Phosphodiesterase 4 in macrophages: relationship between cAMP accumulation, suppression of cAMP hydrolysis and inhibition of [^{3}H]*R*-(−)-rolipram binding by selective inhibitors

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A perplexing phenomenon identified in several tissues is the lack of correlation between inhibition of phosphodiesterase 4 (PDE4) and certain functional responses such as smooth muscle relaxation, gastric acid secretion and cAMP accumulation. Interpretation of these data is complicated further by the finding that function correlates with the ability of PDE4 inhibitors to displace [^{3}H]rolipram [4-(3-cyclopentenyloxy-4-methoxyphenyl)-2-pyrrolidone] from a high-affinity site in rat brain that is apparently distinct from the catalytic centre of the enzyme. We have investigated this discrepancy by using guinea pig macrophages as a source of PDE4 and have confirmed that the ability of a limited range of structurally dissimilar PDE inhibitors (Org 20241, nitraquazone and the enantiomers of rolipram and benafentrine) to increase cAMP content did not correlate with their potency as inhibitors of partly purified PDE4, whereas a significant linear and rank order correlation was found when cAMP accumulation was related to the displacement of [^{3}H]*R*-(−)-rolipram from a specific site identified in macrophage lysates. An explanation for these data emerged from the finding that the IC_{50} values and rank order of potency of these compounds for inhibition of partly purified PDE4 and the native (membrane-bound) form of the same enzyme were distinct. Similarly, no correlation was found when membrane-bound PDE4 was compared with the same enzyme that had been solubilized with Triton X-100. These unexpected results were attributable to a selective decrease in the potency of those inhibitors [nitraquazone, *R*-(−)- and *S*-(+)-rolipram] that interacted preferentially with the rolipram binding site. Indeed, if membrane-bound PDE4 was used as the enzyme preparation, excellent linear and rank order correlations between inhibition of cAMP hydrolysis, displacement of [^{3}H]*R*-(−)-rolipram and cAMP accumulation were found, which improved further in the presence of the vanadyl (Vo)/2.GSH complex. Moreover, using Vo/2.GSH-treated membranes, the IC_{50} values of nitraquazone and the enantiomers of rolipram for the inhibition of PDE4 approached their affinity for the rolipram binding site. Collectively, these data suggest that the rolipram binding site and the catalytic domain on CPPDE4 might represent part of the same entity. In addition, these results support the concept that PDE4 can exist in different conformational states [Barnett, Manning, Cieslinski, Burman, Christensen and Torphy (1995) J. Pharmcol. Exp. Ther. **273**, 674–679] and provide evidence that the cAMP content in macrophages is regulated primarily by a conformer of PDE4 for which rolipram has nanomolar affinity.

INTRODUCTION

It is now recognized that the major mechanism by which cyclic purines (cAMP, cGMP) are inactivated is by enzymic hydrolysis catalysed by a large group of proteins generically known as cyclic nucleotide phosphodiesterases (PDEs; EC 3.1.4.17; EC 3.1.4.35). Since the discovery of these enzymes, evidence has emerged for at least seven PDE families that currently comprise more than 34 similar, but structurally and immunologically distinct, isoenzymes [1,2]. These proteins are in many cases encoded by different genes or, alternatively, represent spliced variants or different transcription products of the same gene [1–4]. Moreover, PDEs exhibit a unique tissue and subcellular distribution, implying that they serve specific and diverse functional roles [5,6]. Although several attempts at classifying PDEs have been made, a new nomenclature was recently introduced [1]. This taxonomy is novel as it is based not only on traditional biochemical criteria (e.g. substrate specificity, sensitivity to allosteric modulators and inhibitors, kinetic and physical characteristics) but also on protein sequencing and analyses of partial and full-length cDNA clones [1,2].

Over the last decade, compounds that display a marked degree of selectivity for one PDE isoenzyme over another have become available and have fuelled the hypothesis that PDE inhibitors could have considerable therapeutic potential in a number of inflammatory diseases including arthritis, atopic dermatitis and allergic asthma [5–9]. Furthermore isoenzyme-selective PDE inhibitors might be associated with a reduced side-effect profile compared with non-selective drugs (e.g. theophylline), which indiscriminately inhibit all PDE isoenzymes [8].

One clinical condition where the development of selective PDE inhibitors is being actively pursued is allergic asthma [6–9]. In particular, inhibitors of PDE4 are receiving considerable interest [6–9]. In spite of this the PDE4 isoenzyme family is poorly

Abbreviations used: PDE, cyclic nucleotide phosphodiesterase; Vo, vanadyl ion; FCS, fetal calf serum; DTT, dithiothreitol; STI, soybean trypsin inhibitor; Org 20241, *N*-hydroxy-4-(3,4-dimethoxyphenyl)-thiazole-2-carboximidamide; nitraquazone, 3-ethyl-1-(3-nitrophenyl)-2,4[1H,3H]-guanazolindione; rolipram, 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone; benafentrine, (6-(*p*-acetamidophenyl)-1,2,3,4,4*a*,10*b*-hexahydro-8,9-dimethoxy-2-methyl-benzo[*c*][1,6]naphthyridine; HBSS, Hanks balanced salt solution.

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understood and exhibits several anomalous characteristics that are not readily explicable. Perhaps the most intriguing of these is the presence of a high-affinity binding site for the anti-depressant PDE inhibitor, rolipram [4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone], that is apparently distinct from the catalytic domain [10]. Evidence that these sites are discrete entities is based on two main observations. First, the rank order of potency of a range of structurally dissimilar PDE inhibitors for the suppression of cAMP hydrolysis and for the displacement of [^{3}H]rolipram is distinct [11]. Secondly, some PDE inhibitors (e.g. rolipram, Ro 20-1724, denbufylline) exhibit considerably higher affinity for the binding site than for the catalytic domain, whereas for others (e.g. dipyridamole, trequinsin) the reverse is true [11–14]. Typically, *R*-(−)-rolipram preferentially binds to its site with very high affinity ($K_d \approx 1$ nM) but is one-hundredth to one-thousandth as potent ($K_i \approx 1\ \mu$M) at inhibiting cAMP hydrolysis [10]. A number of explanations have been advanced for these marked discrepancies, including the possibility that the high-affinity rolipram binding site and the catalytic domain do not reside on the same protein and, in fact, serve different and unrelated biological functions [15,16]. Circumstantial evidence to support this contention is that whereas [^{3}H]rolipram labels a high density of specific binding sites in various brain regions, little, if any, binding is detected in peripheral tissues (including pro-inflammatory and immune cells implicated in the pathogenesis of asthma) despite high levels of PDE4 activity [10,17,18]. A recent investigation [11], however, with genetically engineered PDE4, has confirmed that high-affinity [^{3}H]rolipram binding and catalytic activity are indeed co-expressed by the same gene product, thus prompting speculation about the functional significance of this ill-defined site and why (apparently) it is selectively expressed in brain. An obvious possibility for which conflicting evidence is available is that the high-affinity binding domain allosterically regulates catalytic activity. Although data consistent with this proposal are available [12–14], this is not universally accepted. Indeed, there are at least four salient observations that question the simplicity of this paradigm [17]: (i) low concentrations of rolipram that do not influence catalytic activity but saturate the binding site do not affect the inhibitory potency of other PDE inhibitors, (ii) the stoichiometry of [^{3}H]rolipram binding to purified monomeric PDE4 is substantially less than 1, (iii) the hydrolysis of cAMP conforms to simple Michaelis–Menten kinetic behaviour, and (iv) the pseudo-Hill coefficient for the binding of rolipram to the catalytic and high-affinity domains is not significantly different from unity.

Another perplexing observation concerns the apparent lack of correlation between inhibition of PDE4 and certain functional responses in intact cells including smooth muscle relaxation, gastric acid secretion and cAMP accumulation [12,13,18,19]. Interpretation of these data is complicated further by the finding that a strong linear and rank order correlation exists between these (and other) functional responses and the ability of PDE inhibitors to displace [^{3}H]rolipram from a high-affinity site in rat brain [12–14,18–20]. Collectively, therefore, these data imply that these functional responses are the result of the interaction of PDE4 inhibitors with the high-affinity binding site rather than the catalytic domain of the enzyme.

In view of the controversy that surrounds the high-afinity rolipram binding site and the discrepancies between PDE4 inhibition and function reported in the literature, experiments were performed to resolve whether or not [^{3}H]rolipram specifically labels high-affinity sites in a peripheral tissue and, if so, to determine structure–activity relationships of six distinct PDE inhibitors (Org 20241 [21], nitraquazone [22] and the enantiomers of benafentrine [23] and rolipram [24]) for suppressing PDE4 activity, increasing the cAMP content and displacing [^{3}H]rolipram. In view of the potential therapeutic role of PDE4 inhibitors as anti-inflammatory agents [5–9,14], we performed these studies in macrophages, an antigen-presenting cell type that is central to the induction and perpetuation of inflammatory responses.

EXPERIMENTAL

Drugs and analytical reagents

The following drugs and analytical reagents were used: Percoll and Q-Sepharose were purchased from LKB/Pharmacia (Milton Keynes, Bucks., U.K.). [2,8-^{3}H]cAMP (36 Ci/mmol), [8-^{3}H]cGMP (24 Ci/mmol), [8-^{14}C]adenosine (55 Ci/mmol) and adenosine 3′,5′-cyclic monophospho-2′-*O*-succinyl-3-[^{125}I]iodotyrosyl methyl ester (approx. 2000 Ci/mmol) were supplied by Amersham International (Little Chalfont, Bucks., U.K.), and [^{3}H]*R*-(−)-rolipram (76 Ci/mmol) was from Byk Gulden (Konstanz, Germany). Fetal calf serum (FCS) was obtained from Gibco (Rickmansworth, Herts., U.K.). Org 20241 was donated by Organon Laboratories (Newhouse, Lanarks., Scotland, U.K.) and nitraquazone was from Bayer (Stoke Poges, Berks., U.K.). *R*-(−)-, *S*-(+)- and racemic rolipram, and 4*R*,10*S*-(−)-, 4*S*,10*R*-(+)- and racemic benafentrine were gifts from Schering (Berlin, Germany) and Sandoz (Basel, Switzerland) respectively. Alkaline phosphatase (P-2277), Dowex AG (1 × 8-400 chloride form), calmodulin, isoprenaline and all other reagents were from Sigma (Poole, Dorset, U.K.).

