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Isolation, biochemical characterization and N-terminal sequence of rolipram-sensitive cAMP phosphodiesterase from human mononuclear leukocytes

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Abstract A cyclic AMP specific phosphodiesterase (type IV) was purified 450,000-fold from human peripheral blood mononuclear cells through a sequence of chromatographic steps involving anion exchange, affinity chromatography on a matrix coupled to a derivative of the type IV inhibitor rolipram, and gel filtration. The enzyme showed apparent molecular masses of 70 kDa on gel filtration and 35 kDa on denaturing or native PAGE, indicating a possible dimerization or cleavage under certain conditions. The isoelectric point was 4.6. Kinetic parameters were K_m = 2.2 μM, K_i = 1.2 μM (rolipram) and v_{max} = 80 μmol/min per mg protein. The most probable N-terminal sequence was determined as SLTNTNIPRF, 80% identical to part of the deduced amino acid sequence from cDNA sequences of PDE IV_A and PDE IV_D.

Key words: 3′:5′ Cyclic AMP phosphodiesterase (mononuclear leukocyte, human blood); Rolipram; Sequence homology; Amino acid

1. Introduction

Cyclic nucleotide phosphodiesterases (PDE), which catalyze the hydrolysis of 3′:5′-cyclic nucleotides to the corresponding nucleoside 5′-monophosphates, play an essential role in controlling intracellular levels of cyclic nucleotides and consequently, cell function. Multiple forms of cyclic nucleotides phosphodiesterases differing in apparent molecular mass, chromatographic properties, substrate specificity, interaction with certain antibodies, sensitivity to inhibitors, and mode of regulation have been identified within the same tissue and in some cases within the same cell [1,2]. These isoenzymes are a target for the development of new anti-inflammatory drugs, as indicated by the fact that many pro-inflammatory mechanisms of the immune system can be down-regulated by inhibitors of the type III (cGMP-inhibited PDE) and type IV isoenzymes (rolipram-sensitive PDE, reviewed by Torphy and Undem [3]). Analysis of the PDE complement of relevant cells helps to design the most effective anti-inflammatory compound based on PDE inhibition. In principle, this can be achieved either by homology cloning or by direct biochemical characterisation. Homology cloning in conjunction with detection of corresponding mRNA will yield indication of the type and amount of a protein potentially present cells. Direct characterisation of the isoenzyme activity, albeit labor-intensive, is more informative with respect to the contribution to the overall hydrolysis of cAMP. Additionally the latter approach allows detection of post-translational processing, for example proteolytic cleavage. Human peripheral blood mononuclear cells were chosen for the direct biochemical analysis. This heterogeneous fraction contains monocytes, platelets and B and T lymphocytes. These cells play important roles in the inflammatory response and are therefore primary targets of anti-inflammatory therapy. Whereas monocytes contain mostly type IV PDE, lymphocytes appear to have both type III and IV PDE, whilst the predominant cAMP-hydrolyzing isoenzyme in platelets is PDE-III [3]. In this report we describe the purification and biochemical characterization of the rolipram-sensitive cAMP-hydrolytic activity from mononuclear cells. Additionally this is, to our knowledge, the first account of a succesful determination of the N-terminal amino acid sequence of a type IV PDE.

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Abbreviations: PDE, 3′:5′-cyclic nucleotide phosphodiesterase; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid disodium salt dihydrate; PMSF, phenylmethylsulphonylfluoride; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate.

2. Materials and methods

2.1. Materials

[^{3}H]cAMP (specific activity 28 Ci/mmol), [^{3}H]cGMP (specific activity 5 Ci/mmol), [^{14}C]AMP (specific activity 0.6 Ci/mmol) were obtained from E.I. du Pont de Nemours & Co., (Inc). Snake venom (5′-nucleotidase), Histopaque 1077 and Histopaque 1119 were from Sigma Chemical Co. Dextran T500 and EAH-Sepharose were obtained from Pharmacia. Rolipram (4-[3-cyclopentyloxy-4-methoxyphenyl]-2-pyrrolidone) and the ligand for the affinity column (4-[3-(carboxypropyloxy)-4-methoxyphenyl]-2-pyrrolidone) were kindly provided by Dr. J. Demnitz, Sandoz Preclinical Research Basle, RO 20–1724 (4-[(3-butyloxy-4-methoxyphenyl)methyl]-2-imidazolidinone) was a generous gift from Hoffman-La Roche. SDS-PAGE standard proteins: phosphorylase b, bovine serum albumin, bovine carbonic anhydrase, soybean trypsin inhibitor, and lysozyme were from Bio-Rad. Other reagents were obtained commercially and were of analytical grade.

