Stereospecificity of rolipram actions on eosinophil cyclic AMP-specific phosphodiesterase

John E. SOUNESS and Lisa C. SCOTT

Rhône-Poulenc Rorer Ltd., Dagenham Research Centre, Rainham Road South, Dagenham, Essex RM10 7XS, U.K.

The stereospecificity of rolipram inhibition of particulate cyclic AMP-specific phosphodiesterase (PDE IV) from guinea-pig eosinophils has been investigated. (–)-Rolipram ($IC_{50} = 0.22 \pm 0.08 \mu M$) was 2.5-fold more potent than (+)-rolipram ($IC_{50} = 0.58 \pm 0.05 \mu M$) in inhibiting membrane-bound PDE IV. Solubilization of PDE IV with deoxycholate (0.5%) and NaCl (100 mM) increased rolipram stereospecificity [IC_{50} (–)-rolipram = $0.020 \pm 0.002 \mu M$; IC_{50} (+)-rolipram = $0.33 \pm 0.07 \mu M$]. Partial purification of this solubilized PDE IV by DEAE-trisacryl anion-exchange chromatography reduced the enantiomeric potency difference compared with the pre-chromatographed activity, with (–)-rolipram ($IC_{50} = 0.20 \pm 0.02 \mu M$) being only 2.9-fold more potent than (+)-rolipram ($IC_{50} = 0.57 \pm 0.14 \mu M$). Vanadate–glutathione complex (V–GSH) stimulated membrane-bound PDE IV activity and increased the potency of (–)-rolipram ($IC_{50} = 0.014 \pm 0.006 \mu M$) but not (+)-rolipram ($IC_{50} = 0.32 \pm 0.07 \mu M$). In intact eosinophils, (–)-rolipram ($EC_{50} = 0.19 \pm 0.02 \mu M$) was 10-fold more

potent than (+)-rolipram ($EC_{50} = 1.87 \pm 0.09 \mu M$) in enhancing isoprenaline (10 μM)-stimulated cyclic AMP accumulation. Strong correlations were demonstrated for displacement of [3H]rolipram binding to brain membranes by several PDE inhibitors and their inhibition of solubilized PDE IV ($r = 0.98$, $P < 0.001$, $n = 7$) and stimulation of cyclic AMP accumulation in intact cells ($r = 0.98$, $P < 0.001$, $n = 6$). Rolipram was a relatively weak inhibitor of partially purified pig aortic PDE IV and only slight stereospecificity was exhibited [IC_{50} (–)-rolipram = $1.47 \pm 0.09 \mu M$; IC_{50} (+)-rolipram = $2.73 \pm 0.38 \mu M$]. The results indicate the presence of a partially concealed stereospecific site (Sr) on eosinophil PDE IV possibly similar to the high-affinity rolipram-binding site in brain through which rolipram can potently inhibit enzyme activity. This site, which apparently is not present on partially purified pig aortic PDE IV, is concealed in freshly prepared eosinophil membranes but is exposed by solubilization or V–GSH treatment and is important in regulating intracellular cyclic AMP accumulation in intact cells.

INTRODUCTION

Cyclic AMP-specific phosphodiesterase (PDE IV) is a widely distributed family of enzymes [1,2]. Specific inhibitors of PDE IV, such as rolipram, exert a multiplicity of effects in the central nervous system (CNS) and in peripheral cells and tissues [1,2]. Several studies [3–5] have demonstrated that rolipram is a relatively weak competitive inhibitor of PDE IV; however, whether such an interaction can account for all of its wide-ranging actions is doubtful. For example, a stereospecific high-affinity rolipram-binding site has been demonstrated in brain tissue and may be involved in the CNS actions of rolipram [6,7]. Furthermore, certain peripheral actions of PDE inhibitors, such as bronchodilatation, are more closely correlated with their abilities to displace [3H]rolipram from brain membranes than with inhibition of PDE IV [8].

