Biochemical Pharmacology, Vol. 42, No. 4, pp. 937–945, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

CHARACTERIZATION OF GUINEA-PIG EOSINOPHIL PHOSPHODIESTERASE ACTIVITY

ASSESSMENT OF ITS INVOLVEMENT IN REGULATING SUPEROXIDE GENERATION

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(*Received* 8 *January* 1991; *accepted* 30 *April* 1991)

Abstract—Experiments have been performed to characterize guinea-pig peritoneal eosinophil cyclic nucleotide phosphodiesterase (PDE) activity and establish whether it is involved in regulating superoxide ($.O_2^-$) generation. Eosinophils were found to contain a predominantly membrane-bound cAMP PDE(s) (92.5 ± 2.4% of total activity) which was resistant to solubilization with Triton X-100 (1%). This particulate PDE exhibited complex kinetics (K_m = 1.3 and 31.4 μM) and was unaffected by cGMP (IC_{50} > 100 μM) or $CaCl_2$ (2 mM) + calmodulin (10 units/mL). Little cGMP PDE activity was detected in either the soluble or particulate fractions. Inhibitors of the Ro-20-1724-inhibited (Type IV) cAMP PDE, namely Ro-20-1724 (IC_{50} = 0.92 ± 0.43 μM), rolipram (IC_{50} = 0.20 ± 0.04 μM) and denbufylline (IC_{50} = 0.20 ± 0.01 μM), potently inhibited the particulate cAMP PDE, as did the non-selective inhibitors trequinsin (IC_{50} = 0.11 ± 0.02 μM) and AH-21-132 (IC_{50} = 2.57 ± 0.02 μM). Eosinophil cAMP PDE was resistant to SK&F 94120 (IC_{50} > 1000 μM), the cGMP-inhibited (Type III) cAMP PDE inhibitor, and the cGMP PDE (Type I) inhibitor, zaprinast, was only weakly active (IC_{50} = 35.33 ± 10.74 μM). $.O_2^-$ release from resting cells was potently inhibited by rolipram (IC_{50} = 0.05 ± 0.03 μM) and denbufylline (IC_{50} = 0.06 ± 0.04 μM) but surprisingly, in view of its potent cAMP PDE inhibitory activity, was only weakly decreased by trequinsin (IC_{50} = 8.0 ± 2.7 μM). AH-21-132 (IC_{50} > 10 μM), SK&F 94120 (IC_{50} > 10 μM) and zaprinast (IC_{50} > 10 μM) were without effect. Rolipram and denbufylline alone exerted little effect on cAMP in intact cells but, in the presence of 10 μM isoprenaline, potently increased intracellular accumulation (EC_{50} = 0.45 ± 0.16 and 0.28 ± 0.08 μM, respectively). Trequinsin and AH-21-132 only weakly enhanced isoprenaline-stimulated cAMP accumulation. Although it induced a marked rise in cAMP only in the presence of isoprenaline, rolipram (50 μM) alone was able to increase the activity ratio of cAMP-dependent protein kinase from 0.24 to 0.84. The results suggest that Ro-20-1724-inhibited cAMP PDE plays a role in regulating eosinophil $.O_2^-$ generation. The poor correlation between the PDE inhibitory actions of certain compounds and their effectiveness in elevating cAMP and inhibiting $.O_2^-$ suggests the existence of a barrier impeding access to the enzyme.

Eosinophils play an important role in the host response to helminth parasites [1] and in the pathogenesis of hypersensitivity reactions [2]. In addition, they are a prominent pathological feature of a range of pulmonary diseases—in allergic asthma, blood and lung eosinophil levels are elevated and their numbers correlate with the severity of the disease [3]. The cytotoxic potential of the eosinophil results from its ability to generate reactive oxygen species and release peroxidase as well as cationic proteins. Eosinophil products induce epithelial damage and increase airway reactivity in experimental animals *in vivo* [3], fuelling speculation that they may contribute to the pulmonary hyperreactivity observed in asthmatics. Thus the eosinophil is implicated in the pathogenesis of bronchial asthma and inhibitors of eosinophil function may have potential in the treatment of this and other lung diseases.

Recently, in a preliminary report, Dent *et al.* [4] demonstrated that the β-agonist, salbutamol, and the phosphodiesterase (PDE) inhibitors, rolipram and denbufylline, can inhibit opsonized zymosan-stimulated oxygen radical production from eosinophils. Both rolipram and denbufylline are potent inhibitors of a distinct cAMP PDE which is susceptible to inhibition by low concentrations of Ro-20-1724 (Ro-20-1724-inhibited [Type IV] cAMP PDE) and is resistant to inhibition or stimulation by cGMP [5]. The Type IV enzyme has been identified in other inflammatory cells [6–10] and selective as well as non-selective inhibitors of this enzyme, alone or in combination with adenylate cyclase activators, inhibit the function of a number of inflammatory cells [10–15].

