrate range extending from 0.25 to 10 μM cyclic AMP. Lineweaver–Burke plots of such data were, in all instances, linear (correlation coefficient > 0.998 for regression line analysis of data). $V_{max.}$ and K_m^{cAMP} values were determined from the non-linear fitting of data to the Michaelis equation (see the Materials and methods section; correlation coefficient > 0.998). Separate experiments were done using different preparations for the indicated number of occasions (n). The values for K_m^{cAMP} are given as means with errors as S.D. Dose-dependent inhibition of PDE activity by rolipram was determined in the presence of K_m^{cAMP} concentrations of cyclic AMP over a range of rolipram concentrations from 10 nM to 100 μM (8/10 different concentrations). The concentration of rolipram at which 50% inhibition occurred ($IC_{50}^{rolipram}$) was determined from such plots. The values for $IC_{50}^{rolipram}$ are given as means with errors as S.D. for separate experiments using different preparations. Relative $V_{max.}$ values for RD1 and RPDE-6 were determined, as discussed in the Materials and methods section, using the PDE4A-specific antisera to determine the relative amounts of each of the transfected PDE species in the assays. For comparison, data (*) is also given for RD1 analysed previously in transfected COS cells and in cerebellum membranes [24,25]. Student's t-test was used to analyse the differences observed between RPDE-6 activity in the membrane and cytosol fractions: the relative $V_{max.}$ values‡ were significantly different at $P < 0.001$ as were the IC_{50} values† for rolipram inhibition at $P < 0.01$. For uniformity, all comparative assays were performed in the presence of 0.1% Triton X-100, the concentration required to effect the solubilization of RD1 [25]. This level of detergent did not appear to alter PDE activity (< 6%) change in any instance. For soluble RPDE-6, we did not observe any change in either activity or the values of K_m^{cAMP} and $IC_{50}^{rolipram}$ when either different anti-(C-terminal peptide) sera were employed to immunoprecipitate activity or, alternatively, no antisera were used and determinations made on total cytosol after correcting for endogenous PDE activity observed in cells transfected with a control plasmid (vector only). nd, not determined. $IC_{50}^{rolipram}$ values were not changed by 2–3-fold alterations in protein concentration added to enzyme assays.

		RPDE-6			RD1		Met^{26}-RD1
PDE analysed	Source...	COS cell cytosol	COS cell membranes	Brain cytosol	COS cell membranes	Cerebellum membranes	COS cell cytosol
K_m^{cAMP} (μM)		2.4 ± 0.7 (n = 6)	2.4 ± 0.7 (n = 5)	3.7 ± 1.4 (n = 5)	4.1 ± 2.3 (n = 3)	2.3 ± 0.4 (n = 3) (*)	5.3 ± 3.0 (n = 3) (*)
$IC_{50}^{rolipram}$ (μM)		0.16 ± 0.06 (n = 3)†	1.2 ± 0.5 (n = 5)†	0.12 ± 0.07 (n = 3)	0.4 ± 0.1 (n = 3)	0.7 ± 0.3 (n = 3) (*)	0.6 ± 0.2 (n = 3) (*)
Relative $V_{max.}$		0.31 ± 0.03 (n = 6)‡	0.21 ± 0.02 (n = 6)‡	nd	(1)	nd	2.1 ± 0.2 (n = 3) (*)

of identical size, leads us to suggest that the ~ 109 kDa immunoreactive species found in brain does indeed reflect the PDE4A species encoded for by the RPDE-6 cDNA.

