Human phosphodiesterase 4A: characterization of full-length and truncated enzymes expressed in COS cells

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The type 4 phosphodiesterase (PDE) family comprises four enzymes (4A, 4B, 4C and 4D) that are characterized by their specificity for cAMP and selective inhibition by the anti-depressant drug rolipram {4-[3-(cyclopentoxyl)-4-methoxyphenyl]2-pyrrolidone}. In common with other PDEs, they consist of a central conserved domain associated with catalytic activity in addition to two N-terminal upstream conserved regions (UCR1 and UCR2) that are unique to the type 4 enzymes. We have isolated a 2 kb cDNA encoding a full-length type 4A PDE {HS PDE4A4B [Bolger, Michaeli, Martins, St.John, Steiner, Rodgers, Riggs, Wigler and Ferguson (1993) Mol. Cell Biol. **13**, 6558–6571]} from a human frontal cortex cDNA library. Northern blot analysis showed that the major PDE4A mRNA of 4.5 kb was widely distributed in different human tissues. The recombinant PDE4A expressed in COS cells had a molecular mass of approx. 117 kDa as revealed by SDS/PAGE/Western blotting with a PDE4A-specific antibody and was specific for cAMP with a K_m of 4.8 μM. The enzyme activity was potently inhibited by *R*-rolipram (IC_{50} 204 nM) and showed a 2.7-fold stereoselectivity over the *S* enantiomer. Analysis of the kinetics of inhibition indicated that *R*-rolipram did not behave as a simple competitive inhibitor. Dixon replots suggested that there was more than one mode of interaction consistent with the detection in the enzyme of a high-affinity binding site for *R*-rolipram with a K_d of 2.3 nM. Truncation of the PDE4A enzyme by deletion mutagenesis showed that neither of the UCRs was required for catalytic activity and identified an approx. 71 kDa core enzyme with a K_m for cAMP of 3.3 μM. In contrast with the full-length PDE4A, *R*-rolipram behaved as a simple competitive inhibitor of this form of the enzyme with decreased potency (IC_{50} 1022 nM) and no stereoselectivity. In addition, no high-affinity rolipram-binding site was detected in the truncated enzyme, indicating that this interaction involves sequences upstream of the catalytic domain of the enzyme.

INTRODUCTION

The role of cAMP as a second messenger is well recognized. It is responsible for transducing the effects of a variety of extracellular signals, including hormones and neurotransmitters. The level of intracellular cAMP is regulated through both its synthesis by adenylate cyclases and degradation by cyclic nucleotide phosphodiesterases (PDEs). PDEs form a family of at least seven enzyme isotypes (1–7) that differ in their affinity for cAMP and/or cGMP, subcellular localization and regulation [1,2]. In the same way that receptors controlling the synthesis of cAMP have offered opportunities for developing selective therapeutic agents, the PDEs might afford similar possibilities for drug development. In fact the clinical effects of a number of drugs can be rationalized on the basis of their selectivity for a particular PDE isotype. For example, the cardiotonic drugs milrinone and zaprinast are PDE3 and PDE5 inhibitors respectively [3,4]. The anti-depressant drug rolipram {4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidone} functions as a selective PDE4 inhibitor [5].

The availability of PDE isotype-selective inhibitors has enabled the role of PDEs in a variety of cell types to be investigated. In particular it has been established that PDE4 controls the breakdown of cAMP in many inflammatory cells, for example basophils [6] and eosinophils [7], and that inhibition of this isotype is associated with the inhibition of cell activation. Consequently PDE4 inhibitors are currently being developed as potential anti-inflammatory drugs, particularly for the treatment of asthma [8].

The application of molecular cloning to the study of PDEs has revealed that for each isotype there may be one or more isoforms. For PDE4 it is has been shown that there are four isoforms (A, B, C and D) each coded for by a separate gene in both rodents [9] and man [10]. Alignment of the full-length sequences of both human and rat PDE4s [10,11] has shown three regions of similarity, namely two short upstream conserved regions (UCR1 and UCR2) and a central conserved region associated with catalytic activity [12]. For each isoform there are at least two variants that differ in their N-terminal sequences. For human PDE4B [10,13] and rat PDE4D [14] both long and short versions of the enzyme have been identified. The long-form enzymes comprise both UCR1 and UCR2 in addition to the catalytic region, whereas the short-form enzymes only contain UCR2 upstream of the catalytic region. The functional significance of this variation remains to be established.

The low abundance of the native enzymes from natural sources and their susceptibility to proteolysis has limited the purification of intact enzymes for biochemical characterization. A further difficulty is the co-expression of one or more isoforms in a given cell or tissue source. Thus attention has focused on the expression of cDNAs to produce recombinant enzymes of defined isoform.

Although the isolation of a variety of cDNA clones encoding all four human PDEs has been reported, characterization of the properties of full-length enzymes has been limited [15,16]. For human PDE4A, in particular, there is only one report of the isolation of a full-length cDNA from a foetal brain library, designated as HSPDE4A4B. The enzyme was expressed in yeast in a truncated form (lacking the first 100 amino acid residues) and shown to encode a rolipram-sensitive cAMP-sensitive enzyme. In this report we describe the independent cloning of the

Abbreviations used: PDE, phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine; UCR, upstream conserved region; rolipram, 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidone; Ro20-1724, 4-(3-butoxy-4-methoxybenzyl)2-imidazolidinone; Denbufylline, 1,3-di-n-butyl-7-(2-oxopropyl)-xanthine.

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PDE4A4B variant from a human frontal cortex cDNA library. Further, we have used Northern blot analysis to demonstrate that PDE4A mRNA species are widely expressed in human tissues.

The properties of the full-length PDE4A enzyme were characterized in terms of substrate and inhibitor binding after expression in COS cells. We show that the catalytic activity of a long-form PDE4 enzyme (i.e. comprising both UCR1 and UCR2) is not affected by removing either or both UCRs from the sequence by deletion mutagenesis. However, we observed differences in the sensitivity of the full-length and catalytic-only truncated enzymes to inhibition by selective PDE4 inhibitors including rolipram but not to the non-selective inhibitor 3-isobutyl-1-methylxanthine (IBMX). The decreased sensitivity of the truncated enzyme to rolipram was associated with the loss of a high-affinity [^{3}H]*R*-rolipram-binding site from the enzyme. These results are discussed in relation to the role of the sequences N-terminal to the catalytic domain of PDE4s in modulating their interaction with selective inhibitors.