Drugs were made up as stock solutions of 100 mM in ethanol (rolipram), DMSO (Org 20241, nitraquazone), 0.5 M HCl (benafentrine) or distilled water (isoprenaline) and diluted in the appropriate assay buffer.

Induction, harvesting and purification of macrophages

Macrophages were elicited into the peritoneum of male Dunkin–Hartley guinea pigs (600–1200 g) by weekly intraperitoneal injection of human serum (1 ml per animal), obtained as a by-product of human granulocyte isolations. This procedure led to the production of eosinophil/macrophage-rich peritoneal exudates, substantially or entirely devoid of neutrophils and platelets, within 2–6 weeks; 3–6 days after plasma injection guinea pigs were anaesthetized with ketamine (25 mg/kg) and xylazine (5 mg/kg) and the peritoneal cavity of each animal was lavaged with 50 ml of sterile glucose (5% w/v) injected via a 17G cannula. The lavage fluid was aspirated into conical polypropylene centrifugation tubes and centrifuged at 240 ***g*** for 10 min at 4 °C to pellet the cells. These were then washed in Hanks balanced salt solution (HBSS), pooled and finally resuspended in buffer A (in mM: Pipes 25, pH 7.4, NaCl 110, KCl 5, glucose 5.4) containing Percoll (1.070 g/ml) supplemented with 20% (v/v) FCS.

Macrophages were separated from other cell types by centrifugation of the pooled cell preparation at 1600 ***g*** for 20 min at 18 °C over discontinuous Percoll density gradients (1.070, 1.075, 1.080 and 1.085 g/ml in buffer A) by the method of Gartner [25]. By using this procedure macrophages (more than 95% pure) were recovered from the 1.070/1.075 g/ml Percoll interface. After washing twice in HBSS macrophages were used immediately for cAMP measurements or lysed for PDE and rolipram binding studies (see below).

Preparation of membrane-bound and solubilized fractions

Macrophages in HBSS were centrifuged at 240 ***g*** for 10 min (at 4 °C) and the resulting cell pellet was resuspended at 15×10^6/ml

in buffer B [in mM: Mops 10, pH 7.4, EGTA 1, magnesium acetate 2, dithiothreitol (DTT) 5] in the absence or presence of 1% (v/v) Triton X-100 and supplemented with the proteinase inhibitors PMSF (100 μM), leupeptin (100 μM), bacitracin (100 μg/ml), benzamidine (2 mM) and soybean trypsin inhibitor (STI) (20 μg/ml). After 60 min of incubation at 4 °C the resulting cell lysate was centrifuged at 45000 ***g*** for 30 min at 4 °C. In the absence of Triton X-100, more than 90% of the cAMP hydrolytic activity was retained by the particulate material and was considered membrane-bound. Accordingly the pellet was resuspended in buffer B at the same density (15×10^6 cell equivalents per ml) before PDE activity was measured. In contrast, Triton X-100 treatment of macrophages effectively solubilized more than 96% of the membrane-bound activity and the supernatant was therefore used as the enzyme source.

Resolution of cAMP PDE isoenzymes by Q-Sepharose anion-exchange chromatography

Cells [$(150–400) \times 10^6$] were resuspended at 20×10^6/ml in buffer C (in mM: Bistris 20, pH 6.8, sodium acetate 50, DTT 2, EDTA 1, glucose 5, KCl 400) made 2% (v/v) with Triton X-100 supplemented with proteinase inhibitors leupeptin (100 μM), PMSF (100 μM), STI (20 μg/ml), bacitracin (100 μg/ml) and benzamidine (2 mM). The cells were sonicated (MSE Soniprep 50 with probe attachment) at 20% power for 10 s per 10^8 cells and centrifuged at 45000 ***g*** for 30 min at 4 °C. The supernatant was diluted in 5 mM Bistris [pH 6.8, made 0.2% (v/v) with Triton X-100], to lower the conductivity to less than 8 mS (at 4 °C), and applied at a flow rate of 500 μl/min to a column (Bio-Rad; 1.5 cm internal diam., approx. 10 cm bed volume) of Q-Sepharose pre-equilibrated with buffer D (in mM: Bistris 20, pH 6.8, sodium acetate 50, DTT 2, EDTA 1, glucose 5) made 0.2% (v/v) with Triton X-100. The column was subsequently washed with approx. five bed volumes of Triton X-100-containing buffer D and bound proteins were eluted with a linearly increasing salt gradient running from 50 mM to 1.2 M sodium acetate. The flow rate was adjusted to 250 μl/min and 45–55 fractions were collected (1 ml per 150×10^6 cell equivalents). Each fraction was then assayed for cAMP hydrolytic activity as described below.

Measurement of cAMP PDE activity

cAMP PDE activity was measured with a modification [26] of the method of Thompson and Appleman [27] as described in Dent et al. [28]. The reaction is based on the breakdown of [^{3}H]cAMP by PDE to the corresponding labelled nucleoside 5′-monophosphate, which is subsequently dephosphorylated by alkaline phosphatase. Assays were performed in duplicate at 37 °C and initiated by the addition of 30 μl of enzyme (membrane-bound, solubilized or partly purified) to 270 μl of a reaction cocktail containing (final concentrations) 20 mM triethanolamine, pH 8.0, 5 mM DTT, 500 μg/ml BSA, 5 mM magnesium acetate, 0.25 unit of alkaline phosphatase, 1 mM EGTA, 1 μM cAMP (supplemented with approx. 250000 d.p.m. of [8-^{3}H]cAMP and approx. 5000 d.p.m. of [8-^{14}C]adenosine to estimate recovery) and the PDE inhibitor under evaluation or its vehicle. The reaction was terminated after 30 min by the addition of 1 ml of a mixture of Dowex AG 1 × 8/methanol/water (1:2:1, w/v/v), vortex-mixed and placed in an ice bath until the end of the assay. Samples were then further vortex-mixed for 30 min before being centrifuged at 12000 ***g*** for 5 min at 4 °C. The radioactivity in 750 μl aliquots of the resulting supernatants was determined by liquid-scintillation counting in 2 ml of ACS II scintillant (Amersham) at a counting efficiency of approx. 60%. PDE activity is expressed as the formation of nucleoside 5′-monophosphate from cAMP per min per 10^6 cell equivalents (i.e. the lysate derived from 10^6 cells) at 37 °C after correction for the recovery (approx. 65–85%) of [8-^{14}C]adenosine.

Classification of cyclic nucleotide PDEs

Cyclic nucleotide PDEs are classified according to the nomenclature outlined in Beavo et al. [1]. Thus CPPDE 1 to 7 refer to the Ca^{2+}/calmodulin-dependent, cGMP-stimulated, cGMP-inhibited, cAMP-specific, cGMP-specific, photoreceptor- and rolipram-insensitive, cAMP-specific PDE families respectively, where the prefix CP refers to the species *Cavia porcellus*.

Determination of kinetic constants

cAMP hydrolysis was determined by varying the amount of unlabelled cAMP in the reaction cocktail in the presence of a fixed concentration of radiolabelled cyclic nucleotide tracer, making appropriate corrections for the changes in specific activity of the substrate. K_m and V_{max} were subsequently derived from double-reciprocal plots of these data by the method of least squares. K_i values were then determined by the method of Dixon, with substrate and inhibitor concentrations that spanned the K_m and estimated K_i.

Measurement of amount of cAMP

Freshly prepared macrophages were resuspended at approx. 15×10^6/ml in buffer E (in mM: Hepes 10, pH 7.4, NaCl 124, KCl 4, NaH_2PO_4 0.64, K_2HPO_4 0.66, $NaHCO_3$ 5.2, $CaCl_2$ 1.6, glucose 5.6, $MgCl_2$ 1) and stored on ice until required. Assays performed in duplicate were conducted at 37 °C in a shaking water bath in a total volume of 300 μl and were initiated by the addition of 30 μl of macrophage suspension (approx. 5×10^5 cells) to 240 μl of prewarmed buffer E. To examine the effect of PDE inhibitors on the macrophage cAMP content, compounds (30 μl) were routinely added to the cell suspensions at the concentrations indicated in the text and relevant Figures for 10 min and then for a further 5 min in the presence of the β-adrenoceptor agonist isoprenaline (50 μM). Reactions were terminated by the addition of 300 μl of ice-cold trichloroacetic acid (1 M), centrifugation to precipitate particulate material and neutralization of the supernatants by the method of Downes et al. [29]. Aliquots (500 μl) of the neutalized extracts were acetylated, by the consecutive addition of triethylamine (20 μl) and acetic anhydride (10 μl), and cAMP mass was measured immediately by radioimmunoassay. Briefly, to 200 μl of acetylated sample was added 50 μl of adenosine 3′,5′-monophospho-2-*O*-succinyl-3-[^{125}I]iodotyrosine methyl ester (approx. 2000–3000 d.p.m.) in 0.2% BSA and 100 μl of anti-cAMP antibody in 0.2% BSA. After vortex mixing, samples were incubated overnight at 4 °C and free and antibody-bound cAMP was separated by charcoal precipitation with ice-cold potassium phosphate buffer (100 mM in 0.2% BSA, pH 7.4) and quantified by γ-counting. The detection limit and sensitivity (IC_{50}) of this assay are 10 and 145 fmol of cAMP respectively.

Preparation of the vanadyl/reduced glutathione (Vo/2.GSH) complex

The Vo/2.GSH complex was prepared as described previously [30,31]. Na_3VO_4 and GSH were dissolved in 50 mM Tris/HCl, pH 7.0, immediately before use and mixed 1:1 to give a final concentration of 84 and 168 mM respectively. For use, 10 μl of

the complex was added simultaneously with 30 μl of enzyme preparation to 260 μl of the PDE assay cocktail.