Kinetic properties of RPDE-6

We then compared the properties of this endogenous soluble PDE, immunoprecipitated from brain cytosol by our antisera, with those of the species encoded by the RPDE-6 cDNA. Transfection of COS cells with pSV.SPORT-RPDE-6 led to a profound increase in PDE activity (20- to 25-fold increase; range n = 12), while, as noted before [11], (mock) transfection with the parent plasmid (pSV.SPORT) had no effect (< 3%) on either total PDE activity or the fraction that was inhibited by rolipram [25]. The novel PDE activity seen in pSV.SPORT-RPDE-6-transfected cells was unaffected by the addition of either Ca^{2+}/CaM (100 μM; 20 ng/ml; < 5% change) or 1 mM cyclic GMP (< 5% change). The increase in PDE activity subsequent to transfection with pSV.SPORT-RPDE-6 activity was distributed between both membrane and cytosol compartments with the majority of the activity being in the cytosolic fraction. Thus some 79 ± 4% (errors are S.E.M.; n = 17) of the total novel PDE activity was associated with the cytosol. This compared with some 72 ± 3% (errors are S.E.M.; n = 3) of the total immunoreactivity of the ~ 109 kDa species detected with the anti-(C-terminal peptide) antibody being found in the cytosol. This novel PDE activity found in both membranes and cytosol was potently inhibited by the PDE4-selective inhibitor rolipram (Figure 4; Table 2). The IC_{50} values for inhibition by rolipram were very similar for the cytosolic activities immunoprecipitated from both brain and transfected COS cells although, intriguingly, values for the membrane-bound RPDE-6 activity seen in transfected COS cells seemed to be higher (Figure 4; Table 2). It may be that this difference results from an altered conformation of the enzyme occurring upon membrane association. However, we noted that both membrane and cytosolic forms of RPDE-6 exhibited K_m values for cyclic AMP that were not only similar to each other but also to that exhibited by the native, cytosolic brain enzyme (Figure 4; Table 2). Thus RPDE-6 cDNA appears to encode a PDE4 species in transfected COS cells. In this regard, the novel PDE activity in transfectants was potently inhibited by low concentrations of rolipram, had a low K_m value for cyclic AMP and was insensitive to Ca^{2+}/CaM and to low cyclic GMP concentrations. Such properties are also consistent with the notion that this enzyme reflects the ~ 109 kDa species detected using our PDE4A-specific antisera in brain.

Previously [24], we have shown that the N-terminal domain of RD1 exerts an inhibitory effect on the catalytic activity of the enzyme, as shown by the 2-fold greater relative $V_{max.}$ exhibited by Met^{26}RD1 compared with RD1 itself. Here we see (Table 1) that the much larger N-terminal domain found in RPDE-6 appears to exert a profound inhibitory effect on the 'core' PDE activity, as exemplified by Met^{26}RD1; a species engineered so as to delete the N-terminal spliced domain [24]. Thus membrane-associated RPDE-6 was found to have a relative $V_{max.}$ that was only some 10% of that exhibited by Met^{26}RD1 and some 20% of that observed for RD1. Furthermore, membrane-association itself appeared to have an attenuating effect upon RPDE-6 activity as the relative $V_{max.}$ of membrane-associated RPDE-6 was only ~ 67% of that exhibited by the cytosolic species (Table 2). Thus, as suggested before by us [24], multiple splicing of the PDE4A gene leads to the addition of distinct N-terminal domains that can attenuate the $V_{max.}$ of the enzyme. Not only that, but association of such a domain with membranes appears to lead to a further change in activity (Table 2). This is consistent with our earlier contention [24,25] that, for RD1, membrane association led to conformational and functional changes in RD1.

Pellet association of RPDE-6

As with RD1, when the pellet fractions from either brain or RPDE-6-transfected COS cells were treated with increasing concentrations of NaCl then little or no RPDE-6 immunoreactivity was released (Figure 5a). This indicates that RPDE-6 is neither loosely adsorbed to membranes nor does it bind as a peripheral protein simply through ionic interactions. Intriguingly, RPDE-6 was also resistant to release by the detergent Triton X-100 (Figure 5b). This contrasts markedly with RD1 that was released by very low levels of this detergent [25]. It has, however,