MATERIALS AND METHODS

Isolation of PDE4A cDNA clones

A cDNA clone encoding human PDE4A was isolated by PCR from a cDNA library prepared from mRNA extracted from U937 cells treated with PMA (10 ng/ml for 48 h) with the following primers: forward primer, 5′-ATGTGCCCGTTCCCAGTAACAAGC-3′; reverse primer, 5′-GGATCAGGTAGGGTCTC-3′. This cDNA was inserted into psp65 (Promega, Southampton, Hants., U.K.) to produce the plasmid pDEM1 and sequenced by the chain-termination method with an ABI automated sequencer. A fragment corresponding to the conserved catalytic region of the PDE4 sequence was amplified by PCR from pDEM1 with the following primers: forward primer, 5′-CAGATACCGTGCACAGCC-3′; reverse primer, 5′-TCATCAGGTAGGGTCTCC-3′. The amplified product was cloned into the vector pSP73 (Promega) and labelled with [^{32}P]dCTP (Amersham International, Little Chalfont, Bucks., U.K.) by random priming (Boehringer Mannheim, Lewes, E. Sussex, U.K.). This probe was used to screen a human frontal cortex cDNA library (Clontech, Palo Alto, CA, U.S.A.) by hybridization and washing at moderate stringency (final wash 2 × SSC/0.5 % SDS at 60 °C, where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate). The sequence of the longest cDNA clone obtained, pDEFC1, was determined. The full-length PDE4A cDNA from pDEFC1 and the truncated PDE4A cDNA from pDEM1 were modified at their 5′ ends by PCR to introduce *Hin*dIII restriction enzyme sites followed by the Kozak sequence [17] GCCACC immediately upstream of the first in-frame ATGs. The cDNA species were inserted as *Hin*dIII–*Xba*I fragments into the expression vector pEE7 [18].

Northern blotting

A 3′ untranslated region probe for PDE4A was produced by PCR amplification with HL-60 cell genomic DNA and the following primers: forward primer, 5′-TTTTTAAGCTTGACCTCTGTCCCTGTTCCCCTCC-3′; reverse primer, 5′-TTTTTGGATCCGGCTGGAAGACTGAGCCTGGACC-3′. The probe was radiolabelled with [^{32}P]dCTP by random priming and used to probe human multiple-tissue Northern blots (Clontech). The blots were hybridized for 1 h at 65 °C in Expresshyb® (Clontech), washed for 40 min at room temperature in 2 × SSC,/0.05 % SDS and then for 40 min at 65 °C in 0.1 × SSC/0.1 % SDS. The blots were exposed to X-ray film with intensifying screens at −70 °C for 24 h. A β-actin cDNA was used as a control (Clontech).

Deletion mutagenesis

PCR was used to construct a plasmid (PDB1) containing the first 129 bp (Met-1 to Ile-43) of the PDE4A gene. In addition a 3′ *Bam*HI restriction enzyme site was introduced into the sequence. PCR primers were as follows: forward primer A, 5′-TTTTAAGCTTCCACCATGGAACCCCCGACCGTC-3′; reverse primer B, 5′-TTTTGCGCTGCTGGATCCGGATGGG-3′. Primer B contained a G/T mismatch that introduced a *Bam*HI restriction enzyme site at position 132 bp.

A series of mutants were constructed by PCR with the following pairs of primers, the sequences of which are numbered from the first ATG. Forward primers: C, 5′-TTTTTGGATCCAGCGCCGGGAGTCCTTC-3′ (425–443 bp); D, 5′-TTTTTGGATCCTGTCAGAAGAAACGTGTCAG-3′ (670–689 bp); E, 5′-TTTGGATCCAGCCCATGTCCCAAATCAC-3′ (993–1012 bp); F, 5′-TTTTTGGATCCTGAACATCTTTGCGTGTCGGATTAC-3′ (1135–1160 bp). Reverse primers: G, 5′-TTTTGAATTCATGCATCAACTTTTTCACCCTG-3′ (1012–1034 bp); H, 5′-TTTTGAATTCCTCGAGCACCGACTCATCG-3′ (1496–1514 bp); I, 5′-TTTTTGAATTCATGCATCAACTTTTTCAACCCTGTGATTTGGGACATCAGCGGGGACCGCTTGTTACTG-3′. The sequence underlined in primer I primes over the region 617–638 bp and adds a 5′ tail containing the sequence from positions 999–1031 bp including the *Nsi*I site and causes the juxtaposition of amino acids Leu-211 and Met-342.

The deletion mutants were produced by using the primers in the following combinations: C + G, D + G, E + H, A + I and F + H (Figure 1). The PCR fragments (C + G, D + G, E + H, F + H) were cut with *Bam*HI and *Eco*RI restriction enzymes and cloned into the plasmid (PDB1) described above, to produce a series of intermediate vectors. The PCR fragment A + I was cut with *Hin*dIII and *Eco*RI restriction enzymes and inserted into psp65. After sequencing, the vectors were cut with either *Hin*dIII and *Nsi*I or *Hin*dIII and *Xho*I restriction enzymes and inserted together with either the *Nsi*I–*Xba*I or the *Xho*I–*Xba*I fragment containing the remaining 3′ portion of PDE4A into pEE7 cut with *Hin*dIII–*Xba*I restriction enzymes.

Expression of PDE4A in COS cells

COS 1 cells were grown in Dulbecco's modified Eagle's medium containing 1 mM glutamine, 1 × non-essential amino acids (8.9 mg/l L-alanine, 13.2 mg/l L-asparagine, 19.3 mg/l L-aspartic acid, 14.7 mg/l L-glutamine, 7.5 mg/l-glycine, 11.5 mg/l L-proline and 10.5 mg/l L-serine), 50 units/ml penicillin, 50 μg/ml streptomycin and 10 % (v/v) foetal calf serum (Life Technologies, Paisley, Scotland, U.K.).

Expression plasmids were introduced into COS 1 cells by DEAE-dextran as described by Whittle et al. [19].

COS cells expressing recombinant PDE4A were lysed in 100 mM Tes buffer, pH 7.6, containing 1 % (w/v) Triton X-100 and protease inhibitors (50 μM leupeptin, 1 μM pepstatin, 1 μM PMSF and 2 mM benzamidine). The COS cell lysate was centrifuged at 100000 ***g*** for 30 min and the supernatants were assayed for PDE activity.