Rolipram binding assay

Macrophages (approx. 80×10^6/ml) were resuspended in ice-cold buffer F (in mM: Tris/HCl 10, pH 7.5, $MgCl_2$ 5, DTT 1) supplemented with the proteinase inhibitors PMSF (100 μM), leupeptin (100 μM) and STI (20 μg/ml), mixed end-over-end for 30 min at 4 °C and sonicated (intensity 6 for 5 s/ml). [^{3}H]*R*-(−)-Rolipram binding was subsequently performed in triplicate with a modification of the method of Schneider et al. [10]. Macrophage lysates, or soluble or particulate material (approx. 10^7 cell equivalents in 100 μl), were added to 400 μl of buffer F containing [^{3}H]*R*-(−)-rolipram (0.025–10 nM) and incubated for 60 min at 30 °C. In some experiments the ability of a number of PDE inhibitors to compete with [^{3}H]*R*-(−)-rolipram [2.6 nM; $(8–9) \times 10^5$ d.p.m.] for macrophage subcellular fractions was evaluated. Reactions were terminated by the addition of 2.5 ml of ice-cold buffer E followed by rapid vacuum filtration over Whatman GF/B filters that had been presoaked for at least 120 min in buffer F containing 0.3 % (v/v) polyethyleneimine. Filters were washed again four times with 5 ml of buffer F and the radioactivity retained by each filter was subsequently measured by liquid-scintillation counting in 4 ml of Filtron X (National Diagnostics, Hemel Hempstead, Herts., U.K.) at a counting efficiency of approx. 60 %. Non-specific binding was defined in the presence of 10 μM unlabelled *RS*-rolipram. Preliminary studies revealed that the specific binding of [^{3}H]*R*-(−)-rolipram to macrophage lysates was proportional to cell number (up to 10^7 cell equivalents). Saturation isotherms were analysed on the assumption that the data conformed to either a single or a double hyperbola, and goodness of fit was evaluated by measuring the residual sum of the squares (an index of the variability between the fitted and experimentally derived data). Values for K_d and B_{max} were subsequently determined by Scatchard transformation of the data. Results derived from competition studies were analysed for one or two sites and the goodness of fit was assessed as described above. All IC_{50} values were converted to K_i values with the Cheng and Prusoff [32] correction, to facilitate comparison with values for other ligands reported in the literature.

Statistical analysis

Values in the text and Tables, and data points, represent the means ± S.E.M. for n independent determinations taken from different cell preparations. Concentration–response relationships were analysed by least-squares non-linear iterative regression and pIC_{50} and pEC_{50} values were subsequently interpolated using the PRISM curve-fitting program (GraphPad Software, San Diego, CA, U.S.A.). Where appropriate, Student's t test (two-tailed) was used to assess statistical significance. To establish whether an association existed between cAMP accumulation, inhibition of CPPDE4 and displacement of [^{3}H]*R*-(−)-rolipram, we determined whether there was a significant linear (r^2) or rank order correlation (Spearman's ρ) between these parameters by using the Number Cruncher Statistical System program (Kaysville, UT, U.S.A.). The null hypothesis was rejected when $P < 0.05$.

RESULTS

Effect of PDE inhibitors on membrane-bound CPPDE4

Figure 1 shows the inhibitory effects of the PDE4 inhibitors, *R*-(−)-rolipram, *S*-(+)-rolipram and nitraquazone, and the mixed PDE3/4 inhibitors, Org 20241, 4*R*,10*S*-(−)-benafentrine and 4*S*,10*R*-(+)-benafentrine, on membrane-bound CPPDE4 activity. Although all drugs inhibited cAMP hydrolysis in a concentration-dependent manner (Figure 1 and Table 1), two distinct patterns of activity were observed. Thus the inhibition of CPPDE4 by *R*-(−)-rolipram, *S*-(+)-rolipram and nitraquazone was incomplete (approx. 75 %) at the highest concentration (100 μM) of inhibitor examined, and was described by shallow concentration–response curves (that spanned more than 4 log intervals) with pseudo-Hill coefficients significantly less than unity (Figures 1a, 1b and 1c). In contrast, Org 20241 and the enantiomers of benafentrine essentially abolished CPPDE4 activity under identical experimental conditions, and had relatively steep concentration–response curves (that spanned approx. 2 log intervals) with slope factors not significantly different from unity (Figures 1d, 1e and 1f). Significantly, SK&F 95654 did not inhibit membrane-bound PDE at concentrations up to 30 μM, indicating that the rolipram- and nitraquazone-insensitive activity was not due to PDE3 (results not shown).

An additional anomaly was that the enantiomers of rolipram did not behave stereoselectively in their ability to suppress membrane-bound CPPDE4 under conditions where 4*R*,10*S*-(−)-benafentrine was 22-fold more potent than the 4*S*,10*R*-(+) enantiomer (Table 1).

Partial purification of PDE4 by anion-exchange chromatography

The profile of cAMP PDE activity after anion-exchange chromatography of the total protein extracted from guinea-pig macrophage lysates over Q-Sepharose is shown in Figure 2. In the presence of EGTA two major peaks of rolipram-sensitive cAMP PDE activity were resolved that were eluted at 690 and 880 mM sodium acetate; for the purposes of this study they were arbitrarily designated CPPDE4α and CPPDEβ on the basis of the order in which they were eluted from the column (Figure 2a). No PDE3 activity was detected, as evidenced from the inability of SK&F 95654 (10 μM) and cGMP (10 μM) to affect cAMP hydrolysis (Figure 2b). The properties of CPPDE4α and CPPDE4β will be described elsewhere (J. J. Kelly and M. A. Giembycz, unpublished work).

Of the six inhibitors studied, none distinguished CPPDE4α from CPPDE4β (Table 1) in respect of their rank order of potency ($\rho = 1$) and absolute potency (Tables 1 and 2, and Figure 3a). However, a highly reproducible observation was that the sensitivity of CPPDE4α and CPPDE4β to nitraquazone and, to a smaller extent, the enantiomers of rolipram was reduced 6–30-fold compared with the membrane-bound enzyme (Table 1 and Figures 1a, 1b and 1c). Furthermore, the slopes of the concentration–response curves that described the inhibition of CPPDE4α and CPPDE4β were now comparatively steep, with pseudo-Hill coefficients that approached unity (Figures 1a, 1b and 1c). In contrast, Org 20241 and the enantiomers of benafentrine were equipotent inhibitors of membrane-bound CPPDE4, CPPDE4α and CPPDE4β (Table 1 and Figures 1d, 1e and 1f). The selective decrease in the potency of rolipram and nitraquazone produced by partial purification was associated with a marked change in the rank order of potency of the PDE inhibitors studied (Table 1) such that no relationship existed between inhibition of membrane-bound CPPDE4 and the partly purified forms of the enzyme ($\rho = 0.657$ for both CPPDE4α and CPPDE4β; $P > 0.05$; compare Figures 3a and 3c).

In agreement with data obtained with membrane-bound CPPDE4, the enantiomers of rolipram failed to inhibit CPPDE4α and CPPDE4β stereoselectively, whereas 4*S*,10*R*-(−)-

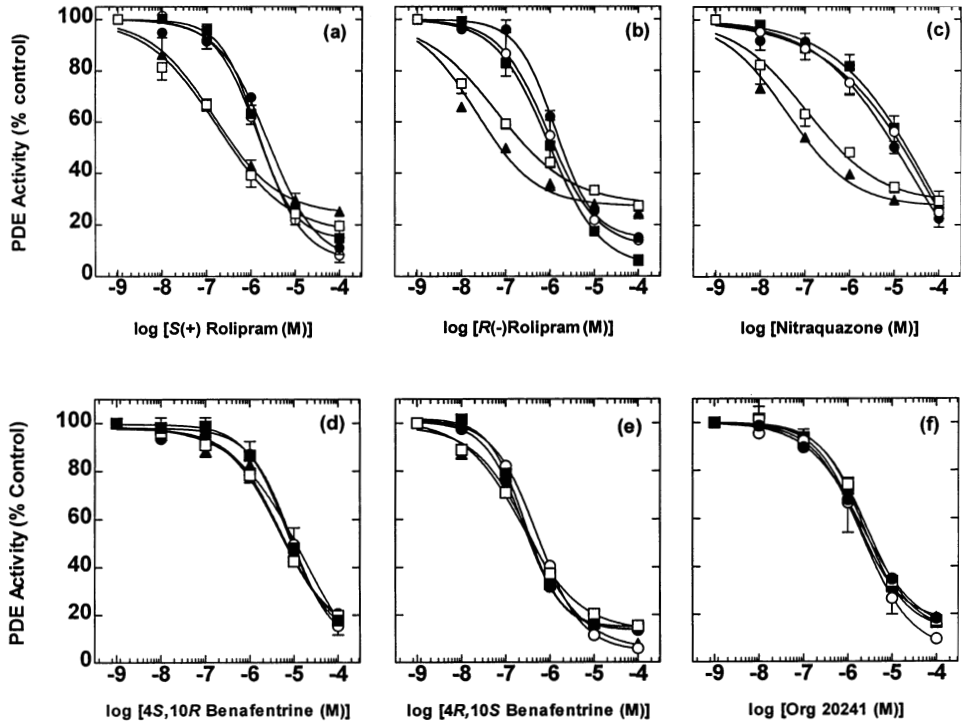

Figure 1 Effect of PDE inhibitors on cAMP hydrolysis

Membrane-bound CPPDE4 (□), Vo.2GSH-treated, membrane-bound CPPDE4 (▲), solubilized CPPDE4 (○), CPPDE4α (■) and CPPDE4β (●) were prepared as described in the Experimental section and concentration–response curves constructed to *S*-(+)-rolipram (**a**), *R*-(−)-rolipram (**b**), nitraquazone (**c**), 4*S*,10*R*-(+)-benafentrine (**d**), 4*R*,10*S*-(−)-benafentrine (**e**) and Org 20241 (**f**) for the inhibition of cAMP hydrolysis. Data represent the means ± S.E.M. for three experiments performed with different cell preparations at a substrate concentration of 1 μM cAMP. See the Experimental section for further details.

Table 1 Effect of selected PDE inhibitors on cAMP hydrolysis catalysed by membrane-bound, detergent-solubilized and partly purified CPPDE4

PDE activity was measured at 37 °C at a substrate concentration of 1 μM cAMP in the absence and presence of six concentrations (1 nM to 100 μM) of the PDE inhibitors shown below. Concentration–response curves were constructed by least-squares non-linear iterative curve-fitting and IC_{50} values were subsequently interpolated. The data represent the means ± S.E.M. for three separate experiments performed with different cell preparations. See the Experimental section for further details.

