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Regulation of human basophil function by phosphatase inhibitors

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1 Okadaic acid, a cell permeant inhibitor of protein serine/threonine phosphatases (PPs), attenuated the IgE-mediated release of the pre-formed mediator, histamine from human basophils in a time- and dose-dependent manner. Optimal inhibition ($77 \pm 4\%$, $P < 0.0001$) of histamine release was observed following a 2 h incubation with 1 μM okadaic acid.

2 Okadaic acid and two analogues of okadaic acid were also studied and were found to inhibit the IgE-dependent release of histamine. Concentrations required to inhibit release by 50% (IC_{50}) were 0.6 μM for okadaic acid and 7.5 μM for okadaol, whereas okadaone was inactive.

3 The structurally-unrelated PP inhibitor, calyculin A, also inhibited IgE-dependent histamine release from basophils dose-dependently and was approximately six fold more potent than okadaic acid.

4 The IgE-mediated generation of sulphopeptidoleukotrienes (sLT) from basophils was inhibited by okadaic acid and related analogues with the following rank order of potency; okadaic acid (approx. IC_{50} 0.3 μM) > okadaol (3 μM) > okadaone (inactive).

5 Okadaic acid, okadaol and okadaone (all at 3 μM) inhibited the IgE-mediated generation of the cytokine interleukin 4 (IL4) from human basophils by $67 \pm 9\%$ ($P < 0.002$), $48 \pm 14\%$ ($P < 0.05$) and $8 \pm 7\%$ ($P = 0.31$), respectively.

6 Extracts of purified human basophils liberated ^{32}P from radiolabelled glycogen phosphorylase and this PP activity was inhibited by $17 \pm 3\%$ ($P < 0.0005$) by a low (2 nM) concentration of okadaic acid and was inhibited by $96 \pm 1\%$ ($P < 0.0001$) by a higher (5 μM) concentration of okadaic acid. Because a low (2 nM) concentration of okadaic acid inhibits PP2A selectively whereas a higher (5 μM) concentration inhibits both PP1 and PP2A, these findings suggest that both PP1 and PP2A are present in basophils.

7 In total these data suggest that PPs are resident in human basophils and that PPs may be important in the regulation of basophil function.

Keywords: Basophils; mediator release; phosphatases; dephosphorylation; okadaic acid

Introduction

Antigenic activation of basophils leads to the release of a wide variety of pro-inflammatory mediators which may be important in the mediation of immediate hypersensitivity responses (Bascom *et al.*, 1988; Charlesworth *et al.*, 1989; Guo *et al.*, 1993). Antigen acts to cross-link surface-bound IgE molecules and the aggregation of IgE receptors initiates a cascade of events leading to basophil secretion. Whilst many of the events leading to secretion have yet to be resolved, a number of studies suggest that protein phosphorylations may be involved (Benhamou & Siraganian, 1992). The importance of protein dephosphorylations in regulating cellular activation is less well established (Paolini *et al.*, 1991).

There are four major classes of protein serine/threonine phosphatase (PP), enzymes which mediate protein dephosphorylation (Cohen, 1989; Cohen & Cohen, 1989; Shenolikar, 1994). These classess can be distinguished according to functional parameters that include, substrate preference, requirement for divalent metal ions and sensitivity to endogenous inhibitor proteins (Cohen, 1989; Cohen & Cohen, 1989). Type 1 PPs show a preference for the β subunit of phosphorylase kinase and are sensitive to the heat stable proteins, inhibitors 1 and 2 whilst the type 2 enzymes preferentially dephosphorylate the α subunit of phosphorylase kinase and are insensitive to inhibitors 1 and 2. PP2A has no divalent metal ion requirement whilst PP2B (also known as calcineurin) and PP2C are dependent on calcium and magnesium, respectively. More recently, several novel PPs have been identified. These PPs include PP3, PP4 and PP5 which retain a degree of structural and functional similarity with PP1 and PP2A, yet are sufficiently different to be regarded as distinct but related species (Honkanen *et al.*, 1991a; Brewis *et al.*, 1993; Chen *et al.*, 1994).

A number of naturally-occurring compounds have been found to act as PP inhibitors. Okadaic acid, microcystin, calyculin A and tautomycin have all been shown to inhibit PP activities in the subnanomolar to nanomolar range (Biajolan & Takai, 1988; Ishihara *et al.*, 1989; MacKintosh & Klumpp, 1990; Honkanen *et al.*, 1991b). The C_{38} polyether, okadaic acid, has been studied most intensively and has proved useful in the classification of PPs. Okadaic acid is a more potent inhibitor of PP2A (IC_{50}, 0.1 nM) than PPI (IC_{50}, 10 nM) whereas PP2B is only affected at far higher concentrations (IC_{50}, > 5 μM) and PP2C is resistant to okadaic acid (Cohen *et al.*, 1989). Thus, by employing appropriate concentrations of okadaic acid it has been possible to identify and to characterize PP activities (Cohen *et al.*, 1989). More recent studies indicate that nanomolar concentrations of okadaic acid inhibit PP3 (Honkanen *et al.*, 1991a), PP4 (Brewis *et al.*, 1993) and PP5 (Chen *et al.*, 1994). These findings would appear to complicate ascribing an okadaic acid-sensitive PP activity to a given PP species. Despite these misgivings, okadaic acid continues to be a useful investigative tool not least because it is cell permeant (Haystead *et al.*, 1989; Hardie *et al.*, 1991).

Okadaic acid and additional cell permeant PP inhibitors have been shown to modulate diverse cellular processes (Co-

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hen *et al.*, 1990). More recently okadaic acid has been shown to inhibit IgE-dependent mediator release from rat peritoneal mast cells (Estevez *et al.*, 1994), human lung mast cells (Peachell & Munday, 1993), human basophils (Botana & MacGlashan, 1993) and to activate RBL-2H3 cells (Sakamoto *et al.*, 1994). In the present study we have investigated the effects of a number of PP inhibitors on mediator release from activated basophils and assessed PP activity present in purified human basophils. Our data indicate that PPs may be important in the regulation of basophil function.