2.2. Phosphodiesterase assay

PDE activity was assayed using a two-step method as described [4] involving conversion of AMP to adenosine with 5′-nucleotidase and separation from unreacted substrate on an anion exchange resin. The volume of the final mixture contained 0.3 ml assay buffer (40 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 3.75 mM β-mercaptoethanol, 0.125 mg/ml BSA, 1 μM [^{3}H]cAMP (10,000 cpm), [^{14}C]AMP (4,000 cpm)) and 0.1 ml of enzyme preparation, diluted in buffer A (see section 2.4) if necessary. Incubations were routinely carried out for 30 min at 37°C. Calmodulin stimulation experiments were done using 2 mM $CaCl_2$ and 1 μM calmodulin.

2.3. Cell isolation and cytosol preparation

All extraction and purification steps were performed at 4°C except for affinity chromatography was performed at room temperature

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(20–25°C) [5]. Buffy coat units prepared from human peripheral venous blood were obtained from the Swiss Red Cross (Berne, Switzerland). Mononuclear cells were prepared by sedimentation of the erythrocytes under unit gravity in the presence of dextran. Separation from granulocytes was achieved by density gradient centrifugation on Histopaque 1077/1119. The cells were then washed three times with PBS to reduce platelet contamination.

Cells were resuspended in homogenization buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 35 μg/ml PMSF, 5 μg/ml aprotinin, 2 mM benzamidine, 0.25 M sucrose, 0.1 M NaCl, 0.25 ml/ml ethylene glycol and 10 mg/ml CHAPS and lysed by gentle shaking, until the suspension was homogeneous (~30 min). In preliminary experiments, cells were sonicated (Branson Cell Sonifier Model B12, 30 s, 50 W). After centrifugation (120,000 × g, 1 h), the supernatant was carefully removed, pooled and immediately applied to Q Sepharose.

2.4. Chromatography

2.4.1. Separation of phosphodiesterase isoenzymes on Q Sepharose. The supernatant was loaded onto a Q Sepharose column (20 ml bed volume) equilibrated with buffer A (10 mM Tris, pH 7.4, 1 mM EDTA, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 5 μg/ml aprotinin, 2 mM benzamidine, 0.25 ml/ml ethylene glycol and 1 mg/ml CHAPS). After loading, the column was washed with 5 bed volumes of buffer A containing 0.1 M NaCl and PDE activity was eluted with a linear gradient in buffer A of NaCl (0.1 to 0.4 M, 15 bed volumes). Fractions (6 ml) corresponding to different PDE activities (against cGMP, cAMP and cAMP plus 3 μM rolipram) were pooled and stored at −20°C prior to further purification.

2.4.2. Affinity chromatography and removal of cAMP on Mono Q. The type IV isoenzyme (showing rolipram-sensitive hydrolysis of cAMP) was then applied to an affinity column of the rolipram analog 4-[3-(carboxypropyloxy)-4-methoxyphenyl]-2-pyrrolidone coupled to EAH-sepharose 4B [5]. After washing with 5 bed volumes of buffer B (buffer A + 0.5 M NaCl, without ethylene glycol), the enzyme was eluted with 3 bed volumes of buffer B containing 1 mM cAMP. After use, the column was regenerated with 6 M urea and stored in 20% ethanol. To remove the cAMP, the eluate from affinity chromatography was diluted 1:4 with buffer C (25 mM Bis-Tris, pH 6.8, 1 mM EDTA, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 5 μg/ml aprotinin, 2 mM benzamidine, and 1 mg/ml CHAPS) and loaded onto a 1 ml Mono Q HR5/5 column (Pharmacia) equilibrated with the same buffer. After washing with 10 bed volumes of buffer C containing 0.125 M NaCl, the PDE-IV activity was eluted with a linear gradient of NaCl (0.125–0.425 M NaCl, 15 bed volumes) in buffer C, collecting fractions (0.5 ml) into tubes containing 0.1 ml ethylene glycol. Fractions were stored at −20°C until use.