	IC_{50} (μM)				
PDE inhibitor	CPPDE4α	CPPDE4β	Detergent-solubilized CPPDE4	Membrane-bound CPPDE4	Membrane-bound CPPDE4 + Vo/2.GSH
S-(+)-Rolipram	2.52 ± 0.2	1.63 ± 0.3	1.83 ± 0.2	0.41 ± 0.06*	0.47 ± 0.07*
R-(−)-Rolipram	1.60 ± 0.2	0.92 ± 0.2	1.04 ± 0.3	0.24 ± 0.04*	0.066 ± 0.001*†
4*S*,10*R*-(+)-Benafentrine	6.01 ± 0.9	6.42 ± 1.2	10.1 ± 3.1	9.41 ± 0.10	11.3 ± 0.71
4*R*,10*S*-(−)-Benafentrine	0.39 ± 0.1	0.28 ± 0.1	0.68 ± 0.2	0.42 ± 0.08	0.47 ± 0.06
Org 20241	3.41 ± 0.4	3.20 ± 0.5	3.20 ± 1.2	3.00 ± 0.31	2.51 ± 0.4
Nitraquazone	15.7 ± 9.3	9.51 ± 2.1	12.6 ± 2.4	0.54 ± 0.19*	0.14 ± 0.02*†

* $P < 0.05$; significantly more potent against CPPDE4 in the membrane-bound conformation than against CPPDE4α, CPPDE4β and detergent-solubilized CPPDE4.
† $P < 0.01$; significantly more potent against membrane-bound CPPDE4 + Vo/2.GSH than against membrane-bound CPPDE4.

benefentrine was respectively 17 and 22 times more potent in inhibiting these enzyme preparations than its antipode (Table 1).

Effect of PDE inhibitors on solubilized CPPDE4

To determine whether the difference in inhibitor sensitivity between membrane-bound and partly purified CPPDE4 was the result of anion-exchange chromatography or simply a consequence of detergent solubilization, the sensitivity of the Triton X-100-solubilized enzyme to the six PDE inhibitors was evaluated. Nitraquazone and the enantiomers of rolipram inhibited solubilized CPPDE4 relatively weakly compared with their potency against the membrane-bound enzyme, but were equipotent against CPPDE4α and CPPDEβ (Table 1 and Figures 1a, 1b and 1c). In contrast, Org 20241 and the enantiomers of benafentrine exhibited similar inhibitory potencies for

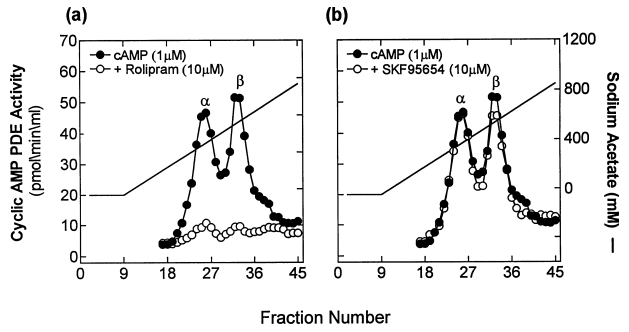

Figure 2 Anion-exchange chromatograms of the cAMP PDEs in guinea-pig macrophages

Total protein was extracted from approx. 2×10^8 macrophages, applied to a Q-Sepharose anion-exchange column and eluted with a linearly increasing salt gradient running from 50 mM to 1.2 M sodium acetate. cAMP hydrolysis was then measured in the absence and presence of rolipram (**a**) and SK&F 95654 (**b**). Data are representative of three determinations performed with different cell preparations. See the Experimental section for further details.

membrane-bound, detergent-solubilized and partly purified forms of the enzyme (Table 1 and Figures 1d, 1e and 1f). Thus the rank order of potency for the inhibition of the solubilized and partly purified CPPDE4 were identical (Table 1, and Figures 1 and 3b), indicating that the process of solubilization was responsible for altering the sensitivity of PDE4 to rolipram- and nitraquazone-like compounds.

The inhibitory potency of the enantiomers of rolipram and benafentrine on solubilized CPPDE4 is given in Table 1. *R*-(−)- and *S*-(+)-rolipram did not display significant stereoselectivity for the inhibition of cAMP hydrolysis. In contrast, the enantiomers of benafentrine exhibited marked stereoselectivity, with 4*R*,10*S*-(−)-benafentrine being approx. 15-fold more potent at inhibiting solubilized CPPDE4 than the 4*S*,10*R*-(+)-enantiomer (Table 1).

Effect of the Vo/2.GSH complex on CPPDE4

Exposure of macrophage membranes to the Vo/2.GSH complex increased the potency of *R*-(−)-rolipram and nitraquazone approx. 4-fold for the inhibition of CPPDE4 (Table 1 and Figures 1b and 1c), without affecting the IC_{50} values of *S*-(+)-rolipram, Org 20241 and the enantiomers of benafentrine

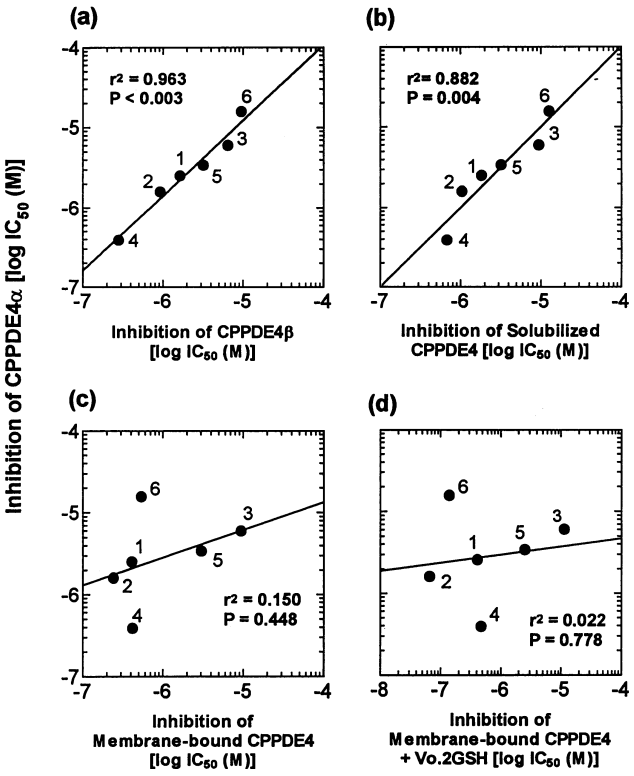

Figure 3 Relationship between inhibition of CPPDE4α with other preparations of macrophage PDE4

Log IC_{50} values were derived for the suppression of CPPDE4α by a range of PDE inhibitors and correlated with the log IC_{50} values derived for the same drugs for the inhibition of CPPDE4β (**a**), solubilized CPPDE4, (**b**), membrane-bound CPPDE4 (**c**) and Vo/2.GSH-treated membrane-bound CPPDE4 (**d**). In all PDE experiments cAMP was used at a substrate concentration of 1 μM. Data represent the means ± S.E.M. for three experiments performed with different cell preparations. Standard error bars are omitted for clarity but are given in Table 1. See the Experimental section for further details. Key: 1, *S*-(+)-rolipram; 2, *R*-(−)-rolipram; 3, 4*S*,10*R*-(+)-benafentrine; 4, 4*R*,10*S*-(−)—benafentrine; 5, Org 20241; 6, nitraquazone. Abbreviation: Vo.2GSH, Vo/2.GSH.

Table 2 Relative selectivity of selected PDE inhibitors in displacing [^{3}H]*R*-(−)-rolipram and inhibiting cAMP hydrolysis catalysed by membrane-bound, detergent-solubilized and partly purified CPPDE4

The PDE inhibitors listed below are ranked according to their ability to displace [^{3}H]*R*-(−)-rolipram over inhibition of CPPDE4. PDE activity was measured at 37 °C at a substrate concentration of 1 μM cAMP. Rolipram binding was conducted at 30 °C in the presence of 2.6 nM [^{3}H]*R*-(−)-rolipram. The relative selectivity of each inhibitor is calculated from the data in Table 1 and represents the mean of three separate experiments performed with different cell preparations. Values less than 1 and more than 1 indicate the fold selectivity for the high-affinity binding site and the catalytic domain respectively. See the Experimental section for further details.

	Inhibition of CPPDE4 (IC_{50}) / Displacementof[^{3}H]*R*-(−)-rolipram[K_i(app)]				
PDE inhibitor	CPPDE4α	CPPDE4β	Detergent-solubilized CPPDE4	Membrane-bound CPPDE4	Membrane-bound CPPDE4 + Vo/2.GSH
R-(−)-Rolipram	533	307	347	80	23.3
Nitraquazone	314	190	252	10.8	2.8
S-(+)-Rolipram	25.2	16.3	18.3	4.1	4.1
Org 20241	2.8	2.7	2.7	2.5	2.1
4*R*,10*S*-(−)-Benafentrine	0.35	0.25	0.62	0.38	0.43
4*S*,10*R*-(+)-Benafentrine	0.18	0.19	0.30	0.28	0.33

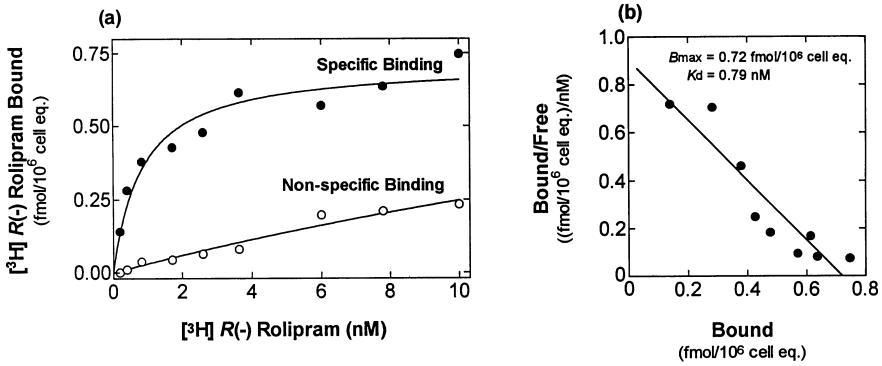

Figure 4 Binding of [^{3}H]*R*-(−)-rolipram to macrophage lysates

Lysates were prepared from frozen macrophages and duplicate aliquots (approx. 8×10^6 cell equivalents) incubated at 30 °C in buffer F containing [^{3}H]*R*-(−)-rolipram (0.25–10 nM). After 60 min the reaction was quenched by rapid vacuum filtration over Whatman GF/B glass fibre filters and the radioactivity retained by each filter was quantified by liquid-scintillation counting. (**a**, **b**) Representative saturation binding isotherm and Scatchard transformation of the specific binding data respectively. Non-specific binding was defined with 10 μM unlabelled *RS*-rolipram. Data are representative of three determinations performed with different cell preparations. See the Experimental section for further details.

