Engineered deletion of the unique N-terminal domain of the cyclic AMP-specific phosphodiesterase RD1 prevents plasma membrane association and the attainment of enhanced thermostability without altering its sensitivity to inhibition by rolipram

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Full-length cDNA for the rat brain rolipram-sensitive cyclic AMP phosphodiesterase (PDE), RD1 was introduced into the expression vector pSVL. COS cells transfected with the recombinant vector pSVL-RD1 exhibited a 30–55% increase in homogenate PDE activity, which was abolished by rolipram (10 μ M). Removal of the first 67 nucleotides of the RD1 cDNA yielded a truncated enzyme called Met²⁶-RD1 which lacked the N-terminal first 25 amino acids. Whereas approx. 75% of RD1 activity was membrane-associated, Met²⁶-RD1 activity was found exclusively in the cytosol fraction. Expression of RD1 nearly doubled membrane-associated PDE activity, while expression of Met²⁶-RD1 increased cytosolic activity by approx. 30%. Membrane RD1 activity was found to be primarily associated with the plasma membrane, was not released by either high concentrations of NaCl or by a 'hypotonic shock' treatment, but was solubilized with low concentrations of Triton X-100. Phase separation of membrane components with Triton X-114 showed partition of RD1 into both the aqueous and detergent-rich phases, whereas Met²⁶-RD1 partitioned exclusively into the

aqueous phase. Both RD1 and Met²⁶-RD1 specifically hydrolysed cyclic AMP; were unaffected by either Ca²⁺/calmodulin or by low cyclic GMP concentrations; exhibited linear Lineweaver–Burke plots with similar K_m values for cyclic AMP (4 μ M); both were potently and similarly inhibited by rolipram (K_i approx. 0.5 μ M) and were similarly inhibited by cilostamide and 3-isobutyl-1-methylxanthine. Thermal inactivation, at 50 °C, showed that while the cytosolic-located fraction of RD1 ($t_{0.5}$ approx. 3 min) and Met²⁶-RD1 ($t_{0.5}$ approx. 3 min) were similarly thermolabile, membrane-bound RD1 was considerably more thermostable ($t_{0.5}$ approx. 11 min). Treatment of both cytosolic RD1 and Met²⁶-RD1 with Triton X-100 did not affect their thermostability, but solubilization of membrane RD1 activity with Triton X-100 markedly decreased its thermostability ($t_{0.5}$ approx. 5 min). The N-terminal domain of RD1 appears not to influence either the substrate specificity or inhibitor sensitivity of this enzyme, but it does contain information which can allow RD1 to become plasma membrane-associated and thereby adopt a conformation which has enhanced thermostability.

INTRODUCTION

Cyclic AMP plays a pivotal role in controlling cellular function. It is synthesized at the cytoplasmic face of the plasma membrane by the enzyme adenylyl cyclase. The activity of this integral membrane protein is modulated by the action of stimulatory and inhibitory transmembrane receptors, whose action is transduced by the guanine nucleotide regulatory proteins G_s and G_i respectively (see Houslay, 1990). The only mechanism for degradation of cyclic AMP is through the action of cyclic AMP phosphodiesterases (PDE). This activity is provided for by a large family of isoforms which differ markedly in their kinetic characteristics, physical properties, sensitivity to a range of inhibitors and ability to be modulated by hormone action (Beavo, 1990; Houslay and Kilgour, 1990). As such these isoforms are poised to play a pivotal role in modulating cellular functioning by controlling the degradation of cyclic AMP. Nevertheless, the precise functional role of specific isoforms has yet to be determined.

Cyclic AMP-PDE activity is found associated with both cytosol and membrane fractions (Houslay and Kilgour, 1990). In hepatocytes, for example, distinct isoforms are found as either peripheral or integral proteins associated with both the plasma membrane and various subfractions of the endoplasmic reticulum. The mechanism whereby various PDEs are targeted to particular membrane sites, or occur in the cytosol, and the functional significance for specific intracellular locations of PDE isoforms is not understood (Pyne et al., 1986, 1987b; Houslay and Kilgour, 1990; Houslay, 1991). However, it has been suggested that membrane attachment may allow 'local' concentrations of cyclic AMP to be manipulated (see Brunton et al., 1981; Houslay and Kilgour, 1990), perhaps giving rise to the type of functional compartmentalization seen in cardiac cells (Brunton et al., 1981). It may also be involved in providing mechanisms for specific PDEs to be regulated by phosphorylation/dephosphorylation reactions (Marchmont and Houslay, 1981; Kilgour et al., 1989; Pyne et al. 1989; Manganiello et al., 1990) as, for example, with the insulin-stimulated and phosphorylated

Abbreviations used: IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase (cyclic nucleotide); RD1, rat brain, rolipram-sensitive cyclic AMP-specific type-IV phosphodiesterase; Met²⁶-RD1, RD1 with the N-terminal 25 amino acids deleted; FCS, fetal-calf serum; DMEM, Dulbecco's modified Eagle's medium; PMSF, phenylmethanesulphonyl fluoride; SV40, simian virus 40; KHEM medium, [50 mM KCl, 50 mM Hepes/KOH (pH 7.2), 10 mM EGTA and 1.92 mM MgCl₂]; DTT, dithiothreitol; DMSO, dimethyl sulphoxide; PNS, post-nuclear supernatant; LDH, lactate dehydrogenase.

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peripheral plasma membrane PDE in hepatocytes which is membrane-located by binding to an integral protein in the plasma membrane (Houslay and Marchmont, 1981) and the 'dense-vesicle' enzyme which is subject to multi-site phosphorylation (Kilgour et al., 1989).

Insight into the molecular nature of PDEs arose originally from studies on *Drosophila melanogaster* which contains two major forms of cyclic nucleotide PDE activity; a Ca^{2+} /calmodulin-dependent PDE and a cyclic AMP-specific PDE (Davis and Kauvar, 1984). Mutants of *Drosophila* carrying lesions on the dunce gene display aberrant learning/memory processes or female sterility and are deficient in the cyclic AMP PDE (see Davis, 1990). The dunce gene has been isolated and shown to encode a cyclic AMP-specific PDE (see Davis, 1990). These observations, therefore, implicate a function for the *dunc* cyclic AMP PDE in neurophysiological and reproductive processes.