Methods

Buffers

PBS contained (mM): NaCl 137, $Na_2HPO_4.12H_2O$ 8, KCl 2.7 and KH_2PO_4 1.5. +PBS additionally contained: $CaCl_2.2H_2O$ 1 mM, $MgCl_2.6H_2O$ 1 mM, glucose 5.6 mM and HSA 30 $\mu g\ ml^{-1}$. PBS-EDTA was PBS supplemented with EDTA (1 mM). The pH of PBS, +PBS and PBS-EDTA were titrated to 7.3

PAG contained (mM): PIPES 22, NaCl 110, KCL 5 and glucose 5.6. HSA (30 $\mu g\ ml^{-1}$) was also added. The pH was titrated to 7.3.

Hypotonic lysis buffer contained: Tris 50 mM, EDTA 1 mM, EGTA 0.1 mM, DTT 0.5 mM, PMSF 50 $\mu g\ ml^{-1}$, soybean trypsin inhibitor 50 $\mu g\ ml^{-1}$, leupeptin 5 $\mu g\ ml^{-1}$ and aprotinin 5 $\mu g\ ml^{-1}$. The pH was titrated to 8.0.

Preparation of inhibitors and stimuli

Okadaic acid, okadaone and okadaol were prepared as 0.5 mM solutions in 10% DMSO. Calyculin A was made up as a 0.1 mM stock solution in 10% DMSO. Microcystin LR was prepared as a 0.1 mM stock solution in 10% methanol. A23187 was prepared as a 10 mg ml^{-1} solution in absolute ethanol. Neat polyclonal goat anti-human IgE antibody, from which all dilutions were prepared, was made up in +PBS. All stock solutions were stored at −20°C with the exception of anti-IgE which was stored at 4°C. The drugs were diluted to the desired concentration in buffer just before use.

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 × *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, UK) and centrifuged (250 × g, 15 min). A basophil-rich layer (5–15% purity) located 1 cm above the 53%/62% interface, was harvested. These cells were washed once in PAG, twice in PBS and then used in experiments investigating the release of either histamine, sulphopeptidoleukotrienes (sLT), or interleukin 4 (IL4). Preparation of basophils by Percoll gradients acts to remove contaminant neutrophils which may influence sLT catabolism (MacGlashan *et al.*, 1986). On occasion, 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 per 100 μl) and incubated (1 h) over ice with monoclonal (IgG_{2A}) mouse anti-human IgE (50 $\mu g\ ml^{-1}$). Cells were then washed twice with PBS-EDTA over ice and incubated (30 min) in PBS-EDTA (2×10^6 basophils per 100 μl) 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 use of 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 between 83 and 99% purity. 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 was carried out immediately after purification, the extracts snap frozen in liquid nitrogen and stored at −80°C for use in PP assays at some later stage.

Mediator release

Basophils (5×10^4 per tube) were added to pre-warmed tubes containing a PP inhibitor or buffer for time periods as indicated in the text. Stimulus was then added and histamine release was allowed to proceed for 45 min at 37°C after which, the tubes were centrifuged (450 × *g*, 4 min, RT). The histamine content of cell supernatants was assessed by a modification (Ennis, 1991) of the automated fluorometric technique of Siraganian (1974). On occasion Percolled basophils (5–9% purity) were used to assess histamine and sLT generation. Basophils ($5-7 \times 10^4$ per tube) were incubated (2 h) in the presence of either a PP inhibitor or buffer as indicated in the text and then challenged (anti-IgE 1 : 3000) and mediator release allowed to proceed for 45 min. Cells were centrifuged (450 × *g*, 4 min, RT) and an aliquot removed for histamine anlaysis. The remaining supernatant was assayed for sLT by EIA. The EIA kit used to determine sLT recognised all three sLT; leukotriene C_4 (LTC_4), LTD_4 and LTE_4. The OD of samples was measured at 450 nm by a Dynatech plate reader. All experiments were performed in duplicate.

IL4 generation

Percolled basophils ($1-2 \times 10^5$ per tube) of enhanced purity (5 to 11%) were incubated in the presence of either PP inhibitors or buffer (2 h, 37°C) and then challenged with anti-IgE (1 : 30000). IL4 generation was allowed to proceed for 4 h at which point the cells were pelleted (450 × *g*, 4 min, RT) and aliquots of the supernatants removed for histamine analysis. The remainder of the supernatant was assayed for IL4 content by EIA. The OD of samples was measured at 450 nm by a Dynatech plate reader.

Phosphatase assays

Lysates of purified basophil preparations, for use in PP assays, were prepared as described previously (Fruman *et al.*, 1992). Purified cells were resuspended in hypotonic lysis buffer (usually $1-1.5 \times 10^6$ basophils in 20 μl) and disrupted by three cycles of freeze/thawing. Following centrifugation (13000 × *g*, 10 min), supernatants were snap frozen in liquid nitrogen and stored at −80°C for use at a later date. Radiolabelled phosphorylase a was prepared (Stewart *et al.*, 1981; Mackintosh, 1993) from phosphorylase b (5 mg ml^{-1}) by incubation in the presence of phosphorylase kinase (200 u ml^{-1}), $MgCl_2$ (4 mM), $CaCl_2$ (0.1 mM), [γ^{32}P]-ATP (0.2 mM; specific activity 7.5×10^5 c.p.m. $nmol^{-1}$) at 37°C for 2 h. Unreacted [γ^{32}P]-ATP was removed by extensive dialysis (36 h) into 50 mM Tris HCl (pH 7.4), 10% (w : v) glycerol, 0.1 mM EGTA and 1 mM DTT. After dialysis, free ATP represented less than 5% of the total ^{32}P label. PP activities were measured by incubation of the ^{32}P-labelled phosphorylase a with extracts of purified basophils (approx. 1.7×10^5 cell equivalents) at 37°C in the dialysis buffer described either in the absence or the presence of okadaic acid. Aliquots of the incubation mixture were removed at 0, 7 and 14 min and added to ice-cold 25% tri-