(Figures 1a, 1d, 1e and 1f). The rank order of inhibitor potency produced by this treatment was distinct from that obtained when membrane-bound, solubilized and partly purified forms of the enzyme were used (Table 1). As with the membrane-bound enzyme, there was no significant linear or rank order correlation between inhibition of Vo/2.GSH-treated, membrane-bound CPPDE4 and CPPDE4α ($r^2 = 0.022$; $\rho = 0.2$; Figure 3d), CPPDE4β and the solubilized enzyme (results not shown).

In Vo/2.GSH-treated membranes, 4*R*,10*S*-(−)-benafentrine was 24-fold more potent than the 4*S*,10*R*-(+) isomer at inhibiting solubilized CPPDE4, which was similar to the stereoselectivity displayed against the membrane-bound and partly purified forms of the enzyme (Table 1). The enantiomers of rolipram also suppressed cAMP hydrolysis in a stereoselective manner [6-fold in favour of the *R*-(−) enantiomer], which was attributable entirely to the selective increase in the potency of *R*-(−)-rolipram produced by Vo/2.GSH.

Exposure of solubilized or partly purified CPPDE4 to Vo/2.GSH did not affect the potencies of any PDE inhibitor tested (results not shown).

Kinetic properties of CPPDE4

The kinetics of cAMP hydrolysis catalysed by partly purified CPPDE4 and the membrane-bound form of the enzyme in the presence of Vo/2.GSH was studied over a substrate concentration range of 0.5 to 100 μM. The hydrolysis of cAMP by each preparation conformed to Michaelis–Menten kinetic behaviour, with K_m values of 13.4 ± 1.2 μM ($n = 7$), 6.4 ± 0.9 ($n = 7$) and 5.4 ± 0.6 μM ($n = 3$) for CPPDE4α, CPPDE4β and membrane-bound CPPDE4 (+Vo/2.GSH) respectively.

To facilitate comparison with other data reported in the literature, the affinities of the enantiomers of rolipram for partly purified CPPDE4 and the membrane-bound form of the enzyme in the presence of Vo/2.GSH were also determined. *R*-(−)- and *S*-(+)-rolipram inhibited cAMP hydrolysis in an apparently competitive manner, with K_i values of 1.34 ± 0.21 and 2.21 ± 0.71 μM, and 0.91 ± 0.26 and 1.23 ± 0.38 μM, for CPPDE4α and CPPDE4β respectively. Similarly, *S*-(+)-rolipram competitively inhibited CPPDE4 in Vo/2.GSH-treated membranes but was 4–6-fold ($K_i = 0.32 \pm 0.02$ μM) more potent than the partly purified forms of the enzyme. In contrast, *R*-(−)-rolipram potently inhibited membrane-bound CPPDE4 in the presence of Vo/2.GSH, with a K_i of 0.04 ± 0.1 μM ($n = 3$), but in a manner that was not strictly competitive (results not shown).

Equilibrium binding of [^{3}H]*R*-(−)-rolipram to macrophage lysates and subcellular fractions

Figure 4 shows a representative saturation isotherm of the binding of [^{3}H]*R*-(−)-rolipram (0.25–10 nM) to guinea-pig peritoneal macrophage lysates. The binding of this radioligand was concentration-dependent, saturable and conformed to a single hyperbola. Non-specific binding increased linearly as a function of radioligand concentration but was very low, amounting to less than 2 % of the total binding at the K_d and approx. 30 % at the B_{max} (Figure 4a). Scatchard transformation of the specific binding data shown in Figure 4(a) yielded a linear plot, indicating that [^{3}H]*R*-(−)-rolipram apparently identified a homogeneous population of non-interacting binding sites (Figure 4b). In three independent experiments performed with different cell preparations the mean equilibrium K_d and B_{max} were 1.01 ± 0.4 nM and 0.76 ± 0.13 fmol per 10^6 cell equivalents respectively.

Differential centrifugation (45000 ***g*** for 30 min at 4 °C) of fresh macrophages that had been lysed hypotonically revealed that [^{3}H]*R*-(−)-rolipram bound exclusively to the particulate fraction (results not shown); binding and PDE4 activity were thus co-localized.

Displacement of [^{3}H]*R*-(−)-rolipram

All of the PDE inhibitors examined displaced [^{3}H]*R*-(−)-rolipram from macrophage lysates in a concentration-dependent manner (Figure 5). Furthermore, *R*-(−)-rolipram and 4*R*,10*S*-(−)-benafentrine were respectively 37-fold and 31-fold more potent than their corresponding enantiomers (Table 2 and Figure 5). *R*-(−)-rolipram and *S*-(+)-rolipram had higher affinity for the rolipram binding site on CPPDE4 than for the inhibition of cAMP hydrolysis; qualitatively identical results were obtained with nitraquazone (compare Figures 5 and 6). Although the selectivity for the catalytic site over the high-affinity binding domain was retained irrespective of the CPPDE4 preparation used, the relative selectivity of these compounds differed markedly, as their potency at inhibiting cAMP hydrolysis was markedly affected by solubilization, partial purification and the Vo/2.GSH

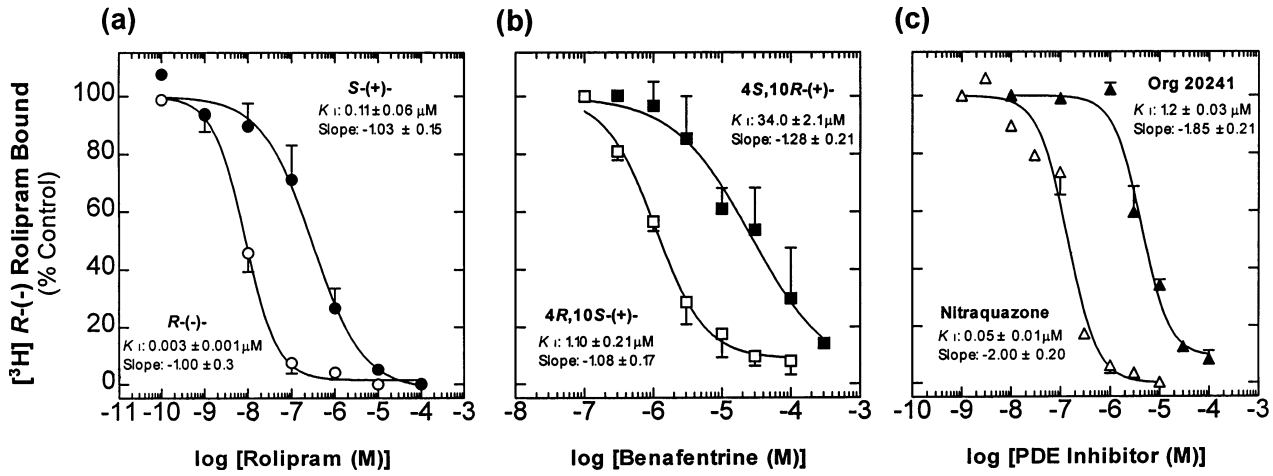

Figure 5 Displacement of [^{3}H]*R*-(−)-rolipram binding to macrophage lysates by selected PDE inhibitors

Lysates were prepared from frozen macrophages and duplicate aliquots (approx. 8×10^6 cell equivalents) incubated at 30 °C in buffer F containing 2.6 nM [^{3}H]*R*-(−)-rolipram and seven or eight concentrations of the PDE inhibitor under investigation, or its vehicle. After 60 min the reaction was quenched by rapid vacuum filtration over Whatman GF/B glass fibre filters and the radioactivity retained by each filter was quantified by liquid-scintillation counting. Equilibrium inhibition constants [K_i(app)] of each PDE inhibitor were subsequently calculated from IC_{50} values by the method of Cheng and Prusoff [32], and pseudo-Hill coefficients were estimated from the slopes of the logarithmically linearized data. (**a**, **b**, **c**) The ability of *R*-(−)-rolipram (○) and *S*-(+)-rolipram (●), 4*S*,10*R*-(−)-benafentrine (□) and 4*R*,10*S*-(+)-benafentrine (■), and nitraquazone (△) and Org 20241 (▲) to displace [^{3}H]*R*-(−)-rolipram. Each data point represents the mean ± S.E.M. for three experiments performed with different cell preparations. See the Experimental section for further details.