The high-affinity rolipram-binding site in brain membranes appears to be associated with PDE IV [7]. Recently, a high-affinity rolipram-binding site similar to that in brain was demonstrated on the cloned human monocyte PDE IV expressed in yeast [9]. The potency order of compounds inhibiting enzyme activity and displacing [3H]rolipram binding is distinct. Furthermore, the affinity of rolipram for the high-affinity binding site is markedly stereospecific, with (–)-rolipram exhibiting > 20-fold greater potency than (+)-rolipram, whereas only a slight (3-fold) enantiomeric potency difference is observed for enzyme inhibition. The significance of the high-affinity rolipram-binding site in regulating enzyme activity remains to

be resolved [9].

A tightly membrane-bound PDE IV is the predominant PDE isoform in guinea-pig peritoneal eosinophils [10]. Inhibition of the membrane-bound enzyme and stimulation of cyclic AMP accumulation by PDE inhibitors in intact cells are poorly correlated [10]; however, a closer correlation exists between inhibition of the deoxycholate/NaCl-solubilized enzyme and stimulation of cyclic AMP accumulation [11]. Solubilization does not affect the inhibitory potencies of several non-selective inhibitors [dipyridamole, trequinsin, AH-21-132, 3-isobutyl-1-methylxanthine (IBMX)] but the IC_{50} values of selective PDE IV inhibitors (rolipram, denbufylline, Ro-20-1724) are decreased by at least 10-fold [11]. Exposure of the membrane-bound PDE IV to vanadate–glutathione complex (V–GSH) also increases the potencies of these selective PDE IV inhibitors but not of non-selective inhibitors [11]. It was proposed that, as well as the catalytic site (Sc), a biologically important high-affinity site for rolipram (Sr) exists on eosinophil PDE IV which is concealed in freshly prepared membranes and exposed by solubilization or V–GSH [11].

To test this hypothesis further, we have investigated the stereospecificity of the action of rolipram on eosinophil PDE IV. The results show that rolipram stereospecificity for the enzyme is markedly increased by solubilization or exposure to V–GSH, indicating that Sr is a stereospecific site. Furthermore, Sr, which is probably important in regulating cyclic AMP accumulation in intact eosinophils, may be similar to the brain high-affinity rolipram-binding site.

MATERIALS AND METHODS

Materials

Cyclic [2,8-³H]AMP (41 Ci/mmol) and cyclic [8-³H]GMP (13.8 Ci/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.). Rolipram [4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone] and AH-21-132 [(+)-*cis*-6-(*p*-acetamidophenyl)-1,2,3,4,4a,10b-hexahydro-8,9-dimethoxy-2-methylbenzo[*c*][1,6]naphthyridine] were synthesized by the Department of Discovery Chemistry, Rhône-Poulenc Rorer Ltd. (Dagenham, Essex, U.K.). Denbufylline [BRL 30892; 1,3-di-*n*-butyl-7-(2'-oxopropyl)xanthine] was a gift from Beecham Pharmaceuticals (Epsom, Surrey, U.K.). Trequinsin (HL-725; 9,10-dimethoxy-2-mesitylimino-3-methyl-3,4,6,7-tetrahydro-2*H*-pyrimido[6,1-*a*]isoquinolin-4-one) was supplied by Hoechst Pharmaceuticals (Hounslow, Middx., U.K.). Ro-20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] was obtained from Roche Products Ltd. (Welwyn Garden City, U.K.). The cyclic AMP radioimmunoassay kit was purchased from NEN Chemicals GmbH. Donor horse serum was purchased from Flow Laboratories Ltd. (Irvine, Scotland, U.K.). All other chemicals were obtained from Sigma Chemical Co., BDH Chemicals (both of Poole, Dorset, U.K.) and Rhône-Poulenc Ltd. (Eccles, Manchester, U.K.).

Male Dunkin-Hartley guinea pigs were purchased from a local supplier.