chloroacetic acid (TCA) to which BSA (0.3 mg ml^{-1}) was added to aid protein precipitation. After centrifugation (15000 × *g*, 3 min) duplicate aliquots of the supernatant, containing the released ^{32}P, were added to liquid scintillant and counted in a Beckman LS 5000 SE liquid scintillation counter.

Materials

The following were purchased from the sources indicated: dimethyl sulphoxide (DMSO), sheep anti-human IgE, calcium ionophore A23187, PIPES (free acid), Percoll, bovine serum albumin (BSA), human serum albumin (HSA), EGTA, ATP, phosphorylase kinase, glycogen, phosphorylase b, dithiothreitol (DTT), aprotinin, PMSF, leupeptin, soybean trypsin inhibitor and Triton X-100 (Sigma Chemical Co., Poole, UK); EDTA, calcium chloride and magnesium chloride (BDH, Poole, UK); dextran (Pharmacia, Nottingham, UK); gentamicin and penicillin/streptomycin, microcystin LR (Gibco BRL, Dundee, UK); okadaic acid, okadaone, okadaol and calyculin A (LC Laboratories, Nottingham, UK); monoclonal (IgG_{2A}) mouse anti-human IgE (Immunotech, Marseilles, France); magnetic beads coated with rat anti-mouse IgG_{2A} antibody (Dynal, Wirral, UK); enzyme immunoassay (EIA) kits for human IL4 (R & D systems, Abingdon, UK); EIA kits for the sulphopeptidoleukotrienes (sLT) LTC_4, LTD_4 and LTE_4 (Amersham, Little Chalfont, UK); [$\gamma^{32}P$]-ATP (ICN Biochemicals, Thame, UK).

Statistics

In order to establish whether drug treatments caused a change in response relative to control untreated sets, Student's paired *t* test was performed. Values of $P<0.05$ were considered significant.

Results

Effect of PP inhibitors on mediator release from human basophils

Our own previous studies in human lung mast cells demonstrated that preincubation (2 h) with either 1 μM okadaic acid or 100 nM calyculin A was maximally effective at inhibiting the IgE-mediated release of histamine (Peachell & Munday, 1993). In an initial series of experiments, human basophils were incubated (2 h) with either okadaic acid (0.03–3 μM) or calyculin A (3–300 nM) before challenge with anti-IgE. Both okadaic acid and calyculin A inhibited the IgE-mediated release of histamine from human basophils in a dose-dependent manner and with respective IC_{50}s of 0.3 μM and 0.05 μM (Figure 1). In the same series of experiments a less active analogue of okadaic acid, nor-okadaone (0.1–10 μM), was ineffective as an inhibitor of histamine release. In a separate series of experiments, microcystin was tested as an inhibitor of anti-IgE-induced histamine release from human basophils. In three experiments, pretreatment (2 h) with microcystin (0.1–10 μM) had no effect on control histamine release (38±7%) whilst in the same experiments, pretreatment with okadaic acid (2 h, 1 μM) or calyculin A (2 h, 100 nM) inhibited control histamine release by 88±5% and 85±5%, respectively.

In order to determine whether 2 h represented a maximally effective preincubation period for each inhibitor, a time course experiment was performed (Figure 2). The data indicate that whereas 2 h was required for okadaic acid to be maximally effective as an inhibitor of histamine release, an equiactive concentration of calyculin A was maximally effective following a 5 min preincubation period.

In addition to attenuating IgE-mediated histamine release, okadaic acid (2 h, 1 μM) inhibited secretion induced by the calcium ionophore A23187 (Figure 3). The inhibition by okadaic acid of histamine release induced by ionophore A23187 was inversely related to the level of control histamine release. Thus although histamine release induced by a submaximal concentration (0.1 μg ml^{-1}) of ionophore A23187 was almost completely abrogated by okadaic acid, a maximal releasing concentration of the ionophore (1 μg ml^{-1}) induced histamine release which was inhibited by 39±9% by the same okadaic acid pretreatment.

In addition to the release of the pre-formed mediator histamine, challenge of human basophils with anti-IgE induces the *de novo* generation of sulphopeptido leukotrienes

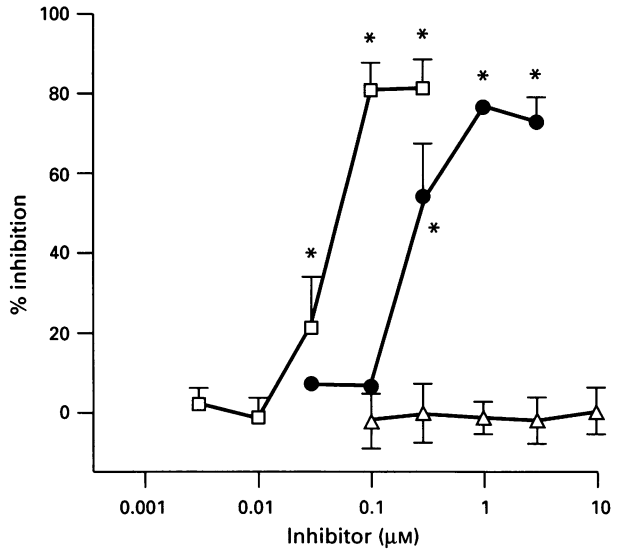

Figure 1 Effect of okadaic acid and calyculin A on anti-IgE-induced histamine release from human basophils. Cells were incubated in either the absence or the presence of increasing concentrations of either okadaic acid (●), calyculin (□) or okadaone (△) for 2 h and then challenged with anti-IgE (1:3000) for a further 45 min after which histamine release was measured. Results are expressed as % inhibition of control histamine release which was 34±5%. Values are means±s.e.mean (vertical lines), $n=6$. Statistically significant ($P<0.05$) inhibition of histamine release is denoted by an asterisk.