With the use of probes derived from the *Drosophila* dunce gene, several similar cDNAs from different species have now been isolated. These include distinct rat PDE cDNAs isolated from rat brain, testis, and Sertoli cell libraries (Davis et al., 1989; Swinnen et al., 1989; Colicelli et al., 1989) and a cDNA isolated from a human monocyte library (Livi et al., 1990; McHale et al., 1991). Such enzymes specifically hydrolyse cyclic AMP, rather than cyclic GMP and have been categorized as type-IV, cyclic AMP-specific PDEs (Beavo, 1990). Alignment of the predicted protein sequence encoded by these dunce-like PDE cDNAs reveals that they share a high degree of sequence similarity within a central region of around 275 residues. The observation that this region contains a perfectly conserved sequence of seven amino acids (Glu-Leu-Ala-Leu-Met-Tyr-Asn), which is also found in the cyclic AMP-binding domain of the RIIa regulatory subunit of the cyclic AMP-dependent protein kinase (Chen et al., 1986), suggests that it may comprise the catalytic domain of the PDE. However, the cDNA sequences diverge substantially at the N- and the C-termini, suggesting the presence of domains, encoded by these cDNAs, which may define points of regulation or of cellular location.

In this study we have analysed a cyclic AMP-specific, type-IV PDE called RD1. The cDNA encoding this enzyme was isolated from a rat brain library by homology cloning using the *dunc* cyclic AMP PDE gene and expressed in yeast (Henkel-Tigges and Davis, 1989). This showed the cDNA to encode a low- K_m cyclic AMP-specific PDE with properties similar to that encoded by the *Drosophila* dunce gene. However, unlike the *Drosophila* cyclic AMP PDE, the rat homologue RD1 was inhibited by very low concentrations of the anti-depressant drug rolipram. This compound has anti-depressant therapeutic properties (Eckmann et al., 1988) and it has been suggested (Henkel-Tigges and Davis, 1989) that the enzyme RD1, which is specifically expressed in brain (Henkel-Tigges and Davis, 1989), may be involved in mood regulation.

Comparison of the sequences of RD1 and cognate type-IV PDEs, such as dunce-like cyclic AMP PDE (DPD) and the *Drosophila* dunce PDE (Davis, 1990) identifies a central region which is well conserved among these enzymes (sequence similarity of approx. 75%). However, over the C-terminal region of approx. 170 amino acids and an N-terminal region of approx. 25 amino acids in RD1 there is very considerable heterogeneity (> 90%). Here, we address the possibility that the unique N-terminal region of RD1 provides a domain which confers functional attributes upon this enzyme. In order to gain insight into the function(s) of this domain, we have expressed the full-length RD1 cyclic AMP PDE and an N-terminal truncated form (Met²⁶-RD1) in the simian virus 40 (SV40)-transformed monkey

kidney COS-1 cell line. Doing this we find that while the major fraction of RD1 PDE expressed in COS-1 cells is membrane-associated, deletion of the N-terminal 25 amino acids of RD1 (Met²⁶-RD1) shifted the intracellular location of the expressed enzyme exclusively to the cytosol fraction. Concomitant with this change in membrane association was a marked difference in the thermostability of the PDEs. Deletion of the N-terminal region of RD1, however, did not alter the K_m value for cyclic AMP hydrolysis, or the K_i values for inhibitor action. We suggest that the N-terminal domain of RD1 serves as a signal for membrane association and the subsequent conferment of enhanced thermostability upon RD1.

MATERIALS AND METHODS

Materials

The rat dunce cDNA was kindly provided by Dr. R. L. Davis, Baylor College of Medicine, Houston, U.S.A. Restriction enzymes, Dulbecco's modified Eagle's medium and fetal-calf serum (FCS) were from Gibco/BRL (Paisley, U.K.). Tris, Hepes, DEAE-dextran (M_r 500000), cytochalasin B, benzamidine hydrochloride, phenylmethanesulphonyl fluoride (PMSF), aprotinin, pepstatin A, antipain, EDTA, EGTA, cyclic AMP, cyclic GMP, Dowex 1X8-400 (chloride form, 200–400 mesh), 3-isobutyl-1-methylxanthine (IBMX), snake venom (*Ophiophagus hannah*), and bovine brain calmodulin were from Sigma Chemical Co. (Poole, Dorset, U.K.). ³H-labelled cyclic AMP and ³H-labelled 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 (DTT), Triton X-100, and Triton X-114 were from Boehringer (U.K.) Ltd. (Lewes, Sussex, U.K.). Triethanolamine was from BDH (Glasgow, U.K.). Glycerol was from Fison (Loughborough, Leics., U.K.). Bradford reagent was from Bio-Rad. Dimethyl sulphoxide (DMSO) was from Koch-Light Ltd (Haverhill, Suffolk, U.K.). Rolipram was a gift from Schering Aktiengesellschaft, Postfach 650311, D-1000 Berlin 65, Germany and cilostamide was a gift from Dr. P. Ellis, Pfizer Central Research, Sandwich, Kent, U.K.

DNA constructs

The rat dunce gene (RD1) cDNA was isolated from pIBI as 2.2 kb *EcoRI* fragment and inserted into the unique *EcoRI* site of pBluescript KS⁺, to give the recombinant vector pKS-RD1. The cDNA was then isolated from pKS-RD1 as a *XhoI*/*BamHI* fragment and directionally cloned into the expression vector pSVL, generating the recombinant vector pSVL-RD1.

To generate the mutant pSVL-Met²⁶-RD1, the RD1 cDNA was isolated from pKS-RD1 as a fragment containing a 5' *HincII* end and a 3' *BamHI* end. The unique *HincII* site lies within the RD1 cDNA just downstream of the first ATG start codon. The *HincII*/*BamHI* fragment was ligated to pSVL via its unique *SmaI* and *BamHI* sites.

Expression in COS-1 cells

The SV40-transformed monkey kidney cell lines (COS-1) was cultured at 37 °C in an atmosphere of 5% CO₂/95% air in a complete growth medium containing DMEM and 10% (v/v) penicillin/streptomycin (100 units/ml) supplemented with 10% (v/v) FCS.