Measurement of PDE activity

Enzyme reactions were performed at pH 7.6 in 50 mM Tes buffer containing 10 mM $MgCl_2$, 3′,5′-cAMP (0.1 μM ^{3}H-labelled, 0.74–1.1 TBq/mmol; Amersham International) and 5′-AMP

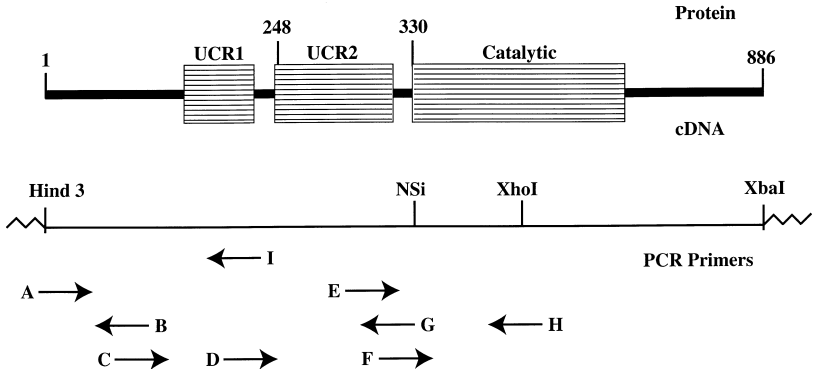

Figure 1 Strategy for construction of PDE4A deletion mutants

Schematic representation of the PDE4A cDNA showing the positions of PCR primers (A–I) used to generate deletions within the N-terminal region. The positions of restriction sites used to produce the deletion mutants are also indicated. The deletion mutants generated with these primers are as follows: PDE4A ΔQ44–L329 (catalytic only), primers E + H; PDE4A ΔG212–P342 (UCR1 + catalytic), primers A + I; PDE4A ΔQ44–T221 (UCR2 + catalytic), primers D + G; PDE4A ΔQ44–S140 (UCR1 + UCR2 + catalytic), primers C + G; PDE4A ΔQ44–G376, primers F + H.

(2.5 μM ^{14}C, 1.85–2.2 GBq/mmol; Amersham International) for 30 min at 30 °C. Sufficient enzyme preparation was added to hydrolyse not more than 20 % of substrate under these conditions. For K_m determinations, unlabelled cAMP was added to achieve substrate concentrations in the range 0.1–20 μM. Reactions were stopped by rapid inactivation of enzyme by the addition of trifluoroacetic acid to a final concentration of 1 %. Substrate and product of reaction were separated as described by Smith et al. [20] and the 5′-[^{3}H]AMP product was analysed by scintillation counting. Correction for losses of 5′-[^{3}H]AMP during separation was made by reference to 5′-[^{14}C]AMP included in the reaction mixture. Enzyme kinetics and inhibition were evaluated with the GRAFIT program (Erithacus Software). K_m values for cAMP were obtained from data fitted to the hyperbolic Michaelis–Menten equation. IC_{50} values were obtained from log dose inhibition curves fitted to the four-parameters logistic equation.

Measurement of [^{3}H]*R*-rolipram binding

R-rolipram was iodinated and dispatched to Amersham International, where it was tritiated by catalytic reduction with palladium charcoal to a specific radioactivity of 851 GBq/mmol.

The ability of [^{3}H]*R*-rolipram to bind to recombinant enzymes was assayed with the method of Schneider et al. [5]. For saturation binding experiments, the concentration of [^{3}H]*R*-rolipram was varied from 0.01 to 30 nM. Sufficient enzyme was used to bind approx. 10 % of the total label at 5 nM. Non-specific binding was established in the presence of 2 μM unlabelled rolipram and was always less than 5 % of the total bound. Thus specific binding was determined by subtraction of non-specific binding from total binding. Assays were performed in either the absence of cAMP or with cAMP at 0.1, 1.0 or 10 μM.

SDS/PAGE and Western blotting

SDS/PAGE was performed by the method of Laemmli [21] with 10 % (w/v) polyacrylamide gels. For Western blotting, proteins were transferred to nitrocellulose and probed with a rabbit polyclonal antiserum raised against a glutathione S-transferase–PDE4A 162-residue C-terminal fragment. Immunoreactivity was detected with alkaline phosphatase-conjugated sheep anti-(rabbit IgG) together with 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium substrate. This antiserum was specific for PDE4A, showing no cross-reactivity with human recombinant PDE4B, C or D. Pre-immune serum did not show any PDE4A immunoreactivity. The glutathione S-transferase fusion protein was constructed by introducing *Bam*H1 sites at positions 2173 bp and 2659 bp of PDE4A and inserting the *Bam*H1 fragment into pGEX2T (Pharmacia Biotech, St Albans, Herts, U.K.). Induction of the fusion protein and purification were performed as previously described [22].

Protein assay

The protein content of COS cells lysates was assayed with bicinchoninic acid reagent (Pierce, Chester, Cheshire, U.K.) with BSA as a standard.

PDE inhibitors

Rolipram and its *R* and *S* enantiomers were synthesized by the Department of Chemistry, Celltech Therapeutics. IBMX and zaprinast were purchased from Sigma (Poole, Dorset, U.K.), 4-(3-butoxy-4-methoxybenzyl)2-imidazolidinone (Ro20-1724) was purchased from Calbiochem (Nottingham, Notts., U.K.), and 1,3-di-n-butyl-7-(2-oxopropyl)-xanthine (Denbufylline) and SKF94120 were gifts from SmithKline Beechams Pharmaceuticals (Epsom, Surrey, U.K.). Inhibitors were dissolved in DMSO and then added to the PDE assay to give a final DMSO concentration of 1 % (v/v).