The aim of the present study was to characterize the PDE subtype(s) in guinea-pig peritoneal eosinophils and attempt to correlate the potency of PDE inhibitors on superoxide ($.O_2^-$) generation with

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† Abbreviations: PDE, cyclic nucleotide phosphodiesterase (EC 3.1.4.17); IC_{50}, concentration giving 50% inhibition; EC_{50}, concentration giving 50% maximal stimulation; cAMP, cyclic adenosine 3′:5′-monophosphate; cGMP, cyclic guanosine 3′:5′-monophosphate; $.O_2^-$, superoxide anion; PAF, platelet activating factor (C16) (1-alkyl-*sn*-glycero-3-phosphocholine).

their activity against the isolated enzyme(s). We have demonstrated that the Type IV PDE is the predominant cAMP hydrolysing enzyme in these cells and that it is tightly membrane bound. Although there is an excellent concordance between the ability of a range of compounds to increase cAMP and reduce $.O_2^-$ generation, certain potent inhibitors of the isolated enzyme only weakly influence these two parameters. We speculate that a barrier exists which impedes the access of certain inhibitors, which potently increase cAMP in other cell types, to the eosinophil PDE.

MATERIALS AND METHODS

Materials. Cyclic [2,8-^{3}H]AMP (41 Ci/mmol), cyclic [8-^{3}H]GMP (13.8 Ci/mmol) and [γ-^{32}P]ATP (30 Ci/mmol) were purchased from Amersham International (Amersham, U.K.). Zaprinast (M&B 22948, 2-*O*-propoxyphenyl-8-azapurin-6-one), rolipram [4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone] and AH-21-132 [(±)-*cis*-6-(*p*-acetamidophenyl) - 1,2,3,4,4a,10*b*-hexahydro-8,9-dimethoxy - 2 - methylbenzo - {*c*}{1,6}naphthyridine] were synthesized by the Department of Pharmaceutical Chemistry, Rhône-Poulenc Rorer Inc. (Dagenham, U.K.). SK&F 94120 [5-(4-acetamidophenyl)pyrazin-2-(1*H*)-one] was a gift from Smith Kline and French Research Ltd (Welwyn, U.K.). Denbufylline (BRL 30892; 1,3-di-*n*-butyl-7-[2′-oxopropyl]-xanthine) was a gift from Beecham Pharmaceuticals (Epsom, 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, U.K.). Ro-20-1724 [1-4-(3-butoxy-4-methoxybenzyl)-2-imidozolidinone] was obtained from Roche Products Ltd (Welwyn Garden City, U.K.). Fura-2/AM was purchased from Cambridge Bioscience (Cambridge, U.K.) and PAF (C16) from Novabiochem Ltd (Nottingham, U.K.). The cAMP radioimmunoassay kit was purchased from NEN Chemicals GmbH. Donor horse serum was purchased from Flow Laboratories Ltd (Irvine, U.K.). All other chemicals were obtained from the Sigma Chemical Co., BDH Chemicals (both of Poole, U.K.) and May & Baker Laboratory Chemicals (Eccles, 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 (i.p.) with 0.5 mL of donor horse serum twice weekly. At least 5 days after the second injection, the guinea-pigs were killed by CO_2 asphyxiation. A ventral incision was made and 30 mL of Hanks buffered salt solution (HBSS) without Ca^{2+} and Mg^{2+} (Gibco, U.K. Ltd, Uxbridge, U.K.) poured into the abdominal cavity. The abdomen was gently massaged for approximately 1 min; the peritoneal exudate was aspirated and centrifuged at 250 *g* for 10 min at 4°. The supernatant was discarded and the pellet washed once (10 mL HBSS) and re-suspended in HBSS. The cell suspension was layered in 1 mL aliquots onto a discontinuous (18.5% and 22.5%, w/v) metrizamide gradient prepared in conical tubes by dissolving metrizamide in Tyrodes buffer (137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.35 mM Na_2HPO_4, 5.5 mM glucose, pH 7.3) containing 0.1% gelatin. The gradients were centrifuged (250 *g*, 20 min, 20°) and the eosinophil-rich cell pellet re-suspended in 10 mL 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%.

Measurement of superoxide generation. Superoxide anion ($.O_2^-$) generation was determined as the superoxide dismutase (SOD) inhibitable reduction of *p*-iodonitrotetrazolium violet (INTV) [16]. Briefly, cells (10^6/well) were incubated in 96-well microtitre plates in 0.25 mL of HBSS containing INTV (0.5 mg/mL) plus other additions for 45 min at 37°. The cells were then centrifuged at 500 *g* for 5 min and the supernatant aspirated. The pellet was solubilized by incubation overnight at room temperature in DMSO containing 0.6 M HCl and the absorbance of the reduced dye measured at 492 nm (Titertek Multiskan MCC/340). The results were expressed in absorbance units.