The cells were plated, 24 h before transfection at a density of 5×10^6 cells per 5 cm-diam. plate. For transfection, the cells were rinsed with 4 ml of serum-free DMEM and the cell monolayer

covered with 0.8 ml of transfection medium containing serum-free DMEM, 50 mM Tris/HCl (pH 8.0), 20 mM Hepes (pH 7.2), 750 µg/ml DEAE-dextran and 5 µg of DNA (Danna and Sompayrac, 1982). The plates were incubated at 37 °C for 4 h. The transfection medium was aspirated and the cells were 'shocked' with 25 % (v/v) glycerol in serum-free DMEM for 1 min at 25 °C. The cells were then rinsed with serum-free DMEM and then 5 ml of DMEM containing 10 % (v/v) serum was added to the plates before the plates were incubated at 37 °C for 72 h. Mock-transfected cells were treated as above but received no DNA. In some instances, however, transfection was done with the 'native' pSVL and this is referred to as pSVL-transfected cells (vector only).

Preparation of cell extracts: cytochalasin procedure

COS cells were seeded on to 24.5 cm × 24.5 cm Nunc plates and grown for 18 h to 50–75 % confluency. The cells were transfected, as described above, with 100 µg of DNA per plate and continually rocked in 10 ml of transfection medium for 4 h. After the cells had been 'glycerol shocked' they were incubated at 37 °C for 72 h in 50 ml of complete growth medium. The cells were prepared from homogenization as follows: the growth medium was removed and the cells incubated in cold KHEM buffer [50 mM KCl, 50 mM Hepes KOH (pH 7.2), 10 mM EGTA and 1.92 mM MgCl₂] containing 20 µM cytochalasin B (Burke and Gerace, 1986) to break down microfilaments, for 45 min at 4 °C. The cells were then washed with 25 ml of 10 mM triethanolamine/0.15 M KCl (pH 7.2) at 4 °C for 10 min. Finally the cells were washed with 15 ml of complete KHEM buffer (KHEM 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). The protease inhibitors were initially dissolved as a 1000 × stock in DMSO before addition to the KHEM buffer. This mixture was further supplemented with 20 µM cytochalasin B. The buffer was drained from the cells, which were then scraped from the culture dish in a final volume of 0.8–1.0 ml per plate and transferred to a glass/glass Dounce homogenizing vessel where they were subjected to 20 passes of the pestle.

Subcellular fractionation

A post-nuclear supernatant (PNS) was prepared by centrifuging the cell homogenate for 5 min at 2000 rev./min (350 *g*_{av}) in a Refrigerated Jouen bench centrifuge at 4 °C. The PNS (1 ml) was then loaded on to the top of a sucrose step-gradient containing a step of complete KHEM-buffered 1.6 M sucrose (1 ml) and a step of 0.5 M sucrose (3 ml). The membranes were harvested from the 0.5/1.6 M sucrose interface after centrifugation for 30 min at 50000 rev./min (25000 *g*_{av}) in an SW55 rotor (Beckman).

The membrane fraction (200 µl) was supplemented with solid sucrose to > 1.6 M before being layered under a 0.6–1.6 M sucrose continuous gradient buffered with complete KHEM. Centrifugation was then performed at 50000 rev./min for 18 h at 4 °C in an SW55 rotor before fractionation and the collection of 200 µl aliquots for assay.

Hypotonic shock procedure

A 'hypotonic shock' procedure, employed by us to release the 'dense-vesicle' PDE (Pyne et al., 1987a), was done with mem-

brane fractions (0.1–0.2 mg of protein) being resuspended in 200 µl of hypotonic buffer [10 mM Tris/HCl (pH 7.4), 1 mM EDTA, and 1 mM KHCO₃], in the presence and absence of protease inhibitors (5 mM benzamidine, 0.2 mM PMSF, 20 µM leupeptin and 0.1 mM DTT). The extract was left on ice for 30 min before centrifugation at 100000 *g* for 1 h at 4 °C. The resulting pellet was resuspended in the same buffer, and both the pellet and supernatant fractions were assayed for PDE activity.

Treatment with high salt concentrations

The high-salt extraction procedure involved membranes (0.1–0.2 mg of protein) being resuspended in KHEM buffer (200 µl) containing a range of NaCl concentrations up to 1 M. The extracts were then left on ice for 30 min before centrifugation at 100000 *g* for 1 h at 4 °C. The resulting pellet was resuspended in buffer containing the appropriate NaCl concentration and the pellet and supernatant fractions assayed for PDE activity.

Solubilization with Triton X-100

Detergent solubilization involved the membranes (0.1–0.2 mg of protein) being resuspended in KHEM (200 µl) containing Triton X-100 (0.2–1 %) and left on ice for 30 min before centrifugation at 100000 *g* for 1 h at 4 °C. The pellet was resuspended in KHEM containing Triton X-100 (0.2–1 %), and both the pellet and supernatant fractions assayed for cyclic AMP-PDE activity.

Phase separation of membrane proteins

This was done using precondensed Triton X-114 as described by Bordier (1981).

Protein assays

The protein concentration in the membrane and cytosolic fractions was determined by the Bradford (1976) method using BSA as a standard. Membranes were solubilized with Triton X-100 before assay. When Triton X-100 was used in the assay it was also included in the standards.

Protein distribution on the gradients was performed as described by Peterson (1977).

PDE assay

Cyclic nucleotide PDE activity was assayed by a modification of the two-step procedure of Thompson and Appleman (1971) and Rutten et al. (1973) as described previously by Marchmont and Houslay (1980). In all cases a freshly prepared slurry of Dowex H₂O (1:2) was used for determination of activities. All assays were conducted at 30 °C and initial rates were taken from linear time courses. Analysis of the effect of Ca²⁺/calmodulin and cyclic GMP on PDE activity, IC₅₀ values for PDE inhibitors and *K*_m values were all determined as described by Lavan et al. (1990). As a routine, *K*_m values were determined using 19 concentrations of cyclic nucleotide ranging from 0.1 µM to 1 mM for at least three different transfection experiments. PDE inhibitors were dissolved in DMSO at a stock concentration of 25 mM and subsequently diluted in 20 mM Tris/HCl (pH 7.4) to provide a range of drug concentrations. IC₅₀ values were determined at 5 µM cyclic AMP from dose-response curves over a range of concentrations, as shown in the appropriate figures.

Routine cyclic AMP PDE activity assays, when analysing distributions and expression, for example, were done at a cyclic AMP substrate concentration of 1.0 µM, unless stated otherwise.