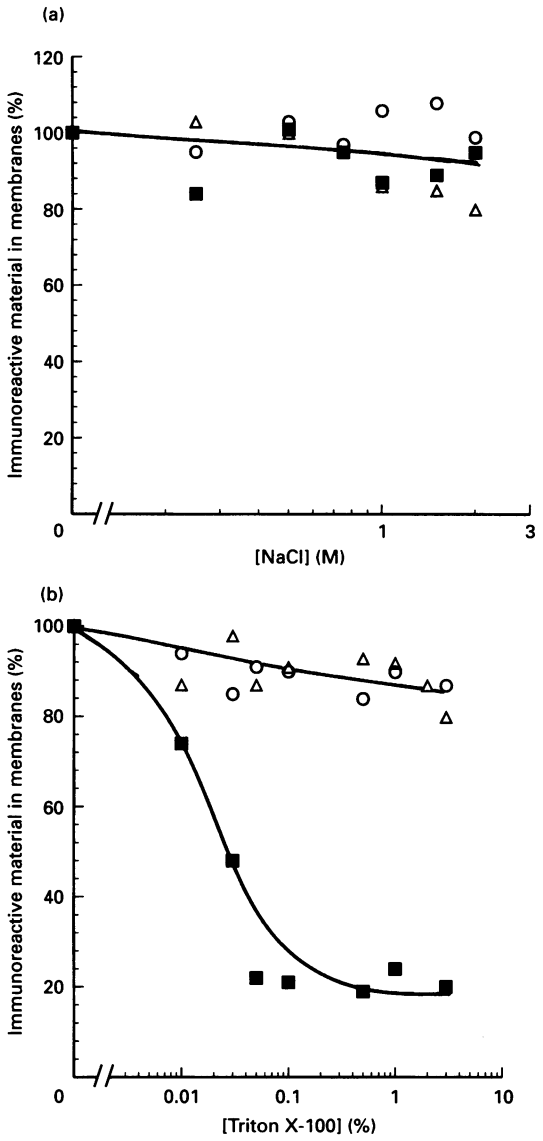

Figure 5 Attempts to release RPDE-6 activity from membranes using high ionic strength and detergent treatment

In (**a**) membranes from both brain and from pSV.SPORT-RPDE-6-transfected COS-7 cells were treated with increasing concentrations of NaCl, incubated on ice and then subjected to high-speed centrifugation (see the Materials and methods section). The membrane-associated immunoreactivity for RPDE-6 is shown for brain (○) and for transfected COS cells (△). Also shown is the RD1 immunoreactivity in brain (■). The amount of pelleted RPDE-6 immunoreactivity was recorded. In (**b**) membranes from both brain and from pSV.SPORT-RPDE-6-transfected COS-7 cells were treated with increasing concentrations of the detergent Triton X-100, incubated on ice and then subjected to high-speed centrifugation (see the Materials and methods section) in order to generate a pellet fraction. This was subjected to SDS/PAGE and immunoblotted using PDE4A anti-(C-terminal peptide) sera. The amount of membrane-associated RPDE-6 immunoreactivity was recorded in brain (○) and in pSV.SPORT-RPDE-6-transfected COS-7 cells (△). Also shown, for comparison, is the amount of RD1 immunoreactivity (■) occurring in brain in the same experiments. These data are typical of experiments performed three times.

been shown that some specialized membrane proteins require both high salt and detergent to effect solubilization [35]. However, even treatment with 1 M NaCl and 4% Triton X-100 failed to render RPDE-6 soluble (< 7% release).

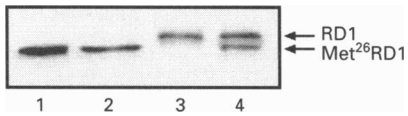

Figure 6 Transfection of COS cells with pSVL-RD1 DNA leads to the expression of membrane-bound RD1 and soluble Met^{26}RD1

Immunoblots of SDS/PAGE gels were done with PDE4A C-terminal antiserum. Analysed were homogenates from COS-7 cells transfected with plasmids carrying either Met^{26}RD1 (track 1) or RD1 itself (track 4). Also, the separated cytosol (track 2) and P2 membrane (track 3) fractions from COS cells transfected to express RD1 were analysed. These data are typical of experiments performed three times. In these experiments, homogenates of RD1-transfected cells showed a doublet, with the upper band attributable entirely to immunoreactivity associated with the P2 pellet and the lower band entirely to immunoreactivity associated with the cytosol fraction. This lower immunoreactive band co-migrated with the single band found in COS cells transfected so as to express Met^{26}RD1.