Measurement of intracellular calcium. Eosinophils (4×10^6 cells/mL) suspended in RPMI 1640 (Gibco, U.K. Ltd) medium containing 2 mM glutamine, 24 mM $NaHCO_3$ and 0.1% (w/v) bovine serum albumin were incubated with Fura-2/AM (0.5 μM) for 30 min at room temperature in the dark. The loaded cells were centrifuged (250 *g*, 5 min) in a M.S.E. Centaur centrifuge, resuspended in fresh RPMI 1640 medium and left to stand in the dark at room temperature for at least a further 20 min before use, to facilitate dye hydrolysis. Immediately prior to use, cells were centrifuged (3000 *g*, 1 min), washed once with 10 mM Hepes, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 5.5 mM glucose, 1 mM $CaCl_2$ and 0.1% (w/v) bovine serum albumin (pH 7.4) and resuspended in 1.5 mL of the same buffer. Intracellular calcium was measured at 37° using a SPEX Fluorolog Fluorimeter with excitation wavelengths of 340 and 380 nm and emission set at 505 nm. The cells were allowed to equilibrate at 37° for 2 min before the start of each experiment [17].

Maximal fluorescence was determined at the end of each experiment by the addition of nonidet-P40 (0.1%, v/v) to lyse the cells and release the dye into the medium which contains 1 mM Ca^{2+}. EGTA (10 mM) was then added to the cuvette to obtain the minimum signal. Ca^{2+} concentration was calculated by the method of Grynkiewicz *et al.* [18]. Cell autofluorescence was subtracted from all traces prior to calculation of Ca^{2+} concentration as were controls for any optical contributions from the test compounds. Dye leakage from the cells over the time course of the assays was not found to be a problem, as divergence of the 340 and 380 nm traces was negligible.

Preparation of subcellular fractions and measurement of cyclic nucleotide phosphodiesterase. Cells ($100–200 \times 10^6$) suspended in HBSS were centrifuged (250 *g*, 10 min, 4°), the supernatant removed and the resulting cell pellet resuspended in 5 mL

homogenization buffer (20 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM EDTA, 0.25 M sucrose, 20 μM *p*-tosyl-L-lycine-chloromethyl ketone, 10 μg/mL leupeptin and 2000 units/mL aprotinin). Cells were homogenized on ice with a Dounce homogenizer (10 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. PDE assays were performed on the homogenate, as well as the cytosolic and particulate fractions.

Attempts to solubilize the membrane-bound cAMP PDE were made by homogenizing the freshly prepared 105,000 *g* particulate fraction in homogenization buffer containing Triton X-100 (1%).

PDE activity was determined by the two-step radioisotope method of Thompson *et al.* [19]. 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/mL of bovine serum albumin. Unless otherwise stated, the concentration of substrate was 2 μM for [^{3}H]cAMP and 1 μM for [^{3}H]cGMP.

The IC_{50} values (concentration which produced 50% inhibition of substrate hydrolysis) for the compounds examined were determined from concentration–response curves in which concentrations ranged from 0.003 μM to 1 mM. At least three concentration–response curves were generated for each agent.

For the determination of V_{max} and K_m values, the concentration of cAMP was varied while the amount of ^{3}H-labelled cAMP remained constant. The data were evaluated by an iterative procedure (computer program written by Dr M. Vlitos, Computer Department, Rhône-Poulenc Rorer Inc.) for the analysis of complex kinetics based on the method of Spears *et al.* [20].

Protein was determined as described by Lowry *et al.* [21] with bovine serum albumin as the standard.

Categorization of PDE isozymes. The nomenclature for the different cyclic nucleotide PDEs adopted in this paper is based on that of Beavo [5].

Measurement of eosinophil cAMP accumulation. For measurement of cAMP, 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 either in the absence or presence of isoprenaline (10 μM). Incubations were terminated with 50 μL of 100% trichloroacetic acid.

The trichloroacetic acid extract was briefly sonicated (10 sec), centrifuged (3000 *g*) for 15 min and the supernatant transferred 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 AMP quantified by radioimmunoassay (RIA, NEN Chemicals GmbH). The DNA content of cell pellets was measured by an automated spectrofluorometric procedure employing Adriamycin [22].

Measurement of cAMP-dependent protein kinase. Eosinophils were suspended in Hepes-buffered Tyrodes solution (pH 7.4) at a concentration of 10^7 cells/200 μL and incubated with test substances for 10 min at 37°. Incubations were terminated by centrifuging cells at 3000 *g* (M.S.E. microfuge) for 20 sec. The incubation buffer was removed and 200 μL of extraction buffer (50 mM KH_2PO_4, pH 6.8, containing 10 mM EDTA, 0.2 mM dithiothreitol, 125 mM NaCl, 0.5 mM 3-isobutyl-1-methylxanthine and 0.2% Triton X-100) were added, after which the mixture was vortexed for 5 sec.