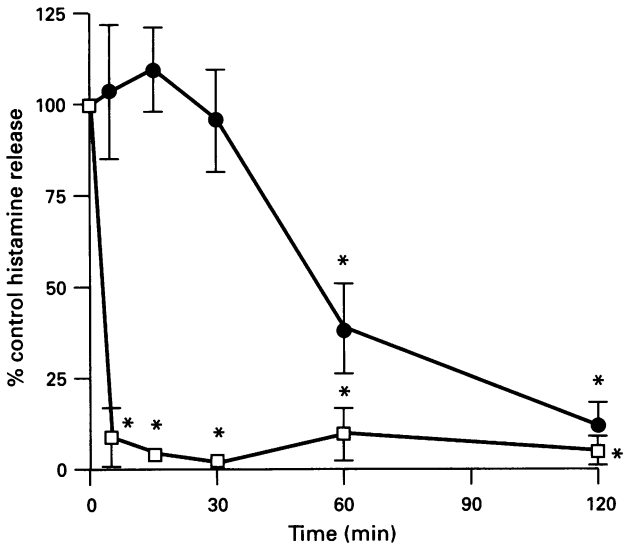

Figure 2 Effect of incubation time on the inhibitory effect of okadaic acid and calyculin. Cells were incubated (5 to 120 min) in either the absence or the presence of okadaic acid (1 μM; ●) or calyculin (100 nM; □) and then challenged with anti-IgE (1:3000) for a further 45 min after which histamine release was measured. Results are expressed as a percentage of the control histamine release which was 33±5%. Values are means±s.e.mean (vertical lines), $n=6$ for okadaic acid and $n=5$ for calyculin. Statistically significant inhibition ($P<0.05$) is denoted by an asterisk.

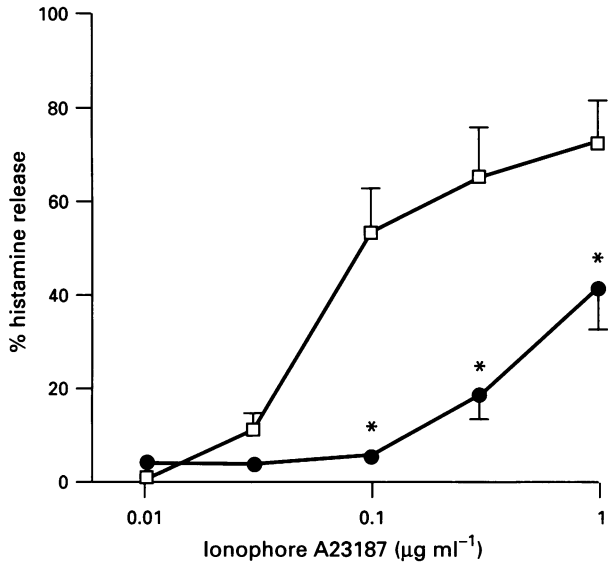

Figure 3 Effect of okadaic acid on histamine release induced by the calcium ionophore, A23187. Cells were incubated in either the absence (□) or the presence (●) of okadaic acid (2 h, 1 μM) and then challenged with increasing concentrations of calcium ionophore, A23187, for a further 45 min for histamine release. Values are the means ± s.e.mean (vertical lines), $n=4$. Statistically significant ($P<0.05$) reductions in ionophore-induced histamine release in the presence of okadaic acid are indicated by asterisks.

(sLT). The effect of preincubation (2 h) with okadaic acid (0.1 – 3 μM) and two less active structural analogues of okadaic acid, okadaol and okadaone (1 – 10 μM), on the IgE-dependent release of histamine and sLT from basophil-enriched preparations (see Methods) was assessed (Figure 4). Okadaic acid was twofold more potent as an inhibitor of sLT generation compared to histamine release (respective IC_{50} values; 0.3 μM and 0.6 μM). Similarly, okadaol was a more potent modulator of sLT generation than of histamine release (respective IC_{50} values; 3 μM and 7.5 μM). Okadaone was ineffective as an inhibitor of either histamine or sLT generation. It should be noted that a discrepancy exists between the IC_{50} values calculated for the inhibition of histamine release by okadaic acid in Figures 1 and 4. Although the reasons for this difference are unknown, basophils used to generate the data in Figure 1 were prepared by dextran sedimentation whereas basophils used to generate the data in Figure 4 were Percolled.

Previous studies by others have demonstrated that long-term (4 h) stimulation of human basophils with anti-IgE leads to the generation of IL4 (MacGlashan *et al.*, 1994; Schroeder *et al.*, 1994). Moreover, these studies demonstrated that the concentration of anti-IgE required to induce maximal generation of IL4 was 10 fold lower than that required to induce optimal levels of histamine release (Schroeder *et al.*, 1994). In preliminary experiments we confirmed these findings and extended these studies to determine the effects of PP inhibitors on IgE-triggered IL4 production from basophil-enriched preparations. Okadaic acid (0.1 – 3 μM) inhibited the anti-IgE (1 : 30000)-induced generation of IL4 from human basophils in a dose-dependent manner with an IC_{50} of 0.3 μM (Figure 5). In the same experiments, okadaol (3 μM) and okadaone (3 μM) were also studied and were found to inhibit IL4 generation by 48 ± 14% ($P<0.05$), and 8 ± 7% ($P=0.31$), respectively. Although aliquots of the supernatants assayed for IL4 were also assayed for histamine release, the conditions used in these experiments (sub-maximal concentrations of anti-IgE, 6 h total incubation period) resulted in levels of histamine release (< 10%) which prevented useful comparison of the effects of okadaic acid on histamine and IL4 production in the same set of experiments.