Preparation of guinea-pig eosinophils

Male Dunkin-Hartley guinea pigs (250–400 g) were injected (intraperitoneally) with 0.5 ml of donor horse serum twice weekly. At least 5 days after the second injection, the guinea pigs were killed by CO₂ asphyxiation. A ventral incision was made and 30 ml of Hanks' buffered salt solution (HBSS) without Ca²⁺ (Gibco U.K. Ltd., Uxbridge, Middx., U.K.) was poured into the abdominal cavity. The abdomen was gently massaged for approximately 1 min; a ventral incision was then made and the peritoneal exudate was aspirated and centrifuged at 250 *g* for 10 min at 4 °C. The supernatant was discarded and the pellet washed once (10 ml of HBSS) and resuspended in HBSS. Portions (1 ml) of the cell suspension were layered on to a discontinuous (18.5% and 22.5%, w/v) metrizamide gradient prepared in conical tubes by dissolving metrizamide in Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 11.9 mM NaHCO₃, 0.35 mM Na₂HPO₄, 5.5 mM glucose, pH 7.3) containing 0.1% gelatin. The gradients were centrifuged (250 *g*, 20 min, 20 °C) and the eosinophil-rich cell pellet was resuspended in 10 ml of HBSS. Total cell counts were determined using a Coulter counter and differential cell counts obtained from cytospin slides fixed in methanol and stained with Wright-Giemsa. Cell viability, as determined by Trypan Blue exclusion, was greater than 99% and eosinophil purity greater than 97%.

Preparation of subcellular fractions

Cells (100 × 10⁶–200 × 10⁶), suspended in HBSS, were centrifuged (250 *g*, 10 min, 4 °C), the supernatant was removed and the resulting cell pellet resuspended in 5 ml of homogenization buffer [20 mM Tris/HCl, pH 7.5, 2 mM MgCl₂, 1 mM dithiothreitol, 5 mM EDTA, 0.25 M sucrose, 20 μM Tosyl-lysylchloromethane (Tos-Lys-CH₂Cl; 'TLCK'), 10 μg/ml leupeptin and 2000 units/ml aprotinin]. Cells were homogenized on ice with a Dounce homogenizer (ten strokes). The homogenate was centrifuged at 105 000 *g* for 60 min, the supernatant was collected and the pellet resuspended in an equal volume of homogenization buffer.

Solubilization of particulate PDE IV

The membrane-bound PDE IV was solubilized by homogenizing freshly prepared membranes with a Dounce homogenizer (ten strokes) in 4 ml of homogenization buffer containing deoxycholate (0.5%) and NaCl (100 mM). The homogenate was centrifuged at 100 000 *g* for 30 min; the supernatant containing the solubilized activity was removed and the pellet resuspended in an equal volume of homogenization buffer. PDE IV and protein were measured in the initial homogenate as well as the cytosolic and particulate fractions.

Partial purification of solubilized PDE IV

Solubilized PDE IV containing 2–4 mg of protein was diluted to 30 ml in column buffer (20 mM Tris/HCl, 2 mM MgCl₂, 1 mM dithiothreitol, 20 μM Tos-Lys-CH₂Cl, 0–2% deoxycholate, pH 7.5). The eluate was applied to a DEAE-trisacryl column (0.7 cm × 1 cm) pre-equilibrated with column buffer. The column was washed with 10 ml of column buffer and PDE activities were eluted with a linear gradient of NaCl (0–0.5 M, 24 ml) in column buffer. The flow rate was 1 ml/min and 1 ml fractions were collected. Assays on the pooled peak fractions were performed immediately after elution from the column.

Partial purification of pig aortic PDE IV

Aortas of freshly slaughtered pigs were clamped with artery forceps, excised, rinsed with water and placed in Hepes-buffered Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.6 mM MgSO₄, 1.2 mM KH₂PO₄, 5.6 mM glucose, 2 mM Hepes, pH 7.4) for transport from the abattoir back to the laboratory. Within 2 h of removal, the aortas were placed on a plastic dissecting mat, any extraneous tissue was trimmed from the outside of the aorta and the artery was cut lengthwise through the thoracic arterial stumps. The artery was then pinned out with syringed needles and excess blood was removed by suction. The endothelial layer was removed by rubbing the intimal surface of the artery with a cotton swab. A criss-cross pattern was cut into the smooth muscle to a depth of 3–4 mm using a scalpel blade. The resulting rectangular strips of smooth muscle were then plucked from the aorta and washed (Krebs solution) and blotted dry. Then 25 g of the smooth muscle strips were cut finely with sharp scissors and homogenized using a Waring Blender (3 × 35 s bursts) in 50–100 ml of an ice-cold solution of 20 mM-Tris/HCl (pH 7.5), 2 mM MgCl₂, 1 mM dithiothreitol, 5 mM EDTA and 1 mg/ml aprotinin. The homogenate was transferred to 50 ml centrifuge tubes and further homogenized on ice using an Ultra-Turrax homogenizer. The homogenate was centrifuged (3000 *g*, 5 min). After removal of the supernatant, the pellet was sonicated (4 × 10 s at 20 s intervals) in a small volume (25–50 ml) of homogenization buffer. The sonicate was then centrifuged (3000 *g*, 5 min), the pellet discarded and the supernatant pooled with that from the first centrifugation step. The pooled supernatants were centrifuged at high speed (100 000 *g*, 1 h, 4 °C). The resulting supernatant (75–150 ml) was filtered (0.45 μm) and protein determined before application to a column (40 cm × 2.4 cm) of DEAE-trisacryl (IBF, Villeneuve La Garenne, France) pre-equilibrated with the same column buffer (without deoxycholate) as was used for partial purification of solubilized eosinophil PDE IV. The column was washed with 500–600 ml of column buffer, and PDE activities were eluted with two successive linear gradients of NaCl (0–200 mM, 400 ml and 200–300 mM, 200 ml) in column buffer. The flow rate throughout was 1 ml/min and 7 ml fractions were collected and assayed. For long-term storage at –20 °C, ethylene glycol was