Thermal denaturation experiments

COS cell lysates (from 2×10^7 cells) were prepared using the cytochalasin procedure and the resulting PNS divided into two parts and treated identically by centrifugation at 100 000 *g* for 1 h at 4 °C to give pellet and supernatant fractions PA and SNA and PB and SNB, respectively. One of the pellet fractions (PA) was resuspended in a complete KHEM buffer and the other in complete KHEM buffer containing 0.2% Triton X-100 (PB). The supernatant collected from the second pellet had Triton X-100 added to a final concentration of 0.2% (SNB). The samples were then left on ice for 30 min. Heat inactivation was done by taking a 100 μ l sample of the appropriate fraction and adding it to 1.4 ml of pre-warmed complete KHEM buffer contained in a glass bijoux, which had been placed on a stirrer in a water-bath kept at 50 °C. Aliquots (50 μ l) were then removed into ice-cold microfuge tubes (1.5 ml) every 30 s over a period of 10 min. These samples were then assayed for cyclic AMP PDE activity at a substrate concentration of 1 μ M.

Assays for marker enzymes

The activity of the Golgi membrane marker, galactosyltransferase, was assayed as described by Bretz and Staubli (1977); activity of the plasma membrane marker, 5'-nucleotidase, by the method of Newby et al. (1975); the mitochondrial membrane marker cytochrome *c* oxidase was assayed as described by Mason et al. (1973); the endoplasmic reticulum marker, rotenone-insensitive NADH cytochrome *c* reductase, was assayed as described by Sottocasa et al. (1967) and the lysosomal enzyme marker, β -hexosaminidase, was assayed as described by Storrie and Madden (1990). Lactate dehydrogenase (LDH) assays were done as described by Houslay and Tipton (1973).

In vitro transcription

RNA was prepared from pKS-RD1 and pKS-Met²⁶-RD1 using T₃ RNA polymerase and components from a Promega (Madison, WI 53711-5399, U.S.A.) *in vitro* transcription system, used according to the manufacturer's instructions.

Cell-free translation

mRNA for RD1 and Met²⁶-RD12 was produced *in vitro* and translation was performed in a final volume of 50 μ l, using rabbit reticulocyte lysate and components from Promega according to the manufacturer's instructions. [³⁵S]Met was included in the reaction to a final concentration of 0.8 mCi/ml. Following translation, samples were subjected to SDS/PAGE and fluorography.

RNA extraction and Northern-blot analysis

Cytosolic RNA was prepared from normal and transfected COS-1 cells (Sambrook et al., 1989) and separated on a 1% (w/v) agarose gel prepared in Mops/EDTA buffer [0.2 M Mops, 50 mM sodium acetate, 10 mM EDTA (pH 8.0) adjusted to pH 7.0] containing 0.66 M formaldehyde. The RNA was transferred to Hybond N⁺ nylon membranes (Amersham International, Amersham Bucks., U.K.), and hybridized as described (Amersham hybridization protocol for Hybond N⁺ membranes) with random-primed ³²P-labelled RD1 cDNA (Feinberg and Vogelstein, 1984).

RESULTS

Expression of full-length and truncated RD1 products in COS-1 cells

For expression studies in COS-1 cells, the full-length RD1 cDNA and a truncated form of the RD1 cDNA, named Met²⁶-RD1, were introduced into the COS cell expression vector pSVL to generate the recombinant vectors, pSVL-RD1 and pSVL-Met²⁶-RD1 respectively, the construct pSVL-Met²⁶-RD1 being generated by deletion of the first 67 nucleotides of RD1. As a consequence of this small 5'-end deletion, then by the first AUG rule (Kozak, 1986) it is predicted that the codon for Met²⁶ will become the first functional translation start site, leading to the effective deletion of 25 amino acids from the N-terminus.

To demonstrate expression of RD1, ³²P-labelled RD1 cDNA was hybridized with RNA isolated from pSVL-RD1-transfected COS-1 cells. This revealed a band of approx. 3.2 kb which was not present in RNA from pSVL/transfected COS-1 cells (Figure 1a). Cells transfected with pSVL-Met²⁶-RD1 were similarly probed and a single band of slightly higher mobility (approx. 3.1 kb) was identified (Figure 1a). *In vitro* translation of RD1 mRNA, obtained by *in vitro* transcription of RD1 cDNA, showed a translation product of *M_r* approx. 68 000 (Figure 1b), corresponding to the *M_r* predicted for RD1 from the open reading frame (Davis et al., 1989). For Met²⁶-RD1 mRNA, a slightly faster migrating product was identified of *M_r* approx. 64 000. These observations are consistent with Met²⁶-RD1 being a protein which lacks the first N-terminal 25 amino acids of RD1 (Figure 1b).

When compared with either mock- or pSVL-transfected cells pSVL-RD1- and pSVL-Met²⁶-RD1-transfected cells expressed

Figure 1 Presence of mRNA for RD1 in transfected cells

To demonstrate expression of RD1 and Met²⁶-RD1, (a) ³²P-labelled RD1 cDNA was hybridized with RNA isolated from vector (pSVL only), pSVL-RD1 and pSVL-Met²⁶-RD1-transfected COS-1 cells. This revealed a band of approx 3.2 kb in pSVL-RD1-transfected COS-1 cells and one of approx 3.1 kb in pSVL-Met²⁶-RD1-transfected COS-1 cells which was not present in RNA from native vector (pSVL)-transfected COS-1 cells. In (b) mRNA for both RD1 (track 1) and Met²⁶-RD1 (track 2) was produced and subjected to *in vitro* translation. This yielded single species of 68 000-*M_r* and 64 000-*M_r* respectively. Results show a typical experiment of one done three times and includes a lane with both samples together (track 3) and a Bromo mosaic virus test translation (track 4).