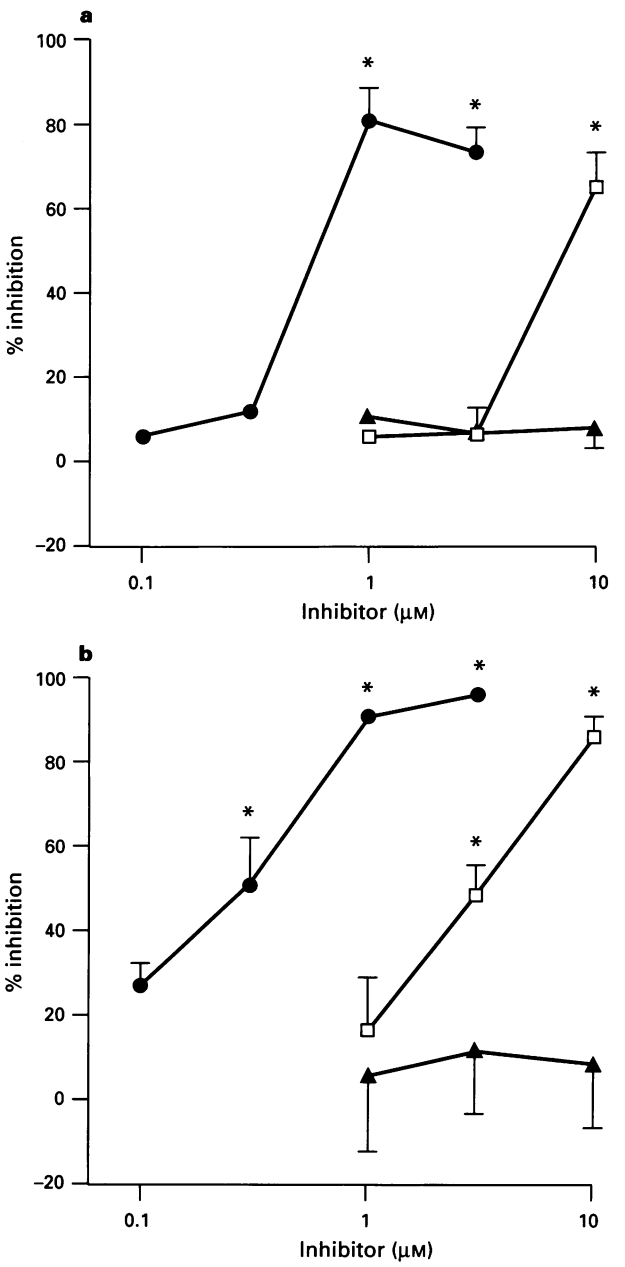

Figure 4 Effect of okadaic acid and analogues on anti-IgE-induced (a) histamine and (b) sulphopeptidoleukotriene (sLT) release from basophil-enriched preparations. Basophils (purities of 5 to 9%) were incubated (2 h) in either the absence or the presence of either okadaic acid (●), okadaol (□) or okadaone (▲) and then challenged with anti-IgE (1 : 3000) for a further 45 min for histamine release. Results are expressed as % inhibition of control histamine release which was 39 ± 5% and control sLT generation 7.6 ± 0.9 ng per 10^6 basophils. Values are means ± s.e.mean (vertical lines), $n=5$. Statistically significant ($P<0.05$) inhibition of mediator release is denoted by an asterisk.

Phosphatase activity in extracts of human basophils

Extracts of purified human basophils liberated ^{32}P from radiolabelled glycogen phosphorylase. PP activity was measured at 0, 7 and 14 min and basophil extracts were diluted in order to obtain linear rates of dephosphorylation over this time course (Figure 6a). In the presence of okadaic acid, linear rates of dephosphorylation were still observed though the extent of dephosphorylation was depressed. These data were reworked to provide rates of dephosphorylation in the presence and absence of okadaic acid (Figure 6b). Thus a low (2 nM) okadaic

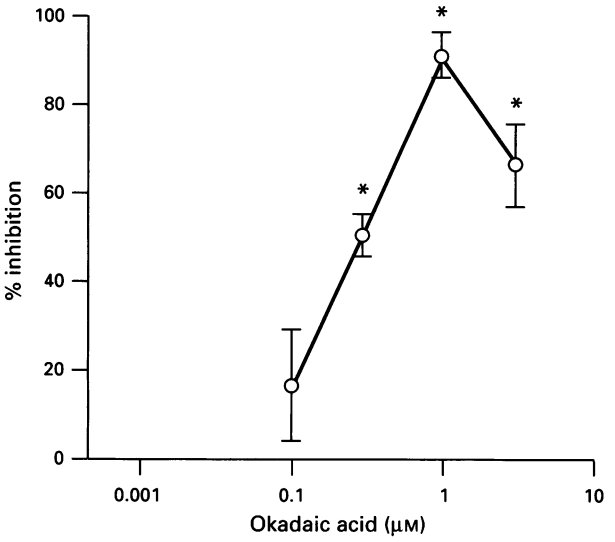

Figure 5 Effect of okadaic acid on anti-IgE-induced interleukin 4 (IL4) generation from human basophils. Partially purified basophils (purities of 5 to 11%) were incubated (2 h) in the absence or presence of okadaic acid and then challenged with anti-IgE (1:30000). IL4 generation was allowed to proceed for 4 h. Results are expressed as % inhibition of control IL4 release which ranged from 52 to 567 pg per 10^6 basophils (mean 198±100 pg per 10^6 basophils). Values are means±s.e.mean (vertical lines), $n=5$. Statistically significant inhibition ($P<0.05$) is denoted by an asterisk.

