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Effects of phosphodiesterase inhibitors on human lung mast cell and basophil function

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1 The non-hydrolysable cyclic AMP analogue, dibutyryl (Bu_2)-cyclic AMP, inhibited the stimulated release of histamine from both basophils and human lung mast cells (HLMC) in a dose-dependent manner. The concentrations required to inhibit histamine release by 50% (IC_{50}) were 0.8 and 0.7 mM in basophils and HLMC, respectively. The cyclic GMP analogue, Bu_2-cyclic GMP, was ineffective as an inhibitor of histamine release in basophils and HLMC.

2 The non-selective phosphodiesterase (PDE) inhibitors, theophylline and isobutyl-methylxanthine (IBMX) inhibited the IgE-mediated release of histamine from both human basophils and HLMC in a dose-dependent fashion. IBMX and theophylline were more potent inhibitors in basophils than HLMC. IC_{50} values for the inhibition of histamine release were, 0.05 and 0.2 mM for IBMX and theophylline, respectively, in basophils and 0.25 and 1.2 mM for IBMX and theophylline in HLMC.

3 The PDE 4 inhibitor, rolipram, attenuated the release of both histamine and the generation of sulphopeptidoleukotrienes (sLT) from activated basophils at sub-micromolar concentrations but was ineffective at inhibiting the release of histamine and the generation of both sLT and prostaglandin D_2 (PGD_2) in HLMC. Additional PDE 4 inhibitors, denbufylline, Ro 20-1724, RP 73401 and nitraquazone, were all found to be effective inhibitors of mediator release in basophils but were ineffective in HLMC unless high concentrations (1 mM) were employed.

4 Neither 8-methoxymethyl IBMX (PDE 1 inhibitor), zaprinast (PDE 5 inhibitor) nor a range of PDE 3 inhibitors (siguazodan, SKF 94120, SKF 95654) were effective inhibitors of mediator release from either basophils or HLMC.

5 In basophils, rolipram acted to potentiate the inhibitory effects of the adenylate cyclase activator, forskolin, whereas in HLMC, rolipram failed to potentiate the inhibitory effects of forskolin.

6 Extracts of purified HLMC and basophils hydrolysed cyclic AMP. IBMX (100 μM) inhibited the PDE activity in basophil extracts by $67\pm7\%$ ($P<0.0001$) and in HLMC extracts by $63\pm9\%$ ($P<0.0005$). The hydrolysis of cyclic AMP by basophil extracts was inhibited by the selective PDE inhibitors (all at 10 μM), rolipram ($56\pm8\%$, $P<0.0001$) and the mixed PDE 3/4 inhibitor, Org 30029 ($47\pm9\%$, $P<0.01$), whereas 8-methoxymethyl IBMX, siguazodan and zaprinast were ineffective. In HLMC, rolipram, Org 30029, 8-methoxymethyl IBMX, siguazodan and zaprinast all inhibited the hydrolysis of cyclic AMP by extracts to a significant ($P<0.05$) and similar extent (approximately 25% inhibition at 10 μM).

7 In total, these data suggest that modulation of the PDE 4 isoform can regulate basophil responses whereas an association of the PDE 4 isoform with the regulation of HLMC function remains uncertain.

Keywords: Mast cells; basophils; phosphodiesterases; cyclic AMP; mediator release; rolipram

Introduction

The primary mechanism by which cyclic nucleotides are inactivated is thought to occur by the action of phosphodiesterases (PDEs). At least five and perhaps as many as eight different classes of PDE have been identified based on structural and functional criteria (Beavo & Reifsnyder, 1990; Beavo *et al*., 1994; Nicholson & Shahid, 1994). Of these the calcium/calmodulin-activated (PDE 1), the guanosine 3′:5′-cyclic monophosphate (cyclic GMP)-activated (PDE 2), the cyclic GMP-inhibited (PDE 3), the adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-specific (PDE 4) and the cyclic GMP-specific (PDE 5) classes are the best characterized.

A variety of inhibitors of PDEs are in existence (Beavo & Reifsnyder, 1990; Nicholson *et al*., 1991). These fall into two major categories: (a) classical non-specific inhibitors of PDE activity, such as theophylline and isobutyl-methylxanthine (IBMX) and (b) selective inhibitors of PDE isoenzymes. Included in this latter class are compounds such as zaprinast (PDE 5 inhibitor), SKF 94120 (PDE 3 inhibitor) and rolipram (PDE 4 inhibitor). Selective inhibitors can be particularly valuable in attempts to identify and to characterize PDE isoforms that may be resident in either tissues or cell types (Torphy & Undem, 1991). In this context, a large number of studies employing isoform-selective drugs has emerged aimed at identifying the prominent isoform of PDE that regulates a given process. For example, functional studies in which isoform-selective inhibitors were used have shown that both PDE 3 and PDE 4 may be important in relaxing airways smooth muscle and that PDE 4 is involved in regulating the activity of a number of inflammatory cell types (Beavo & Reifsnyder, 1990; Nicholson & Shahid, 1994). From the therapeutic standpoint, these studies may be potentially important as they could serve to identify targets at which novel drugs might be directed. Within the framework of this endeavour, a determination of the isoforms of PDE regulating the responses of those cells more intimately involved in allergic responses could be informative.

It has been recognized for some time that methylxanthines can inhibit the release of histamine from basophils (Lichtenstein & Margolis, 1968). A large number of studies has consequently demonstrated that compounds such as theophylline

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and IBMX are inhibitory to basophil and human lung mast cell (HLMC) function and, indeed, several studies have investigated the effects of selective inhibitors of PDE isoforms in basophils (Frossard *et al*., 1981; Peachell *et al*., 1992). These studies indicate that the cyclic AMP-specific PDE (PDE 4) is the major isoform that regulates basophil function. Additional studies in a variety of alternative inflammatory cell types indicate that PDE 4 is the predominant isoform of PDE in these cells. For example, PDE 4 has been shown to regulate responses in eosinophils (Dent *et al*., 1991; Souness *et al*., 1991; Hatzelman *et al*., 1995), macrophages (Turner *et al*., 1993), mononuclear cells (Essayan *et al*., 1994; Hichami *et al*., 1995), and neutrophils (Nielson *et al*., 1990). In the present work, we have performed a comparative study between basophils and HLMC to determine the effects of non-selective and selective PDE inhibitors on these cells. Our data reinforce the notion that PDE 4 is the major isoform in basophils. However, the PDE 4 class does not appear to regulate HLMC responses.