2.4.3. Gel filtration on Superose 12. Fractions showing rolipram-sensitive cAMP hydrolysis were pooled, concentrated to a volume of 0.5 ml on Centricon-3 membranes (Amicon) and applied to a 12 ml HR 10/30, Superose 12 column (Pharmacia) running at a flow rate of 0.2 ml/min with buffer A containing 0.1 M NaCl. Fractions (250 μl) were collected into tubes containing 50 μl ethylene glycol and stored at −20°C.

2.4.4. Numerical procedures. Standard enzyme kinetic models were fitted to experimental data using the method of non-linear least-squares regression analysis as supplied by the RS/1 software (Bolt Beranek and Newman Inc.).

2.4.5. Other procedures. SDS-gel electrophoresis was performed on 12.5% Phastgels (Phastsystem, Pharmacia). The separation and silver staining were carried out according to the manufacturer's instructions. Active fractions from the gel filtration were pooled, concentrated to 0.14 ml on Microcon-3 membranes (Amicon), and applied to a 2 × 8 mm piece of Problott membrane (Applied Biosystems). After air-drying, the membrane was directly loaded into an automatic sequencer model 477A (Applied Biosystems) and the N-terminal amino acid sequence determined according to Edman [6,7]. Protein concentration was determined as described by Bradford [8].

3. Results

3.1. Enzyme extraction and purification

PDE activity was released from human mononuclear cells by detergent lysis with minimal mechanical intervention. By comparison with sonication the recovery was approximately 2 times lower, however it appeared that purity was increased, especially in the later steps. More than 90% of the activity remained in the supernatant after centrifugation (120,000 × g, 1 h). Storage at −20°C in the presence of 25% (v/v) ethylene glycol prevented the loss of activity seen in experiments without ethylene glycol. The use of a buffer containing 0.1% (w/v) CHAPS [9] in all chromatographic steps resulted in improved yields.

3.1.1. Separation of PDE-isoenzymes on Q Sepharose. The first purification step separated PDE isoenzymes from the bulk of the protein using anion exchange chromatography. PDE isoenzymes were identified on the basis of substrate specificity and inhibition of cAMP hydrolysis by rolipram. The elution profile (Fig. 1) shows 3 peaks of PDE activity, eluting at 170 mM (peak 1, selective hydrolysis of cGMP, PDE-V), 260 mM (peak 2, selective hydrolysis cAMP inhibited by rolipram, PDE-IV), and 340 mM (peak 3), rolipram-insensitive cAMP-hydrolyzing activity. This peak eluted at the same position as cGMP-inhibited cAMP PDE from human platelets (PDE-III, data not shown). Peaks 2 and 3 accounted for 67% and 28% of the total recovered cAMP-PDE activity, respectively. The activity present in all peaks was independent of calcium-calmodulin. The fractions of peak 2 were pooled and stored at −20°C for further purification.

3.1.2. Affinity chromatography. Irrespective of the selectivity of rolipram seen in enzyme assays, the rolipram-based affinity matrix possesses significant binding capacity for PDE-III at 0.25 M NaCl. This material can be eliminated by elution with buffer containing 0.5 M NaCl. Selective elution of type IV PDE is then achieved with buffer containing 0.5 M NaCl and 1 mM cAMP. Excess unlabelled cAMP in the elution buffer prevents accurate determination of PDE activity and must be removed by anion exchange chromatography. In first experiments this step was carried out at pH 7.4, and contaminating bands with pI ≥ 7 were found at later steps. These could be eliminated by reducing the pH of the elution buffer to pH 6.8, resulting in a step yield of about 67% with a 11,000-fold increase of specific

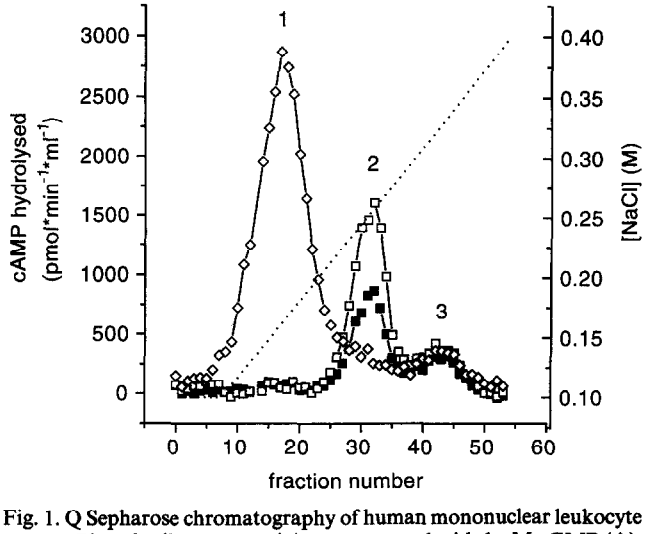

Fig. 1. Q Sepharose chromatography of human mononuclear leukocyte extract. Phosphodiesterase activity was assayed with 1 μM cGMP (◇), with 1 μM cAMP (□), and with 1 μM cAMP + 3 μM rolipram (■). NaCl-gradient (----).