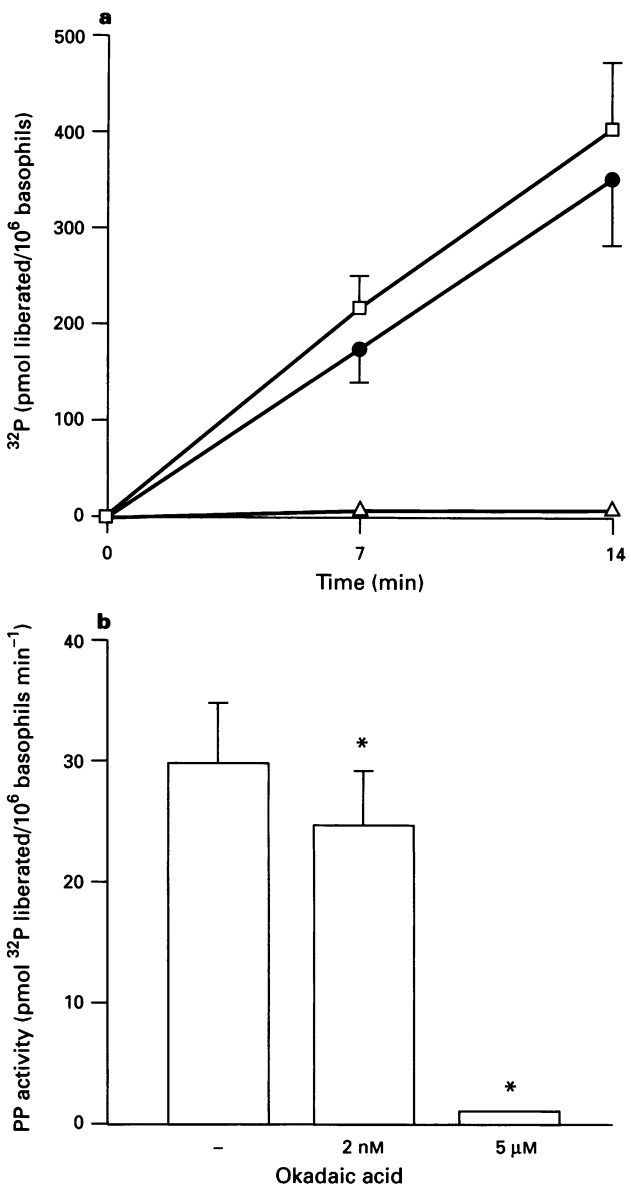

Figure 6 Effect of okadaic acid on the dephosphorylation of radiolabelled glycogen phosphorylase by basophil extracts. (a) Dephosphorylation of radiolabelled phosphorylase by basophil extracts was measured over 14 min in either the absence (□) or the presence of either 2 nM (●) or 5 μM (△) okadaic acid. (b) Data from (a) have been converted to rates of dephosphorylation. Values are means±s.e.mean (vertical lines), $n=8$. Statistical significance ($P<0.0005$ at least) is indicated by an asterisk. Basophil purities were 92±3%.

acid concentration inhibited total PP activity by 17±3% ($P<0.0005$) whereas a higher (5 μM) concentration of okadaic acid inhibited total PP activity by 96±1% ($P<0.0001$). Because 2 nM okadaic acid should inhibit PP2A selectively whereas 5 μM okadaic acid should inhibit both PP1 and PP2A (Cohen *et al.*, 1989), these data could suggest that 17% of the total PP activity present in basophil extracts is due to PP2A and 79% of the total activity due to PP1.

Okadaol (3 μM), as well as okadaic acid (3 μM), inhibited the PP activity present in extracts of basophils whereas okadone (3 μM) was ineffective (Figure 7). In these experiments PP activity was inhibited by 92±4% ($P<0.001$), 24±3% ($P<0.01$) and −6±6% ($P=0.31$) by okadaic acid, okadaol and okadaone, respectively.

Discussion

The importance of PPs and of protein dephosphorylation in a variety of cellular processes has been highlighted by the use of cell-permeant PP inhibitors such as okadaic acid in intact cells (Haystead *et al.*, 1989; Hardie *et al.*, 1991). In the current study, we have investigated a potential role for PPs in the regulation of human basophil function using okadaic acid, and additional PP inhibitors.

Okadaic acid and calyculin A, two structurally-unrelated PP inhibitors, attenuated the IgE-mediated release of histamine from human basophils in a dose-dependent manner. Submicromolar concentrations of the PP inhibitors were effective although calyculin A was, approximately, six fold more potent than okadaic acid. These data on the effects of PP inhibitors on histamine release correspond with the relative potencies of these compounds as inhibitors of isolated PPs (Ishihara *et al.*, 1989). Although the IC_{50} concentrations required to inhibit mediator release were at least 10 fold higher than those required to inhibit isolated PP, it is possible that there may be a need to expose cells to higher extracellular concentrations of a PP inhibitor in order to attain appropriately elevated concentrations intracellularly (Hardie *et al.*, 1991). However, it should be noted that the concentration range used in the present study is comparable to concentrations employed by others in alternative cell systems (Karaki *et al.*, 1989; Haystead *et al.*, 1990; Taffs *et al.*, 1991; Lu *et al.*, 1992).