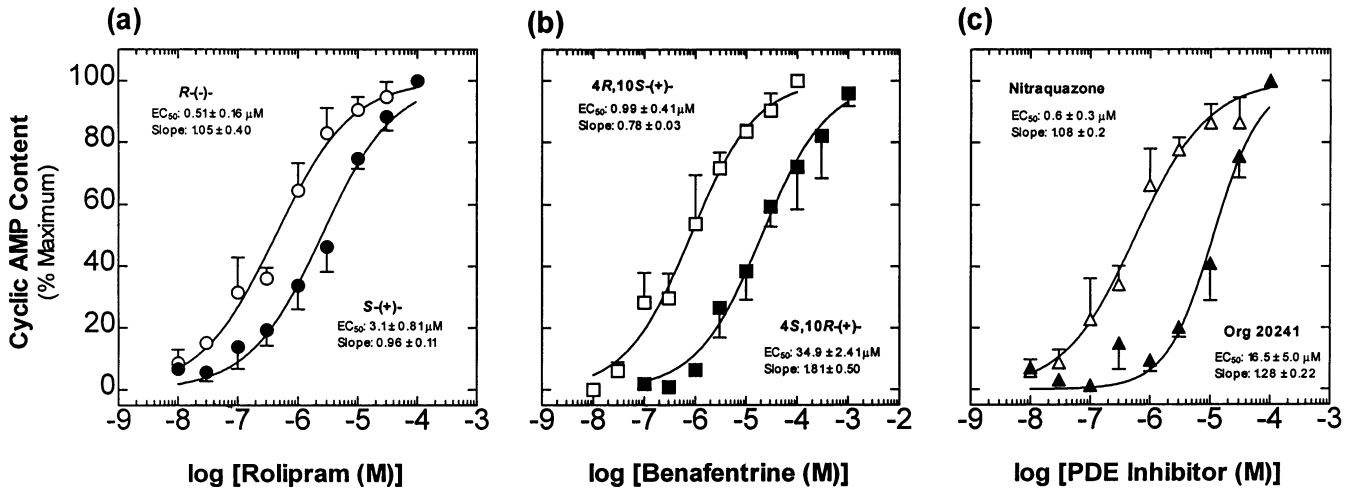

Figure 6 Effect of selected PDE inhibitors on isoprenaline-induced cAMP accumulation

Aliquots containing 5×10^5 freshly purified macrophages were added to buffer E at 37 °C containing the PDE inhibitor (10 nM to 100 μM) under investigation, or its vehicle. After 10 min, isoprenaline (30 μl; 50 μM) was added and the incubation allowed to continue for a further 5 min. An equal volume of trichloroacetic acid (1 M) was added and cAMP subsequently extracted and measured by radioimmunoassay. Concentration–response curves were constructed by least-squares non-linear iterative curve-fitting and EC_{50} values were subsequently interpolated. (**a**, **b**, **c**) The enhancement of isoprenaline-induced cAMP accumulation by *R*-(−)-rolipram (○) and *S*-(+)-rolipram (●), 4*S*,10*R*-(−)-benafentrine (□) and 4*R*,10*S*-(+)-benafentrine (■), and nitraquazone (△) and Org 20241 (▲). The cAMP levels in resting macrophages and in macrophages exposed to a maximally effective concentration of PDE inhibitor were: *S*-(+)-rolipram, from 3.6 ± 0.8 to 14.9 ± 1.8 fmol per 10^6 cells; *R*-(−)-rolipram, from 3.3 ± 0.4 to 14.6 ± 2.6 fmol per 10^6 cells; 4*S*,10*R*-(+)-benafentrine, from 4.1 ± 0.5 to 12.3 ± 2.2 fmol per 10^6 cells; 4*R*,10*S*-(−)-benafentrine, from 4.6 ± 1.1 to 16.3 ± 2.7 fmol per 10^6 cells, nitraquazone, from 4.8 ± 0.8 to 21.0 ± 3.1 fmol per 10^6 cells; Org 20241, from 2.9 ± 0.4 to 11.2 ± 0.8 fmol per 10^6 cells. Each data point represents the mean ± S.E.M. for four experiments performed with different cell preparations. See the Experimental section for further details.

complex (Tables 1 and 2). Indeed, in Vo/2.GSH-treated membranes, the IC_{50} of nitraquazone, *R*-(−)-rolipram and *S*-(+)-rolipram for the inhibition of cAMP hydrolysis approached their K_i values for the displacement of [^{3}H]*R*-(−)-rolipram (see Figures 5 and 6). This was best illustrated with reference to nitraquazone, which was approx. 300 times more active at displacing [^{3}H]*R*-(−)-rolipram from macrophage lysates than at inhibiting CPPDE4α, but only three times more potent when membrane-bound CPPDE4 was assayed in the presence of the Vo/2.GSH complex (Table 2).

Org 20241 also displayed a modest degree (2–3-fold) of selectivity for the high-affinity rolipram binding site whereas the enantiomers of benafentrine were, conversely, 3–5-fold catalytic site-selective (Figures 5 and 6). Because the potency of these compounds was unaffected by solubilization, partial purification and Vo/2.GSH (Figures 1d, 1e and 1f), there was no difference

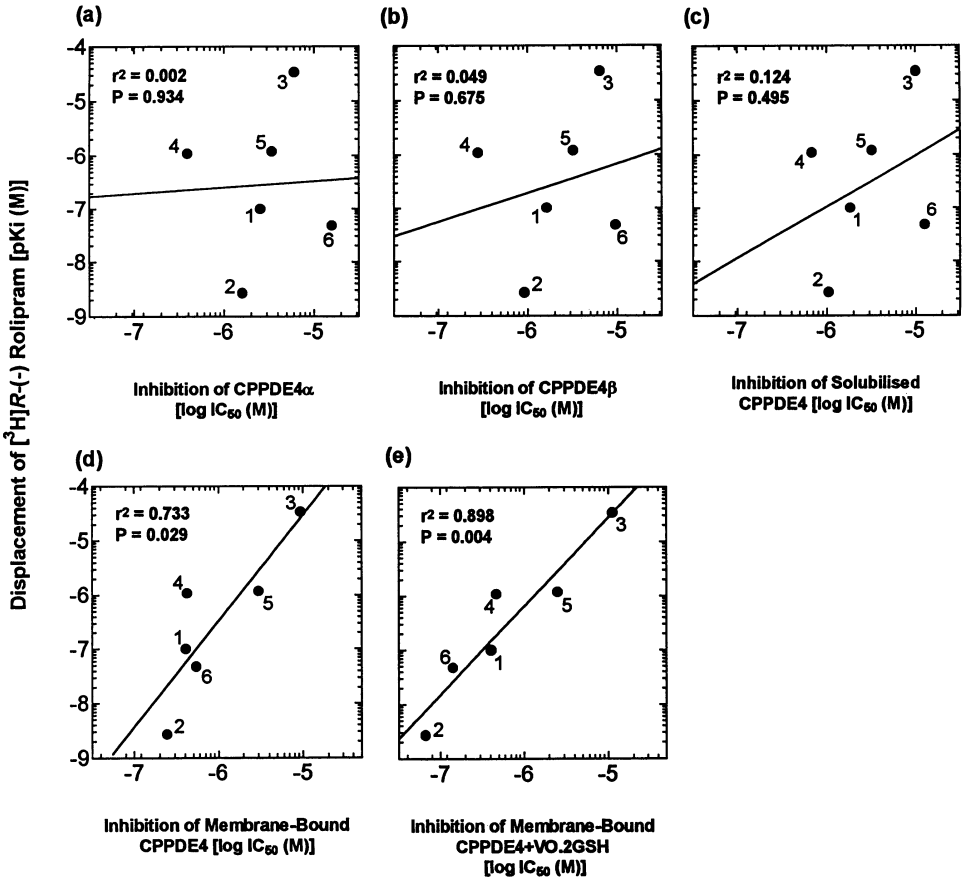

Figure 7 Relationship between displacement of [^{3}H]*R*-(−)-rolipram and inhibition of CPPDE4 activity by selected PDE inhibitors

Log K_i(app) values for the displacement of [^{3}H]*R*-(−)-rolipram from macrophage lysates were derived by interpolation from response curves constructed for seven to nine concentrations of the PDE inhibitors under investigation and correlated with the log IC_{50} values derived for the same drugs for the inhibition of CPPDE4α (**a**), CPPDE4β (**b**), solubilized CPPDE4 (**c**), membrane-bound CPPDE4 (**d**) and Vo/2.GSH-treated membrane-bound CPPDE4 (**e**). In all PDE experiments cAMP was used at a substrate concentration of 1 μM. Data represent the means ± S.E.M. for three experiments performed with different cell preparations. Standard error bars are omitted for clarity but are given in Table 1 and Figure 5. See the Experimental section for further details. Key: 1, *S*-(+)-rolipram; 2, *R*-(−)-rolipram; 3, 4*S*,10*R*-(+)-benafentrine; 4, 4*R*,10*S*-(−)-benafentrine; 5, Org 20241; 6, nitraquazone. Abbreviation: VO.2GSH, Vo/2.GSH.

in the ratio of CPPDE4 activity to binding under any conditions (Table 2).

The binding of [^{3}H]*R*-(−)-rolipram to macrophage lysates was similarly prevented by the inclusion of exogenous cAMP in the assay cocktail with a K_i of 29.1 ± 9.3 μM ($n = 3$). However, the affinity of cAMP is likely to be an underestimate owing to significant metabolism of cyclic AMP by the PDE4 in the preparation (results not shown).

cAMP accumulation

The ability of the PDE inhibitors to potentiate isoprenaline-induced cAMP accumulation is shown in Figure 6. Each inhibitor studied increased the cAMP content 5–7-fold, with *R*-(−)-rolipram and 4*R*,10*S*-(−)-benafentrine being approx. 6-fold and 35-fold more potent than their corresponding enantiomers respectively (Figure 6).

Relationship between CPPDE4 activity and [^{3}H]*R*-(−)-rolipram binding

To establish whether an association existed between inhibition of CPPDE4 activity and displacement of [^{3}H]*R*-(−)-rolipram, we assessed whether there was a significant linear or rank order correlation between these two parameters. As illustrated in Figure 7, there was no linear ($r^2 < 0.12$) or rank order correlation ($\rho < 0.35$) between displacement of [^{3}H]*R*-(−)-rolipram and inhibition of CPPDE4α, CPPDE4β and solubilized CPPDE4 (Figures 7a, 7b and 7c). In contrast, a good linear ($r^2 = 0.733$) and rank order ($\rho = 0.828$) correlation was found between inhibition of membrane-bound CPPDE4 and displacement of [^{3}H]*R*-(−)-rolipram (Figure 7d), which improved ($r^2 = 0.898$; $\rho = 0.942$) when the membranes were pretreated with the Vo/2.GSH complex (Figure 7e).

Relationship between CPPDE4 activity and cAMP accumulation

A similar analysis was performed to determine whether an association existed between inhibition of CPPDE4 activity and potentiation of isoprenaline-induced cAMP accumulation. No apparent relationship existed ($r^2 < 0.17$; $\rho = 0.314$) between these two parameters when CPPDE4α, CPPDE4β and solubilized CPPDE4 were used as the enzyme source (Figures 8a, 8b and 8c), whereas a highly significant ($P < 0.002$) linear and rank order correlation was obtained when cAMP accumulation was related to inhibition of membrane-bound CPPDE4 activity in the absence ($r^2 = 0.864$; $\rho = 0.771$) or presence ($r^2 = 0.922$; $\rho = 1$) of the Vo/2.GSH complex (Figures 8d and 8e).