RESULTS

Sequence of human PDE4A

By using the published sequence data of Livi et al. [23], an approx. 2 kb PDE4A cDNA was initially isolated from the human monocytic cell line U937 treated with PMA. Sequencing of this cDNA revealed significant differences between this cDNA and that reported by Livi et al. [23]: in particular, the reading frame at the 5′ end of the cDNA differed such that the initiating methionine identified by Livi et al. was not in-frame with the remainder of the sequence. In fact the first in-frame methionine

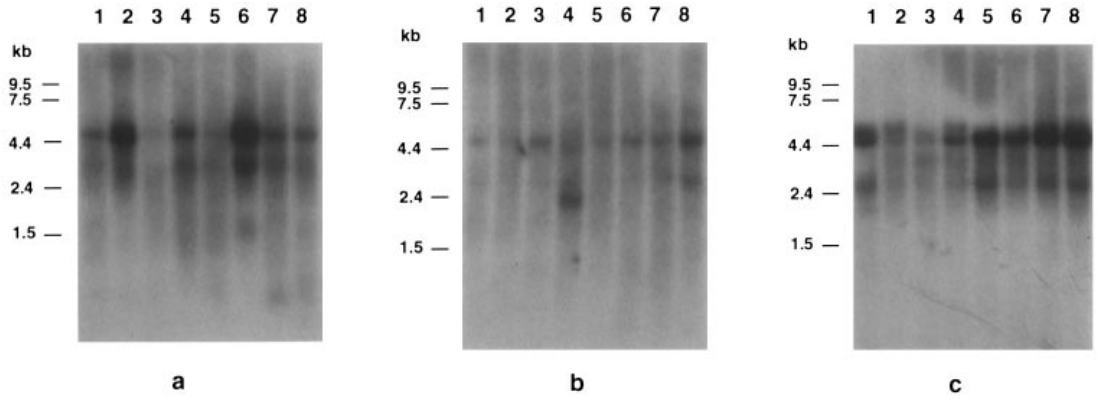

Figure 2 Human tissue distribution of PDE4A mRNA species

Northern blots containing poly(A)$^+$ mRNA extracted from various human tissues (Clontech) were probed with a ^{32}P-labelled PCR fragment of PDE4 corresponding to a non-conserved 3′ non-coding region, then stripped and re-probed with a ^{32}P-labelled β-actin cDNA (result not shown). Each lane contained 2 μg of poly(A)$^+$ RNA from the following tissues. (**a**) Lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas. (**b**) Lane 1, spleen; lane 2, thymus; lane 3, prostate; lane 4, testes; lane 5, ovary; lane 6, small intestine; lane 7, colon; lane 8, peripheral blood leucocytes. (**c**) Lane 1, amygdala; lane 2, caudate nucleus; lane 3, corpus callosum; lane 4, hippocampus; lane 5, hypothalamus; lane 6, substantia nigra; lane 7, subthalamic nuclei; lane 8, thalmus. The positions of size markers are shown at the left of each panel.

occurred 48 residues downstream of this point. A fragment of this cDNA clone was used to isolate a longer PDE4A cDNA from a human frontal cortex cDNA library. This cDNA comprised a 2658 bp open reading frame encoding a protein of 886 residues, plus 128 bp 5′ and 65 bp 3′ untranslated regions. The sequence initially obtained from U937 cells was entirely contained within this cDNA. The PDE4A sequence obtained from human brain mRNA was identical with that recently reported by Bolger et al. [10] and designated HSPDE4A4B (GenBank accession no. L20965), with the following minor differences: G for A at 724 bp producing a Met to Ile change, and a silent change G for A at 1238 bp. It seems likely that the cDNA isolated from U937 cells and that reported by Livi et al. [23] represent partial PDE4A cDNA species.

Distribution of PDE4A mRNA in human tissues

Human multiple-tissue blots were probed with a 3′-untranslated region DNA fragment of the PDE4A mRNA. The results shown in Figure 2 indicated that the major PDE4A mRNA was approx. 4.5 kb, consistent with a previous report (Livi et al. [23]). Additional mRNA species of approx. 3 kb were also detected. Although PDE4A mRNA species were detected in a variety of tissues, their relative abundance varied. Thus brain, skeletal muscle and peripheral blood leucocytes showed the highest levels of the major 4.5 kb mRNA. Interestingly a 2.4 kb mRNA seemed unique to testes.

The PDE4A mRNA species were also present in all regions of the brain that were probed, although again the levels of expression seemed to vary. Thus the highest levels were found in the subthalamus, hypothalamus, thalamus and substantia nigra. RNA loading was confirmed by stripping and rehybridizing the Northern blot with a β-actin probe (results not shown).

Expression of recombinant PDE4A in COS cells

PDE4A was produced by transient expression in COS cells. Cells were lysed by treatment with 1 % Triton X-100, and the 100 000 ***g*** supernatant was assayed for PDE activity without further purification. Lysates of cells transfected with the PDE4A expression vector showed a 20–30-fold elevation of cAMP PDE activity compared with mock-transfected cells. Typically, the cAMP-specific PDE activity of the PDE4A-transfected cells was 549 ± 96 (S.E.M.) pmol of cAMP hydrolysed/min per mg of total cell lysate protein ($n = 6$), compared with 20.7 ± 2.49 pmol/min per mg ($n = 3$) from mock-transfected cells. Mock transfection of COS cells did not alter the level of endogenous PDE activity, which was inhibited more than 95 % by 10 μM rolipram, indicating that it was largely of the PDE4 type. All PDE4A activity measurements were routinely corrected for the residual COS cell PDE activity. Analysis of the transfected cells by SDS/PAGE and Western blotting with a PDE4A-specific antiserum revealed an immunoreactive 117 kDa polypeptide (Figure 3). This molecular mass was greater than the 98 kDa calculated

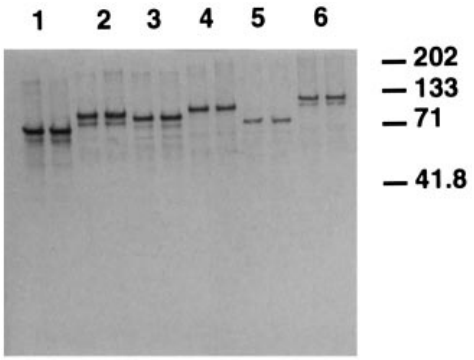

Figure 3 Western blot of recombinant PDE4A constructs expressed in COS cells

DNA species encoding the following constructs were transfected into COS cells and lysates analysed for expression of recombinant PDE4s by immunoblotting: lane 1, PDE4A ΔQ44–L329 (catalytic only); lane 2, PDE4A ΔG212–P342 (UCR1 + catalytic); lane 3, PDE4A ΔQ44–T221 (UCR2 + catalytic); lane 4, PDE4A ΔQ44–S140 (UCR1 + UCR2 + catalytic); lane 5, PDE4A ΔM1–T247; lane 6, PDE4A (full length); lane 7, mock-transfected cells. The numbers at the right are the sizes (in kDa) of molecular mass standards run in parallel. Duplicates of each sample were run to demonstrate consistency of gel loading.