Figure 2 Distribution of cyclic AMP PDE activity between membrane and cytosol fractions and the ability of Triton X-100 to elicit the release of RD1 from membranes of COS-1 cells transfected with pSVL-RD1

(a) Cells were transfected, harvested and disrupted using the cytochalasin procedure as described in the Materials and methods section. The homogenate was centrifuged at 100 000 *g* for 60 min at 4 °C in order to isolate membrane and cytosol fractions. Assay of cyclic AMP PDE activity was done with 1.0 μ M cyclic AMP as substrate. The range of distributions of activity between membrane (□) and cytosol (■) fractions is shown above. The range of endogenous cyclic AMP PDE activity was 71–84 pmol/min per mg of protein and 116–135 pmol/min per mg of protein for the membrane and cytosolic fractions respectively, obtained from mock-transfected cells ($n = 10$ separate mock transfections). The activity of Met²⁶-RD1, found in the cytosol, was some 30–46 pmol/min per mg of protein and for RD1 in the membrane fraction, some 66–69 pmol/min per mg of protein. The percentage distribution, between the membrane and cytosolic fractions, of total activity for mock transfection (identical to endogenous) and for cells transfected with either RD1 or Met²⁶-RD1 is shown here. Results give the average for ten separate transfection experiments in each instance, with the range of distribution of the membrane/cytosol activities being 24–32%/76–68% for the endogenous COS cell activity, 71–78%/29–22% for RD1 and 0–3%/100–97% for Met²⁶-RD1. In (b) and (c), detergent solubilization involved resuspending membranes (0.1–0.2 mg of protein) in (200 μ l) of KHEM buffer containing Triton X-100 (0–0.2%). They were then left on ice for 30 min prior to centrifugation at 100 000 *g* for 1 h at 4 °C. The pellet was resuspended in KHEM containing Triton X-100 (0.2–1%), and both the pellet and supernatant fractions assayed for cyclic AMP PDE activity. Results are shown for the mock-transfected cells (b) and for cells which had been transfected with pSVL-RD1 (c) and thus expressed RD1 activity. In both instances we show the appearance of solubilized activity (●) and the residual activity remaining in the cell pellet (○). The experimental data shown are for the typical result of experiments done four times using different transfected cell preparations.

markedly elevated levels of cyclic AMP PDE activity; it being increased in homogenates by some 30–55%. No such difference, in cyclic AMP PDE activity, between pSVL-RD1- and mock-transfected cells was evident (< 4% difference) if assays were done in the presence of the PDE-IV-type selective inhibitor rolipram (10 μ M).

Homogenates were subjected to a simple high-speed pelleting procedure and the PDE activities of the cytosol and particulate fractions assayed. This revealed a major difference in the intracellular distributions of the full-length and truncated RD1 products, RD1 and Met²⁶-RD1 (Figure 2a). In the case of the full-length RD1 product, the majority of the transfected activity was detected in the membrane fraction, whereas the truncated RD1 product was located solely in the cytosolic fraction (Figure 2a). Analysis of cyclic AMP PDE activity, assayed with 1.0 μ M cyclic AMP, showed that expression of Met²⁶-RD1 elevated cytosolic PDE activity some 26–34% and expression of RD1 elevated membrane PDE activity some 82–93% and cytosolic PDE activity some 17–21% (range; $n = 10$).

It should be noted that in native COS-1 cells and in pSVL-transfected COS-1 cells, the membrane fraction did not exhibit any significant (< 4%) rolipram (10 μ M)-inhibitable PDE activity. Such rolipram-inhibitable cyclic AMP PDE activity was only apparent after transfection of the cells with either the recombinant vector pSVL-RD1 or pSVL-Met²⁶-RD1. Thus all the increase in membrane cyclic AMP PDE activity seen in pSVL-RD1-transfected cells compared with that of pSVL-transfected cells was fully abolished (> 96%) upon rolipram (10 μ M) addition. Furthermore, endogenous COS-1 cell PDE activity, in both membrane and cytosol fractions, differed from RD1 and Met²⁶-RD1 in being considerably more sensitive to inhibition by the PDE-III-selective compound cilostamide (Table 1).

RD1 cyclic AMP PDE is associated with the membrane fraction

Using the cytochalasin/homogenization procedure no latent LDH activity was evident, indicating that sealed vesicles containing entrapped cytosolic material were not produced by this homogenization method. RD1 activity was not released (< 5% change) by treatment of membranes, isolated under iso-osmotic conditions, by incubating for periods of up to 30 min with a range of NaCl concentrations (0.5–2.0 M) (results not shown). This contrasts with the peripheral plasma membrane cyclic AMP PDE found in hepatocytes which was released at NaCl concentrations of between 0.2 M and 0.5 M (Marchmont and Houslay, 1980). A 'hypotonic shock' procedure has been shown to release a cyclic GMP-inhibited type-II PDE from the endoplasmic reticulum of hepatocytes and adipocytes (Pyne et al., 1987a) but, using such an approach, we were unable to effect the solubilization of RD1. Treatment of membranes with low concentrations (0.2%, w/v) of the non-ionic detergent Triton X-100 solubilized both endogenous PDE activity (Figure 2b) and that of RD1 (Figure 2c).

Solutions of the non-ionic detergent Triton X-114 can be induced to separate into detergent-rich and aqueous phases at 30 °C (Bordier et al., 1981). This can be exploited to determine whether proteins are truly integral species which have significant hydrophobic surfaces inserted into the lipid bilayer. Such proteins will partition exclusively into the detergent phase, whereas peripheral and cytosolic proteins will accumulate in the aqueous phase. Performing such experiments with membrane-bound RD1 showed that 50–56% of its activity was found in the aqueous phase, with the remainder in the detergent-rich phase (range, $n = 3$). In contrast, Met²⁶-RD1 activity was found exclusively in

Table 1 Inhibitor and substrate kinetic constants for endogenous and transfected cyclic AMP PDEs

Analysis of substrate utilization by cyclic AMP was done over a substrate range of 0.1 μM to 1 mM using determinations at 19 different concentrations. Assays were done in triplicate in each instance. In the case of RD1, for both membrane-associated and cytosolic forms, and also for Met²⁶-RD1, linear Lineweaver–Burke and Hofstee plots were obtained. Analysis of these allowed us to derive K_m values which are shown below together with S.E.M.s based upon experiments performed three times with different transfected cells. Endogenous cyclic AMP PDE activity showed non-linear kinetic plots for cyclic AMP utilization. These were analysed as before (Marchmont and Houslay, 1980, 1981) in order to obtain limiting values of K_m (1' and 2'). Analysis of inhibition was done at the K_m concentration of cyclic AMP for the RD1 species (4 μM). The ranges of concentrations employed were 0.005 μM –50 μM for rolipram, 0.1 μM –200 μM for cilostamide and 0.1 μM –200 μM for IBMX, with determinations measured using between eight and 11 different concentrations of inhibitor. This allowed the IC_{50} values for inhibition of activity to be calculated. As analyses were done at the K_m concentration of cyclic AMP for the RD1 species, then the IC_{50} values for these competitive inhibitors equate to their K_i values for RD1 and Met²⁶-RD1. This is not true for the endogenous PDE activity analysed here, which represents a complex mixture of isoforms. In such instances data are IC_{50} values, indicating the degree of inhibition ensuing using 4 μM cyclic AMP as substrate, and can be used for comparison with the inhibition effect upon transfected-PDE activities assayed at an identical substrate concentration. These are all given in μM with errors as S.D. for three experiments performed using different transfections. N.I., indicates not inhibited (< 5% change) by concentrations of rolipram up to 100 μM . $\neq$ indicates not applicable as the 2' column is only appropriate where the two limiting values for the K_m of cyclic AMP are given.

