British Journal of Pharmacology (1998) 125, 1336–1344
http://www.stockton-press.co.uk/bjp

Pharmacology of [^{3}H]-pyrilamine binding and of the histamine-induced inositol phosphates generation, intracellular Ca^{2+}-mobilization and cytokine release from human corneal epithelial cells

[1,2,3]N.A. Sharif, [1,2]T.K. Wiernas, [1,2]B.W. Griffin & [1]T.L. Davis

[1]Molecular Pharmacology Unit, Alcon Laboratories, Inc (R2-19), 6201 South Freeway, Fort Worth, Texas 76134-2099 and [2]Department of Pharmacology, University of North Texas Health Sciences Center, Fort Worth, Texas, U.S.A.

1 We recently reported on the successful generation of immortalized (CEPI-17-CL4) cells from primary human corneal epithelial (P-CEPI) cells which exhibited phenotypic, immunohistochemical and metabolic characteristics akin to the P-CEPI cells.

2 The aims of the present studies were to investigate the ligand binding and functional coupling of the histamine receptors to various biochemical and physiological systems in the P-CEPI and CEPI-17-CL4 cells and to relate these findings to the normal and/or pathophysiological role of histamine on the human ocular surface.

3 Specific [^{3}H]-pyrilamine binding to CEPI-17-CL4 cell homogenates comprised >93% of the total binding and represented interaction with an apparent single population of high affinity ($K_d = 3.76 \pm 0.78$ nM; $n = 4$) and saturable ($B_{max} = 1582 \pm 161$ fmol g^{-1} tissue) number of histamine-1 (H_1) receptor binding sites on CEPI-17-CL4 cell homogenates. The H_1-receptor selective antagonists, pyrilamine ($K_i = 3.6 \pm 0.84$ nM, $n = 4$) and triprolidine ($K_i = 7.7 \pm 2.6$ nM, $n = 3$), potently displaced [^{3}H]-pyrilamine binding, while the H_2- and H_3-receptor selective antagonists, ranitidine and clobenpropit, were weak inhibitors (K_is > 13 μM).

4 Histamine induced phosphoinositide (PI) hydrolysis 2.7–4.4 fold above basal levels and with a potency of 14.9 ± 4.9 μM ($n = 9$) and 4.7 ± 0.2 μM ($n = 9$) in P-CEPI and CEPI-17-CL4 cells, respectively. Histamine-induced PI turnover was antagonized by H_1-receptor selective antagonist, triprolidine, with a potency (K_i) of 3.2 ± 0.66 nM ($n = 10$) and 3.03 ± 0.8 nM ($n = 4$) in P-CEPI and CEPI-17-CL4 cells, respectively, but weakly effected by 10 μM cimetidine and clobenpropit, H_2- and H_3-receptor antagonists. The PI turnover response was attenuated by pre-treatment of the cells with the selective phospholipase C inhibitor, U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) ($IC_{50} = 4.8 \pm 2.4$ μM, $n = 3$).

5 Histamine stimulated intracellular Ca^{2+} ($[Ca^{2+}]_i$) mobilization in CEPI-17-CL4 cells with a potency of 6.3 ± 1.5 μM ($n = 4$). The histamine-induced $[Ca^{2+}]_i$ mobilization was reduced by about 28% following pre-incubation of the cells with 4 mM EGTA. While triprolidine completely inhibited histamine-induced $[Ca^{2+}]_i$ mobilization, it did not influence the bradykinin-induced $[Ca^{2+}]_i$ mobilization response.

6 Histamine (EC_{50}s = 1.28–2.77 μM, $n = 3$–4) concentration-dependently stimulated the release of interleukin-6 (IL-6), IL-8 and granulocyte macrophage colony-stimulating factor, but it did not significantly alter release of tumour necrosis factor-α, PGE_2 or collagenase-1 (matrix metalloproteinase-1; MMP-1) from CEPI cells. However, IL-1 (10 ng ml^{-1}), foetal bovine serum (10%) and phorbol-12-myristate-13-acetate (3 μg ml^{-1}) were effective positive control secretagogues of all the cytokines, PGE_2 and MMP-1, respectively, from these cells.

7 It is concluded that the CEPI cells express H_1-histamine receptors which are positively coupled to PI turnover and $[Ca^{2+}]_i$ mobilization which may be directly or indirectly responsible for the release of various cytokines from these cells at physiologically and/or pathologically relevant concentrations.