Table 1 Kinetic and inhibitor profile of full-length and truncated PDE4A

K_m values for cAMP were determined over a broad concentration range (0.01–50 μM) from data fitted to the hyperbolic Michaelis–Menten equation. Relative V_{max} values were obtained for the two enzymes by using the anti-(PDE4A) antiserum and Western blotting to normalize the amount of each enzyme in the assay. IC_{50} values were obtained at 0.1 μM cAMP from log dose–inhibition curves fitted to the four-parameters logistic equation. Values are means $\pm$ S.D. for at least three separate experiments. Statistically significant differences between IC_{50} values for the two enzymes were determined with Student's unpaired *t* test; * $P < 0.01$; ** $P = 0.0006$.

Parameter	PDE4A (full-length)	PDE4A ΔQ44–L329 (catalytic only)
K_m cAMP (μM)	4.8 $\pm$ 0.2	3.3 $\pm$ 0.3
K_m cGMP (μM)	> 1000	> 1000
V_{max} cAMP (nmol/min per mg)	19.6 $\pm$ 3.7	20.1 $\pm$ 3.2
	IC_{50} (nM)	
Inhibitor	PDE4A (full-length)	PDE4A ΔQ44–L329 (catalytic only)
R-rolipram	204 $\pm$ 32	1022 $\pm$ 141**
S-rolipram	549 $\pm$ 44	1136 $\pm$ 167*
Ro20-1724	1450 $\pm$ 97	4154 $\pm$ 228*
Denbufylline	295 $\pm$ 58	550 $\pm$ 61*
IBMX	10550 $\pm$ 269	12500 $\pm$ 311

from the amino acid sequence but was consistent with that observed in the following human cell lines by Western blotting: U937 (promonocyte), Jurkat J6 (T-cell lymphoma) and U87 (glioblastoma). The fact that PDE4A contains stretches of polyproline sequences might contribute to its aberrant gel mobility.

COS-cell-expressed PDE4A was selective for cAMP with a K_m of 4.8 μM (Table 1). Lineweaver–Burk plots for the hydrolysis of cAMP were linear over a wide range of substrate concentrations (0.01–50 μM), indicative of simple Michaelis–Menten kinetics. The recombinant enzyme was insensitive to cGMP, Ca^{2+}/calmodulin, SKF94120 (a PDE3 inhibitor) and zaprinast (a PDE5 inhibitor). However, the enzyme was potently inhibited by a number of PDE4-selective inhibitors, including the archetypal inhibitor, rolipram (Table 1).

It was noted that the log dose–inhibition curve for *R*-rolipram was shallow, giving a Hill number of 0.69 (results not shown). In addition, from the double-reciprocal plots (Figure 4a) the mechanism of inhibition by this compound did not seem to be simple competitive. The change in slope of the double-reciprocal plot of inhibition was smaller than expected, giving rise to a slope replot that was concave (Figure 4b). Similarly the Dixon [24] replot for *R*-rolipram was non-linear (Figure 4c). These findings were indicative of PDE4As having more than one class of binding site for *R*-rolipram capable of inhibiting catalytic activity. The concave nature of the slope replot also argues against the enzyme binding more than one molecule of rolipram, as the latter case would be expected to give rise to convex slope replots [23]. The results are consistent with those previously reported for a partial PDE4A (starting at Met-265) expressed in yeast [25], but contrast with the results of Wilson et al. [26] (for a similarly truncated PDE4A enzyme), who noted that rolipram behaved as a simple competitive inhibitor of this enzyme preparation. More recently Huston et al. [27] reported that racemic rolipram was a partial competitive inhibitor of human recombinant PDE4A (HSPDE4A4B) expressed as a particulate enzyme in COS7 cells