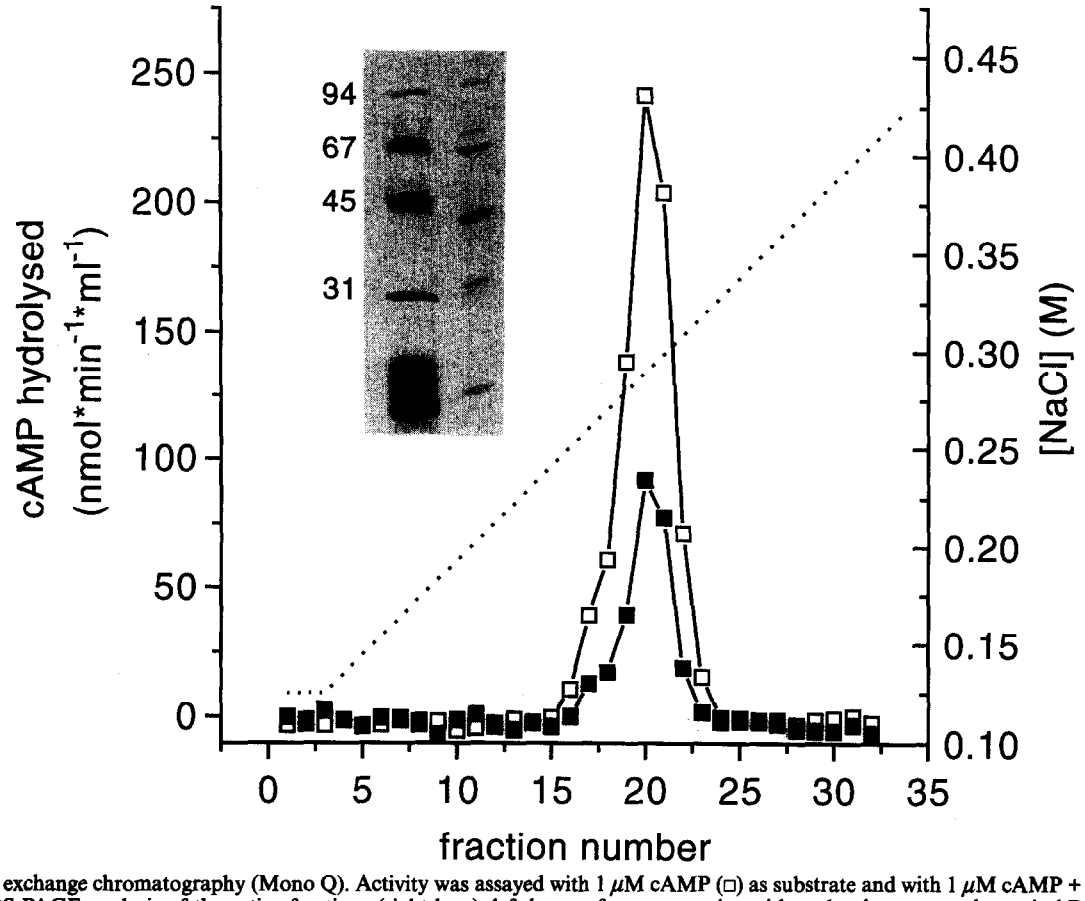

Fig. 2. Anion exchange chromatography (Mono Q). Activity was assayed with 1 μM cAMP (□) as substrate and with 1 μM cAMP + 3 μM rolipram (■). Inset: SDS-PAGE analysis of the active fractions (right lane), left lane: reference proteins with molecular masses shown in kDa.

activity. The overall recovery was 45% with a 130,000-fold purification. SDS-PAGE analysis (inset of Fig. 2) revealed 5 proteins with apparent molecular masses of 35, 67, 100, 31 and 18 kDa (in decreasing order of intensity).