Keywords: Cornea; corneal epithelial cells; histamine; PI turnover; calcium mobilization; cytokines; matrix metalloproteases

Introduction

Histamine is a major inflammatory mediator released from human conjunctival mast cells following irritation or allergic provocation of the conjunctiva (Berdy *et al*., 1991; Leonardi *et al*., 1992; Abelson & Schaefer, 1993). Irani *et al*. (1990) have estimated the existence of 10^4 mast cells in the human conjunctiva and Proud *et al*. (1990) have demonstrated the presence of 0.5 μM histamine in the tear fluid of human subjects following an ocular allergic reaction. While the blood vessels, sensory nerve-endings (Allansmith & Ross, 1988; Abelson & Schaefer, 1993; Proia, 1994) and conjunctival epithelial cells (Sharif *et al*., 1996a; 1997a; Gamache *et al*., 1997) appear to represent the major sites of action of the released histamine to elicit and/or amplify the inflammatory reaction, culminating in oedema, itching and redness and thus allergic conjunctivitis (Berdy *et al*., 1991; Abelson & Schaefer, 1993; Higorani & Lightman, 1995), the corneal epithelial cells may also play a role in the inflammatory cascade on the ocular surface as do epithelial cells in other organs (Sauder, 1990; McGee *et al*., 1992; Marini *et al*. 1992; Bedard *et al*., 1993). Indeed, human corneal epithelial (CEPI) cells express various pro-inflammatory cytokines including interleukin-8 (IL-8)

[3] Author for correspondence at Alcon.

(Cubitt *et al*., 1993), IL-1, IL-6, IL-8, granulocyte macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor-α (TNF-α) (Sharif *et al*., 1997b), and are capable of secreting these cytokines in response to surfactants (Faquet *et al*., 1997), and are also capable of generating free radicals (e.g. nitric oxide) in response to bacterial toxins (Eckmann *et al*., 1993; Zanvit *et al*., 1997). Furthermore, the levels of the cytokines, IL-1 and IL-8, are elevated in the corneas of patients with pseudophakic bullous keratopathy (Rosenbaum *et al*., 1995).

While the symptoms associated with seasonal allergic conjunctivitis involving the eyelids and conjunctiva can now be treated effectively with new generation anti-histamines such as emedastine (Emedine™; Sharif *et al*., 1994; Yanni *et al*., 1994), and a dual pharmacophore antihistamine/anti-allergic like olopatadine (Patanol™; Sharif *et al*., 1996b; Yanni *et al*., 1997) with reduced or minimal side-effects, the prognosis for corneal inflammatory/allergic conditions (e.g. atopic kerato-conjunctivitis) is less good at present (Higorani & Lightman, 1995; Reim *et al*., 1997; Ben Ezra *et al*. 1997). In view of the above observations, the CEPI cells were hypothesized to be additional targets for the histamine released from conjunctival mast cells during an ocular allergic reaction or an injurious situation. Therefore, the goals of the present studies were to explore the presence of histamine receptors on primary CEPI (P-CEPI) and previously SV40-virus-immortalized CEPI cells (CEPI-17-CL4) (Sharif *et al*., 1997b; Wiernas *et al*., 1997) and to characterize their pharmacology using ligand binding and second messenger assays. Additional aims were to determine the coupling of the histamine receptors on human CEPI cells to other physiological and pathological (e.g. inflammatory) response mechanisms including the secretion of cytokines, prostaglandin E_2 and matrix metalloproteinase-1 (MMP-1; collagenase-1).

Methods

Human CEPI cell isolation and cultures

The procedures for isolating and culturing normal, primary human CEPI (P-CEPI) cells have been previously described (Wiernas *et al*., 1997; Sharif *et al*., 1997b). In brief, human corneas were aseptically dissected from cadaver eyes within 8–12 h of death and transported from eye blanks in ice-cold Dexol® or Optisol® corneal preservation medium. Corneas from different donors were kept separate and rinsed in phosphate buffered saline (PBS) before being exposed to dispase at 10 u ml^{-1} in 50% Hanks buffered salts and keratinocyte growth medium (KGM) containing 0.05 mM calcium at 4°C for 24–48 h. The KGM was prepared by adding bovine pituitary extract (30 $\mu g\ ml^{-1}$), hydrocortisone (0.5 $\mu g\ ml^{-1}$), amphotericin B (0.05 $\mu g\ ml^{-1}$)/gentamicin (50 $\mu g\ ml^{-1}$), insulin (5 $\mu g\ ml^{-1}$), transferrin (10 $\mu g\ ml^{-1}$), murine epidermal growth factor (10 $\mu g\ ml^{-1}$) and 0.05 mM $CaCl_2$ to keratinocyte basal medium (KBM). Following this incubation, the epithelium was gently removed with a scalpel blade. This tissue was then gently triturated to produce individual cells which were then washed in Dulbecco's modified Eagle medium (DMEM) containing 10% foetal bovine serum using a low speed centrifugation/re-suspension procedure. The CEPI cell pellet was resuspended in low calcium (0.05 mM) KGM medium, the cells plated into T25 flasks (previously coated with collage type IV and fibronectin) and incubated at 37°C under a humidified atmosphere of 95% air/5% CO_2. The culture medium was changed 24 h later and then every 2 days thereafter. The cells became confluent in approximately 10 days at which point they were sub-cultured (passage 1; P1) by rinsing with PBS, incubating in dispase for up to 1 h until the cells detached, washing in DMEM by centrifugation and plated on collagen-coated 24-well plates. All experiments on P-CEPI cells were performed with the P1 cells.