Methods

Buffers

Phosphate buffered saline (PBS) was employed in these studies. −PBS contained (mM): NaCl 137, $Na_2HPO_4.12H_2O$ 8, KCl 2.7, KH_2PO_4 1.5. PBS was −PBS which additionally contained: $CaCl_2.2H_2O$ 1 mM, $MgCl_2.6H_2O$ 1 mM, glucose 5.6 mM, bovine serum albumin (BSA) 1 mg ml^{-1} and DNase 15 μg ml^{-1}. +PBS was −PBS additionally supplemented with: $CaCl_2.2H_2O$ 1 mM, $MgCl_2.6H_2O$ 1 mM, glucose 5.6 mM and human serum albumin (HSA) 30 μg ml^{-1}. PBS-EDTA was −PBS supplemented with EDTA (1 mM). The pH of all PBS buffers was titrated to 7.3.

PAG contained (mM): PIPES 22, NaCl 110, KCl 5, glucose 5.6. HSA (30 μg ml^{-1}) was also added. The pH was titrated to 7.3.

Hypotonic lysis buffer contained: Tris 50 mM, EDTA 1 mM, EGTA 0.1 mM, dithiothreitol (DTT) 0.5 mM, phenylmethylsulphonyl fluoride (PMSF) 50 μg ml^{-1}, soybean trypsin inhibitor 50 μg ml^{-1}, leupeptin 5 μg ml^{-1}, aprotinin 5 μg ml^{-1} and Triton X-100 0.1% (v:v). The pH was titrated to 8.0.

Preparation of inhibitors

Theophylline (6 mM), IBMX (2 mM), SKF 94120 (5-[4-acetamidophenyl]-2-[1H]-pyrazinone) (1 mM), SKF 95654 (5-methyl-6-[4-4-oxo-1,4-dihydropyridin-1-yl)phenyl]-4,5-dihydro-3(2H)-pyridazinone) (1 mM), siguazodan (1 mM), dibutyryl (Bu_2)-cyclic AMP (10 mM) and Bu_2-cyclic GMP (10 mM) were prepared fresh daily by being directly dissolved in buffer. Nitraquazone, (−)-rolipram, denbufylline, RP 73401 (3-cyclopentyloxy-N-[3,5-dichloro-4-pyridyl]-4-methoxybenzamide), Ro 20-1724 (4-[3-butoxy-4-methoxy-benzyl]-2-imidazolidinone), Org 30029 (N-hydroxy-5,6-dimethoxy-benzo[b]thiophene-2-carboximidamide HCl) and 8-methoxymethyl IBMX (all at 100 mM) were dissolved in dimethyl sulphoxide (DMSO) and stored frozen in appropriate aliquots. Zaprinast (100 mM) was dissolved initially in 1 M NaOH and then further diluted in buffer to give a stock solution (1 mM) that was stored at 4°C and prepared weekly. Forskolin (10 mM) was dissolved in ethanol and stored at −20°C. Just before use, a small aliquot of this stock solution was removed and diluted appropriately.

Isolation and purification of human basophils

Mixed leukocyte preparations were obtained from whole blood by dextran sedimentation. Briefly, 50 ml of venous blood was mixed with 12.5 ml of 6% dextran and 5 ml of 100 mM EDTA, then allowed to sediment for 90 min at room temperature. The upper buffy coat layer was removed, cells were recovered by centrifugation ($120 \times g$, 8 min) and washed twice with PBS. These mixed cell preparations were used in some of the histamine release experiments.

Basophil-enriched preparations were obtained by Percoll density gradient centrifugation. Briefly, either whole venous or buffy coat (provided by the National Blood Service Trent Centre) blood was layered over a two-step discontinuous Percoll gradient consisting of 15 ml of 62% Percoll overlaid with 15 ml of 53% Percoll prepared in 50 ml 'Leucosep' tubes (Greiner, Dursley, U.K.) and centrifuged ($250 \times g$, 15 min). A basophil-rich layer (5–15% purity) located 1 cm above the 53% and 62% interface was harvested. These cells were purified further by immunomagnetic bead separation. The basophil-rich fraction (containing $3-5 \times 10^6$ basophils) was washed twice in PAG and once in PBS-EDTA, resuspended in PBS-EDTA (2×10^6 basophils 100 μl^{-1}) and incubated (1 h) over ice with monoclonal (IgG_{2A}) mouse anti-human IgE (50 μg ml^{-1}). Cells were then washed twice with PBS-EDTA over ice and incubated (30 min) in PBS-EDTA (2×10^6 basophils 100 μl^{-1}) containing Dynal magnetic beads coated with a rat anti-mouse IgG_{2A} antibody at a ratio of beads to cells of 4 to 1. The magnetic fraction was harvested, by a Dynal MPC-1 magnet, and washed (5×1 ml) with ice cold PBS-EDTA and the magnetically adherent cells counted with alcian blue to determine basophil purities (Gilbert & Ornstein, 1975). This fraction typically contained $1-3 \times 10^6$ basophils at purities of 80 to 99%. Although the possibility exists that basophils may have been activated by this method of purification, all procedures were carried out in the cold and preparation of extracts, for use in PDE assays, was carried out immediately after purification.

Isolation and purification of HLMC

Mast cells were isolated from human lung tissue by a modification of the method described by Ali and Pearce (1985). Macroscopically normal tissue from lung resections of patients with carcinoma was stripped of its pleura and chopped vigorously for 15 min with scissors in a small volume of −PBS buffer. The chopped tissue was washed over a nylon mesh (100 μm pore size; Cadisch and Sons, London, U.K.) with 0.5–1 l of −PBS buffer to remove lung macrophages. The tissue was reconstituted in PBS (10 ml g^{-1} tissue) containing collagenase Ia (350 u ml^{-1} PBS) and agitated by using a water-driven magnetic stirrer immersed in a water bath set at 37 °C. The supernatant (containing some HLMC) was separated from the tissue by filtration over nylon mesh. The collagenase-treated tissue was then reconstituted in a small volume of PBS buffer and disrupted mechanically with a syringe. The disrupted tissue was then washed over nylon gauze with PBS (300–600 ml). The pooled filtrates were sedimented ($120 \times g$, room temperature, 8 min), the supernatant discarded and the pellets reconstituted in PBS (100 ml). The pellet was washed a further two times. HLMC were visualized by microscopy with an alcian blue stain (Gilbert & Ornstein, 1975). Of the total cells, 3–13% were mast cells. This method generated 2 to 9×10^5 HLMC g^{-1} tissue. HLMC prepared in this manner were used in mediator release experiments. For PDE assays, HLMC were purified further by immunomagnetic bead separation. The protocol (i.e. incubation times, buffers, cell numbers) for immunomagnetic bead separation was essentially the same as that described for basophils except that a monoclonal (IgG_1) anti-c-kit antibody and Dynal beads coated with rat anti-mouse IgG_1 were employed. These methods generated HLMC purities of 72 to 95% with cell yields of between 1 to 3×10^6 HLMC.