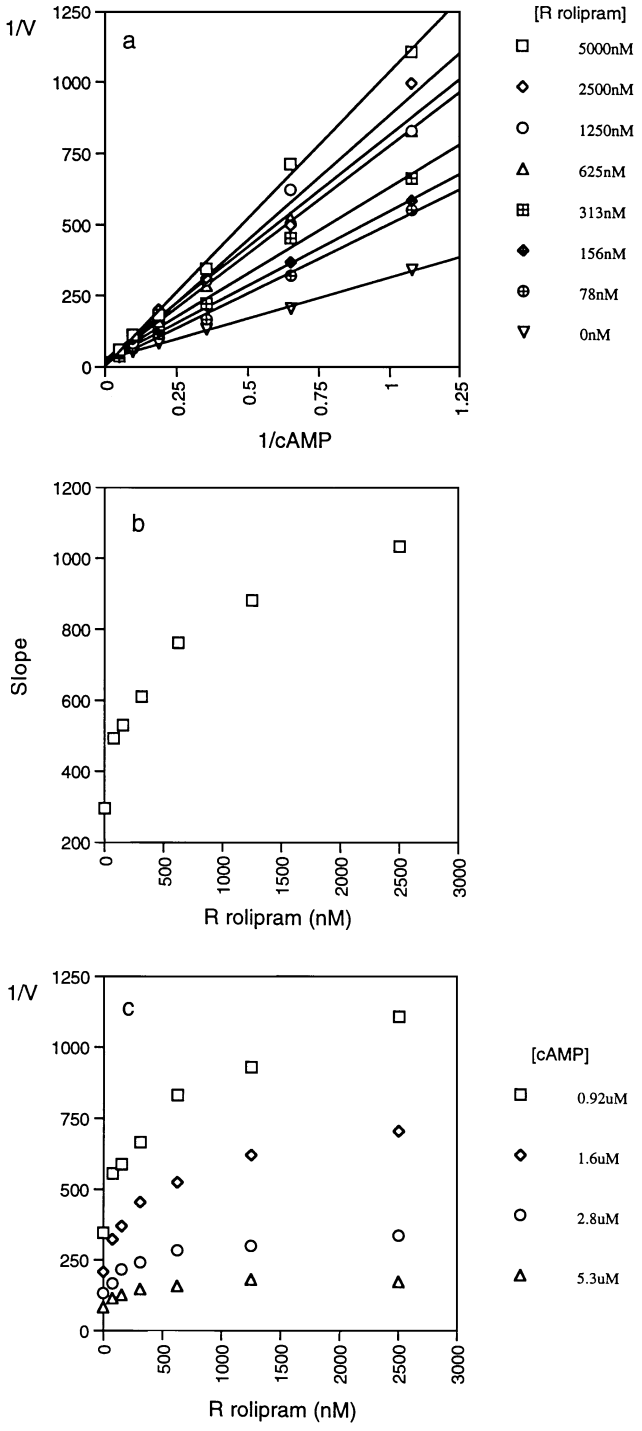

Figure 4 Kinetics of *R*-rolipram inhibition of PDE4A

Shown are double-reciprocal plots of cAMP hydrolysis against cAMP concentration (**a**), a replot of the slopes of the double-reciprocal plot against rolipram concentration (**b**) and a Dixon replot (**c**). Velocity is in arbitrary units. For graphical presentation, the *x*-axis in (**a**) is shown only up to 1.25 μM although the full data set was used in the linear analysis; in (**c**) only data for 0.92–5.3 μM cAMP are shown. The data are representative of those obtained from three separate experiments.

with an IC_{50} of 0.195 μM. This is very similar to the value that we have obtained for a Triton X-100-solubilized preparation of the same enzyme. In contrast, rolipram was shown to be a

Table 2 Catalytic activity of PDE4A deletion mutants expressed in COS cells

A series of deletions were made to the N-terminal region of human PDE4A by using PCR. The first and last amino acid residues of the sequences that were deleted are given. The domain organization of the resulting PDE4A enzymes are also shown in parentheses. Lysates were prepared from transfected COS cells and assayed for PDE activity and protein content as described in the Materials and methods section. The background PDE activity in non-transfected cells was subtracted from the activities in the transfected cells.

Construct	Specific activity (pmol/min per mg)
Met 1A (full-length)	400.0
PDE4A ΔQ44–S140 (UCR1 + UCR2 + catalytic)	510.0
PDE4A ΔQ44–T221 (UCR2 + catalytic)	610.0
PDE4A ΔG212–P342 (UCR1 + catalytic)	640.0
PDE4A ΔQ44–L329 (catalytic only)	590.0
PDE4A ΔQ44–G376	0

competitive inhibitor of the cytosolic enzyme with an IC_{50} of 1.6 μM. Although the Hill coefficient and Dixon plot for *R*-rolipram inhibition of the COS-cell-expressed PDE4A obtained in the present study are also consistent with partial competitive inhibition, we observed 100 % inhibition of the PDE4A enzyme at 10 μM rolipram (Figure 4). This complete inhibition is in contrast with the observations of Huston et al. [27] and suggest that *R*-rolipram is not a partial competitive inhibitor of the PDE4A as expressed in this study.

Mapping functional domains

A series of N-terminal deletion mutants of the full-length PDE4A were constructed and evaluated after transient expression in COS cells (Figure 1). Each deletion contained a common 123 bp 5′ coding sequence to ensure as far as possible that each sequence would be translated with the same efficiency. All the constructs were expressed at approximately the same level as judged by SDS/PAGE/Western blotting (Figure 3). Samples were assayed for PDE activity to investigate the effect of the deletions on catalysis. The results indicated that removal of both or either of the two UCR had no effect on the enzyme activity (Table 2). However, activity was lost on deletion of Gln-330 to Leu-377 from the enzyme. This result is consistent with that previously reported for rat PDE4D [12], indicating that this might be a general feature of PDE4s.

The properties of the catalytic-only version of PDE4A (ΔQ44–L329) were compared with the full-length enzyme (Table 1). The K_m values for cAMP were similar for both enzyme preparations. However, whereas the truncated and full-length enzymes were equally sensitive to the non-selective PDE inhibitor IBMX, the former was less sensitive to the PDE4-selective inhibitors and in particular rolipram (Table 1). The 5-fold difference in IC_{50} values (204 nM compared with 1022 nM) was shown to be highly statistically significant ($P = 0.0006$) with the unpaired Student *t* test. In further contrast with the full-length enzyme, *R*-rolipram gave a Hill number close to unity against the truncated enzyme and behaved as a simple competitive inhibitor. This was indicated by linear double-reciprocal plots over a range of substrate and inhibitor concentrations (Figure 5a) together with linear slope (Figure 5b) and Dixon replots (Figure 5c). It was also noted that the stereoselective inhibition of the full-length enzyme by *R*-rolipram relative to *S*-rolipram was lost against the truncated enzyme, where both enantiomers were equipotent (Table 1).