Immortalized CEPI cells

The SV40 virus-induced immortalization and the initial genetic, morphological and preliminary biochemical and pharmacological characterization of the primary (P-CEPI) and the latter immortalized (CEPI-17-CL4) cells have been recently described (Sharif *et al*., 1997b). These and other studies (Sharif *et al*., 1997b; Wiernas *et al*., 1997) have established that CEPI-17-CL4 cells represent the P-CEPI cells well in terms of the above-mentioned parameters, but especially in relation to the coupling of certain receptors, especially bradykinin receptors, to phospholipase C (PLC) (Sharif *et al*., 1997b; Wiernas *et al*., 1997; 1998). Accordingly, the general term CEPI cells will be used from here onward to generically denote the corneal epithelial cells.

[^{3}H]-pyrilamine binding studies

Due to the limited availability and slow growth characteristics of human P-CEPI cells (Kahn *et al*., 1993; Araki-Sasaki *et al*., 1995) and the small amount of corneal tissue that is potentially obtainable from cadaver eyes, all binding experiments were performed with CEPI-17-CL4 cells which could be grown in large quantities. These immortalized cells (currently at passage # 195) are particularly well suited for such studies. Total particulate CEPI-17-CL4 (passage # 37-130) cell homogenates were prepared by gently dislodging confluent cells from culture flasks with a rubber policeman, gently homogenizing with a Polytron (5 s, setting 3), harvesting by centrifugation ($30{,}000 \times g$/20 min at 4°C) and resuspending by gentle homogenization in 50 mM sodium potassium phosphate buffer (pH 7.5) at 30 mg wet weight tissue ml^{-1}. The histamine-1 (H_1) receptor-subtype binding assays were performed according to previously published methods (Sharif *et al*., 1994; 1996b). Briefly, [^{3}H]-pyrilamine (1–2 nM; 50 μl) was incubated with 10–20 mg ml^{-1} of washed CEPI-17-CL4 cell membranes in the presence or absence of unlabeled test compounds in a total volume of 500 μl in polypropylene tubes. The non-specific binding was determined with 5 mM histamine or 10 μM triprolidine, both compounds yielding the same results. The assays were conducted at 23°C for 40 min and then terminated by rapid vacuum filtration over Whatman GF/B glass fibre filters pre-wetted in 0.3% polyethyleneimine using 2×6 ml of ice-cold 50 mM TrisHCl buffer (pH 7.4) on a Tomtek cell harvester. The radioactivity bound to filters was determined on a Wallac 'Big-Spot' beta-counter with four samples being simultaneously counted. Binding data were evaluated using a non-linear, iterative curve-fitting program incorporating a logistics function (Bowen & Jerman, 1995) to derive drug affinity values. Binding data were analysed as described below.

PI turnover studies

PI turnover studies were performed as previously described (Wiernas *et al*., 1997; Sharif *et al*., 1997a,b). In brief, CEPI cells (approximately 2×10^5 cells/well) cultured in 24-well plates were incubated with [^{3}H]-myo-inositol (2 μCi ml^{-1}) in DMEM for 24 h at 37°C in order to label the cell membrane

lipids. The medium was then aspirated and the cells exposed to histamine in DMEM/F-12 medium (+15 mM HEPES buffer) containing 10 mM LiCl for 60 min at 37°C in order to facilitate the accumulation of [^{3}H]-inositol phosphates ([^{3}H]-IPs) (Berridge *et al*., 1982). To determine the effects of histamine antagonists, the latter drugs were added to the cells 30 min prior to the addition of histamine. The medium was aspirated at the end of the incubation, and the assay terminated by the addition of 1 ml of ice-cold 0.1 M formic acid and the [^{3}H]-IPs quantified by standard ion exchange chromatography (Berridge *et al*., 1982) and liquid scintillation spectrometry.