Mediator release

Histamine release experiments were performed in +PBS buffer. Histamine release was initiated immunologically with anti-IgE. Lower concentrations of anti-IgE are required to obtain optimal levels of secretion in basophils (1 : 3000) as compared

to HLMC (1 : 300). Secretion was allowed to proceed for 45 (basophils) or 25 (HLMC) min at 37°C after which time the cells were pelleted by centrifugation ($400 \times g$, room temperature, 3 min). Histamine released into the supernatant was determined by a modification (Ennis, 1991) of the automated fluorometric method of Siraganian (1974) and, when appropriate, an aliquot of the supernatant was removed and stored frozen for either sulphopeptidoleukotrienes (sLT) or prostaglandin D_2 (PGD_2) analysis by enzyme immunoassay (EIA). When inhibitors were employed, the drugs were incubated with cells at 37°C for 15–20 min, as indicated in the text, before the addition of stimulus and then samples were processed as indicated above. Total histamine content was determined by lysing aliquots of the cells with 1.6% perchloric acid. Cells incubated in buffer alone served as a measure of spontaneous histamine release (<6%). Histamine release was thus expressed as a percentage of the total histamine content after the spontaneous histamine release had been subtracted. In experiments with HLMC in which high ($\geqslant 100$ μM) concentrations of some of the PDE inhibitors were used and which were prepared as stock solutions in DMSO, the vehicle by itself also inhibited mediator release. In all experiments, therefore, DMSO dilutions were also included and the effect of DMSO dilutions on mediator release was determined. In all the data presented, any inhibitory effects of DMSO have been subtracted.

Assay for PDE activity

Hydrolysis of cyclic AMP by extracts of purified basophils and HLMC was determined essentially according to methods which have been described elsewhere (Reeves *et al*., 1987). Cell extracts wre prepared by treatment of purified cells with a hypotonic lysis buffer (Fruman *et al*., 1992). These extracts were used in PDE assays. Typically, the reaction was conducted in 0.1 ml of a mixture containing 50 mM Tris HCl (pH 7.5), 5 mM $MgCl_2$, 50 μM [^{14}C]-5′-AMP (400 d.p.m. nmol^{-1}) as carrier and to determine percentage recovery of product, 1 μM [^{3}H]-cyclic AMP (4000 d.p.m. pmol^{-1}) and cell extract ($0.05-0.1 \times 10^6$ cell equivalents) with or without an inhibitor of PDE. The reaction was initiated by the addition of enzyme and was terminated after 30 min by placing reaction vessels in a 100°C heating block followed by transfer to an ice bath. Cyclic nucleotide substrates were separated from 5′-nucleotide products by adding 0.5 ml of 0.1 M HEPES buffer (pH 8.5) containing 0.1 M NaCl to each sample and the whole sample applied to a polyacrylamide-boronate gel column (0.5 g Affi-gel 601) previously equilibrated in the HEPES, NaCl buffer. The unreacted cyclic nucleotides were eluted with 10 ml of the HEPES, NaCl buffer and the 5′-monophosphate products were eluted with 10 ml of 0.25 M acetic acid into a scintillation vial containing 10 ml of Lumasafe (Lumac LSC, Groningen, The Netherlands) scintillation cocktail. Radioactivity was measured by scintillation spectrometry. Recovery of [^{14}C]-5′-AMP carrier was 65 to 85%. An identical protocol was used to assess cyclic GMP PDE activity except that [^{3}H]-cyclic GMP was used rather than radiolabelled cyclic AMP.

Materials

The following were purchased from the sources indicated; anti-human IgE, Bu_2-cyclic AMP, Bu_2-cyclic GMP, BSA, cyclic AMP, cyclic GMP, collagenase, DMSO, HSA, forskolin, IBMX, theophylline, PIPES (free acid), Percoll, EGTA, DTT, aprotinin, PMSF, leupeptin, soybean trypsin inhibitor and Triton X-100 (all Sigma, Poole, U.K.); EDTA, calcium chloride and magnesium chloride (BDH, Poole, U.K.); 8-methoxymethyl IBMX (LC Laboratories, Woburn, U.S.A.); Ro 20-1742 (Biomol Research Labs, Plymouth Meeting, U.S.A.); dextran (Pharmacia, Nottingham, U.K.); Affi-gel 601, Tris (Bio-Rad, Hemel Hempstead, U.K.); monoclonal (IgG_1) mouse anti-human c-kit, monoclonal (IgG_{2A}) mouse anti-human IgE (Immunotech, Marseilles, France); magnetic beads coated with rat anti-mouse IgG_{2A} or IgG_1 antibody (Dynal, Wirral, U.K.); enzyme immunoassay (EIA) kits for PGD_2 (Cayman Chemicals Company, Michigan, U.S.A.); EIA kits for the sulphopeptideoleukotrienes (sLT), LTC_4, LTD_4 and LTE_4, [^{14}C]-5′-AMP, [^{3}H]-cyclic AMP, [^{3}H]-cyclic GMP (Amersham, Little Chalfont, U.K.).

The following items were gifts: zaprinast, SKF 94120, SKF 95654, siguazodan and denbufylline (Dr J.R.S. Arch, SKB); (−)-rolipram, nitraquazone and RP 73401 (Dr R.G. Sturton, Bayer); Org 30029 (Dr C.D. Nicholson, Organon).

Statistics

The statistical significance of drug-related effects was analysed by comparing control and treated cells by use of Student's t test for paired data. Values were considered significant at the $P<0.05$ level.

Results

Effects of PDE inhibitors on mediator release

In an initial series of experiments, the effects of the cell-permeant, non-hydrolysable cyclic nucleotide analogues, dibutyryl (Bu_2)-cyclic AMP and Bu_2-cyclic GMP on the IgE-mediated release of histamine from basophils and HLMC were determined (Figure 1). The data indicate that Bu_2-cyclic AMP inhibited histamine release with approximate IC_{50} values of 0.8 and 0.7 mM from basophils and HLMC respectively whereas Bu_2-cyclic GMP was ineffective in both cell types.

The classical, non-specific PDE inhibitors, IBMX and theophylline, inhibited the IgE-mediated release of histamine from both basophils and HLMC (Figure 2). IBMX was a more potent inhibitor than theophylline of IgE-mediated histamine release in both cell types and the effects of both PDE inhibitors were more pronounced in basophils than in HLMC. IC_{50} values for the IBMX inhibition of histamine release from basophils and HLMC were 0.05 and 0.25 mM, respectively. IC_{50} values for the theophylline inhibition of histamine release from basophils and HLMC were 0.2 and 1.2 mM, respectively.