The cAMP-dependent protein kinase activity of eosinophil Triton X-100 extracts was determined by a modification of the procedure previously described by Hunt *et al.* [23] in which incorporation of ^{32}P from [γ-^{32}P]ATP into the synthetic specific substrate, Kemptide, was measured. Briefly, 20 μL of the eosinophil extract was added to an assay mixture (total volume 100 μL) containing 100 μM Kemptide, 0.2 mM [γ-^{32}P]ATP (10 μCi/mL), 2 mM $MgCl_2$, 10 mM KH_2PO_4 (pH 6.8), 0.1 mM EDTA, 10 mM aminophylline, 0.1% β mercaptoethanol and 160 mM NaF in the presence and absence of 20 μM cAMP and/or synthetic cAMP-dependent protein kinase inhibitor (Sigma Chemical Co.) (50 μL/mL). Incubations were allowed to proceed for 15 min before the reaction was stopped by pipetting 75 μL of the assay mixture onto sheets of Whatman P81 phosphocellulose ion-exchange paper which were then washed extensively in 75 mM phosphoric acid and, once finally with 95% ethanol. The sheets were allowed to dry thoroughly and individual squares cut out, placed in scintillation vials and ^{32}P measured by liquid scintillation spectrometry after addition of scintillation cocktail (Lumagel, May & Baker Laboratory Chemicals). The extent of cAMP-dependent protein kinase activation was expressed as the activity ratio, which is the ratio of specific activity in the absence of added cAMP to that in the presence of sufficient cAMP to fully activate the enzyme (−cAMP/+cAMP activity ratio).

Statistical analysis. Data are presented as means ± SEM and analysed by Student's unpaired *t*-test. Values were considered to be statistically significant when $P < 0.05$.

RESULTS

Phosphodiesterase activity

As shown in Table 1, eosinophils contain a predominantly membrane-bound (92.5 ± 2.4%) cAMP PDE. This activity was not influenced by either cGMP (1 μM) or calcium (2 mM) plus calmodulin (10 units/mL) (data not shown). cGMP PDE activity was low with 80 ± 7.5% of the enzyme(s) being associated with the particulate fraction. The activity of cGMP PDE was also uninfluenced by addition of calcium plus calmodulin.

Initial attempts to solubilize the particulate cAMP PDE in order to partially purify and further characterize the enzyme proved unsuccessful. Treatment of eosinophil membranes with Triton X-100 (1%) which solubilized 47 ± 9.8% of (N = 3)

Table 1. cAMP and cGMP PDE activities in subcellular fractions of guinea-pig eosinophils

Preparation	PDE activity (pmol/min/mg protein)	
	cAMP	cGMP
Homogenate	18.41 ± 3.22	0.75 ± 0.27
Cytosolic fraction	29.43 ± 6.75 (7.5%)	3.97 ± 1.73 (20.0%)
Particulate fraction	47.56 ± 9.15 (92.5%)	2.13 ± 0.80 (80.0%)

Subcellular fractions were prepared and PDE activity measured as described in Materials and Methods. cAMP PDE was assayed with 2 μM substrate and cGMP PDE with 1 μM substrate. The values in parentheses indicate the percentage of total PDE activity associated with a particular subcellular fraction. The results represent means ± SEM (N = 3).

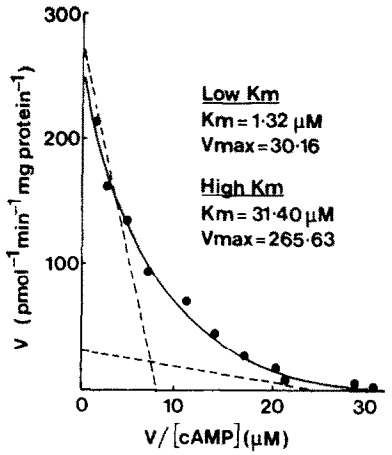

Fig. 1. Kinetic analysis of guinea-pig particulate cAMP PDE. An eosinophil particulate fraction was prepared and initial PDE velocities measured as described in Materials and Methods. Eadie–Hofstie analysis was performed on data from the means of duplicate assays in a typical experiment. The dashed lines represent the results of an iterative procedure for deriving kinetic constants for two enzymes acting on the same substrate.

particulate proteins failed to dislodge any of the cAMP PDE activity from membranes. For this reason, all further studies were performed on the particulate enzyme.

Kinetic analysis of the particulate cAMP PDE by the method of Spears *et al.* [20] revealed high- (K_m = 1.3 μM, V_{max} = 30 pmol/min/mg) and low-affinity (K_m = 31 μM, V_{max} = 265 pmol/min/mg) components as shown in the Eadie–Hofstee plot presented in Fig. 1.