The incubation period required in order to observe maximal inhibition of histamine release was quite different for calyculin compared to that for okadaic acid (5 min and 2 h, respectively). These data could suggest that okadaic acid and calyculin A gain entry into the cell at different rates or, once inside the cell, access to or efficacy at distinct targets may differ. However, both okadaic acid and calyculin A are lipophilic and are thought to traverse the plasma membrane readily (Haystead *et al.*, 1989; Ishihara *et al.*, 1989). It is interesting that, whilst okadaic acid and calyculin are roughly equipotent against PP2A (approx. IC_{50}s, 0.5 to 1 nM), calyculin is more

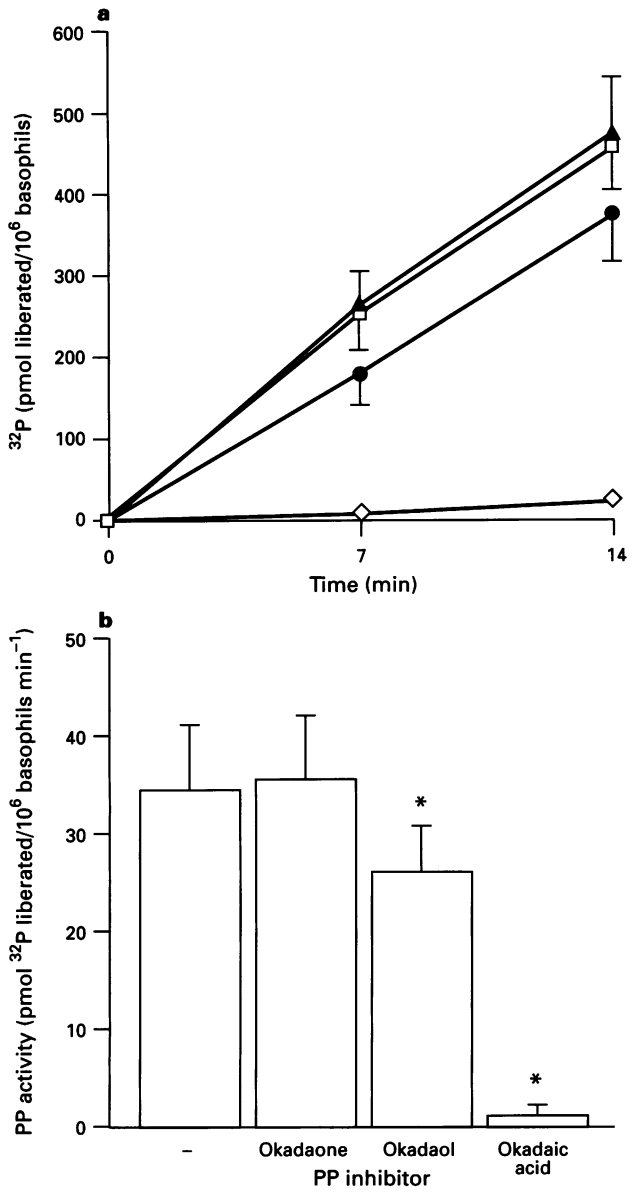

Figure 7 Effect of phosphatase (PP) inhibitors (3 μM) on the dephosphorylation of radiolabelled glycogen phosphorylase by basophil extracts. (a) Dephosphorylation of radiolabelled phosphorylase by basophil extracts was measured over 14 min in either the absence (□) or the presence of okadaic acid (◇), okadaol (●) and okadaone (▲). (b) Data from (a) have been converted to rates of dephosphorylation. Values are means ± s.e.mean (vertical lines), $n=3$. Statistical significance ($P<0.01$ at least) is indicated by an asterisk. Basophil purities were 98 ± 1%.

potent than okadaic acid as an inhibitor of PP1 in isolated enzyme preparations (respective IC_{50}s, 2 and 60 to 500 nM) (Ishihara *et al.*, 1989). Since lower concentrations of calyculin A are required to inhibit PP1, it might be expected that calyculin A reaches intracellular concentrations capable of inhibiting PP1 more rapidly than okadaic acid. This may explain the different incubation periods required for calyculin A and okadaic acid to inhibit histamine release maximally. The data, therefore, suggest that PP1 may be important in regulating basophil function although the possibility that PP2A might also be involved cannot be excluded. Alternatively, calyculin A and okadaic acid may inhibit basophil histamine release by different mechanisms.

In addition to inhibiting anti-IgE-induced histamine release, okadaic acid attenuated histamine release induced by the calcium ionophore, A23187. Ionophore A23187 evokes histamine

Table 1 IC_{50} values for the inhibition of mediator release by okadaic acid and analogues

	IC_{50} (μM)[a]		
	Histamine	*sLT*	*IL4*
Okadaic acid	0.6	0.3	0.3
Okadaol	7.5	3	3[b]
Okadaone	inactive	inactive	inactive

[a]IC_{50} values for the inhibition of the IgE-mediated release of histamine, sulphopeptidoleukotrienes (sLT) and interleukin 4 (IL4) from basophil-enriched cell preparations. The approximate IC_{50} values were calculated from Figures 3 (histamine and sLT) and 5 (IL4).
[b]The IC_{50} value for okadaol inhibition of IL4 generation was not established from a dose-response curve and the value provided is, therefore, very approximate. A concentration of 3 μM okadaol inhibited IL4 generation by 48 ± 14%, $n=5$, $P<0.05$.

release by facilitating the direct translocation of extracellular calcium into the cell. Thus, the ionophore mimics the increase in intracellular calcium associated with IgE-mediated cell activation, an event which is thought to be necessary for mediator release (MacGlashan & Guo, 1991). That okadaic acid inhibited histamine release induced by the calcium ionophore suggests that okadaic acid might act at sites distal to the entry of calcium into the cell. Support for this contention is provided by studies demonstrating that okadaic acid attenuates IgE-mediated histamine release from human basophils without affecting calcium entry into the cell (Botana & MacGlashan, 1993).