Selective inhibitors of PDE were also studied. Previous work in basophils had demonstrated that rolipram (PDE 4 inhibitor) was an effective inhibitor of histamine release whereas neither zaprinast (PDE 5 inhibitor) nor SKF 95654 (PDE 3 inhibitor) was effective (Peachell *et al*., 1992). In HLMC, rolipram, SKF 95654 and zaprinast were not effective as inhibitors of the IgE-mediated release of histamine (Figure 3). Because the PDE 4 isoform has been shown to be important in the regulation of the activity of a number of different inflammatory cell types, further studies were performed with additional PDE 4-selective inhibitors. Rolipram, denbufylline and Ro 20-1724 were found to inhibit histamine release from basophils (Figure 4). However, Ro 20-1724 was approximatley one hundred fold less active than either rolipram or denbufylline. All three compounds were ineffective in HLMC except at very high (1 mM) concentrations (Figure 4).

The effectiveness of a drug to inhibit histamine release from both basophils and HLMC can often be inversely dependent on the level of control release. Thus, higher levels of histamine release are, often, less well modulated by an inhibitor. Because optimal releasing concentrations of anti-IgE had been used in the previous experiments, further studies were performed to determine whether sub-optimal levels of control release, induced by a variety of anti-IgE concentrations, could be modulated more effectively by either IBMX or rolipram in both basophils and HLMC. The data indicate that the inhibitory effects of IBMX were sensitive to the level of control release in both cell types (data not shown). The effectiveness of rolipram to inhibit histamine release in basophils was also influenced by the magnitude of the control secretion (Figure 5). Thus, although the reduction in histamine release by rolipram was similar at all concentrations of anti-IgE, this translated as

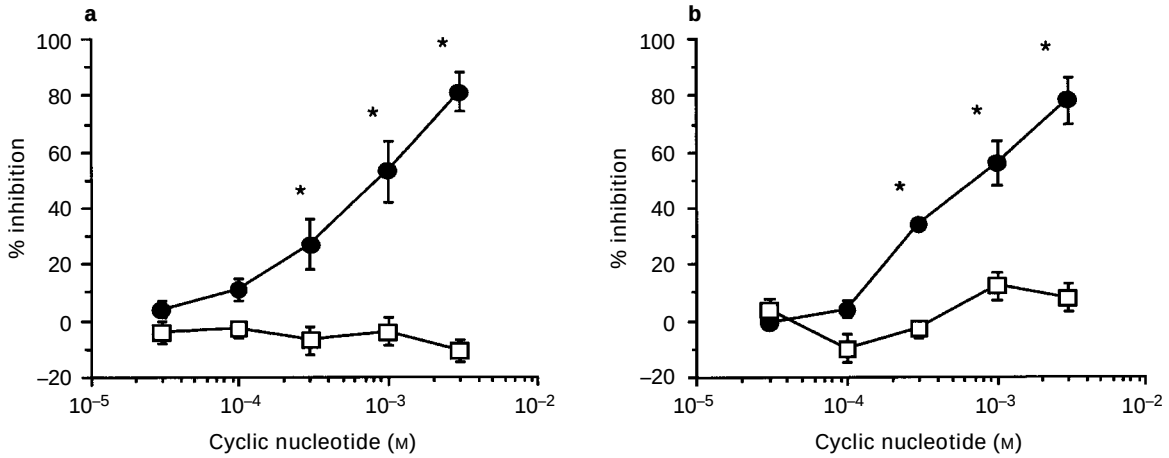

Figure 1 Effect of dibutyryl Bu_2-cyclic AMP (●) and Bu_2-cyclic GMP (□) on basophils (a) and HLMC (b). Cells were incubated for 20 min with either of the cyclic nucleotide analogues before challenge with anti-IgE (1 : 3000, basophils; 1 : 300, HLMC). Histamine release was allowed to proceed for 45 (basophils) or 25 (HLMC) min. Results are expressed as the % inhibition of the control histamine releases which were 48±4% (basophils) and 26±7% (HLMC). Statistically significant ($P<0.05$ at least) levels of inhibition are indicated by an asterisk. Values are means, $n=4$ (basophils) and $n=5$ (HLMC); vertical lines show s.e.mean.

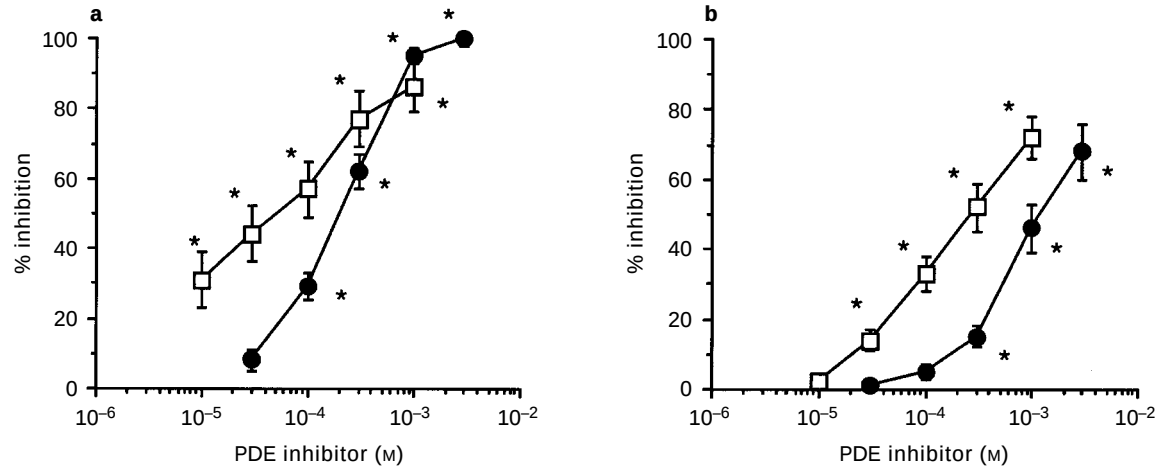

Figure 2 Effect of non-selective PDE inhibitors on histamine release. The effects of either IBMX (□) or theophylline (●) on the release of histamine from basophils (a) or HLMC (b) were determined. Cells were incubated for 15 min with a PDE inhibitor before challenge with anti-IgE (1 : 3000, basophils; 1 : 300, HLMC) for a further 45 (basophils) or 25 (HLMC) min. Results are expressed as the % inhibition of the control releases which were 41±8% (basophils) and 31±5% (HLMC). Statistically significant ($P<0.05$ at least) levels of inhibition are indicated by an asterisk. Values are means, $n=5$ (basophils) and $n=7$ (HLMC); vertical lines show s.e.mean.

greater levels of inhibition at lower levels of control release. For example, histamine release from basophils induced by a low (1 : 100000) concentration of anti-IgE was inhibited by rolipram by 40±3% whereas a higher (1 : 3000) concentration of anti-IgE, inducing higher levels of secretion, was inhibited by 17±4%. In HLMC, rolipram was ineffective even at low levels of control histamine release (Figure 5).