Lack of release by Triton X-100 suggests that association is not driven solely by hydrophobic interactions between membrane lipids. However, if association was with an integral membrane protein then one would have expected solubilization to occur. That this did not occur might imply that RPDE-6 can bind to cytoskeletal structures that are associated with membranes and that such remnants were not disaggregated upon detergent solubilization of membranes [35,36]. Consistent with this we found that both brain-derived RPDE-6 (Figure 1a) and RPDE-6 expressed in COS cells (Figure 2a) were found similarly associated with both the low-speed (P1) and the high-speed (P2) membrane fractions as well as cytosol. As such there appears to be a fundamental difference in the mode of membrane/pellet association between the two splice variants RD1 and RPDE-6. Indeed, there is no sequence homology at all evident between the membrane targeting domain of RD1 and the 256-amino-acid N-terminal domain of RPDE-6 [10]. Thus it is highly intriguing that the splice junction for the rat PDE4A gene seems to be at a 5′ position that allows for the domain swopping of N-terminal regions which can confer distinct targeting properties on these two splice variants.

pSVL-RD1-transfected COS cells express both RD1 and Met^{26}RD1

It should be noted that we have previously reported that transfection of COS cells with pSVL-RD1 yielded an increase in both membrane and cytosol PDE activity, with the major fraction (~ 85%) being membrane-bound [25]. On this basis we had suggested that RD1 may partition between cytosol and membrane compartments [25]. However, our recent studies using chimeric proteins formed from a fusion between the N-terminal 25 amino acids of RD1 and the normally soluble, bacterial protein CAT indicated that such chimeras were almost exclusively membrane-associated in both COS-cell expression and *in vitro* reconstitution analyses [26]. Such studies [26] also highlighted that the residue Met^{26} in RD1 was within a suboptimal Kozak sequence that, in over-expression systems like COS cells, could lead to products formed by a second start of initiation occurring at this point. As we have noted from truncation studies [25] that Met^{26}RD1 is a soluble protein, then it is quite possible that the so-called 'cytosolic RD1' activity reported in COS cell transfections done with pSVL-RD1 was Met^{26}RD1. In order to address this we have analysed, on the same immunoblot, the homogenate, membrane and cytosol fractions of COS cells transfected with pSVL-RD1 (Figure 6) alongside the cytosol fraction from cells transfected with pSVL-Met^{26}RD1. We can see

Table 3 The relative magnitude of PDE4 activities in various brain regions

Cytosol and membrane fractions were isolated from the various brain areas and each assayed for 'total' PDE activity using 1 μM cyclic AMP as substrate. An approximation of the total PDE4 activity was made by determining the degree of inhibition that could be achieved in each fraction using 10 μM rolipram. This acts as a selective inhibitor of PDE4 activity at such concentrations [1,4,5,20,37,38]. In dose-effect studies done on the various fractions, except for pituitary where no inhibition ($< 4\%$) was evident at concentrations up to 100 μM rolipram, inhibition of PDE activity due to rolipram attained a plateau at 10 μM rolipram with half-maximal effects being seen over a range from 1–4 μM. The degree of inhibition caused by 10 μM rolipram under such conditions allowed us to express such a 'rolipram-inhibitable component' as a percentage of the total PDE activity; this is referred to as the percentage PDE4 activity (% PDE4) in the Table. These data are taken from our previous study [22]. Using antisera to immunoprecipitate RPDE-6 from the cytosol and a mixture of both RPDE-6 and RD1 from membranes we then expressed their activities as a percentages of both the total PDE4 activity and the total PDE activities in both the cytosol and the membrane fractions. Also expressed is the percentage of total PDE activity found in the membrane compared with the cytosol components, taken from [22]; the fraction of the total homogenate activity that the PDE4A family (RD1 + RPDE-6) contributes in various brain regions. Such data are given as means with errors as S.D. for $n = 3$ separate experiments using different brain extracts. Abbreviations: ne, no activity evident; na, not applicable.