Microcystin, an alternative PP inhibitor which is structurally-unrelated to either calyculin or okadaic acid, was found to be ineffective as an inhibitor of IgE-mediated histamine release from human basophils. Microcystin has been shown to be effective in some intact cells (e.g. liver cells) (Rutter *et al.*, 1991) and not in others (e.g. fat cells) (Eriksson *et al.*, 1990) and this activity may be related to either the presence or the absence, respectively, of a specialized transporter (Rutter *et al.*, 1991). It is probable, therefore, that this specialized transporter is not present in basophils.

In addition to okadaic acid, okadaol and okadaone were assessed for effects on the release of histamine and the generation of sLT. The rank order of activity for the inhibition of either mediator was okadaic acid > okadaol > okadaone. Okadaic acid was 10 fold more potent than okadaol at modulating the release of either mediator whereas okadaone was ineffective (Table 1). This rank order for the inhibition of the release of mediators parallels the activity of these compounds as inhibitors of isolated PPs (Nishiwaki *et al.*, 1990). Okadaic acid was 2 fold more potent as an inhibitor of sLT generation than histamine release and a similar shift in potency was observed for okadaol suggesting that the pathway leading to sLT generation is more readily modulated by PP inhibitors than the pathways leading to histamine release. A number of studies have demonstrated that, in basophils, sLT generation can be attenuated more effectively than histamine release by a variety of different inhibitors with varying specificities (e.g. Peachell *et al.*, 1988; Warner & MacGlashan, 1990) indicating that this effect is not unique to PP inhibitors.

Recent studies have demonstrated that IgE-mediated activation of human basophils can lead to the generation of the cytokine IL4 (MacGlashan *et al.*, 1994; Schroeder *et al.*, 1994). Basophils may, therefore, be important in the mediation of allergic diseases because IL4 has been shown to promote the generation of IgE (Del-Prete *et al.*, 1988). In the present study, okadaic acid, okadaol and oakadaone and in that order of decreasing activity, were found to attenuate the IgE-mediated generation of IL4 from human basophils. The IC_{50} concentration for okadaic acid to inhibit IL4 generation compares very favourably with the IC_{50} concentration required to inhibit

sLT generation and is in reasonable agreement with the IC_{50} value for the inhibition of histamine release (Table 1). These data may suggest that okadaic acid acts at a common target(s) in order to modulate both the generation of sLT and the synthesis of IL4 and, perhaps, the release of histamine as well.

Extracts of purified basophils liberated ^{32}P from radiolabelled glycogen phosphorylase and this PP activity was inhibited by okadaic acid. Because okadaic acid preferentially inhibits PP2A at low (2 nM) concentrations whereas a higher (5 μM) concentration of okadaic acid would be expected to inhibit both PP2A and PP1, the relative contribution of PP2A and PP1 in a tissue/cell system can be established (Cohen *et al.*, 1989). Utilizing this method, the data indicate that 17% of the total PP activity in basophil extracts is due to PP2A whereas 79% of the PP activity is due to PP1. The apparent ratio (79 : 17) of PP1 : PP2A activity observed for basophils is not too dissimilar with our previous findings in human lung mast cells in which the ratio of PP1 : PP2A activity was 60 : 18 (Peachell & Munday, 1993). Because PP2A is thought to be six fold less active than PP1 against phosphorylase (Tung *et al.*, 1985) these data may underestimate the relative contribution of PP2A to total PP activity. A contribution of either PP2B or PP2C in this assay would not be expected because of the presence of EDTA which would limit the availability of divalent cations which are necessary for the activity of both PP2B and PP2C.

The effects of both okadaol and okadaone, as well as okadaic acid, on the PP activity present in basophil extracts were also assessed. Whereas a high (3 μM) concentration of okadaic acid inhibited PP activity by 93%, okadaol, at the same concentration, inhibited PP activity by 24% and okadaone was inactive. This order of activity for the inhibition of PP activity parallels the effects of PP inhibitors on mediator relesae. The extent of inhibition by okadaol of PP activity in extracts was lower than might have been expected based on the effects this analogue on the generation of both sLT and IL4. However, it has been shown that structural modification to okadaic acid lowers the affinity of the modified analogue for PP2A relative to PP1 (Takai *et al.*, 1992). An alternative study (Nishiwaki *et al.*, 1990) has shown that 1 μM okadaol inhibits PP2A activity by 41% whereas this concentration of okadaic acid inhibits PP2A completely and PP1 close to maximally (Cohen *et al.*, 1989). These considerations may explain why okadaol is less active as an inhibitor of PP activity in basophil extracts than might have been predicted.

Whilst the data clearly implicate PPs as important in the regulation of basophil activity it is not possible to establish, based on these data alone, which PP is involved. In addition to PP1 and PP2A, several novel mammalian PPs, PP3, PP4 and PP5, have been shown to be sensitive to okadaic acid and act on substrates known to be dephosphorylated by PP1 and PP2A (Honkanen *et al.*, 1991a; Brewis *et al.*, 1993; Chen *et al.*, 1994). However, these novel PPs appear to be involved in cell growth and proliferation and may be less important in regulating the activity of fully-differentiated cells, such as basophils. Nevertheless, it seems probable that one or more species of okadaic acid-sensitive PP is involved in the regulation of basophil activity.

The data presented in this manuscript show that PP inhibitors can attenuate histamine release, sLT and IL4 generation from human basophils implicating PPs as important in the regulation of these basophil functions. We have confirmed that PPs are constitutively-associated with basophils and that these PP activities are okadaic acid-sensitive providing the mechanism for the observed attenuation of basophil function. These data may serve to identify PPs as potential targets for therapeutic intervention in order to contain the undesirable consequences of basophil activation.

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