Intracellular Ca^{2+}-mobilization studies

Histamine-stimulated mobilization of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in CEPI-17-CL4 cells was studied using the standard Fura-2 fluorescent Ca^{2+} chelator method (Grynkiewicz *et al*., 1985) on stirred suspensions of cells in cuvets (Griffin *et al*., 1997; 1998; Wiernas *et al*., 1998). The methods used for the CEPI cells have been extensively detailed in a recent publication (Wiernas *et al*., 1998). Specific details pertaining to the histamine-related studies using this technique include the following. For the determination of potential antagonist effects, each test compound (6–15 μl) was added to the cell suspension (1.5 ml) for 20 min prior to adding histamine. EGTA (1 M stock) was utilized to chelate extracellular calcium and, therefore, define the importance of extracellular Ca^{2+} influx for the effect of histamine on $[Ca^{2+}]_i$. A selective phospholipase C inhibitor (U73122; (1-(6-((17β-3-methoxyestra-1,3,5(10-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione)) was utilized to block the formation of IPs and, thus, help establish the importance of this pathway for the effect of histamine on $[Ca^{2+}]_i$. The H_1-receptor selective antagonist, triprolidine, was utilized to confirm that histamine-induced $[Ca^{2+}]_i$ release can be blocked with an appropriate H_1-receptor antagonist.

Cytokine release studies

These studies were performed as previously described (Wiernas *et al*., 1998). Briefly, confluent P-CEPI or CEPI-17-CL4 cells were incubated for 24 h at 37°C with 5 μl of various concentrations of histamine (0.01 μM–1 mM final) in 0.5 ml of low calcium (serum-free) DMEM medium per well. Interleukin-1α (IL-1α; 10 ng ml^{-1} final) was used as a positive control. Following this incubation period, commercially available ELISA kits were used according to the manufacturer's directions to quantify the levels of various cytokines (interleukin-6, {IL-6}, interleukin-8 {IL-8} and granulocyte macrophage colony stimulating factor {GM-CSF}) released into the incubation medium *via* spectrophotometry at 450 nm using a series of reference standards of known concentration using a microplate reader. All the kits used in the current studies employ the quantitative sandwich enzyme immunoassay procedure. The limits of detection (sensitivity) for each cytokine were as follows: IL-6 0.7 pg ml^{-1}, IL-8 6 pg ml^{-1}, GM-CSF $<$2.8 pg ml^{-1}, TNF-α $<$0.2 pg ml^{-1}.

PGE_2 release studies

Once again, these studies were performed as previously described (Wiernas *et al*., 1998). PGE_2 released from P-CEPI and CEPI-17-CL4 cells following stimulation by various agonists was determined using a commercially available radioimmunoassay (RIA) kit according to the manufacturer's instructions. The cell stimulation was similar to that described above for cytokine release except that the incubation period was 60 min and 10% foetal bovine serum was used as a positive control. All standards and reagents supplied in the kit were prepared as stipulated in the RIA kit instructions. The limits of detection (sensitivity) of the PGE_2 RIA was 0.44 pg well^{-1}.

Collagenase-1 release studies

The procedures used for these studies with P-CEPI and CEPI-17-CL4 cells were basically identical to those utilized for the cytokine release studies (also see Wiernas *et al*., 1998). The only differences were the use of KGM medium, PMA (3 μg ml^{-1}) as the positive control and a commercially available collagenase-1 (Matrix Metalloproteinase-1; MMP-1) ELISA kit. This kit also utilizes the quantitative sandwich enzyme immunoassay procedure mentioned above with the MMP-1 levels being determined *via* spectrophotometry using a series of reference standards of known concentration. The kits were utilized according to the manufacturer's instructions. The limits of detection (sensitivity) of the MMP-1 ELISA kit was 1.7 ng ml^{-1}.