3.1.3. Gel filtration on Superose 12. On a gel filtration column, enzyme activity migrated with an apparent molecular mass of about 70 kDa, approximately at the same position as BSA (Fig. 3). With this step, a further 3-fold purification was achieved with a yield of 95%. The overall recovery was 43% with a 450,000-fold purification. SDS-PAGE analysis of the active fractions (41–49) showed that the band at 35 kDa corresponded in intensity to the protein and activity peaks on gel filtration (Fig. 3, inset). There was no visible staining corresponding to a protein of 70 kDa. The active fractions were pooled and stored at −20°C in the presence of 20% (v/v) ethylene glycol for further analysis. Data from a typical preparation are summarized in Table 1.

3.2. Biochemical properties of the purified enzyme

Purified type IV PDE hydrolyzed cAMP, but not cGMP at concentrations up to 10 μM. Hydrolysis of cAMP (1 μM) was inhibited by rolipram and RO 20–1724 with IC_{50} values of 1.3 μM and 7.9 μM, respectively (data not shown). When assayed with substrate and rolipram concentrations ranging from 0.1 to 10 μM and 0 to 3 μM, respectively, the purified enzyme showed behavior compatible with Michaelis–Menten kinetics. Fitting the simple model of competitive inhibition (interaction of inhibitor with the free enzyme only, parameters v_{max} and K_i) yielded a K_m of 2.2 μM and a v_{max} of 0.37 μmol/min/ml at 37°C. By using an estimated protein concentration of 5 μg/ml, obtained by comparison with standard proteins on SDS-PAGE,

Table 1
Purification summary of cAMP specific rolipram-sensitive phosphodiesterase from $2.3 \cdot 10^{11}$ human mononuclear cells

Step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Purification (-fold)	Yield (%)
Supernatant 120,000 × g	5,300	990	0.19	1	100
Q Sepharose	300	660	2.20	12	67
Rolipram-Sepharose, Mono Q	0.017*	450	25,000	130,000	45
Superose 12	0.005*	430	86,000	450,000	43

*Protein concentrations were estimated from SDS-PAGE.