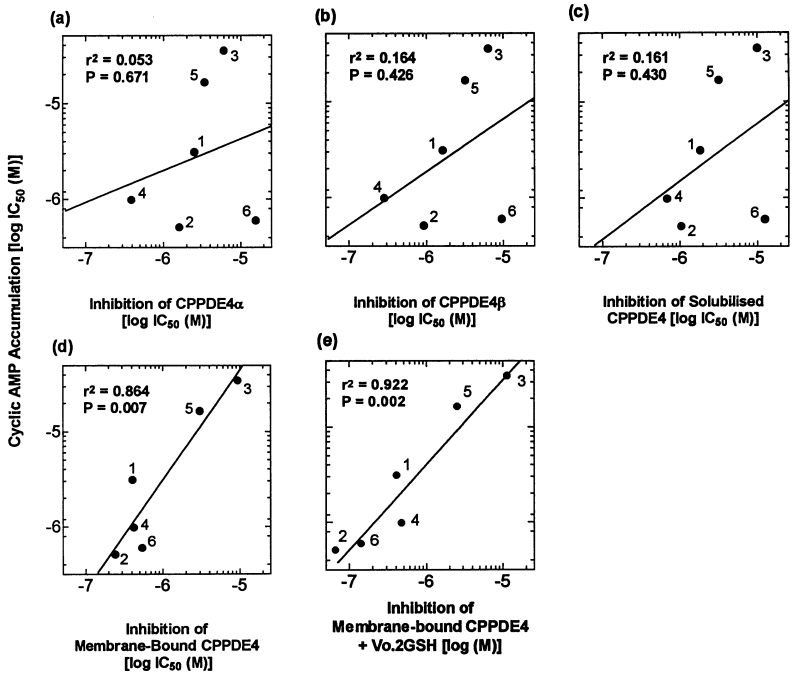

Figure 8 Relationship between potentiation of isoprenaline-induced cAMP accumulation and inhibition of CPPDE4 activity by selected PDE inhibitors

Log EC_{50} values for the potentiation of isoprenaline-induced cAMP accumulation were derived by interpolation from response curves constructed for eight concentrations of the PDE inhibitors under investigation and correlated with the log IC_{50} values derived for the same drugs for the inhibition of CPPDE4α (**a**), CPPDE4β (**b**), solubilized CPPDE4 (**c**), membrane-bound CPPDE4 (**d**) and Vo/2.GSH-treated membrane-bound CPPDE4 (**e**). In all PDE experiments cAMP was used at a substrate concentration of 1 μM. Data represent the means $\pm$ S.E.M. for three experiments performed with different cell preparations. Standard error bars are omitted for clarity but are given in Table 1 and Figure 6. See the Experimental section for further details. Key: 1, *S*-(+)-rolipram; 2, *R*-(−)-rolipram; 3, 4*S*,10*R*-(+)-benafentrine; 4, 4*R*,10*S*-(−)-benafentrine; 5, Org 20241; 6, nitraquazone. Abbreviation: Vo.2GSH, Vo/2.GSH.

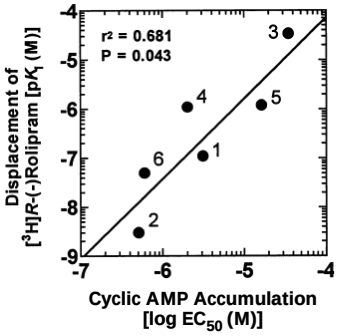

Figure 9 Relationship between displacement of [^{3}H]*R*-(−)-rolipram and potentiation of isoprenaline-induced cAMP accumulation by selected PDE inhibitors

Log K_i(app) values for the displacement of [^{3}H]*R*-(−)-rolipram from macrophage lysates were derived by interpolation from response curves constructed for seven to nine concentrations of the PDE inhibitors under investigation and correlated with the log EC_{50} values derived for the same drugs for the potentiation of isoprenaline-induced cAMP accumulation. Data represent the means $\pm$ S.E.M. of three experiments performed with different cell preparations. Standard error bars are omitted for clarity but are given in Figures 5 and 6. See the Experimental section for further details. Key: 1, *S*-(+)-rolipram; 2, *R*-(−)-rolipram; 3, 4*S*,10*R*-(+)-benafentrine; 4, 4*R*,10*S*-(−)-benafentrine; 5, Org 20241; 6, nitraquazone.

Relationship between [^{3}H]*R*-(−)-rolipram binding and cAMP accumulation

Regression analysis of [^{3}H]*R*-(−)-rolipram displacement by the six PDE inhibitors studied against cAMP accumulation is shown in Figure 9. A high significant linear and rank order correlation was found between affinity of the compounds for the binding site and their ability to increase the cAMP content in intact cells ($r^2 = 0.681$; $\rho = 0.943$; $P < 0.05$).

DISCUSSION

A striking feature of this study was that the potencies of six PDE inhibitors to inhibit partly purified CPPDE4 and to elevate the cAMP content in intact guinea-pig macrophages were distinct. Thus there was no linear or rank order correlation between these two parameters. These results are consistent with the apparent lack of relationship between inhibition of PDE4 and an array of cAMP-mediated functional responses in other systems (see the Introduction section). An additional enigma was the demonstration of a significant relationship between cAMP accumulation and the ability of the same PDE inhibitors to interact with the high-affinity rolipram binding site. Similar findings in other systems [12,13,18–20] have prompted the contention that the rolipram binding site, rather than the catalytic domain of the enzyme, mediates these effects of PDE inhibitors. A major problem that confounds this hypothesis is that high-affinity rolipram binding has been difficult to identify in peripheral tissues, suggesting that the expression of this domain might be tissue-specific. The results of this study, however, clearly demonstrate that [^{3}H]*R*-(−)-rolipram bound with high affinity ($K_d = 1$ nM) to an apparently homogeneous class of sites in guinea-pig macrophage lysates with characteristics similar to the binding of [^{3}H]rolipram to rat brain homogenates ($K_d = 1.2$ nM) and human recombinant PDE4A expressed in *Saccharomyces cerevisiae* ($K_d = 1.0$ nM) [10,11]. Moreover, differential centri-

fugation of macrophage lysates revealed that [^{3}H]*R*-(−)-rolipram bound exclusively to the membrane fraction, where approx. 90% of the catalytic activity was expressed. These results are entirely consistent with the recent demonstration that cAMP hydrolysis and high-affinity rolipram binding are properties of the same gene product [11] and provide evidence that the rolipram binding site is expressed in a peripheral tissue.

The identification of a domain in macrophage lysates for which rolipram had high affinity allowed a detailed assessment of the relationship between inhibition of PDE4, rolipram binding and cAMP accumulation in the same cell. In complete agreement with previous reports [12,13,18–20], there was a significant linear and rank order correlation between cAMP accumulation and displacement of [^{3}H]*R*-(−)-rolipram, providing further evidence that the high-affinity binding site is the functional 'receptor' for PDE inhibitors in this cell. A perplexing finding, however, and one that has important implications with regard to the functional role of the high-affinity rolipram binding domain, was the lack of linear and rank order correlations between the potency of a range of PDE inhibitors to suppress membrane-bound CPPDE4, and partly purified and solubilized preparations of the same enzyme. Inspection of the concentration–response data illustrates that enzyme solubilization was responsible for this anomaly. Indeed, solubilization itself selectively decreased the potency of those PDE inhibitors [nitraquazone, *R*-(−)-rolipram, *S*-(+)-rolipram] that preferentially interacted with the high-affinity rolipram binding site. The significance of this observation is that good linear and rank order correlations were obtained when cAMP accumulation was related to displacement of [^{3}H]*R*-(−)-rolipram and also inhibition of membrane-bound PDE4. Moreover these relationships improved further when membrane-bound CPPDE4 was measured in the presence of Vo/2.GSH. Although it is currently unclear how Vo/2.GSH modifies PDE4 (see [30,31]) these findings suggest that in intact cells native PDE4 exists in a conformation similar to the membrane-bound enzyme measured in the presence of Vo/2.GSH. Taking these data together, we believe that it is unnecessary to invoke the presence of two functionally distinct domains (i.e. catalytic and binding) on PDE4 and suggest that cAMP is hydrolysed at a single catalytic site that can adopt at least two conformations for which rolipram-like compounds have either high (nanomolar) affinity, which represents the form of the enzyme recognized by [^{3}H]rolipram in binding studies, or relatively low (micromolar) affinity, which represents the form of PDE4 identified by rolipram in enzyme kinetic studies. This interpretation satisfactorily explains the conflicting reports in the literature regarding the potency of rolipram as a PDE4 inhibitor (see, for example, [11,28,33–36]) and suggests that the procedure used to extract the enzyme can alter the native conformation of the protein and so destroy the relationship between functional responses and inhibition of cAMP hydrolysis. However, it is important to emphasize that these results could also be explained if PDE4 expressed two distinct sites, the affinity of one of which is altered by a conformational change of the enzyme. Although we cannot unequivocally exclude this possibility, the finding that the K_m values of cAMP for partly purified and membrane-bound forms of the enzyme were essentially identical and that cAMP displaced [^{3}H]*R*-(−)-rolipram from macrophage lysates could argue against this interpretation. Regardless of the precise relationship between the catalytic and [^{3}H]rolipram binding sites, these findings clearly have important implications for the purification of PDE4 and for the evaluation of new PDE inhibitors.

A number of important questions arise from the above discussion. In particular, is there evidence that PDE4 can adopt different conformations in intact cells and, if so, what conformer does CPPDE4 adopt in macrophages? Barnette et al. [18,37] have provided pharmacological evidence that PDE4 exists in different active conformers in a number of different cells that are suggested to subserve distinct functional roles. This conclusion was suggested by the fact that certain responses (e.g. inhibition of respiratory burst in guinea-pig eosinophils, suppression of the generation of tumour necrosis factor α from human monocytes) evoked by PDE4 inhibitors relate more closely to the suppression of cAMP hydrolysis (indicative of an interaction with a low-affinity conformer of PDE4) than to the displacement of [^{3}H]rolipram (indicative of an interaction with a high-affinity conformer of PDE4) [37,38], whereas for others (e.g. enhancement of gastric acid secretion in rabbit parietal cells, relaxation of guinea-pig tracheal smooth muscle) the reverse is true [18,19]. Direct biochemical evidence for distinct conformations of PDE4 was also published recently [39].