Materials

The reagents, chemicals, drugs and materials used in the present studies were purchased or were gifts from the following sources: Optisol® or Dexol® from Chiron Ophthalmics, (Irvine, CA, U.S.A.); dispase from Collaborative Biomedical, (Bedford, MA, U.S.A.); KGM from Clonetics Corp. (San Diego, CA, U.S.A.); DMEM and DMEM/F-12 media, BSS, trypsin/EDTA, phosphate-buffered saline without Ca^{2+} or Mg^{2+} and Hanks' BSS from Life Technologies, (Grand Island, NY, U.S.A.); culture plates from Corning/Costar, (Cambridge, MA, U.S.A.); Becton Dickenson, (Oxnard, U.S.A.), CAFNC-matrix from Biological Research Faculty and Facility, Inc., (Ijamsville, MD, U.S.A.); BK was from Peninsula Labs., (Belmont, CA, U.S.A.); histamine, pyrilamine, triprolidine, ranitidine and clobenpropit were from Research Biochemicals, Int. (Natick, MA, U.S.A.); MMP-1 ELISA kits, PGE_2 RIA kits and [^{3}H]-myo-inositol (15-17 Ci mmol^{-1}) from Amersham Corp., (Arlington Heights, IL, U.S.A.); Cytokine ELISA kits from R & D Systems, (Minneapolis, MN, U.S.A.); [^{3}H]-pyrilamine (24.4 Ci mmol^{-1}) from New England Nuclear (NEN), (Boston, MA, U.S.A.); Fura-2 AM from Molecular Probes, (Eugene, OR, U.S.A.); AG1 $\times$ 8 resin, Econo-columns and sodium dodecyl sulphate from Biorad, (Richmond, CA, U.S.A.); BSA, digitonin, formic acid, ammonium formate, LiCl, polyethylenimine and trichloroacetic acid from Sigma, (St. Louis, MO, U.S.A.); PMA from L.C. Laboratories, (San Diego, CA, U.S.A.); gentamicin, penicillin/streptomycin from Gibco/BRL, (Grand Island, NY, U.S.A.); U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2.5-dione) and U73343 (1-(6-((17β-3-methoxyestra-1,3, 5(10)-trien-17-yl) amino)hexyl)–2,5-pyrrolidine-dione) from Biomol Research Laboratories, (Plymouth Meeting, PA, U.S.A.); Ecolume scintillation cocktail from ICN, (Costa Mesa, CA, U.S.A.); Opti-Fluor scintillation cocktail from Packard Instrument Co., (Meriden, CT, U.S.A.); Origin Scientific Graphics software package from Microcal, (Northampton, MA, U.S.A.).

Data analysis

The original data (d.p.m. bound; d.p.m./well; nM $[Ca^{2+}]_i$ mobilized) were analysed using a non-linear, iterative curve-

fitting computer program (Bowen & Jerman, 1995) and Origin® software (Sharif *et al*., 1996b; 1997b) incorporating a logistic function. The competition data for pyrilamine vs [^{3}H]-pyrilamine were processed using the 'EDBA' suite of computer programs (McPherson, 1983a,b) to perform Scatchard analysis and thus derive the apparent receptor affinity (K_d) and apparent density (B_{max}) parameters. The initial inhibition constants (IC_{50}s) for various compounds competing for [^{3}H]-pyrilamine binding were converted to equilibrium dissociation constants (K_is) using the standard Cheng-Prusoff equation (Cheng & Prusoff, 1973).

Agonist potency (EC_{50}) was defined as the concentration of the compound required to stimulate 50% of the maximal PI turnover response. Individual concentration-response curves for each agonist were analysed as above to obtain the potency values. However, in order to present composite data for each compound from several experiments (e.g. Table 1), the data were normalized and presented as a percentage of the maximal specific binding, or for functional assays, basal (unstimulated) levels of [^{3}H]-IPs or $[Ca^{2+}]_i$ were set to zero and the maximally-stimulated levels of [^{3}H]-IPs or $[Ca^{2+}]_i$ at the top of each of the concentration-response curves were set to 100%. All intermediate levels of [^{3}H]-IPs accumulation or $[Ca^{2+}]_i$ were then calculated as a percentage of the maximal stimulation. Some data were presented as histograms to depict the concentration-response relationship for histamine or the relative activity of reference compounds. Statistical analysis utilized the standard ANOVA with post-test procedure for determining the level of significance between the basal responses and the magnitude of the effects of the test compounds. A P value of <0.05 indicated the minimal level of statistical significance between the afore-mentioned parameters. The potency of triprolidine for inhibiting histamine-induced PI turnover was using the following equation:

$$K_i = IC_{50}/1 + (\text{histamine concentration})/(EC_{50} \text{ of histamine}).$$

Origin® Scientific Graphics software package was utilized to construct the figures shown.