	K_m values (μ M)						
	RD1			Endogenous-cytosol		Endogenous-membrane	
	Membrane	Cytosol	Met ²⁶ -RD1	1'	2'	1'	2'
Rolipram	0.4 ± 0.1	0.5 ± 0.2	0.6 ± 0.2	0.9 ± 0.2	≠	N.I.	≠
Cilostamide	199 ± 31	240 ± 37	178 ± 17	31 ± 9	≠	59 ± 5	≠
IBMX	26 ± 8	25 ± 6	20 ± 3	21 ± 3	≠	29 ± 4	≠
Cyclic AMP	4.1 ± 2.3	3.8 ± 1.8	5.3 ± 3.0	1.9 ± 0.7	13.1 ± 2.6	0.73 ± 0.24	12.7 ± 2.3

the aqueous phase (> 97%; $n = 3$ separate experiments using different transfection experiments).

Subcellular localization of RD1

The intracellular localization of RD1 in rat brain is unknown as the enzyme has yet to be isolated and characterized from that source. Since we have shown here that a major fraction of RD1 activity is membrane bound, an attempt was made to see whether RD1 was associated with a particular membrane fraction. In order to do this, cells were homogenized using the cytochalasin procedure and the step-gradient membrane fraction isolated from the PNS was fractionated by loading it under a density gradient of sucrose and floating the membranes to their buoyant densities (Figure 3). Analysis of the distribution of membrane-associated RD1 activity showed a clearly defined peak (Figure 3) which banded at 1.43 M sucrose whether or not the membranes from pSVL-RD1-transfected cells were loaded at either the top or bottom of the gradient. This distribution closely followed the profile of the plasma membrane marker 5'-nucleotidase and was quite clearly different from the markers for mitochondria, Golgi, endoplasmic reticulum and lysosomes (Figure 3). However, a small shoulder was evident which co-migrated with the major fraction of the Golgi marker galactosyltransferase (Figure 3).

In certain cells it has been indicated that a fraction of the 5'-nucleotidase activity may be endoplasmic reticulum-associated (Farquhar and Palade, 1981), the majority of this activity formed a single peak on the gradient which clearly migrated differently from the major peak of the endoplasmic reticulum marker (Figure 3). We note that the plasma membrane, but not other cellular membranes, from COS cells migrated at a higher buoyant density than is found for many cell lines (see Munday and Warren, 1992). This was not due to any aberrant isopycnic behaviour, as the distribution of the various marker enzymes was identical whether membrane samples were loaded at the bottom or the top of the gradient.

Kinetic properties

RD1 and Met²⁶-RD1 appear to have similar kinetic properties. Thus, whereas COS-1 cells expressing either RD1 or Met²⁶-RD1

showed an increase in PDE activity with cyclic AMP as substrate (see above), we observed no change (< 3%) in PDE activity using cyclic GMP. The cyclic AMP-hydrolysing activity of these enzymes was not activated by the addition of Ca^{2+} /calmodulin, nor was it altered by the presence of low concentrations (2 μM) of cyclic GMP (< 4% change).

The membrane-bound and cytosolic forms of RD1-PDE exhibited linear double-reciprocal plots for cyclic AMP utilization, as did the soluble Met²⁶-RD1 activity, indicating that these enzymes obeyed normal Michaelis–Menten kinetics. All three enzymes exhibited very similar K_m values for cyclic AMP, which were in the sub-micromolar range (Figure 4; Table 1). In contrast, analysis of endogenous COS-1 cell PDE activity yielded markedly non-linear double-reciprocal plots which, undoubtedly, reflected the presence of multiple forms of PDE activity exhibiting different K_m values for the hydrolysis of cyclic AMP (Figure 4; Table 1).

Dose–effect curves were performed for rolipram, a selective inhibitor for certain members of the type-IV cyclic AMP-specific PDEs (Davis et al., 1989; Swinnen et al., 1989; Beavo, 1990; Conti and Swinnen, 1990), the non-selective cyclic AMP-PDE inhibitor IBMX (Beavo, 1990) and also for cilostamide, a selective inhibitor of type-III, cyclic GMP-inhibited cyclic AMP-specific PDEs (Pang et al., 1988). As can be seen (Figure 5; Table 1), there was no difference between Met²⁶-RD1 and the membrane-bound and soluble forms of RD1 in their response to these three very different compounds. Each was inhibited by rolipram at extremely low concentrations, with IBMX and cilostamide eliciting inhibition at much higher concentrations.

In native COS-1 cells and in mock-transfected COS-1 cells, the endogenous PDE activity of the membrane fraction did not exhibit any significant (< 4%) rolipram (10 μM)-inhibitable PDE activity and was inhibited by lower concentrations of cilostamide than was found for RD1 (Figure 5; Table 1). Endogenous cytosolic PDE activity was also more sensitive to inhibition by cilostamide than Met²⁶-RD1 (Table 1).

Thermostability of RD1 and Met²⁶-RD1

The thermostability of RD1 was assessed by incubating

Figure 3 Sucrose-density-gradient analysis of membrane-bound RD1 activity

This was carried out three times as described in the Materials and methods section and a typical result is shown here. Activities of marker enzymes and of RD1 are shown as distribution (%) of that total activity applied to the gradient. In each panel the activity of the particular marker enzyme (□) and RD1 (●) is shown for comparison. Shown in (a) is the plasma membrane marker, 5'-nucleotidase; (b) the Golgi marker, galactosyltransferase (GT); (c) the endoplasmic reticulum marker, rotenone-insensitive NADH cytochrome *c* reductase; (d) the lysosomal marker, β -hexosaminidase and in (e) the mitochondrial marker, cytochrome oxidase.