added to a final concentration of 30 % (v/v). Activity was stable for several weeks under these conditions.

Measurement of PDE activity

PDE activity was determined by the two-step radioisotopic method of Thompson et al. [12]. The reaction mixture contained 20 mM Tris/HCl (pH 8.0), 10 mM $MgCl_2$, 4 mM 2-mercaptoethanol, 0.2 mM EGTA and 0.05 mg of BSA/ml. Unless otherwise stated, the concentration of substrate was 1 μ M.

The IC_{50} values for the compounds examined were determined from concentration–response curves in which concentrations ranged from 3 nM to 200 μ M. At least three concentration–response curves were generated for each agent.

For the determination of K_m values, the concentration of cyclic AMP was varied while the amount of 3H -labelled cyclic AMP remained constant. Apparent K_m values were calculated from Lineweaver–Burk plots by linear regression analysis.

Protein was determined as described by Lowry et al. [13] with BSA as standard.

Categorization of PDE isoenzymes

The nomenclature for the different cyclic nucleotide PDEs adopted in this paper is based on that of Beavo and Reifsnnyder [14].

Measurement of [3H]rolipram binding to brain membranes

($\pm$)-Rolipram was brominated in CCl_4 and dispatched to Amersham International where it was tritiated by catalytic reduction with palladium and charcoal. The specific radioactivity of the [3H]rolipram was 24.7 Ci/mmol.

Brain membranes were prepared and the binding assay was performed with 10 nM [3H]rolipram and samples of brain tissue fractions corresponding to 500 μ g of tissue as described by Schneider et al. [7].

Measurement of eosinophil cyclic AMP accumulation

For measurement of cyclic AMP, freshly prepared eosinophils (1×10^6 cells/ml) were preincubated in HBSS containing Ca^{2+} and Mg^{2+} . To test the effects of PDE inhibitors, compounds were routinely added to cell suspensions at the concentrations indicated for 10 min after which incubations were continued for a further 2 min in the presence of isoprenaline (10 μ M). Incubations were terminated with 50 μ l of 100 % (v/v) trichloroacetic acid. The trichloroacetic acid extract was briefly sonicated (10 s), centrifuged (3000 g) for 15 min and the supernatant removed to a clean tube. Trichloroacetic acid was removed with three washes of water-saturated ether (5 vol.). The last traces of ether were removed by gassing with nitrogen, and sodium acetate (pH 6.2) was added to a final concentration of 50 mM. Samples were acetylated and cyclic AMP quantified by radioimmunoassay (kit from NEN Chemicals GmbH).

Preparation of Na_3VO_4 -GSH complex (V-GSH)

Preparation of V-GSH was by the method of Souness et al. [15].

Separation of the stereoisomers of rolipram

The stereoisomers of rolipram were separated as described by Schneider et al. [7].