Inhibition of particulate cAMP PDE activity

Ro-20-1724 (IC_{50} = 0.92 ± 0.43 μM), rolipram (IC_{50} = 0.2 ± 0.04 μM) and denbufylline (IC_{50} = 0.2 ± 0.01 μM), which have been described as selective inhibitors of Type IV cAMP PDE [5, 24], all potently inhibited the eosinophil cAMP PDE activity. Other inhibitors of the eosinophil enzyme included the non-selective compounds trequinsin

Table 2. Effects of various inhibitors on the activity of guinea-pig eosinophil, particulate cAMP PDE

Inhibitor	IC_{50} (μM)	
Rolipram	0.20 ± 0.04	(N = 5)
Dipyridamole	11.67 ± 1.20	(N = 3)
Denbufylline	0.20 ± 0.01	(N = 3)
Trequinsin	0.11 ± 0.02	(N = 3)
AH-21-132	2.57 ± 0.02	(N = 3)
SK&F 94120	>1000	(N = 3)
Aminophylline	124.00 ± 53.95	(N = 5)
IBMX	3.20 ± 0.28	(N = 3)
Enprofylline	250.00 ± 2.50	(N = 3)
Ro-20-1724	0.92 ± 0.43	(N = 3)
Zaprinast	35.33 ± 10.74	(N = 3)

A particulate fraction was prepared and PDE activity (2 μM cAMP substrate) measured as described in Materials and Methods in the presence of increasing concentrations (0.003–1000 μM) of inhibitors. The results represent means ± SEM.

(IC_{50} = 0.1 ± 0.02 μM), dipyridamole (IC_{50} = 11.7 ± 1.2 μM), 3-isobutyl-1-methylxanthine (IC_{50} = 3.2 ± 0.3 μM) and the recently described anti-asthma agent, AH-21-132 [25] (IC_{50} = 2.6 ± 0.02 μM). The cGMP PDE inhibitor, zaprinast (IC_{50} = 35.3 ± 10.7 μM), exhibited only weak inhibitory activity as did the anti-asthma drugs, aminophylline (IC_{50} = 124 ± 54.1 μM) and enprofylline (IC_{50} = 250 ± 2.5 μM). cGMP did not inhibit activity (IC_{50} > 1000 μM); nor did SK&F 94120, the cGMP-inhibited (Type III) cAMP PDE inhibitor (IC_{50} > 1000 μM). These data are presented in Table 2. The dose–response curves for rolipram and denbufylline are shallow in comparison with those for the non-selective inhibitors, trequinsin, AH-21-132 and dipyridamole, and even at high concentrations (100 μM) there was incomplete inhibition of cAMP PDE activity (Fig. 2).

Effects of PDE inhibitors on eosinophil cAMP accumulation

Incubation of eosinophils with PDE inhibitors alone resulted in only modest increases in cAMP accumulation; for example, a 10 min incubation of

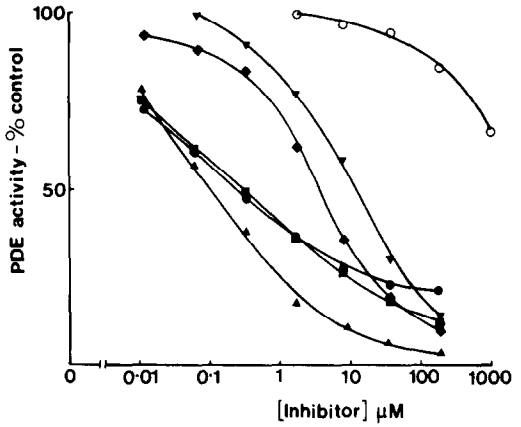

Fig. 2. Inhibition of particulate cAMP PDE by various compounds. A particulate fraction was prepared and PDE activity (2 μM substrate) assayed, as described in Materials and Methods in the presence of the indicated concentrations of rolipram (■), denbufylline (●), trequinsin (▲), dipyridamole (▼), AH-21-132 (◆) and SK&F 94120 (○). The results represent the means of 3 experiments.

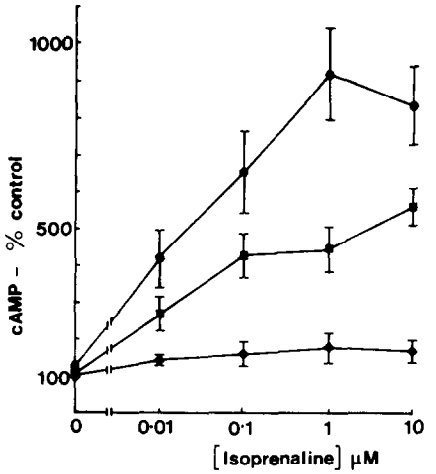

Fig. 3. Potentiation of isoprenaline-induced cAMP accumulation by rolipram. Eosinophils were preincubated without (◆) or with rolipram at 0.5 μM (■) or 50 μM (●) for 10 min before exposure to isoprenaline for a further 2 min, after which incubations were terminated by addition of trichloroacetic acid (5%). Control cells (no additions) contained 0.13 ± 0.05 pmol cAMP/μg DNA (N = 3). The results represent means ± SEM (vertical bars) of 3 separate experiments.

cells with rolipram (50 μM) elicited a small (0.17 ± 0.02 to 0.25 ± 0.02 pmol/μM DNA, N = 12) but significant ($P < 0.01$) elevation of cAMP levels. Because of this poor response, the effects of PDE inhibitors on cAMP accumulation in the presence of isoprenaline were determined. Exposure of cells to isoprenaline (10 μM) alone for 2 min elicited a small increase in cAMP (0.17 ± 0.02 to 0.29 ± 0.03 pmol/μg DNA); however, following a 10 min preincubation with rolipram (50 μM), a greater than 500% elevation in cAMP levels could be observed (0.29 ± 0.02 to 1.27 ± 0.09 pmol/μg DNA, N = 12) with isoprenaline. The effect of increasing concentrations of rolipram on the isoprenaline dose–response curve is shown in Fig. 3.