Results

[^{3}H]-pyrilamine binding

Specific [^{3}H]-pyrilamine binding to washed total particulate homogenates of CEP-17-CL4 cells comprised >93% of the total binding, was of high affinity ($K_d = 3.76 \pm 0.78$ nM, $n = 4$) and reflected interaction with a finite number of saturable binding sites ($B_{max} = 1582 \pm 161$ fmol g^{-1} wet weight) (e.g. Figure 1a). Various histamine receptor subtype-selective antagonists concentration-dependently inhibited [^{3}H]-pyrilamine binding (Figure 1b) with relative affinities indicative of labelling of H_1-receptors. Thus: for pyrilamine the

Table 1 Affinity of various histamine antagonists competing for [^{3}H]-pyrilamine binding to CEPI-17-CL4 cell homogenates

Compound	*Affinity* (K_i, nM)	*Hill coefficient* (nH)
Pyrilamine	3.63 ± 0.84	1.0 ± 0.06
Triprolidine	7.67 ± 2.6	0.83 ± 0.14
Clobenpropit	13,300 ± 7100	0.84 ± 0.23
Ranitidine	> 32,400	–

Data are means ± s.e.mean from 3–4 experiments. The Hill coefficients were not significantly different from unity ($P > 0.05$).

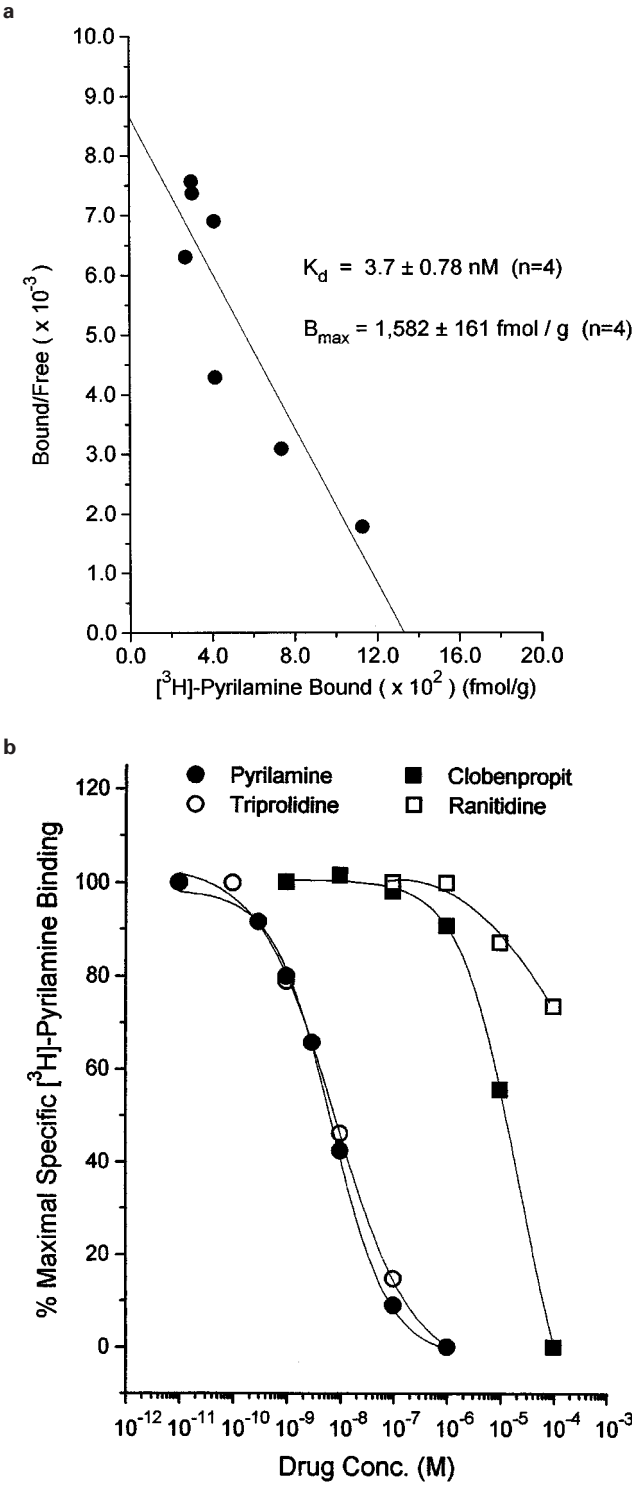

Figure 1 Scatchard analysis of specific [^{3}H]-pyrilamine binding (a) and competition curves (b) for various histamine antagonists competing for specific [^{3}H]-pyrilamine binding to washed CEPI-17-CL4 cell homogenates. The data for unlabelled pyrilamine competing for [^{3}H]-pyrilamine binding were transformed into Scatchard plots and the K_d and B_{max} values obtained from the computer-fitted plots (a). Various histamine antagonists (10 pM – 100 μM) were tested for their ability to compete for [^{3}H]-pyrilamine binding. Inhibition curves were generated (b) and the IC_{50} and K_i values calculated from the computer-fitted plots (Table 1). Data shown are from a single representative experiment. Composite data from several experiments are shown in Results and Table 1.