RESULTS

Effect of solubilization on the stereospecificity of rolipram inhibition of PDE IV

As described previously [11], deoxycholate (0.5 %) plus NaCl (100 mM) solubilized almost all (> 95 %) of the PDE IV activity in guinea-pig eosinophil membranes, whereas less than 50 % of the total particulate proteins were liberated. Lineweaver–Burk plots (Figure 1) show that the kinetics of the bound particulate and solubilized PDE IVs differ substantially. The bound enzyme displayed marked non-linear kinetics with high- ($K_{mapp} = 1 \mu$ M) and low- ($K_{mapp} = 6 \mu$ M) affinity components. In contrast, the Lineweaver–Burk plot of the solubilized PDE was almost linear. Extrapolation of the higher- and lower-affinity components of the plot revealed K_{mapp} values of 4 μ M and 20 μ M.

In previous studies [10,11], an iterative procedure for the analysis of complex kinetics was utilized for the determination of K_m and V_{max} values. Being uncertain of the reasons for the complex kinetics of the particulate eosinophil PDE IV, we have contented ourselves in the present communication with calculating apparent K_m values by extrapolation of Lineweaver–Burk

Figure 1 Lineweaver–Burk plots of the bound particulate (a) and solubilized (b) PDE IV activities in the absence (○) and presence (●) of V-GSH

A suspension of eosinophil membranes in 2 ml (2 mg of protein/ml) of homogenization buffer was prepared. Of this, 1 ml was diluted 1:1 in homogenization buffer and 1 ml of homogenization buffer containing deoxycholate (1 %) and NaCl (200 mM) was added to the remainder. PDE IV was solubilized as described in the Materials and methods section. PDE IV activity in equivalent volumes of the bound particulate (a) and solubilized (b) preparation was measured over a range of cyclic AMP concentrations in the absence (○) and presence (●) of GSH (2.8 mM) plus Na_3VO_4 (1.4 mM), which were mixed together and added to the assay mixture immediately before enzyme addition. Substrate concentration and velocity (v) are expressed in μ M and pmol/min per ml respectively. The apparent K_m values were as follows: particulate PDE, 1 μ M and 6 μ M; particulate PDE + V-GSH, 4 μ M and 20 μ M; solubilized PDE, 4 μ M and 20 μ M; solubilized PDE + V-GSH, 6 μ M and 20 μ M.

Figure 2 Effects of solubilization and V-GSH on the PDE IV inhibitory potencies of the (+)- and (-)-enantiomers of rolipram

PDE activity ($1 \mu\text{M}$ substrate) in bound particulate (a), solubilized (b) and V-GSH-treated bound particulate (c) preparations was measured in the presence of the indicated concentrations of (+)-rolipram (●) and (-)-rolipram (■). The results represent the means $\pm$ S.E.M. of three separate experiments.

plots. For this reason, the $K_{m_{app}}$ values presented in this paper differ slightly from the values presented previously [11].

Only slight stereospecificity of rolipram against membrane-bound PDE IV was observed [IC_{50} (+)-rolipram = $0.58 \pm 0.05 \mu\text{M}$; IC_{50} (-)-rolipram = $0.22 \pm 0.08 \mu\text{M}$]; however, (-)-rolipram ($IC_{50} = 0.020 \pm 0.002 \mu\text{M}$) was 17-fold more potent against solubilized PDE IV than (+)-rolipram ($IC_{50} = 0.33 \pm 0.07 \mu\text{M}$) (Figure 2).

Effect of V-GSH on the stereospecificity of rolipram inhibition of PDE IV

As with the solubilized enzyme, the Lineweaver-Burk plot of the V-GSH-stimulated membrane-bound PDE IV was almost linear ($K_{m_{app}} = 4 \mu\text{M}$ and $20 \mu\text{M}$) (Figure 1). V-GSH had little effect on the affinity of the solubilized PDE IV ($K_{m_{app}} = 6 \mu\text{M}$

Figure 3 Lineweaver-Burk plot of the partially purified solubilized PDE IV

Substrate concentration and velocity (v) are expressed in μM and pmol/min per ml respectively. The apparent K_m values, calculated from the non-linear plot, were $1 \mu\text{M}$ and $5 \mu\text{M}$.

Figure 4 Inhibition of partially purified PDE IV by (+)- and (-)-rolipram

PDE activity ($1 \mu\text{M}$ substrate) was measured in the presence of the indicated concentrations of (+)-rolipram (●) and (-)-rolipram (■). The results represent means $\pm$ S.E.M. ($n = 3$).

and $20 \mu\text{M}$) and only a slight increase in V_{max} was observed (Figure 1).