The increase in cAMP induced by a combination of rolipram (50 μM) and isoprenaline (10 μM) remained constantly elevated for at least 30 min (data not shown). The cAMP remained cell-associated with little being extruded. The effect of rolipram could be reduced by washing cells exposed to the PDE inhibitor prior to incubation with isoprenaline. Propanolol (10 μM), the β-antagonist, rapidly reversed the increase in cAMP produced by rolipram (50 μM) and isoprenaline (1 μM) even in their continued presence (data not shown).

In dose–response studies (Fig. 4), rolipram (EC_{50} = 0.45 ± 1.16 μM) and denbufylline (EC_{50} = 0.28 ± 0.08 μM) potently increased isoprenaline-induced cAMP accumulation, the maximum increase being from 0.4 ± 0.1 (isoprenaline alone) to 1.8 ± 0.5 and 2.6 ± 0.7 pmol/μg DNA, respectively, at 100 μM. In contrast, trequinsin (EC_{50} = 4.3 ± 2.0 μM) and AH-21-132 ($EC_{50} > 10$ μM) only weakly amplified the cAMP response to isoprenaline. In a separate set of experiments, Ro-20-1724 (EC_{50} = 1.2 ± 0.08 and IBMX ($EC_{50} > 10$ μM) were also shown to increase isoprenaline-stimulated cAMP levels whilst zaprinast (50 μM), dipyridamole

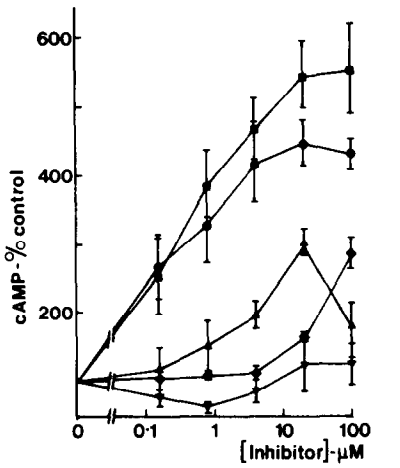

Fig. 4. Enhancement of isoprenaline-induced cAMP accumulation by various PDE inhibitors. Cells were preincubated in the presence of the indicated concentrations of rolipram (■), denbufylline (●), trequinsin (▲), dipyridamole (▼), or AH-21-132 (◆) for 10 min before exposure to isoprenaline (10 μM) for a further 2 min. Control cells (isoprenaline alone) contained 0.38 ± 0.05 pmol cAMP/μg DNA (N = 12). The results represent the means ± SEM (vertical bars) of at least 3 separate experiments.

(100 μM), SK&F 94120 (100 μM) and aminophylline (50 μM) were without effect.

Effects of PDE inhibitors on $.O_2^-$ generation

Incubation of guinea-pig eosinophils in HBSS for

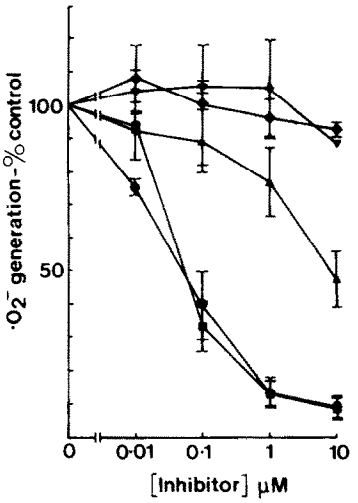

Fig. 5. Inhibition of $.O_2^-$ generation from resting eosinophils. $.O_2^-$ release from resting eosinophils was measured in the presence of the indicated concentrations of rolipram (■), denbufylline (●), trequinsin (▲), dipyridamole (▼) and AH-21-132 (◆) as described in Materials and Methods. $.O_2^-$ generation from resting cells was 1.38 ± 0.09 arbitrary units/min/10^6 cells. The results represent the mean ± SEM (vertical bars) of at least 3 separate experiments.

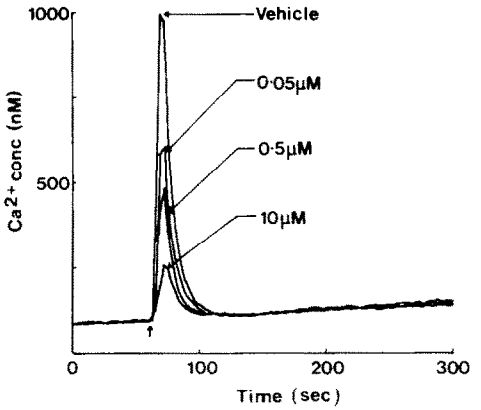

Fig. 6. Rolipram inhibition of PAF-induced increase of intracellular Ca^{2+}. Cells were incubated with the indicated concentrations of rolipram for 15 min prior to addition of PAF (0.01 μM). The results represent a typical trace. The arrow indicates time of PAF addition.