membranes at 50 °C and then taking samples for assay of cyclic AMP-PDE activity. The membrane-bound RD1 activity decayed as a single exponential, as indicated by the linear semi-logarithmic plots of remaining activity against time (Figure 6). This is consistent with a single enzyme activity being analysed. The half-life of the PDE activity of membrane-bound RD1 was of the order of 11 min (Table 2). In marked contrast with this, although Met²⁶-RD1 activity also decayed as a single activity, its activity was considerably more thermolabile, as indicated by a half-life of around 3–4 min (Table 2; Figure 6). Exposure of cytosolic Met²⁶-RD1 to the detergent Triton X-100 had no discernible effect on either the activity of the enzyme (< 5%) or on its thermostability (Table 2; Figure 6). However, when the membrane-bound RD1 activity was solubilized with Triton X-100 then the thermostability of this activity was markedly reduced, yielding a half-life of approx. 5 min (Figure 6; Table 2). While this figure approached that seen for the half-life of the cytosolic RD1 activity and also for Met²⁶-RD1 activity it was, nevertheless, significantly ($P < 0.005$; $n = 4$) larger than the values obtained for the decay of the PDE activities derived from

Figure 4 Kinetics of hydrolysis of cyclic AMP by RD1, Met²⁶-RD1 and endogenous COS cell cyclic AMP PDE activity

(a) Lineweaver–Burke analysis of Met²⁶-RD1 activity (■) together with the activities of RD1 in its cytosolic (s/n) form (○) and in its membrane-bound (pellet) form (▲). The plot is typical of one done at least three times for different transfection experiments. The lowest substrate concentration shown here is 0.67 μ M, in order that the common intercept at $-1/K_m$ could be clearly shown. However, assays have been done with concentrations as low as 0.05 μ M for these enzyme activities, yielding linear plots (see Table 1). Endogenous COS cell cyclic AMP PDE activity was subjected to Lineweaver–Burke analysis for (b) membrane (■) and (c) cytosolic (●) fractions. Plots are typical of ones performed on three occasions using different cell preparations.

the cytosol, even when they were analysed in the presence of Triton X-100.

The thermostabilities of RD1 and Met²⁶-RD1 were further examined at 55 °C. At this temperature, activities decayed distinctly and extremely rapidly (Figure 6c) with, as at 50 °C, the

Figure 5 Action of the selective inhibitor rolipram upon RD1, Met²⁶-RD1 and endogenous COS cell cyclic AMP PDE activity

Analysis of the degree of inhibition of cyclic AMP PDE activity was done at a substrate concentration which reflected the K_m value for the transfected enzymes ($4.0 \mu\text{M}$). Activities examined were RD1 in membrane/pellet ($\blacktriangle$) and cytosolic (s/n) ($\circ$) forms, Met²⁶-RD1 ($\blacksquare$) and the endogenous COS cell activity in the cytosol ($\triangle$) and membrane fractions ($\square$). Data are typical of those found in three different transfection experiments.

activity of Met²⁶-RD1 being significantly ($P < 0.005$; $n = 3$) more thermostable than that of RD1 (Table 2).

DISCUSSION

Transfection of COS-1 cells with pSVL-RD1 elicited a marked enhancement of homogenate PDE activity. RD1, which we have expressed here in a mammalian cell line, exhibited a similar K_m for cyclic AMP and K_i for rolipram to those reported for RD1 activity expressed in yeast (Henkel-Tigges and Davis, 1989). In addition, we show here that RD1 activity was potently inhibited by the non-selective PDE inhibitor IBMX, but was relatively insensitive to the action of the type-III-PDE inhibitor cilostamide (Figure 6; Table 1). Furthermore, we show that RD1 activity was cyclic AMP-specific, was not activated or inhibited by low concentrations of cyclic GMP, and was unaffected by Ca^{2+} /calmodulin addition. RD1 is thus a typical member of the 'rolipram-sensitive' subset of type-IV-PDE activity (Beavo, 1990).

Disruption of COS cells and separation of total membranes

Figure 6 Thermostability of Met²⁶-RD1 and both membrane-bound and cytosolic forms of RD1

Results show semi-logarithmic plots for the decay of cyclic AMP PDE activity with time when enzyme fractions are incubated at elevated temperatures. The plots shown are typical of ones performed at least three times using different transfection experiments. (a) The decay, at 50°C , of Met²⁶-RD1 PDE activity ($\blacksquare$) together with the activities of RD1 in its cytosolic (s/n) form ($\circ$) and in its membrane-bound (pellet) form ($\blacktriangle$). In (b) is shown the decay, at 50°C , of PDE activity for Met²⁶-RD1 ($\blacksquare$) and the cytosolic form of RD1 (s/n) ($\circ$) performed in the presence of Triton X-100 (TX-100), as well as the Triton X-100-solubilized membrane-bound RD1 activity ($\blacktriangle$). (c) shows the decay, at 55°C , of PDE activity for membrane-bound ($\blacksquare$) and cytosolic (s/n) forms ($\circ$) of RD1 and also for Met²⁶-RD1 ($\bullet$).

from the cytosolic fraction allowed us to make the novel observation that the majority (75%) of RD1-PDE activity was membrane bound, with the remaining 25% of RD1 activity located in the cytosolic fractions (Figure 1). The membrane-

Table 2 Thermal denaturation constants for transfected cyclic AMP PDE activities

The rate of thermal inactivation of PDE activity was assessed as described in the Materials and methods section using 1 μ M cyclic AMP as substrate. Enzyme activities analysed were that of RD1, in its membrane-associated and cytosolic states, and also that of cytosolic Met²⁶-RD1 activity. For studies done at 50 °C, analyses were performed upon native enzymes and also for enzyme preparations which had been treated with 0.2% Triton X-100. This concentration of detergent was sufficient to elicit the solubilization of membrane-bound RD1 and thus a 100 000 *g* supernatant fraction of detergent-solubilized membrane RD1 was used. PDE activity was assessed over a range of times, as indicated in Figure 7, and half-lives calculated by regression analysis of plots of log [cyclic AMP PDE activity remaining (%)] versus time. The data below shows the half-lives, together with errors given as $\pm$ S.D., for three separate experiments using different cell transfections. Abbreviation: not determined.