Although rolipram was found to be ineffective as an inhibitor of histamine release in HLMC, the possibility that rolipram might act to enhance the inhibitory response of an adenylate cyclase activator was investigated in both HLMC and basophils. In basophils (Figure 6a), rolipram enhanced the inhibitory response to forskolin in a greater than additive fashion, whereas in HLMC (Figure 6b), rolipram was less effective at enhancing the forskolin inhibition of histamine release. In contrast, IBMX was as effective at enhancing the inhibitory effects of forskolin in both basophils and HLMC. In basophils, forskolin (10 μM) inhibited histamine release by 14±4%, IBMX (10 μM) by 19±4% and both compounds in combination by 48±3% ($n=4$). In HLMC, forskolin (10 μM) inhibited histamine release by 13±2%, IBMX (30 μM) by 10±5% and both compounds together by 41±6% ($n=4$).

Previous studies in basophils indicated that a combination of a PDE 3 inhibitor (SKF 94120) with a PDE 4 (rolipram) inhibitor could cause synergistic increases in the extent of inhibition of histamine release (Peachell *et al*., 1992). In the present study, a combination of SKF 94120 (1 μM) with rolipram (30 μM) was ineffective at inhibiting histamine release in HLMC (inhibition with SFK 94120 alone, 2±5%.; rolipram alone, 14±5%; SKF 94120 plus rolipram, 8±5%; $n=5$). Similar results were obtained with alternative PDE 3 inhibitors such as siguazodan with and without rolipram (data not shown). Alternative combinations of PDE inhibitors (zaprinast + SKF 94120; zaprinast + rolipram; zaprinast + rolipram + SKF 94120) were all ineffective at inhibiting IgE-mediated histamine release from HLMC (data not shown).

It has previously been demonstrated that the generation of products of arachidonic acid from stimulated HLMC and basophils is more effectively modulated by cyclic AMP-active compounds than the release of histamine (Peachell *et al*., 1988; Undem *et al*., 1988). The effects of a wide variety of PDE inhibitors were assessed on the release of histamine and the generation of sLT in basophils (Table 1) and the release of histamine and the liberation of both sLT and PGD_2 in HLMC (Table 2). The data indicate that not one of the selective PDE

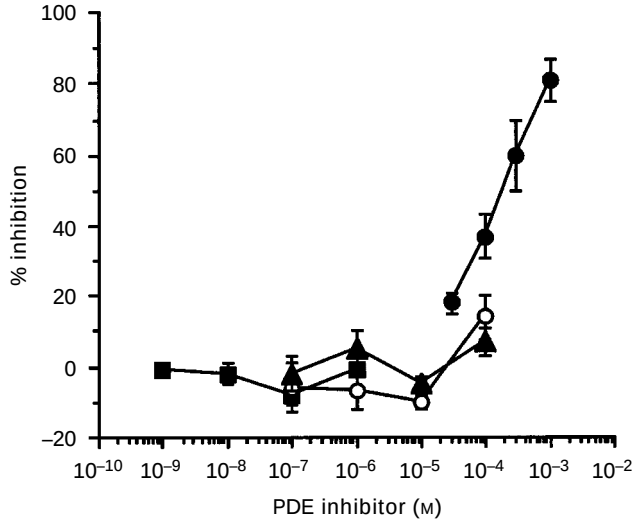

Figure 3 Effects of selective PDE inhibitors on HLMC. HLMC were incubated (15 min) with either rolipram (○), zaprinast (▲), SKF 95654 (■) or IBMX (●) before challenge with anti-IgE (1 : 300) for a further 25 min. Values are expressed as the % inhibition of the control histamine release which was 33±6%. Statistically significant ($P<0.05$ at least) levels of inhibition were obtained at all concentrations of IBMX. Values are means, $n=4$; vertical lines show s.e.mean.

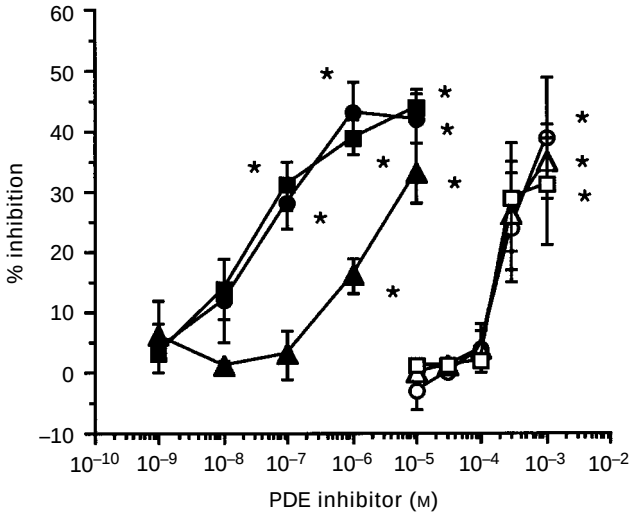

Figure 4 Effects of selective PDE 4 inhibitors on HLMC and basophils. Basophils (solid symbols) or HLMC (open symbols) were incubated (15 min) with either rolipram (●, ○), Ro 20-1724 (▲, △), or denbufylline (■, □) before challenge with anti-IgE (1 : 3000, basophils; 1 : 300, HLMC) for a further 45 (basophils) or 25 (HLMC) min. Values are expressed as the % inhibition of the control histamine releases which were 38±5% (basophils) and 30±6% in HLMC. Statistically significant ($P<0.05$ at least) levels of inhibition are indicated by an asterisk. Values are means, $n=6$ (basophils) and $n=4$ (HLMC); vertical lines show s.e.mean.

inhibitors affected either the release of histamine or the generation of sLT from HLMC to a significant ($P>0.05$) extent. Whereas the majority of the PDE inhibitors tested had no effect on the generation of PGD_2 from HLMC, compounds with activity at PDE 3 (siguazodan and Org 30029) inhibited PGD_2 generation from activated HLMC. All of the PDE 4 inhibitors and the mixed PDE 3/4 inhibitor, Org 30029, inhibited the release of histamine and the generation of sLT from basophils.