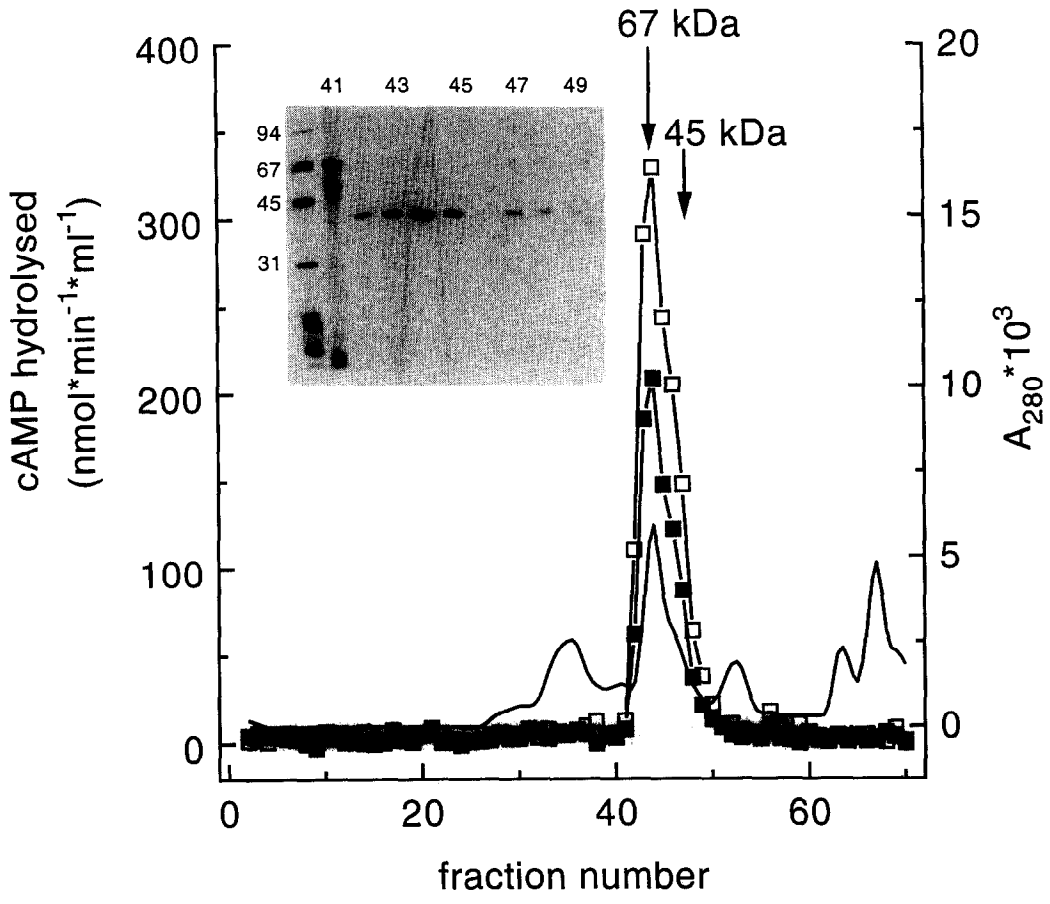

Fig. 3. Chromatography on Superose 12. Activity was assayed with 1 μM cAMP (□) as substrate and with 1 μM cAMP + 3 μM rolipram (■). A_{280} is shown as (—). Inset: arrows indicate the elution position of BSA (67 kDa) and ovalbumin (45 kDa), respectively. Fraction 46 was lost during the concentration step.

a specific v_{max} of 80 μmol/min/mg protein was calculated. The inhibitory constant for the competitive model was estimated as 1.2 μM (Table 2). Alternative models of inhibition were also evaluated: The model assuming interaction of the inhibitor with the enzyme–substrate complex only ('un-competitive', parameters v_{max} and K_j) fitted the data less closely, as evidenced by a considerably larger sum of squared residuals, and by visual examination of the fit (not shown). On the other hand, the model assuming interactions of the inhibitor with both free enzyme and enzyme–substrate complex ('non-competitive', parameters v_{max}, K_i and K_j) showed a slightly smaller sum of squared residuals than the competitive model. Within experimental error, the K_m, K_i and v_{max} were similar for the competitive and non-competitive models. For the non-competitive model, the additional parameter K_j was estimated at 17 μM with a large standard error of 10 μM. These results indicate that interaction of rolipram with the enzyme–substrate complex, if present, has little effect on the hydrolysis of cAMP by PDE-IV. This is also supported by the essentially straight lines of the Lineweaver–Burk plot shown in Fig. 4 and the Dixon replot (inset of Fig. 4). Parameters estimated from these plots were similar to those obtained by nonlinear fitting (v_{max} = 0.4 μmol·min^{-1}·ml^{-1}, K_m = 2.5 μM, K_i = 1.9 μM)

3.3. N-terminal sequence

Determination of the N-terminal amino acid sequence of the purified PDE-IV has been difficult to date due to the low abundance of this isoenzyme in most tissues and to its sensitivity to proteolysis. Another reason for this difficulty may be that many cell types contain several isoenzymes of the type IV family [10–12], in theory requiring selection of a tissue or cell containing predominantly one isoenzyme in order to obtain a homogeneous preparation of PDE-IV using standard purification methods.

Table 2
Model parameters for inhibition of cAMP hydrolysis by rolipram

	V_{max} (μmol·min^{-1}·ml^{-1})	K_m (μM)	K_i (μM)	K_j (μM)	Σ (residuals)^2 ($\times 10^{-3}$)
Competitive	0.37 (0.01)	2.2 (0.2)	1.2 (0.1)		5.4
Un-competitive	0.46 (0.03)	4.4 (0.5)		3.0 (0.4)	12
Non-competitive	0.39 (0.01)	2.5 (0.2)	1.6 (0.3)	16.9 (10.1)	5.1

Standard errors are in parentheses.

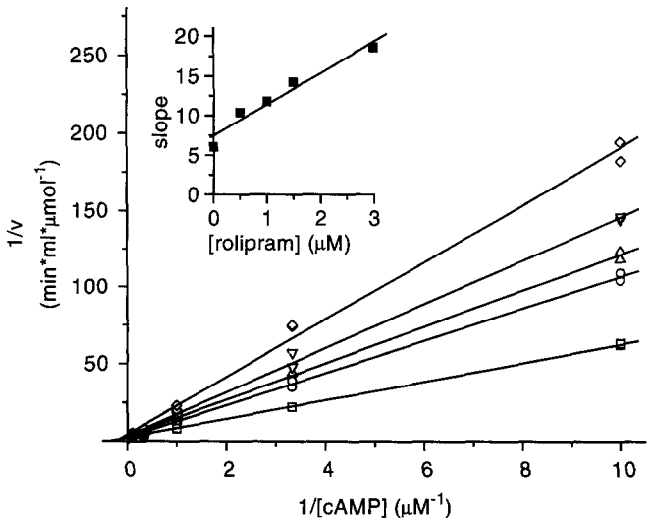

Fig. 4. Lineweaver–Burk analysis of cAMP hydrolysis by pooled active fractions from Mono Q chromatography. Rolipram concentrations were 0 (□), 0.5 (○), 1 (△), 1.5 (▽), and 3 µM (◇). In the inset, slopes of the lines from the Lineweaver–Burk plot are plotted against inhibitor concentration (Dixon replot).