Marked stereospecificity of rolipram was observed against the V-GSH-stimulated bound particulate PDE IV, with the (-)-enantiomer ($IC_{50} = 0.014 \pm 0.006 \mu\text{M}$) being 23-fold more potent than the (+)-enantiomer ($IC_{50} = 0.32 \pm 0.07 \mu\text{M}$) (Figure 2).

Effect of partial purification of solubilized PDE IV on the stereospecificity of rolipram

As described previously [11], solubilized PDE IV was eluted as a single peak from a DEAE-trisacryl column. The recovery of the enzyme was 150%, indicating perhaps the presence of an endogenous inhibitor in the solubilized membrane preparation. As with the bound particulate PDE IV, but in contrast with the solubilized prechromatographed enzyme, pronounced non-linear kinetics were displayed by the partially purified PDE IV ($K_{m_{app}} = 1 \mu\text{M}$ and $5 \mu\text{M}$) (Figure 3).

Only slight rolipram stereospecificity was observed against the partially purified PDE IV compared with the prechromatographed

Figure 5 Enhancement of isoprenaline-induced cyclic AMP accumulation in intact eosinophils by (+)- and (-)-rolipram

Cells were preincubated for 10 min in the presence of the indicated concentrations of (+)-rolipram (●) and (-)-rolipram (■) before exposure to isoprenaline ($10 \mu\text{M}$) for a further 2 min. Control cells contained 3.0 ± 0.15 pmol of cAMP/ 10^6 cells. The results represent means $\pm$ S.E.M. ($n = 3$).

Figure 6 Inhibition of solubilized PDE IV (b) and stimulation of cyclic AMP accumulation in intact eosinophils (a) as functions of displacement of [^3H]rolipram binding to brain membranes

Eosinophil cyclic AMP data are expressed as EC_{50} values ($-\log \text{concn.}$), PDE IV data as IC_{50} values ($-\log \text{concn.}$) and rolipram-binding-displacement data as IC_{50} values ($-\log \text{concn.}$). Each point is the mean of at least three determinations. Regression analysis demonstrated that PDE IV inhibitory activity as a function of rolipram-binding displacement is highly significant ($r = 0.98$, $P < 0.001$, $n = 7$). Stimulation of cyclic AMP accumulation as a function of rolipram-binding displacement is also highly significant ($r = 0.98$, $P < 0.001$, $n = 6$). ○, Rolipram; ●, denbufylline; ■, Ro-20-1724; ▲, trequinsin; □, AH-21-132; ▽, IBMX; ◇, dipyrindamole.

enzyme, with the (-)-enantiomer ($\text{IC}_{50} = 0.20 \pm 0.02 \mu\text{M}$) being only 2.7-fold more potent than the (+)-enantiomer ($\text{IC}_{50} = 0.57 \pm 0.14 \mu\text{M}$) (Figure 4).

Figure 7 DEAE-trisacryl chromatography of PDE activity of the 100000 g supernatant fraction from pig aortic smooth muscle

A 100000 g cytosolic fraction from pig aortic smooth muscle was prepared and chromatographed as described in the Materials and methods section. Cyclic GMP PDE activity was determined using $1 \mu\text{M}$ cyclic GMP (●, ▲) in the presence of EGTA ($100 \mu\text{M}$) with (●) or without (▲) $2 \text{ mM CaCl}_2 + 2.5$ units of calmodulin. Cyclic AMP PDE activity was determined using $1 \mu\text{M}$ cyclic AMP substrate in the absence (■) or presence (◆) of $1 \mu\text{M}$ cyclic GMP. For further studies, fractions 71–73 were pooled for PDE IV activity. —, [NaCl].

Interestingly, another selective PDE IV inhibitor, denbufylline, was 6-fold less potent against the partially purified PDE IV ($\text{IC}_{50} = 0.20 \pm 0.01 \mu\text{M}$, $n = 3$) than against the prechromatographed enzyme ($\text{IC}_{50} = 0.035 \pm 0.009 \mu\text{M}$, $n = 3$). In contrast dipyrindamole and AH-21-132 displayed greater potencies against the partially purified PDE IV (results not shown).