Cyclic nucleotide PDE activity in cell extracts

Extracts of purified HLMC and basophils were found to hydrolyse cyclic AMP. The PDE activity present in extracts of both cell types was inhibited dose-dependently and equipotently (IC_{50} value of 0.04 mM for both cell types) by IBMX (Figure 7). In basophils, rolipram and Org 30029 both inhibited PDE activity to a significant ($P<0.05$) extent whereas 8-methoxymethyl IBMX, zaprinast and siguazodan were ineffective (Figure 8). In contrast, the PDE activity in HLMC was inhibited to a significant ($P<0.05$) and to a similar degree by 8-methoxymethyl IBMX, zaprinast, siguazodan, rolipram and Org 30029. Previous studies indicated that extracts of basophils contain modest levels of cyclic GMP hydrolytic activity (Peachell *et al.*, 1992). In the present study, in two of three experiments, essentially negligible levels of cyclic GMP hydrolysis were detected in extracts derived from purified HLMC and in the third experiment 0.17 pmol of cyclic GMP were hydrolysed per min by 10^6 HLMC. In all three experiments investigating the potential hydrolysis of cyclic GMP, the same HLMC extracts hydrolysed cyclic AMP (mean±s.e.mean, 0.9±0.1 pmol cyclic AMP hydrolysed min^{-1} by 10^6 HLMC).

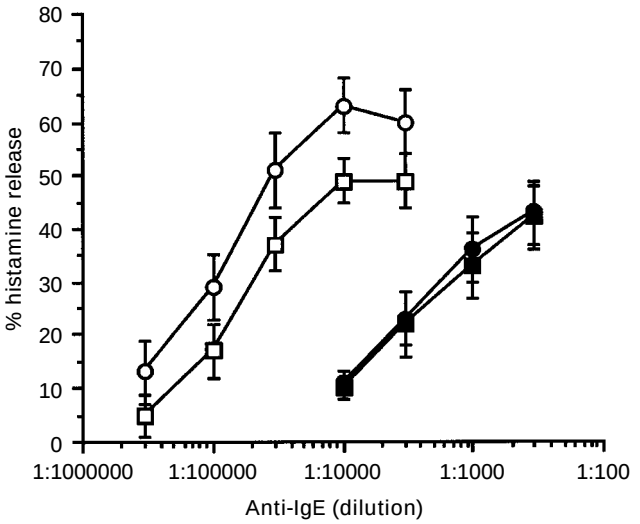

Figure 5 Effect of rolipram on sub-optimal levels of mediator release. Basophils (open symbols) or HLMC (solid symbols) were incubated (15 min) either with (□, ■) or without (○, ●) rolipram (30 μM) before challenge with a range of anti-IgE concentrations for a further 25 (HLMC) or 45 (basophils) min. Statistically significant ($P<0.05$) reductions in basophil histamine release were obtained for rolipram at all concentrations of anti-IgE. Values are means, $n=7$ (basophils) and $n=9$ (HLMC); vertical lines show s.e.mean.

Discussion

A large number of studies indicates that PDE 4 is important in regulating the activity of a wide variety of inflammatory cell types (Barnes, 1995). A major aim of the present study was to establish whether PDE 4 is important in regulating responses in HLMC and basophils.

Initial studies in which non-selective PDE inhibitors were employed indicated that both IBMX and theophylline inhibit the stimulated release of histamine in a dose-dependent manner from both basophils and HLMC. These data suggest that inhibition of PDE can lead to the attenuation of secretory responses in both cell types. Inhibition of PDE would be expected to cause increases in cyclic AMP and alternative studies indicate that treatment of either basophils or HLMC with IBMX causes intracellular elevations in cyclic AMP (Peachell *et al.*, 1988). However, both of the non-selective PDE inhibitors were approximately five to six fold more potent in

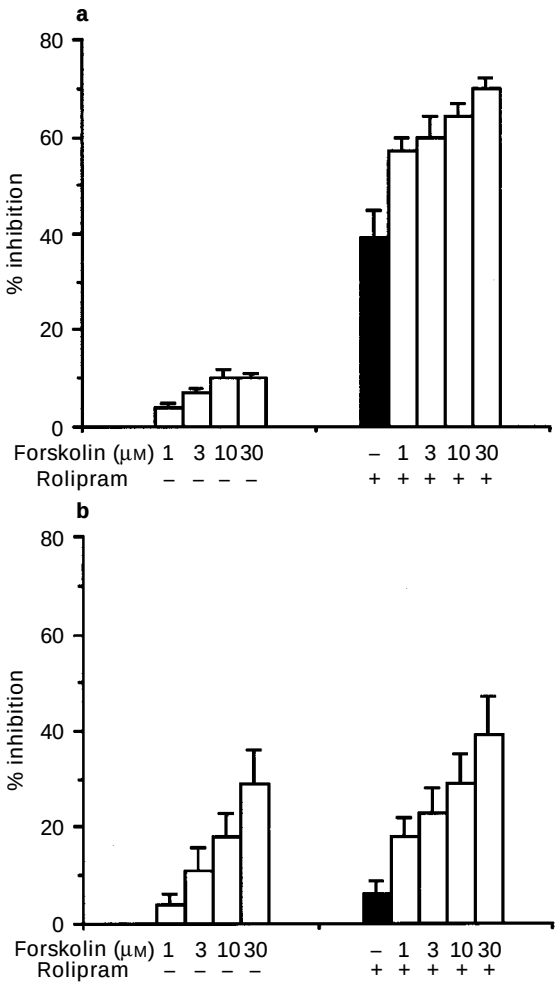

Figure 6 Effect of rolipram on inhibition by forskolin of histamine release from basophils (a) and HLMC (b). Cells were incubated with (+) or without (−) rolipram (0.3 μM, basophils; 30 μM, HLMC) for 10 min and then for an additional 15 min with or without forskolin before challenge with anti-IgE (1:3000, basophils; 1:300, HLMC) for a further 45 (basophils) or 25 (HLMC) min. Values are expressed as the % inhibition of the control histamine releases which were 60±4% in basophils and 40±8% in HLMC. Values are means± s.e.mean, $n=4$ (basophils) and $n=6$ (HLMC).

Table 1 Effects of PDE inhibitors on the release of histamine and the generation of sulphopeptideleukotrienes (sLT) from basophils

		% inhibition	
Inhibitor	*Class*	*Histamine*	*sLT*
Theophylline	NS	28±5*	35±7*
IBMX	NS	62±6*	71±13*
8-Me-IBMX	1	4±2	0±5
Siguazodan	3	5±2	−7±3
Rolipram	4	40±8*	44±12*
RP 73401	4	29±5*	35±11*
Nitraquazone	4	44±7*	54±8*
Zaprinast	5	6±1	−8±4
Org 30029	3/4	32±7*	49±9*

Basophils were incubated with a given PDE inhibitor for 15 min before challenge with anti-IgE (1:30000) for 45 min to induce histamine release. All of the selective inhibitors were used at a concentration of 10 μM, except theophylline and IBMX which were used at 100 μM. Results are expressed as the % inhibition of the control histamine release which was 38±9% and the control sLT generation which was 5.4±0.2 ng per 10^6 basophils. Asterisks indicate statistically significant ($P<0.05$) levels of inhibition. Values are means±s.e.mean, $n=4$. NS stands for non-selective. 8-Me-IBMX, stands for 8-methoxymethyl IBMX.

basophils than in HLMC. These data may suggest that basophils are more readily modulated by cyclic AMP. However, this contention is not supported by studies with the non-hydrolysable analogue of cyclic AMP, Bu_2-cyclic AMP, which was equipotent as an inhibitor of histamine release in basophils and HLMC.