Comparison of the N-terminal sequence of mononuclear cell PDE-IV with sequences deduced from human recombinant cDNA of isoenzymes IV_A to IV_D shows identical amino acids at 6 to 8 out of 10 corresponding positions (Fig. 5). As PDE-IV_C is least similar to the N-terminal peptide found, this form is probably not present in mononuclear cAMP PDE. This conclusion is supported by the absence of PDE-IV_C mRNA in human white blood cells as analyzed by reverse-transcriptase polymerase chain reaction [13].

It is theoretically possible that the N-terminal peptide sequence is derived from a mixture of PDE-IV_A, IV_B and IV_D. This however is not consistent with the data presented. Thus, based on the presence of Thr at position 3 of PDE-IV_D, this isoenzyme should contribute more to the peptide sequence than the other forms. Similar reasoning can be applied to positions 5 (PDE-IV_B) and 6 (PDE-IV_A).

4. Discussion

By combining anion exchange chromatography and gel filtration with affinity chromatography on rolipram–Sepharose, the low K_m cAMP specific, rolipram-sensitive PDE (PDE-IV, competitive K_i for rolipram 1.2 µM) was purified ~ 450,000-fold from human blood cells in 43% overall yield. The molecular mass of mononuclear cell cAMP specific PDE, as determined by gel filtration, was about 70 kDa, while SDS-PAGE analysis showed a molecular mass of about 35 kDa, similar to non-denaturing PAGE analysis (data not shown). This may indicate that two molecules of 35 kDa either dimerize under certain conditions, or are cleaved while retaining enzymatic activity. Similarly, Thompson et al. [14] have observed a decrease of apparent molecular mass after storage (24 h at 4°C) or freezing and thawing.

Hydrolysis of cAMP and its inhibition by rolipram obey the simple Michaelis–Menten model. The K_m value for cAMP hydrolysis estimated from our data (2.2 µM) is close to published values for the high-affinity component of lymphocyte cAMP hydrolysis [15,16]. For PDE-IV expressed from cDNA clones, the values are also similar [17,18] or slightly higher, in the range of 7 to 10 µM [11]. Maximum specific activity values (specific v_{max}) cited in the literature are in the range of 0.5 nmol/min/mg protein for lymphocyte PDE [15] and in the 50 to 300 nmol/min/mg range for cloned PDE-IV expressed in yeast [11] and COS-cells [17], respectively. Thus, even allowing for the uncertainty associated with estimation of such low quantities of protein, our value of 80 µmol/min/mg is considerably higher than the reference values. This large specific activity may reflect a combination of the high purification factor and low activity loss obtained with the present purification procedure (Table 1).

Sequence comparison between the mononuclear cAMP specific PDE and the four human recombinant cAMP specific phosphodiesterases (IV_A [11,17], IV_B [11,19,20], IV_C [13] and IV_D [11,12]) shows at least two amino acids changes (PDE_A and PDE_D, Fig. 5). The possibility that alternative splicing might lead to such differences in sequence seems unlikely, as the exon boundaries are identical for rat PDE IV_B and IV_D [21], and none of these exon boundaries is found within the corresponding N-terminal decapeptide of mononuclear PDE IV. Although polymorphism may account for some inter-individual variation in cDNA sequences, our N-terminal sequence is representative of a large population of donors. It is unlikely that the N-terminal sequence shown in Fig. 5 is simply derived from a (nearly equimolar) mixture of several peptides, since the data presented are not consistent with such an assumption. Based on our data, we cannot exclude that the final preparation contains small amounts of other PDE-IV isoenzymes. However, it appears that we have obtained the N-terminal sequence of a form of PDE-IV which, on average, is the most abundant species in mixed mononuclear leukocytes from large numbers of individuals. The relative contributions of the different cell types (mainly B and T lymphocytes and monocytes) to the amounts of PDE-IV isoenzymes remains the subject of further investigations, as does the extent of polymorphism found in the population.