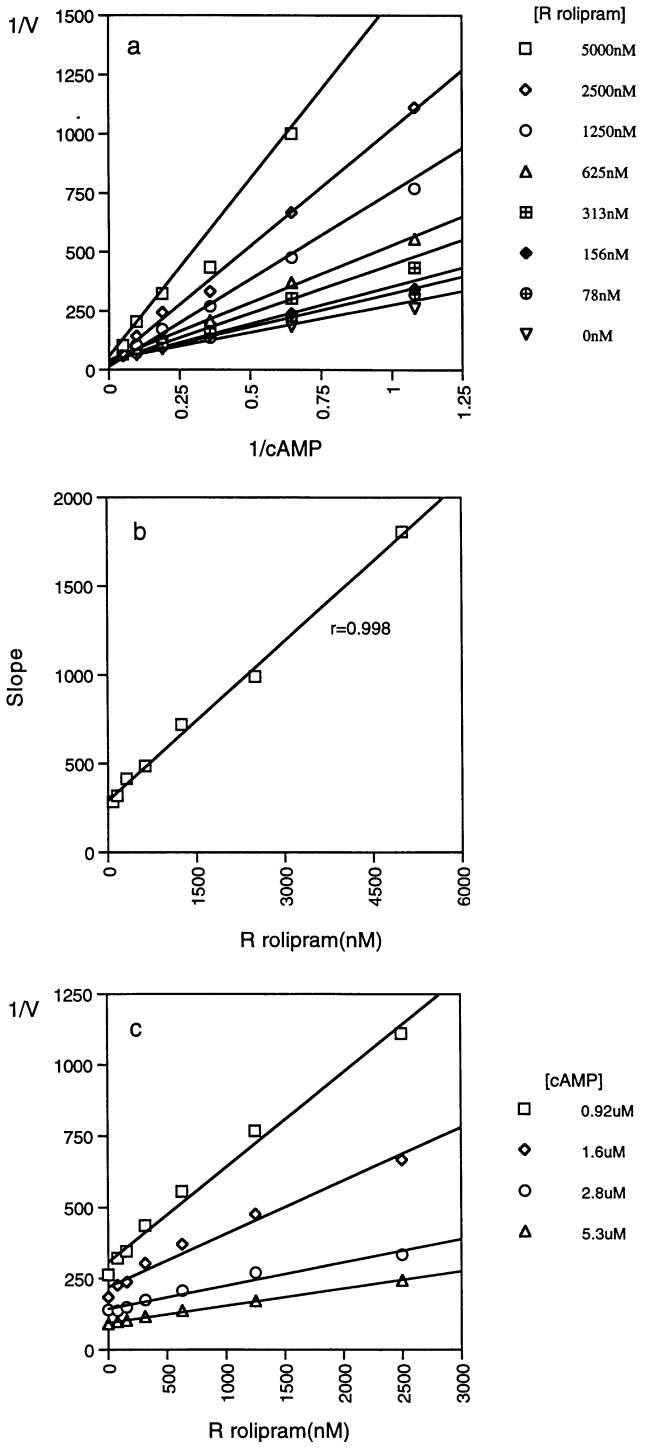

Figure 5 Kinetics of *R*-rolipram inhibition of PDE4A ΔQ44–L329

Shown are double-reciprocal plots of cAMP hydrolysis against cAMP concentration (**a**), a replot of the slopes of the double-reciprocal plot against rolipram concentration (**b**) and a Dixon replot (**c**). Velocity is in arbitrary units. For graphical presentation, the *x*-axis in (**a**) is shown only up to 1.25 μM although the full data set was used in the linear analysis; in (**c**) only data for 0.92–5.3 μM cAMP are shown. The data are representative of those obtained from three separate experiments.

Rolipram-binding site

It has previously been shown that recombinant PDE4s [13] can bind [^{3}H]rolipram with high affinity. This has been correlated

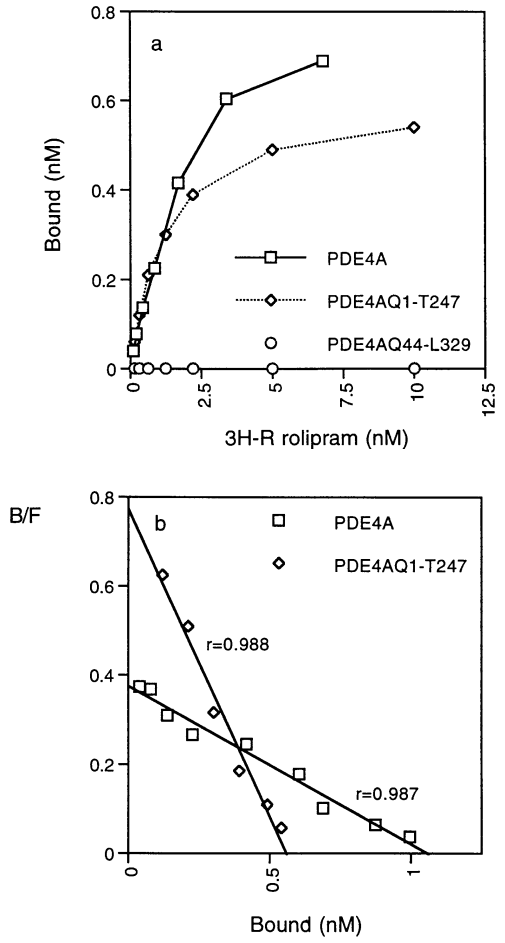

Figure 6 [^{3}H]*R*-rolipram binding to full-length and truncated PDE4A enzymes

(**a**) Saturation of [^{3}H]*R*-rolipram binding to full-length PDE4A (□), PDE4AΔQ1–T247 (◇) and PDE4A ΔQ44–L329 (○); (**b**) Scatchard analysis of [^{3}H]*R*-rolipram binding to full-length PDE4A (□) and PDE4AΔQ1–T247 (◇). Recombinant enzymes were incubated with [^{3}H]*R*–rolipram (0.01–30 nM). For graphical presentation, only data up to 10 nM are shown for the saturation binding curve. The results of the Scatchard analysis were analysed by the ENZFITTER computer program and gave K_d values of 2.3 ± 0.21 (mean ± S.E.M.) and 2.6 ± 0.76 nM (mean ± S.E.M.) for full-length PDE4A and PDE4AΔQ1–T247 respectively. The data are representative of those obtained from three separate experiments.

with the earlier identification of a high-affinity rolipram-binding site in brain membranes that is stereoselective for the *R* enantiomer of rolipram [5]. The ability of the COS-expressed full-length and the truncated enzymes PDE4A ΔM1–T247 (pDEM1 cDNA insert lacking UCR1) and PDE4A ΔQ44–L329 (catalytic domain only) to bind to rolipram with high affinity was measured in a filter-binding assay. Equivalent amounts of all three enzymes were assayed. The full-length enzyme and the enzyme truncated to Met-248 both expressed a high-affinity binding site for *R*-rolipram with a K_d of 2.3 ± 0.21 (S.D.) nM ($n = 3$) and 2.6 ± 0.76 nM ($n = 3$) respectively. The B_{max} values were 4.7 ± 0.91 pmol/mg ($n = 3$) for the full-length enzyme and 2.5 ± 0.37 pmol/mg ($n = 3$) for the enzyme truncated to Met-248 (Figure 6a). A linear Scatchard analysis coupled with a Hill coefficient of 1.0 suggested a single class of non-interacting sites (Figure 6b). The binding constants were not altered by inclusion of cAMP in the assay up to a concentration of 10 μM (results not shown). In contrast, the enzyme truncated to the catalytic domain (PDE4A ΔQ44–L329) did not demonstrate a high-affinity binding site for *R*-rolipram (Figure 6a). Inclusion of 100-fold more of the latter enzyme in the assay also failed to show binding of [^{3}H]*R*-rolipram.

Taken together, these results indicate that the sequence N-terminal to the catalytic domain has no effect on the affinity of PDE4A for its substrate, cAMP, but exerts a significant effect on the ability of the enzyme to interact with certain inhibitors, as exemplified by *R*-rolipram.