$K_i = 3.62 \pm 0.84$ nM; triprolidine, $K_i = 7.67 \pm 2.6$ nM; ranitidine and clobenpropit, $K_i > 13$ μM (all $n = 3-4$) (Table 1; Figure 1b).

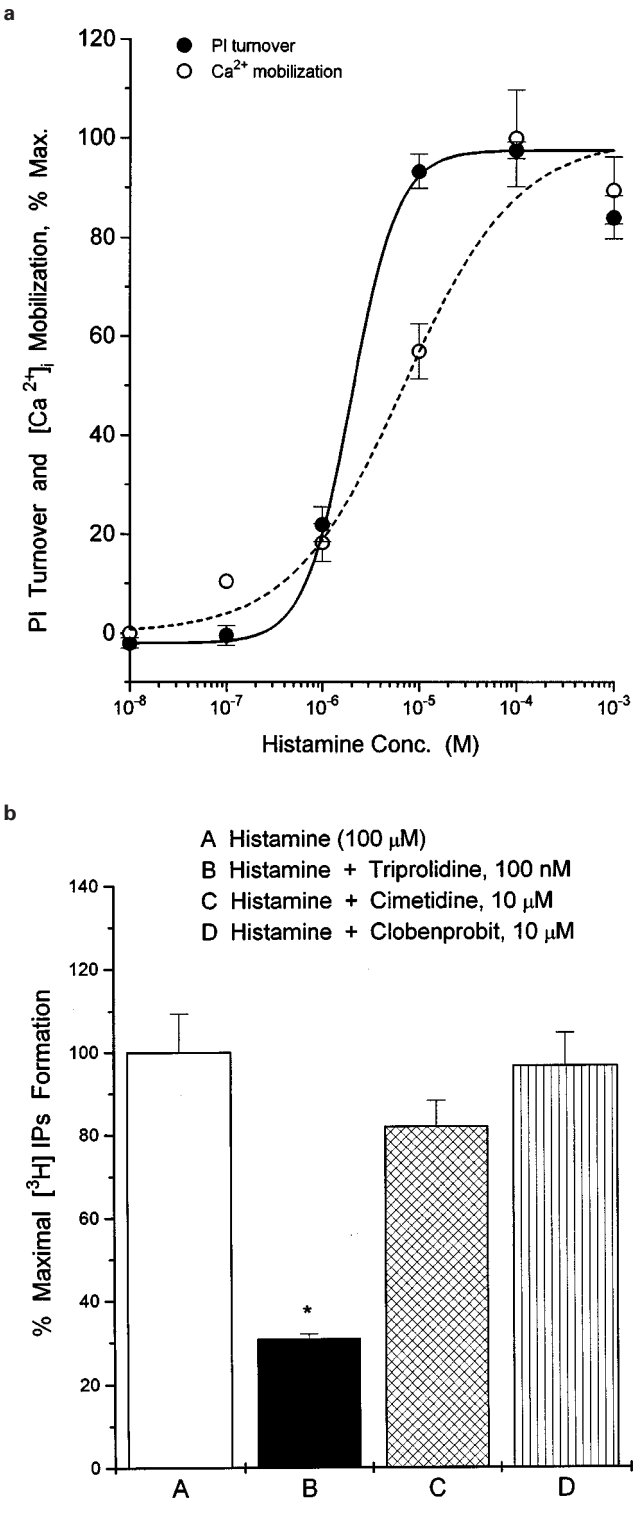

Figure 2 Histamine-induced PI turnover and $[Ca^{2+}]_i$ mobilization in CEPI cells. (a) shows the concentration-response curves for histamine in stimulating PI turnover and $[Ca^{2+}]_i$ mobilization and (b) shows the antagonistic effects of triprolidine (100 nM), cimetidine (10 μM) and clobenpropit (10 μM) on the PI response in CEPI-17-CL4. Data were represented as the percentage of the maximal response induced by histamine. Data are mean from nine experiments (vertical line show s.e.mean) for PI turnover and from up to four experiments for the $[Ca^{2+}]_i$ mobilization studies. Similar results were obtained for P-CEPI cells (data not shown). *$P < 0.05$ relative to the control histamine-induced response by ANOVA with a post-test.