Stereospecificity of rolipram actions on cyclic AMP accumulation in intact cells

(-)-Rolipram ($\text{EC}_{50} = 0.19 \pm 0.02 \mu\text{M}$) was 10-fold more potent than (+)-rolipram ($\text{EC}_{50} = 1.87 \pm 0.09 \mu\text{M}$) in enhancing isoprenaline ($10 \mu\text{M}$)-induced accumulation of cyclic AMP in intact eosinophils (Figure 5).

Relationship between inhibition of eosinophil PDE IV, cyclic AMP accumulation in intact eosinophils and ($\pm$)-[^3H]rolipram binding to brain membranes

Although we were unable to measure specific [^3H]rolipram binding to eosinophil membranes, a strong significant correlation was observed for potency of several compounds in inhibiting rolipram binding to brain membranes and solubilized (prechromatographed) PDE IV ($r = 0.98$, $P < 0.001$, $n = 7$) (Figure 6b). Only a weak correlation existed between displacement of rolipram binding and inhibition of eosinophil-bound particulate PDE IV ($r = 0.71$, $P < 0.01$, $n = 7$), and no correlation with pig aortic PDE IV ($r = 0.14$, $P = 0.76$, $n = 7$) was observed. A highly significant correlation was observed between displacement of rolipram binding and elevation of eosinophil cyclic AMP levels ($r = 0.98$, $P < 0.001$, $n = 6$) (Figure 6a).

Lack of stereospecificity of rolipram inhibition of pig aortic PDE IV

DEAE-trisacryl chromatography of a 100000 g cytosolic fraction of pig aorta resolved four peaks of activity which, in order of elution, corresponded to cyclic GMP-specific PDE (PDE V) (K_m

Figure 8 Inhibition of partially purified pig aortic PDE IV by (+)- and (-)-rolipram

PDE activity (1 μ M substrate) was measured in the presence of the indicated concentrations of (+)-rolipram (●) and (-)-rolipram (■). The results represent the means $\pm$ S.E.M. of three separate experiments performed in duplicate.

cyclic GMP = 3 μ M), Ca²⁺-dependent PDE (PDE I) (K_m cyclic GMP = 4 μ M; K_m cyclic AMP = 4 μ M), cyclic GMP-inhibited PDE (PDE III) (K_m cyclic AMP = 1 μ M) and cyclic AMP-specific PDE (PDE IV) (Figure 7). The Lineweaver-Burk plot for PDE IV was linear and the $K_{m,app}$ calculated to be 3 μ M (results not shown).

Only slight rolipram stereospecificity against pig aortic PDE IV was observed and the potencies of the (-)-enantiomer ($IC_{50} = 1.47 \pm 0.09 \mu$ M) and (+)-enantiomer ($IC_{50} = 2.73 \pm 0.38 \mu$ M) were lower than against eosinophil PDE IV (Figure 8).

DISCUSSION

Stereospecificity is a variable feature of the multifold actions of rolipram. In certain *in vivo* CNS models, (-)-rolipram is 15-fold more potent than (+)-rolipram in antagonizing reserpine-induced hypothermia in mice or inducing head twitches in rats [6]. Stereospecificity is apparent in other *in vivo* CNS actions of rolipram [16–19]. However, in *in vitro* and *in vivo* pulmonary models, only a slight enantiomeric potency difference (2–3-fold) is observed on inhibition of mediator release and smooth muscle relaxation, and the manifestation of this depends on the conditions employed [20]. Although rolipram is a relatively weak competitive inhibitor of PDE IV from various sources [3–5], its CNS actions appear to be relative to interaction at a high-affinity binding site (K_D approx. 2 nM). Both *in vitro* and *in vivo* binding studies have shown (-)-rolipram to be 15–30-fold more potent than (+)-rolipram in displacing [³H]rolipram from brain membranes [6,7,21] which is in agreement with the pharmacological tests for antidepressant activity. Furthermore, the therapeutic plasma concentrations of rolipram are 2–20 nM [22] which are much lower than its inhibitory potency against brain PDE IV. A stereospecific high-affinity rolipram-binding site similar to that in brain is coexpressed with the cloned human monocyte PDE IV expressed in yeast [9]. There is little correlation between the structure-activity relationships of a limited number of compounds in their abilities to inhibit human monocyte PDE IV activity and compete for [³H]rolipram binding [9].