Region	Stem	Cerebellum	Cortex	Hippocampus	Hypothalamus	Mid-brain	Pituitary	Striatum
Cytosol								
PDE4 (%)	35±3	32±8	38±9	30±1	41±3	38±10	ne	36±3
RPDE-6 (as % PDE4)	ne	ne	14±3	5±2	10±2	< 1	ne	8±2
(PDE4A + PDE4B) (as % PDE4)	0	0	35±5	40±7	27±5	< 1	na	21±4
Membrane								
PDE4 (%)	45±9	56±6	55±8	64±7	45±5	44±7	ne	55±6
(RD1 + RPDE-6) (as % PDE4)	51±7	23±5	20±2	50±8	31±2	29±8	ne	31±9
(PDE4A + PDE4B) (as % PDE4)	100±10	69±14	61±9	97±16	71±9	49±11	na	101±14
Homogenate								
PDE4A (as % of total)	17±5	5±2	9±4	12±4	9±4	3±1	0	11±2
(PDE4A + PDE4B) (as % of total)	28±5	12±4	21±5	26±8	20±4	6±3	na	22±6

from such analyses that the immunoreactive species found in the cytosol of COS cells transfected with pSVL-RD1 co-migrated with that found in homogenates of cells transfected with pSVL-Met[26]RD1 and that this species was smaller (76 kDa) than the component found in the membranes of pSVL-RD1 transfected cells (Figure 6). Furthermore, analysis of homogenates of pSVL-RD1-transfected cells allowed us to identify the expression of a closely migrating doublet, reflecting the expression of both RD1 and Met[26]RD1 (Figure 6). It would seem that the increase in soluble, cytosolic PDE activity seen in COS cells transfected with pSVL-RD1 was due to Met[26]RD1 expression rather than to that of RD1. Indeed, this would explain why the novel cytosolic PDE activity found in pSVL-RD1-transfected cells not only exhibited a dramatically different half-life for thermal denaturation from that shown for membrane-bound (RD1) activity, but that this value was identical to that seen for cells transfected to express Met[26]RD1 [25]. Thus we believe that the N-terminal domain of RD1 confers an exclusive membrane localization upon this splice variant, in contrast to that seen for RPDE-6. Certainly we believe that no significant level of soluble RD1 occurs in brain as shown from our inability both to immunoprecipitate PDE activity from cerebellum homogenates (Table 1) and to detect any ~ 79 kDa immunoreactive species in soluble fractions from various brain regions (Figure 3) using our anti-(RD1 C-terminal peptide) sera. Thus we believe that usage of this suboptimal Kozak sequence at Met[26] (RD1 numbering) is likely to be confined to overexpressing systems such as those found in COS cells and is not seen *in vivo*.

Distribution of RPDE-6 and RD1 in various brain regions

We have shown previously that both cytosolic and membrane-bound PDE4B activity are differentially expressed in various brain regions [22]. This would appear also to be the case for both RD1 and for RPDE-6 (Figure 3). However, as mentioned above, no region was evident where RPDE-6 was expressed but not RD1 (Figure 3). The major locations of these two PDE4A species appeared to be cortex, hypothalamus and striatum. The next major sources were hippocampus and mid-brain with small amounts of membrane-bound RD1, but not RPDE-6, found in brain stem and cerebellum (Figure 3; Table 1). As noted in analyses of the PDE4B forms [22], the pituitary was bereft of either of these PDE4A species. Additionally, either no or very little cytosolic PDE4A and PDE4B activities were found in brain stem, cerebellum and mid-brain. Consistent with our distribution studies (Table 2), in RNase protection analyses, Bolger et al. [10] were able to identify transcripts for both RPDE-6 and RD1 in cortex, while in both cerebellum and brain stem they were only able to detect transcripts for RD1. This provides additional support for our attributing the two species detected in brain by the PDE4A C-terminal antisera to RD1 and RPDE-6.