Fraction	Half-life in the absence of Triton X-100 (min)			Half-life in the presence of Triton X-100 (min)		
	Membrane RD1	Cytosolic RD1	Met ²⁶ -RD1	Membrane RD1	Cytosolic RD1	Met ²⁶ -RD1
<i>t</i> _{0.5} (50 °C)	10.9 $\pm$ 1.4	2.2 $\pm$ 0.6	3.5 $\pm$ 0.9	5.2 $\pm$ 0.7	2.7 $\pm$ 0.8	3.3 $\pm$ 0.6
<i>t</i> _{0.5} (55 °C)	4.2 $\pm$ 0.5	2.0 $\pm$ 0.3	1.1 $\pm$ 0.3	n.d.	n.d.	n.d.

associated RD1 activity was not loosely absorbed, as it could not be removed by repeated washing of the membranes, and was not due to the presence of soluble enzyme entrapped in vesicles. Furthermore, RD1 did not appear to be membrane-associated by virtue of ionic interactions as it was not released by simply increasing the ionic strength with NaCl (Figure 3). However, the solubilization of RD1 activity could be achieved using low concentrations of the detergent Triton X-100 (Figure 3). From such observations it would appear that RD1 has neither the characteristics of a simple peripheral protein nor, by virtue of its appearance in active form in both membrane and cytosol fractions, does it appear to behave as a simple integral membrane protein.

Subcellular fractionation of COS cells showed that RD1 fractionated with a profile and buoyant density which were very similar to those of the plasma membrane fraction, as identified using 5'-nucleotidase as a marker (Figure 3), although a small shoulder on the major peak may be indicative of the association of a fraction with the Golgi apparatus. We would thus tentatively conclude that the membrane-bound RD1 activity is primarily plasma membrane associated.

To explore the possibility that the unique N-terminal region of RD1 may encode a domain which endows RD1 with particular properties, we made a construct which would effect the deletion of the N-terminal 25 amino acids from RD1, producing a protein which we have called Met²⁶-RD1. Intriguingly, Met²⁶-RD1 activity was found exclusively in the cytosol of COS cells (Figure 2a). That a different distribution for Met²⁶-RD1 was found, compared with RD1, is consistent with our contention that the membrane-associated RD1 activity did not represent entrapped enzyme. Comparison of the enzymic characteristics of Met²⁶-RD1 with RD1 showed that the *K*_m for cyclic AMP and the *K*_i for various inhibitors were identical for these two enzymes as was, indeed, the case for the soluble RD1 activity found in the cytosol (Table 1). This suggests that the folding of the enzyme, during biosynthesis, had been relatively unaffected by the deletion of this N-terminal domain, and that membrane association had little effect on the affinity of inhibitors for the active site. It would thus seem unlikely that RD1 becomes membrane associated by virtue of its polypeptide chains being inserted into the membrane core, with the N-terminal domain providing a signal sequence. For, if this was to occur then one might expect the folding of the cytosolic and integral PDEs to be markedly different, and hence also their activities; this is clearly not the case. Certainly no support for RD1 being a normal integral membrane protein can be derived from either the inspection of the sequence of this enzyme or the application of hydrophobicity analysis, where no

hydrophobic domain of sufficient size to provide even a single transmembrane segment can be found.

The basis for membrane attachment for RD1 remains to be elucidated. One possibility is that RD1 is synthesized in the cytosol as a soluble species which is then subjected to post-translational modification, allowing it to become associated with the membrane fraction. Such a modification may take the form of, for example, acylation (Spiegel, 1990). This would account for the fact that RD1 was found to partition between aqueous and detergent-rich phases of Triton X-114, as has been noted for other proteins which become membrane associated by virtue of their acylation (McIlhinney et al., 1987). A fraction of RD1 activity may thus be anchored to the bilayer rather than having proteinaceous domains integrated into it. Certainly, a variety of essentially hydrophilic proteins which are synthesized on soluble ribosomes, have been shown to be targeted to the plasma membrane through the covalent attachment of lipid moieties at either their N- or C-terminal domains (see Spiegel et al., 1991). It is thus possible that the covalent attachment of lipid moieties at the N-terminal domain of RD1 could effect its membrane association. While myristoylation of a glycine residue close to the N-terminus has been shown to provide a plasma membrane-association signal for a variety of proteins (see Spiegel et al., 1991), no such motif appears in RD1 (Davis et al., 1989). However, it is possible that either or both of the two cysteine groups within the N-terminal domain of RD1, which are deleted in the construction of Met²⁶-RD1, might provide a site for acylation, perhaps by the addition of palmitate (see Olson, 1988). As native RD1 has yet to be isolated from rat brain and examined, it remains to be seen whether native RD1 occurs in a membrane-bound state. However, low-*K*_m PDE activity has been observed associated with the rat brain membranes (de Mazancourt and Giudicelli, 1988) as have rolipram-binding sites (Schneider et al., 1986).

A further dramatic difference between the soluble and membrane-bound forms of RD1 relates to their markedly different thermostabilities. Whereas RD1, in its membrane-bound state, was relatively thermostable at 50 °C, cytosolic RD1 and Met²⁶-RD1 showed considerable and similar thermostability (Table 2; Figure 6). The enhanced thermostability of RD1 would seem to be attributable to membrane attachment rather than being conferred *per se* by the N-terminal domain of this enzyme. Thus we infer that the marked thermostability of the cytosolic fraction of RD1 parallels that of Met²⁶-RD1 rather than that of membrane-bound RD1 (Figure 6; Table 2), and that the detergent solubilization of RD1 leads to a marked reduction in its thermostability (Table 2; Figure 6). This reduced thermostability,

seen upon the solubilization of RD1, was unlikely to be due to the action of Triton X-100 *per se* as this detergent did not alter the thermostability of either cytosolic RD1 or Met²⁶-RD1 (Table 2; Figure 6). Intriguingly, that the detergent-solubilized RD1 expressed a half-life for decay which was slightly different from both cytosolic RD1 and Met²⁶-RD1 may indicate that these species are indeed subtly different. This might be a consequence of the postulated post-translational modification (acylation) of membrane-bound RD1.

Here then, for the first time, we can attribute a specific function to the N-terminal domain of the rolipram-sensitive type-IV PDE, RD1. For this domain would appear to contain information which can lead to the plasma membrane association of this enzyme. Once effected, this association, in turn, confers a marked enhancement of the thermostability of RD1. The reason for the membrane association of a fraction of RD1 activity remains to be elucidated. It may serve to effect the compartmentalization of cyclic AMP within the cell or it may stabilize RD1 within the cell. As RD1, which occurs exclusively in rat brain (Davis et al., 1989), and the cognate enzyme in *Drosophila*, the *dunc* PDE, is thought to be involved in learning processes (Davis, 1990), then it may be that alteration of the distribution of RD1 between membrane and cytosol could be of importance in memory function.

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