DISCUSSION

PDE4A is one of a family of four enzymes implicated in the control of cAMP metabolism in pro-inflammatory cells and the central nervous system. The functional significance of this apparent redundancy remains to be established. However, it is clear that expression of the different isoforms is regulated resulting in tissue/cell-specific expression patterns [28]. In man, PDE4A seems to be widely distributed among different cells and tissues as indicated by Northern blot analysis (reported here). In addition PDE4A cDNA clones have been isolated from a promonocytic cell line, U937 [23], T lymphocyte [29] and brain tissue [10]. Although in this study the PDE4A was isolated from a frontal cortex cDNA library, Northern blot analysis showed that PDE4A mRNA was expressed in a variety of different brain regions. Therefore PDE4A might have a ubiquitous role in controlling cAMP levels.

In addition to the four isoforms of PDE4, cDNA cloning has shown that there is more than one mRNA for each isoform. In particular, long and short versions of some of the enzymes have been identified. In rat, PDE4A activity results from both a long-form enzyme equivalent to the human enzyme described here and a short-form enzyme [30]. So far no evidence for a short-form version of PDE4A in man has been found. However, our detection of a number of different PDE4A mRNA species suggests that further variants might occur.

The long and short forms of PDE4 differ in the presence or absence of UCR1. To investigate the functional significance of these domains we performed deletion mutagenesis of PDE4A. The results indicate that neither of the UCRs modulates catalytic activity. In fact a core enzyme was identified beginning around Glu-330 that retained full catalytic activity, exhibiting similar K_m and V_{max} values for cAMP to those of the full-length enzyme. Removal of a further 47 amino acid residues to Leu-377 resulted in complete inactivation of the enzyme.

However, whereas the regions upstream of this catalytic core did not seem to modulate the interaction of the enzyme with cAMP, they did show a profound effect on the interaction with certain inhibitors, particularly rolipram. The truncated enzyme (PDE4A ΔQ44–L329) was not stereoselective for the enantiomers of rolipram, displaying an IC_{50} for inhibition of approx. 1 μM. In contrast the full-length enzyme was more sensitive to rolipram, particularly the *R* enantiomer (IC_{50} for *R*-rolipram, 0.204 μM; IC_{50} for *S*-rolipram, 0.549 μM). There were also significant mechanistic differences in the way in which *R*-rolipram inhibited the two forms of PDE4A. The results obtained with [^{3}H]*R*-rolipram demonstrated that the full-length PDE4A but not the truncated enzyme possessed a high-affinity binding site for *R*-rolipram. These observations can be interpreted in the light of previous studies of the interaction between rolipram and PDE4 enzymes as discussed below.

It has been reported that solubilization of a PDE4 activity from guinea-pig membranes resulted in an increased sensitivity of the enzyme activity to inhibition by rolipram but not to non-selective inhibitors including IBMX [31]. Rolipram also demon-

strated an increased stereoselectivity in its binding [32]. To explain these findings, Souness and co-workers [31,32] proposed the existence of two sites on the enzyme with which inhibitors could interact, namely a site associated with catalysis (Sc) and a distinct high-affinity stereoselective site (Sr). Solubilization of the guinea-pig eosinophil PDE4 is presumed to result in the increased expression of the Sr site, perhaps by a change in conformation, thus leading to an increase in the potency of some PDE4 inhibitors. In support of this model, the ability of some recombinant PDE4 enzymes to bind [^{3}H]*R*-rolipram with high affinity has been documented [25]. However, the significance of this binding activity with respect to PDE4 catalytic activity has been unclear. In this study we have demonstrated that those inhibitors (*R*-rolipram, Ro20-1724 and Denbufylline) that are known to bind at the Sr site [25] displayed weaker inhibitory activity towards the enzyme (PDE4A ΔQ44–L329) that lacked this site. In contrast, the activity of IBMX, an inhibitor reported to have equal affinity for the Sr and catalytic sites [31], was equivalent against both PDE4A and PDE4A ΔQ44–L329. Thus the data reported here provide evidence that binding at the Sr site is inhibitory to catalytic activity.

What, then, is the nature of the Sr site? Our observations with the full-length and truncated enzymes indicate that the Sr site involves sequences upstream of the catalytic domain of the enzyme, implicating the UCRs. We have shown that a PDE4A enzyme truncated to Met-248, i.e. lacking the entire UCR1, also displays the Sr site. It has also been shown that a short-form PDE4B enzyme that naturally lacks the UCR1 sequence displays high-affinity binding of rolipram [13]. Therefore it seems that the UCR2 sequence is required to form this binding site. It is conceivable that the Sr site does not represent a discrete sequence but rather a conformational state of the enzyme that influences the way in which certain inhibitors are able to bind to the active site as originally proposed by Torphy et al. [25]. In other words, PDE4 enzymes can exist in at least two different conformations, both of which hydrolyse cAMP with the same K_m but differ markedly in the affinity with which certain inhibitors such as rolipram bind to the catalytic site.

PDE4A ΔQ44–L329 therefore represents the 'low-affinity' state with respect to rolipram binding. The question arises as to the conformational state of the full-length enzyme because the IC_{50} for rolipram inhibition, although less than that of the truncated enzyme, is still greater than the K_d for the high-affinity rolipram-binding site. To explain this in the context of the two-conformations model of PDE4s we propose that the full-length enzyme derived from COS cells consists of a mixture of low-affinity and high-affinity states, presumably with a relative low stoichiometry of high-affinity binding to catalytic activity. Insufficient enzyme is produced from COS cells to enable purification of the PDE4A enzyme and hence determination of this stoichiometry.

Recently Houslay and co-workers [27] have suggested that the ability of PDE4s to express more than one active conformation distinguished by their sensitivity to inhibition by rolipram might be related to the subcellular localization of the enzymes. Thus the expression in COS cells of the human PDE4A enzyme resulted in the localization of a proportion of the activity to a particulate fraction of the cells. This was detected as a cortical staining by immunocytochemistry. As mentioned in the Results section, this particulate enzyme proved to be more sensitive to rolipram inhibition than the cytosolic enzyme. Interestingly the converse was observed for the rat PDE4A homologue [30]. Nevertheless these observations raise the intriguing possibility that association with accessory molecules might be involved in determining the active conformation of the PDE4 enzymes *in situ*.

We are currently investigating the production of the enzyme from other expression systems with a view to obtaining pure PDE4A enzyme. This would enable the stoichiometry of high-affinity rolipram binding relative to catalytic activity to be determined and further the exploration of the possibility of completely 'locking' the enzyme into the high-affinity form.

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