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                                  1              5                 10
MNL PDE-IV                        Ser-Leu-Thr-Asn-Thr-Asn-Ile-Pro-Arg-Phe-
PDE-IVA (human) [17]               . - . -Asn- . -Ser- . - . - . - . - . -
PDE-IVB (human) [19,20]            . - . -Asn- . - . -Ser- . -Ser- . - . -
PDE-IVc (human) [13]               . - . -Pro-Thr-Ala-Ala- . - . - . - . -
PDE-IVD (human) [12]               . - . - . - . -Ser-Ser- . - . - . - . -
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Fig. 5. Homology of the N-terminal amino acid sequence of human mononuclear PDE-IV with partial deduced amino acid sequences of the four groups of human cDNA clones.

In summary, the data presented in this paper suggest that human mononuclear cells contain a rolipram-sensitive (type IV) PDE similar but not identical to PDE-IV_A, PDE-IV_D or PDE-IV_B.

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References

[1] Beavo, J.A. (1988) Adv. Second Messenger Phosphoprotein Res. 22, 1–38.
[2] Conti, M., Jin, S.-L.C., Monaco, L., Repaske, D.R. and Swinnen, J.V. (1991) Endocr. Rev. 12, 218–234.
[3] Torphy, T.J. and Undem, B.J. (1991) Thorax 46, 512–523.
[4] Thompson, W.J., Terasaki, W.J., Epstein, P.M. and Strada, S.J. (1979) Adv. Second Messenger Phosphoprotein Res. 10, 69–92.
[5] Fougier, S., Némoz, G., Prigent, A.F., Marivet, M., Bourguignon, J.-J., Wermuth, C. and Pacheo, H. (1986) Biochem. Biophys. Res. Commun. 138, 205–214.
[6] Brandt, W.F. and Frank, G. (1988) Anal. Biochem. 168, 314–323.
[7] Edman, P. and Begg, G. (1967) Eur. J. Biochem. 1, 80–91.
[8] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[9] Matuo, Y., Nishi, N., Matsumoto, K., Miyazaki, K., Susuki, F. and Nishikawa, K. (1991) Methods Enzymol. 198, 511–518.
[10] Shelton, E.R., Obernolte, R., Bhakta, S., Alvarez, R., Kalfayan, V. and Jamagin, K. (1992) J. Cell. Biochem. 16B, 231.
[11] Bolger, G., Michaeli, T., Martins, T., St. John, T., Steiner, B., Rodgers, L., Riggs, M., Wigler, M. and Ferguson, K. (1993) Mol. Cell. Biol. 13, 6558–6571.
[12] Baecker, P.A., Obernolte, R., Bach, C., Yee, C. and Shelton, E.R. (1994) Gene 138, 253–256.
[13] Engels, P., Fichtel K. and Lübbert, H. (1994) FEBS Lett., in Press.
[14] Thompson, W.J., Ross, C.P., Pledger, W.J., Strada, S.J., Banner, R.L. and Hersch, E.M. (1976) J. Biol. Chem. 2512, 4922–4929.
[15] Wedner, H.J., Chan, B.Y., Parker, C.S. and Parker, C.W. (1979) J. Immunol. 123, 725–732.
[16] Robicsek, S.A., Blanchard, D.K., Djeu, J.Y., Krzanowski, J.J., Szentivanyi, A. and Polson, J.B. (1991) Biochem. Pharmacol. 42, 867–877.
[17] Livi, G.P., Kmetz, P., McHale, M.M., Cieslinski, L.B., Sathe, G.M., Taylor, D.P., Davis, R.L., Torphy, T.J. and Balcarek, J.M. (1990) Mol. Cell. Biol. 10, 2678–2686.
[18] Torphy, T.J., Stadel, J.M., Burman, M., Cieslinski, L.B., McLaughlin, M.M., White, J.R. and Livi, G.P. (1992) J. Biol. Chem. 267, 1798–1804.
[19] McLaughlin, M.M., Cieslinski, L.B., Burmanm, M., Torphy, T.J. and Livi, G.P. (1993) J. Biol. Chem. 268, 6470–6476.
[20] Obernolte, R., Bhakta, S., Alvarez, R., Bach, C., Zuppan, P., Mulkins, M., Jarnagin, K. and Shelton, E.R. (1993) Gene 129, 239–247.
[21] Monaco, L., Vicini, E. and Conti, M. (1994) J. Biol. Chem. 269, 347–357.