45 min at 37° elicited a time-dependent reduction of INTV that was inhibited (82 ± 4%) by superoxide dismutase (100 μg/mL). This phenomena was confirmed using ferricytochrome C as the reduced species and is indicative of $.O_2^-$ production.

Whereas rolipram (IC_{50} = 0.05 ± 0.03 μM) and denbufylline (IC_{50} = 0.06 ± 0.04 μM) potently inhibited spontaneous production of $.O_2^-$, trequinsin (IC_{50} = 8.0 ± 2.7 μM) was a relatively weak inhibitor of $.O_2^-$ generation and AH-21-132 and dipyridamole (Fig. 5), as well as SK&F 94120 and zaprinast (cGMP PDE inhibitor), were without effect at concentrations up to 10 μM.

That the effects of the PDE inhibitors are mediated by cAMP is supported by the observation that dibutyryl cAMP (IC_{50} = 75 μM) and salbutamol (IC_{50} = 6 μM) also inhibited incubation-induced $.O_2^-$ generation. Since isoprenaline is a free radical scavenger, its effect on $.O_2^-$ generation was not investigated.

Intracellular calcium measurement

To determine whether the generation of $.O_2^-$ from resting eosinophils was a consequence of a general cellular activation resulting from preparative procedures, we measured the intracellular calcium level, changes in which are implicated in activation secretion-coupling in eosinophils [26]. Fura-2 studies demonstrated that the intracellular calcium level in resting cells was low (<0.1 μM) and a calcium transient was generated by PAF (0.0001–1 μM) (see Ref. 17). The increase in intracellular calcium elicited by PAF (0.01 μM) was inhibited dose-dependently by rolipram (Fig. 6) and also reduced by 10 μM denbufylline (70% inhibition of peak response) and 10 μM trequinsin (80% inhibition of peak response). AH-21-132 (20 μM) did not influence the calcium signal induced by PAF (data now shown). The effect of dipyridamole on the calcium level could not be tested because it fluoresced.

Effects of rolipram and isoprenaline on eosinophil cAMP-dependent protein kinase

A 10 min incubation of eosinophils with rolipram (50 μM) increased the activity ratio of the cAMP-dependent protein kinase from 0.24 ± 0.04 to 0.84 ± 0.25 (N = 4). Isoprenaline (10 μM) also significantly increased activity (0.37 ± 0.03) and a combination of rolipram and isoprenaline maximally stimulated the enzyme. These results are presented in Table 3.

DISCUSSION

Eosinophils can now be added to the list of inflammatory cells in which a Ro-20-1724-inhibited (Type IV) cAMP PDE plays an important role in regulating cAMP levels and functional parameters. The Type IV enzyme is the only clearly demonstrable PDE activity in guinea-pig peritoneal eosinophils, as the lack of effect of cGMP and calcium/calmodulin on PDE activity indicates that cGMP-stimulated-(Type II), cGMP-inhibited-(Type III) and calcium/calmodulin-stimulated-(Types 1b and c) PDEs are absent from this cell type. Furthermore, there are only low levels of cGMP hydrolytic activity in the particulate or soluble fractions indicating that this second messenger may not play an important role in regulating eosinophil function.

As in human neutrophils [10], the bulk (93%) of the cAMP PDE is membrane-bound and potently inhibited by the Type IV PDE inhibitors, rolipram and denbufylline but not by type III PDE inhibitors such as SK&F 94120 (Fig. 2). The enzyme(s) was resistant to Triton X-100 (1%) treatment which

Table 3. Stimulation of cAMP-dependent protein kinase activity ratio by rolipram and isoprenaline

Addition	cAMP-dependent protein kinase activity ratio
None	0.23 ± 0.03
Rolipram	0.82 ± 0.13*
Isoprenaline	0.43 ± 0.07*
Rolipram + isoprenaline	1.01 ± 0.05†

Eosinophils were incubated with or without isoprenaline (10 μM) and/or rolipram (50 μM) for 10 min. Incubations were terminated and protein kinase activity measured as described in Materials and Methods. The specific activity of cAMP-dependent protein kinase in control cells was 82.6 ± 20.8 pmol/min/mg protein.

The results represent means ± SEM (N = 4).

* $P < 0.05$, † $P < 0.01$ for significant difference from control.

solubilized 47% of the particulate proteins, suggesting that the cAMP PDE is an integral component of eosinophil membranes. Its exact intracellular location is unknown but it is tempting to speculate that, as with other particulate PDEs [27, 28], the PDE activity of eosinophils is under the influence of extracellular humoral agents. Certainly, increased leukocyte PDE activity has been observed in response to inflammatory agents and mitogens [9, 29].