In a similar manner to that done previously by us [22], we have attempted to gauge the relative magnitude of the contribution of RD1 and RPDE-6 in various brain regions to total PDE4 activity (Table 3). This was assessed using rolipram, a compound that serves as a potent and selective inhibitor of PDE4 enzymes. Enzymes of the PDE4 family are potently inhibited in a competitive fashion by rolipram with K_i values of the order of 0.4–4 μM [3–5,12–14,18,24,25]. In contrast, other PDE isoforms are either insensitive to rolipram or only very weakly inhibited with K_i values greater than 100 μM having been noted [3–5,20,37,38]. We can then consider that an approximation of the amount of PDE4 activity present in crude extracts will be reflected in the fraction of the total PDE activity that can be inhibited by low concentrations of rolipram. In such comparative analyses we routinely used a cyclic AMP substrate concentration of 1 μM that is not only below both the K_m for cyclic AMP for PDE4 species but is also below the activation threshold for protein kinase A and is similar in magnitude to that observed in a number of cells under resting/basal conditions (see refs. [1] and [39]). Of course, in intact cells, any activation of adenylate cyclase would elevate intracellular cyclic AMP levels, causing the relative activities of various PDE isoforms to change dependent upon their K_m and $V_{max.}$ values [39]. However, while the similarity in the K_m values for the various PDE4 species makes it probable that their relative activities will stay constant over a range of cyclic AMP concentrations, changes in their relative activities is likely to occur with respect to other PDE families whose activities

have different K_m values and may be affected by distinct regulatory processes [1–5]. Additionally, these brain regions contain a variety of cell types that will further complicate the issue concerning relative expression of PDE isoforms and the nature of their regulatory mechanisms. With these constraints in mind, our analyses may, however, give insight into the possible significance of these PDE4 splice variants to cyclic AMP metabolism in the various brain regions. Doing this we can see that, in the cytosol, RPDE-6 contributes between 5 and 14% of the PDE4 activity in the cortex, hippocampus and striatum (Table 3). In membranes, however, the combined actions of RPDE-6 and RD1 can contribute up to ~ 50% of the total PDE4 activity in brain stem and hippocampus and between 20 and 30% in all other regions save the pituitary (Table 3). Thus RD1 and RPDE-6 appear to provide a major component of the PDE4 activity in membranes. Taken together with the results of our previous studies [22], analysing the PDE4B activities in brain membranes, it would seem that the combined activities of the PDE4A and PDE4B isoforms make up the major fraction of the membrane PDE4 activity (Table 3). Indeed, in the case of striatum, the PDE4A and PDE4B families appear to provide the entire PDE4 activity (Table 3). We suggest that it is likely that the residual PDE4 activity in the other brain regions is due, primarily, to the activity of members of the PDE4D family. Our reason for proposing this is because strong signals for transcripts of PDE4D have been found in brain, whereas transcript levels for PDE4C appear either to be non-existent or vanishingly small [10,40]. Interestingly, analysis of PDE4A and PDE4B activities in cytosol show that while these isoforms contribute between 20 and 40% of the total PDE4 activity in cortex, hippocampus, hypothalamus and striatum, they do not contribute to soluble PDE4 activity found in the brain stem, cerebellum and mid-brain (Table 3). Thus it is our overall impression that, for cytosol, the major PDE4 activity is likely to be provided by members of the PDE4D family whereas, for membranes, the PDE4A and PDE4B activities appear to predominate.

Conclusion

In this study we have characterized the activity of a novel PDE4A splice variant RPDE-6 and provided evidence that is consistent with its expression in brain. Interestingly, RPDE-6 appears to exhibit a different distribution pattern, between various brain regions, compared with the other PDE4A splice variant, RD1. Unlike RD1, which appears to be exclusively membrane-bound, RPDE-6 is found both in the cytosol and associated with particulate fractions. The different subcellular distribution pattern between these two forms appears to be conferred by their distinct N-terminal domains, which result from alternative splicing. These domains also differentially regulate enzymic activity by attenuating the $V_{max.}$ of the reaction. Such differences between RD1 and RPDE-6 are likely to reflect distinct functional roles performed by these two PDE4 splice variants. This implies that their distinct intracellular location, coupled with an ability to regulate their activity via their N-terminal domains, may be of some importance to the control of cellular functioning. In this regard, the design of selective inhibitors for the PDE4 family for therapeutic use might not only have to consider the four gene families but also the various splice variants found within them. Similarly, any investigations aimed at determining aberrant functioning of these enzymes in disease states should also take into account any change in their subcellular localization.

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