According to the criteria described in Barnette et al. [18,37], guinea-pig macrophage PDE4 apparently exists as a high-affinity conformer. This contention is supported by the fact that (1) [^{3}H]*R*-(−)-rolipram bound specifically to macrophage membranes with a K_d of approx. 1 nM, (2) significant linear and rank order correlations were obtained between cAMP accumulation and displacement of [^{3}H]*R*-(−)-rolipram from macrophage membranes, and (c) no apparent relationship was found when cAMP was related to the inhibition of solubilized and partly purified forms of the enzyme (which we propose exist in a low-affinity form). In addition, when membrane-bound CPPDE4 activity was measured in the presence of the Vo/2.GSH complex (conditions that favour a high-affinity conformer of the enzyme), excellent linear and rank order correlations were obtained for cAMP accumulation, displacement of [^{3}H]*R*-(−)-rolipram binding and inhibition of cAMP hydrolysis.

It is important to stress, however, that the stereoselectivity (approx. 6-fold) of rolipram for potentiating isoprenaline-induced cAMP accumulation was significantly less than predicted, indicating that it may be too simplistic to envisage a situation in which macrophages express exclusively a high-affinity conformer of PDE4. Indeed, the rolipram binding studies performed with guinea-pig macrophages (this study) and those conducted with rat brain membranes [10] and human recombinant PDE4A [11] predict that *R*-(−)-rolipram should be 20 to 40 times more potent than its respective antipode at inhibiting the high-affinity form of CPPDE4. It is entirely conceivable, however, that high- and low-affinity forms of CPPDE4 co-exist in cells either through interconversion of a single enzyme or through the expression of distinct PDE4 isogene products. Alvarez et al. [39] have reported that human recombinant PDE4D3 can exist in at least two (high and low) affinity states, which can be distinguished pharmacologically with compounds that preferentially interact with the rolipram binding site. These apparent conformational states of the enzyme can be regulated by cAMP-dependent phosphorylation, which increases the inhibitory potency of some PDE inhibitors. Although it is currently unclear whether inhibition of phosphorylated HSPDE4D3 by PDE inhibitors reflects their interaction at the high-affinity domain identified in binding studies, it remains an attractive possibility because phosphorylation affects only the potency of those inhibitors that preferentially exhibit affinity towards the high-affinity rolipram binding site. Experiments are currently in progress to determine the extent to which phosphorylation is responsible for the differences in potency of rolipram-like compounds reported in this study.

If Vo/2.GSH-treated membrane-bound CPPDE4 represents a high-affinity conformation of the enzyme, then an important discrepancy that merits discussion is why nitraquazone and *S*-

(+)- and *R*-(−)-rolipram were less potent at inhibiting cAMP hydrolysis than at displacing [^{3}H]*R*-(−)-rolipram from macrophage lysates. Given the above discussion, an entirely plausible explanation could reflect the ability of rolipram and nitraquazone to interact with both high- and low-affinity conformations of PDE4. Indeed, this concept is supported by the shallow concentration–response curves that described the inhibition of membrane-bound preparations of CPPDE4 (which gave IC_{50} values less than predicted if the PDE inhibitors acted solely at the high-affinity site) and the apparently complex kinetics of inhibition that *R*-(−)-rolipram displayed on this PDE4 preparation.

In conclusion, the results presented here suggest that the high-affinity rolipram binding site and the catalytic domain on CPPDE4 might represent part of the same entity and that the cAMP content in these cells is regulated primarily, although not exclusively, by a high-affinity conformation of CPPDE4 that is selectively inhibited by rolipram and related compounds. The finding that a highly significant linear and rank order correlation between cAMP accumulation, displacement of [^{3}H]*R*-(−)-rolipram binding and inhibition of cAMP hydrolysis by a range of inhibitors is obtained only when membrane-bound PDE4 is measured in the presence of Vo/2.GSH demonstrates that the conformation of the enzyme is a critical determinant of inhibitor potency.

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REFERENCES

1 Beavo, J. A., Conti, M. and Heaslip, R. J. (1994) Mol. Pharmacol. **46**, 399–405
2 Giembycz, M. A. and Kelly, J. J. (1994) in Methylxanthines and Phosphodiesterase Inhibitors in the Treatment of Airways Disease (Piper, P. J. and Costello, J., eds.), pp. 27–80, Parthenon Publishing, London
3 Conti, M., Nemoz, G., Sette, C. and Vincini, E. (1995) Endocrine Rev. **16**, 370–389
4 Beavo, J. A. and Reifsnyder, D. H. (1990) Trends Pharmacol. Sci. **11**, 150–155
5 Nicholson, C. D., Challiss, R. A. J. and Shahid, M. (1991) Trends Pharmacol. Sci. **12**, 19–27
6 Giembycz, M. A. and Souness, J. E. (1996) in Immunopharmacology of Allergic Disease (Townley, R. G. and Agarwal, D. K., eds.), Marcel-Dekker, New York, in the press
7 Giembycz, M. A. (1992) Biochem. Pharmacol. **43**, 2041–2051
8 Torphy, T. J. and Undem, B. D. (1991) Thorax **416**, 521–513
9 Dent, G. and Giembycz, M. A. (1995) Clin Immunother. **3**, 423–437
10 Schneider, H. H., Schmiechen, R., Brezenski, M. and Seidler, J. (1986) Eur. J. Pharmacol. **127**, 105–115
11 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
12 Souness, J. E., Maslen, C. and Scott, L. C. (1992) FEBS Lett. **302**, 181–184
13 Souness, J. E. and Scott, L. C. (1993) Biochem. J. **291**, 389–395
14 Giembycz, M. A. and Souness, J. E. (1994) in Bronchitis V (Postma, D. and Geritsen, J., eds.), pp. 318–330, Van Gorcum, Assen
15 Schultz, J. E. and Schmidt, B. H. (1986) Naunyn Schmiedeberg's Arch. Pharmacol. **333**, 376–384
16 Saccomano, N. A., Vinick, F. J., Koe, B. K., Nielson, J. A., Whalen, W. M., Moletz, M., Phillips, D., Thadieo, P. F., Jung, S., Chapin, D. S., Lebel, L. A., Russo, L. L., Heleveg, D. A., Johnson, J. L., Ives, J. F. and Williams, I. H. (1991) J. Med. Chem. **34**, 291–298
17 Torphy, T. J., DeWolf, D. E., Green, D. W. and Livi, G. P. (1993) Agents Actions Suppl. **43**, 51–71
18 Barnette, M. S., Grous, M., Cieslinski, L. B., Burman, M., Christensen, S. B. and Torphy, T. J. (1995) J. Pharmacol. Exp. Ther. **273**, 1396–1402
19 Harris, A. L., Connell, M. J., Ferguson, E. W., Wallace, A. M., Gordon, R. J., Pagani, E. D. and Silver, P. J. (1989) J. Pharmacol. Exp. Ther. **251**, 199–206
20 Schmiechen, R., Schnider, H. H. and Wachtel, H. (1990) Psychopharmacology **102**, 17–21
21 Nicholson, C. D., Shahid, M., Bruin, J., Barron, E., Spiers, I., de Boer, J., van Amsterdam, R. G. M., Zaagsma, J., Kelly, J. J., Dent, G., Giembycz, M. A. and Barnes, P. J. (1995) J. Pharmacol. Exp. Ther. **274**, 678–687
22 Glaser, T. and Traber, J. (1984) Agents Actions **15**, 341–348
23 Giembycz, M. A. and Barnes, P. J. (1991) Biochem. Pharmacol. **42**, 667–677
24 Schwabe, U., Miyake, M., Ohga, Y. and Daly, J. J. (1976) Mol. Pharmacol. **12**, 900–910
25 Gartner, I. (1980) Immunology **40**, 133–136
26 Schwartz, J. P. and Passoneau, J. V. (1974) Proc. Natl. Acad. Sci. U.S.A. **71**, 3844–3848
27 Thompson, W. J. and Appleman, M. M. (1971) Biochemistry **10**, 311–316
28 Dent, G., Giembycz, M. A., Evans, P. M., Rabe, K. F. and Barnes, P. J. (1994) J. Pharmacol. Exp. Ther. **271**, 1167–1174
29 Downes, C. P., Hawkins, P. T. and Irvine, R. F. (1986) Biochem. J. **238**, 501–506
30 Souness, J. E., Thompson, W. J. and Strada, S. J. (1985) J. Cyclic Nucleotide Protein Phosphorylation Res. **10**, 383–396
31 Thompson, W. J., Tan, B. H. and Strada, S. J. (1991) J. Biol. Chem. **266**, 17011–17019
32 Cheng, Y. C. and Prusoff, W. H. (1973) Biochem. Pharmacol. **22**, 3099–3108
33 Elliott, K. R. F. and Leonard, E. J. (1989) FEBS Lett. **254**, 291–295
34 Shahid, M., van Amsterdam, R. G. M., de Boer, J., ten Berge, R. E., Nicholson, C. D. and Zaagsma, J. (1991) Br. J. Pharmacol. **104**, 471–477
35 Dent, G., Giembycz, M. A., Rabe, K. F. and Barnes, P. J. (1991) Br. J. Pharmacol. **103**, 1339–1346
36 Swinnen, J. V., Tsikalas, K. E. and Conti, M. (1989) J. Biol. Chem. **266**, 18370–18377
37 Barnette, M. S., Manning, C. D., Cieslinski, L. B., Burman, M., Christensen, S. B. and Torphy, T. J. (1995) J. Pharmacol. Exp. Ther. **273**, 674–679
38 Semmler, J., Wachtel, H. and Endres, S. (1993) Int. J. Immunopharmacol. **15**, 409–413
39 Alvarez, R., Sette, C., Yang, D., Eglen, R. M., Wilhelm, R., Shelton, E. R. and Conti, M. (1995) Mol. Pharmacol. **48**, 616–622

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