Previous studies in basophils, in which a number of selective inhibitors of PDE were employed, indicated that rolipram, a PDE 4 inhibitor, was the only compound capable of inhibiting the stimulated release of histamine from basophils (Peachell *et*

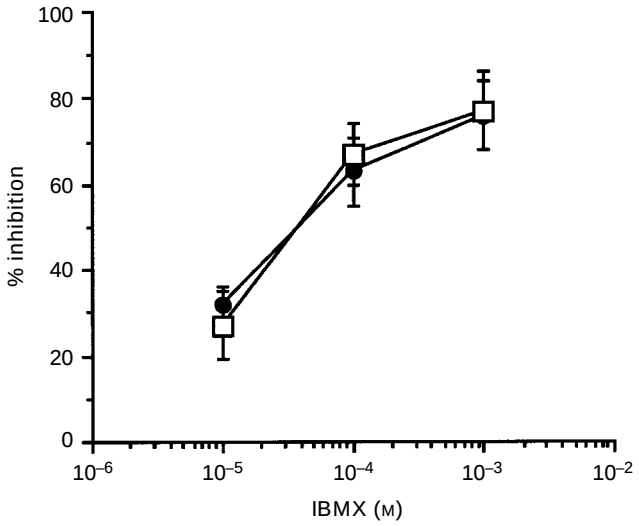

Figure 7 Effect of IBMX on PDE activity in extracts of either basophils (□) or HLMC (●). Extracts of cells were prepared by hypotonic lysis and the effects of IBMX on the PDE activity were determined. Results are expressed as the % inhibition of the control PDE activities which were 0.8±0.1 pmol cyclic AMP hydrolysed by 10^6 basophils and 1.5±0.3 pmol cyclic AMP hydrolysed by 10^6 HLMC. All values are statistically significant ($P<0.05$). Values are means, $n=5$ (basophils) and $n=6$ (HLMC); vertical lines show s.e.mean. Mean purities were 90±4% (basophils) and 86±5% (HLMC).

Table 2 Effects of PDE inhibitors on the release of histamine and the generation of both sulphopeptideleukotrienes (sLT) and PGD_2 from HLMC

		% inhibition		
Inhibitor	*Class*	*Histamine*	*sLT*	*PGD_2*
Theophylline	NS	18±3	27±3*	28±9*
IBMX	NS	46±7*	98±1*	92±2*
8-Me-IBMX	1	4±3	6±4	14±4
Siguazodan	3	6±3	22±11	26±6*
Rolipram	4	3±2	7±4	1±1
RP 73401	4	11±3	16±9	7±6
Nitraquazone	4	8±3	39±22	0±1
Zaprinast	5	10±4	11±7	11±7
Org 30029	3/4	2±2	25±11	38±9*

HLMC were incubated with a given PDE inhibitor for 15 min before challenge with anti-IgE (1:1000) for 25 min to induce histamine release. All of the selective inhibitors were used at a concentration of 10 μM, except theophylline and IBMX which were used at 100 μM. Results are expressed as the % inhibition of the control histamine release which was 40±5%, the control sLT generation which was 6.8±0.9 ng per 10^6 HLMC and the control PGD_2 generation which was 165±55 ng per 10^6 HLMC. Asterisks indicate statistically significant ($P<0.05$) levels of inhibition. Values are means ±s.e.mean, $n=6$ (histamine) and $n=4$ (sLT and PGD_2). NS, stands for non-selective. 8-Me-IBMX, stands for 8-methoxymethyl IBMX.

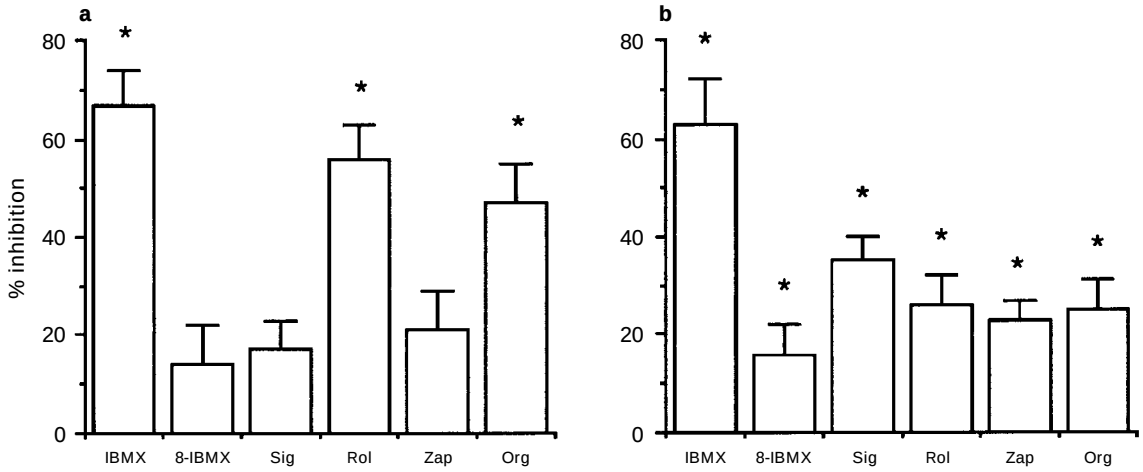

Figure 8 Effect of PDE inhibitors on cyclic AMP hydrolysis in extracts of either basophils (a) or HLMC (b). Extracts of cells were prepared by hypotonic lysis and the effects of IBMX (100 μM) and selective inhibitors (all at 10 μM) 8-methoxymethyl IBMX (8-IBMX), siguazodan (Sig), rolipram (Rol), zaprinast (Zap) and Org 30029 (Org) on the PDE activity were determined. Results are expressed as the % inhibition of the control PDE activities which were 1.1 ± 0.3 pmol cyclic AMP hydrolysed by 10^6 basophils and 1.5 ± 0.2 pmol cyclic AMP hydrolysed by 10^6 HLMC. Statistically significant ($P < 0.05$ at least) levels of inhibition are indicated by an asterisk. Values are means $\pm$ s.e.mean, $n = 5$ (basophils) and $n = 6$ (HLMC). Mean purities were $91 \pm 4\%$ (basophils) and $85 \pm 4\%$ (HLMC).

al., 1992). In the present study, none of the selective inhibitors employed, including rolipram, had any effect on the release of histamine from HLMC unless very high (1 mM) concentrations were used. These findings differ from studies in the guinea-pig in which rolipram was found to inhibit the antigen-induced release of mediators from tracheal mast cells (Underwood *et al*., 1993).