We recently proposed the existence of a biologically important high-affinity site (Sr) distinct from the catalytic site (Sc) on

eosinophil PDE IV at which rolipram can potently interact [11]. The increased rolipram stereospecificity induced by solubilization or V-GSH activation reported herein supports this contention. Changes in rolipram stereospecificity are related to the altered kinetic properties of PDE IV revealed under the different conditions to which the enzyme was exposed. The untreated membrane-bound PDE IV displayed marked non-linear kinetics and in this preparation minimal rolipram stereospecificity was observed. PDE IV kinetics changed markedly upon solubilization or when activated by V-GSH. In both cases, Lineweaver-Burk plots were almost linear, with the higher-affinity component disappearing completely. Under these conditions, increased potency of (-)-rolipram and marked stereospecificity were observed, with the (-)-enantiomer being 17–23-fold more potent than the (+)-enantiomer. Interestingly, partial purification of the solubilized PDE IV by anion-exchange chromatography resulted in the reappearance of non-linear kinetics similar to those of the membrane-bound enzyme. The enantiomeric potency difference of rolipram against partially purified PDE IV was greatly decreased in comparison with the prechromatographed enzyme, once again being similar to that displayed against the bound particulate enzyme.

These results may be interpreted in the context of the two-site model [11]. In freshly prepared membranes, Sr is partially concealed or positioned so that it exerts little influence on catalytic activity. Minimal stereospecificity is observed since rolipram actions on catalytic activity would be via a relatively weak action at Sc. Solubilization and treatment with V-GSH, by inducing conformational changes in PDE IV, as revealed by the altered kinetic properties, exposes the stereospecific Sr or brings it into a position, possibly closer to Sc, whereby it can exert a much greater influence on enzyme activity. Subjecting the enzyme to anion-exchange chromatography once again changes the conformation of PDE IV to one similar to that in freshly prepared membranes, so reducing the influence of Sr on catalysis.

A 10-fold greater potency of (-)-rolipram compared with (+)-rolipram in increasing cyclic AMP accumulation is displayed in intact eosinophils. This, coupled with the very close correlation between the potencies of several compounds in inhibiting solubilized PDE and elevating intracellular cyclic AMP [11], suggests that the native PDE IV may resemble more closely the solubilized or V-GSH-activated enzymes than the untreated membrane-bound PDE IV. Whether intracellular substances exist within eosinophils which can influence PDE IV activity in a manner similar to V-GSH awaits further investigation.

The evidence for the existence of the putative Sr prompts speculation concerning its nature. Although we have been unable to measure specific [³H]rolipram binding to eosinophil membranes (results not shown), strong correlations were demonstrated between inhibition of solubilized PDE IV, stimulation of cyclic AMP accumulation in intact eosinophils and displacement of [³H]rolipram binding to brain membranes, suggesting that Sr may be similar to the brain high-affinity rolipram-binding site.

Whether stereospecificity is a feature of rolipram action on all PDE IVs is uncertain. The pig aortic PDE IV displays properties similar to those reported for partially purified preparations from other sources [3–5]. In agreement with these previous studies, rolipram was a relatively weak (IC_{50} approx. 2 μ M) inhibitor of pig aortic PDE IV. No stereospecificity was apparent in rolipram action on pig aortic PDE IV. Rolipram is a weak effector of aortic smooth muscle and in this tissue PDE III and PDE IV inhibitors exhibit synergistic effects on contractility [23]. Perhaps, in this system, rolipram acts purely as a competitive PDE IV inhibitor. Alternatively, in view of the altered properties of the

eosinophil PDE IV induced by the treatments detailed above, the rigorous homogenization procedures and the subsequent chromatography may have destroyed Sr or completely eliminated its influence on catalytic activity.

In conclusion, the results suggest the existence of a stereospecific site on eosinophil PDE IV at which rolipram can interact with great potency. This putative site appears to be important in regulating the function of intact eosinophils and may be similar to the high-affinity rolipram-binding site in brain. The location of this site in relation to the catalytic site awaits further investigation.

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