As in neutrophils [10], complex kinetics are exhibited by eosinophil particulate cAMP PDE (Fig. 1) with low-affinity and high-affinity components being evident. Whether this indicates the existence of more than one eosinophil particulate cAMP PDE or one enzyme in two affinity states awaits further investigation. The shallow dose–response curves of potent inhibitors and the inability of these compounds to totally abolish activity even at high (1000 μM) concentrations may be supportive of the former contention; however, complex kinetics might be a characteristic of this PDE and until the enzyme(s) has been solubilized and attempts made to purify the activity, this issue will remain unresolved.

Incubation of eosinophils resulted in spontaneous generation of $.O_2^-$ which was linear for 45 min (data not shown) and was potently inhibited by rolipram and denbufylline (Fig. 5). The nature of this phenomenon is unclear but $.O_2^-$ generation does not appear to be a consequence of the method of cell purification since eosinophils prepared on Percol or Metrizamide gradients spontaneously generated $.O_2^-$. The induction of peritoneal eosinophilia in guinea-pigs requires a repeated sensitization and boosting regime, usually to foreign proteins (horse serum or human albumin) or Polymixin B. It is likely, therefore, that the sensitization procedure itself caused partial activation of the recruited cells *in situ* and an enhanced reactivity *ex vivo*, with adherence or other interaction with the plate over the relatively long (45 min) incubation period contributing to the production of oxygen radicals in the absence of any other stimulus. In this regard, we have observed varying numbers of hypodense eosinophils banding on the gradient interface above the eosinophil pellet (unpublished data), suggesting *in situ* activation of at least one population of cells which was discarded from study in the experiments reported here (see Materials and Methods). However, this release of $.O_2^-$ proceeded in the absence of elevated intracellular Ca^{2+}, which has been implicated in eosinophil activation processes [26]. In addition, low concentrations of PAF (10 nM) induced a transient elevation (<0.1 to 1 μM) in Ca^{2+} (Fig. 4). Interestingly, the Ca^{2+} transient induced by PAF was markedly attenuated by rolipram (Fig. 6) and other PDE inhibitors (data not shown). Although Ca^{2+} is not necessarily required for $.O_2^-$ generation [26], which can proceed in the absence of any Ca^{2+} transients, these results suggest that Ca^{2+}-dependent eosinophil functions would be susceptible to inhibition by PDE inhibitors.

High concentrations of PAF (>1 μM) caused a short-lived increase in $.O_2^-$ generation (data not shown) and the effects of PDE inhibitors on this response are currently under investigation.

The potent inhibition of $.O_2^-$ generation from eosinophils by PDE inhibitors is likely to be due to elevation of cAMP since the membrane permeant analogue of cAMP, dibutyryl cAMP, and the β-agonist, salbutamol, also inhibited $.O_2^-$ release. Surprisingly, trequinsin, which was equipotent with rolipram and denbufylline in inhibiting eosinophil particulate cAMP PDE, only weakly reduced $.O_2^-$ generation and AH-21-132, another moderately potent PDE inhibitor, had no effect at concentrations up to 10 μM (Fig. 5). The reasons for these apparent anomalies are not known—one possible explanation is that another, as yet unidentified, mechanism mediates the actions of rolipram and denbufylline. Alternatively, access of trequinsin and AH-21-132 to the intracellular location of the cAMP PDE may be impeded. This latter notion is supported by the finding that, in contrast to denbufylline and rolipram, trequinsin and AH-21-132 only weakly enhanced isoprenaline-induced cAMP accumulation (Fig. 4). That AH-21-132 and trequinsin are comparatively weak effectors of the functions of intact eosinophils is surprising—trequinsin is a very potent inhibitor of platelet aggregation [30] and both compounds are more effective stimulators of endothelial cAMP accumulation than rolipram [6].

Although denbufylline and rolipram alone potently inhibited $.O_2^-$ generation (Fig. 5) they exerted only

a very weak stimulatory effect on cAMP in the absence of isoprenaline (Fig. 4), suggesting that only small changes in cAMP are needed to activate cAMP-dependent protein kinase with subsequent inhibition of $.O_2^-$ generation. This, indeed, was demonstrated to be the case since incubation of eosinophils with rolipram (50 μM), which elevated cAMP by only 47% (see Results, increased the activity ratio of the cAMP-dependent protein kinase almost maximally from 0.24 ± 0.84 (Table 3).

In summary, guinea-pig peritoneal eosinophils contain a particulate Ro-20-1724-inhibited (Type IV) cAMP PDE. Some, but not all, inhibitors of this enzyme reduced $.O_2^-$ generation, probably through a mechanism involving elevation of cAMP and activation of cAMP-dependent protein kinase. The failure of certain inhibitors to reduce $.O_2^-$ release and increase cAMP may be due to a barrier, possibly at the level of the plasma membrane, impeding their access to the enzyme.

Acknowledgements—The authors wish to express their thanks to Miss L. Scott for her excellent technical assitance, Dr T. J. Brown for helpful criticism of the work and Dr M. Palfreyman for synthesizing rolipram and AH-21-132. We also acknowledge the help of Dr M. Vlitos who wrote the computer program for analysing complex enzyme kinetics.

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