In addition to rolipram, alternative PDE 4-selective compounds were assessed for effects on histamine release from HLMC and basophils. HLMC were globally unresponsive to all of the PDE 4 inhibitors tested, whereas these same compounds were all effective inhibitors of histamine release in basophils. Interestingly, Ro 20-1724 was a hundred fold less potent than rolipram as an inhibitor of histamine release from basophils. Differences in the relative potencies of PDE 4-selective inhibitors have been obtained in different systems. Most notably, the PDE 4-selective inhibitor, RP 73401, has been shown to be, respectively, three fold and seventy fold more potent than rolipram as an inhibitor of eosinophil (Souness *et al*., 1995) and monocyte (Souness *et al*., 1996) function. The suggestion has been made that differences in the relative potencies of PDE 4-selective inhibitors in a variety of inflammatory cells may be due to differences in either the conformational nature of PDE 4 or the complement of PDE 4 subtypes in the cell (Barnette *et al*., 1995; Souness *et al*., 1996).

Further strategies were employed to determine whether rolipram might act to inhibit responses in HLMC under appropriate conditions. For example, mediator release from HLMC induced by sub-optimal concentrations of stimulus was unaffected by rolipram. In contrast, rolipram was a more effective inhibitor of mediator release from basophils when sub-optimal concentrations of stimulus were used to induce secretion. Moreover, in HLMC, rolipram did not enhance the inhibitory effects on histamine release of the adenylate cyclase activator, forskolin. In contrast, forskolin was a more effective inhibitor of secretion in basophils when used in the presence of rolipram. Again, these data argue against a role for PDE 4 in the regulation of HLMC responses.

It has been demonstrated that cyclic AMP-active compounds can inhibit the stimulated generation of products of arachidonic acid more potently than the release of histamine in both HLMC and basophils (Peachell *et al*., 1988; Undem *et al*., 1988). The effects, therefore, of a number of PDE 4 inhibitors and additional selective PDE inhibitors on the release of histamine and the generation of both sLT and PGD_2 in HLMC was assessed. None of the PDE inhibitors tested had any significant effect on the generation of sLT and the release of histamine from HLMC. In contrast, all the PDE 4 inhibitors tested and the mixed PDE 3/4 inhibitor, Org 30029, were effective inhibitors of sLT generation and histamine release from basophils. These data further argue against a role for PDE 4 in the regulation of HLMC responses. In point of fact, it would seem more likely that PDE 3 is important in regulating HLMC responses because siguazodan and Org 30029, compounds with activity directed at PDE 3, attenuated PGD_2 generation from stimulated HLMC. These data may suggest that PDE 3 is closely coupled to the regulation of PGD_2 generation although, clearly, rather more work would be required to substantiate this possibility.

Studies in broken cell preparations indicated that a cyclic AMP hydrolytic activity could be detected in both HLMC and basophils. In basophil extracts, this activity was inhibited by rolipram and Org 30029 whereas 8-methoxymethyl IBMX, siguazodan and zaprinast were ineffective. These data are consistent with the presence of PDE 4 in basophils. However, in HLMC extracts, the cyclic AMP hydrolytic activity was inhibited modestly and to a similar degree by 8-methoxymethyl IBMX, rolipram, zaprinast, Org 30029 and siguazodan. It is possible that, in these experiments, the PDE inhibitors are acting non-selectively because the compounds were used at a high concentration (10 μM). However, it is noteworthy that neither zaprinast, 8-methoxymethyl IBMX, nor siguazodan had any effects on PDE activity in basophil extracts when used at this concentration. These data indicate that, whereas rolipram inhibits the cyclic AMP hydrolytic activity selectively in basophil extracts, rolipram does not demonstrate a selective inhibitory effect on the cyclic AMP hydrolytic activity in HLMC extracts.

It is interesting to note that IBMX was equiactive at inhibiting the cyclic AMP hydrolytic activity in extracts derived from either HLMC or basophils yet IBMX was five fold more potent as an inhibitor of histamine release in basophils than in HLMC. Similarly, theophylline was six fold more potent as an inhibitor of histamine release in basophils than in HLMC. These data may suggest either that IBMX and theophylline gain entry into basophils more readily or that basophils are more sensitive to PDE inhibition than HLMC. Based on these differences, it might be predicted that if a PDE 4 inhibitor were to be effective in HLMC, it would be less active in HLMC than

in basophils. However, rolipram was approximately 5,000 times less potent as an inhibitor of histamine release in HLMC than in basophils. These considerations further argue against a role for PDE 4 in the regulation of HLMC responses.

Although a preponderance of evidence suggests that PDE 4 does not regulate HLMC responses, it remains a possibility that, under certain situations, PDE 4 may be important in HLMC. For example, in certain disease states different PDE profiles may exist. It has been demonstrated that mononuclear cells isolated from individuals with atopic dermatitis are more responsive to rolipram than cells from individuals without atopic dermatitis (Chan & Hanifin, 1993; Banner *et al*., 1995). Alternatively, consequences of therapy could also influence PDE profiles. A recent study indicates that exposure of a human monocyte cell line (U937) to salbutamol, a β-adrenoceptor agonist, leads to the upregulation of PDE 4 activity (Torphy *et al*., 1992). Because bronchodilator β-adrenoceptor agonists continue to be a mainstay in the therapeutic management of asthma, the possibility exists that asthmatics may possess an altered profile of PDE activity compared to non-asthmatics. Thus, disease states and the consequences of therapy could influence the profile of PDE activity in cells such as the HLMC and the basophil.

It should be noted that, in the present study, mast cells isolated from lung parenchyma have been used exclusively. In view of a large body of work which indicates that mast cells isolated from different sites can display functional heterogeneity (Pearce, 1983), the possibility exists that the responses to PDE inhibitors of parenchymal mast cells may not necessarily reflect those of alternative subsets of lung mast cells such as bronchial mast cells or mast cells derived from bronchoalveolar lavage.

In summary, the present work has established that PDE 4-selective inhibitors attenuate basophil but not HLMC responses. This suggests that PDE 4 is important in regulating basophil function whereas the nature of the PDE isoform(s) which regulates HLMC responses remains uncertain.

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