PI turnover

Histamine induced the production of [^{3}H]-IPs in P-CEPI (potency, $EC_{50} = 14.9 \pm 4.9$ μM, $n = 9$) and CEPI-17-CL4 ($EC_{50} = 4.7 \pm 0.2$ μM, $n = 9$) cells in a concentration-dependent manner (e.g. Figure 2a). The apparent difference in the histamine potency in the two cell-types was not statistically different ($P > 0.05$). The histamine-induced PI turnover in both cell-types was substantially ($\geqslant 90\%$) blocked by nanomolar concentrations of triprolidine, an H_1-receptor selective antagonist, but not significantly influenced by cimetidine or clobenpropit, H_2- and H_3-receptor selective antagonists (Figure 2b). The potency, determined from concentration-response curves, of triprolidine for inhibiting histamine-stimulated PI turnover was: $K_i = 3.2 \pm 0.66$ nM in P-CEPI cells; $K_i = 3.03 \pm 0.8$ nM in CEPI-17-CL4 cells ($n = 4-10$) (e.g. Figure 3a). The specific PLC inhibitor, U73122, inhibited histamine-induced PI turnover ($IC_{50} = 4.8 \pm 2.4$ μM, $n = 3$) (Figure 3b), while an inactive analogue, U73343 (10 nM – 10 μM) was without any effect (data not shown).

$[Ca^{2+}]_i$ mobilization

Histamine stimulated mobilization of $[Ca^{2+}]_i$ in CEPI-17-CL4 cells ($EC_{50} = 6.3 \pm 1.5$ μM; $n = 4$) which correlated reasonably well with its potency in stimulating PI turnover (e.g. Figure 2a). Representative tracings of $[Ca^{2+}]_i$ mobilized in response to control and test agents are shown in Figure 4a. The response to a maximally effective concentration of histamine (100 μM) was greater than that to a maximally effective concentration of BK (1 μM) (Figure 4a). However, while triprolidine (10 μM) blocked the histamine-induced $[Ca^{2+}]_i$ mobilization it did not influence the BK-induced response (Figure 4a, panel B). Similar results were obtained in P-CEPI cells (data not shown). The specific PLC inhibitor, U73122 (4 μM) effectively prevented histamine-induced $[Ca^{2+}]_i$ mobilization (Figure 4b). However, U73343 (a known inactive analogue of U73122) neither inhibited BK- nor histamine-induced $[Ca^{2+}]_i$ mobilization (data not shown).

In order to determine the contribution of extracellular Ca^{2+} to the histamine-induced $[Ca^{2+}]_i$ mobilization in CEPI-17 cells, the effect of calcium chelator EGTA (4 mM final) was investigated. EGTA appeared to reduce the histamine-induced $[Ca^{2+}]_i$ mobilization by about 28% in CEPI-17-CL4 cells (Figure 4b).

Cytokine release

Histamine concentration-dependently stimulated the secretion of IL-8, IL-6 and granulocyte macrophage colony-stimulating factor (GM-CSF) from CEPI cells with potencies of 1.28 ± 0.32 μM for IL-8, 2.77 ± 1.61 μM for IL-6 and 1.45 ± 0.53 μM for GM-CSF ($n = 4$ for each) (e.g. Figure 5). Histamine appeared not to significantly effect tumour necrosis factor-α (TNF-α) release (data not shown). However, IL-1 (10 ng ml^{-1} final) potently stimulated the release of all four cytokines from these cells by 1.8 ± 0.14 to 2.51 ± 0.9 fold above basals ($n = 4$ for each cytokine; $P < 0.05$). Typically, the basal levels of the cytokines were: IL-8 = 903 – 1510 pg ml^{-1}, IL-6 = 244 – 270 pg ml^{-1}, GM-CSF = 12 – 19 pg ml^{-1}, TNF-α = 0.35 – 0.73 pg ml^{-1}.

PGE_2 and collagenase-1 release

Histamine (0.01 μM – 1 mM) had no significant effect on the release of either PGE_2 (Figure 6a) or collagenase-1 (MMP-1)

(Figure 6b) from CEPI cells. However, 10% foetal bovine serum stimulated PGE_2 release (Figure 6a) and the phorbol ester (PMA ; 3 μg ml^{-1} final) stimulated MMP-1 release when tested in parallel with histamine (Figure 6b).

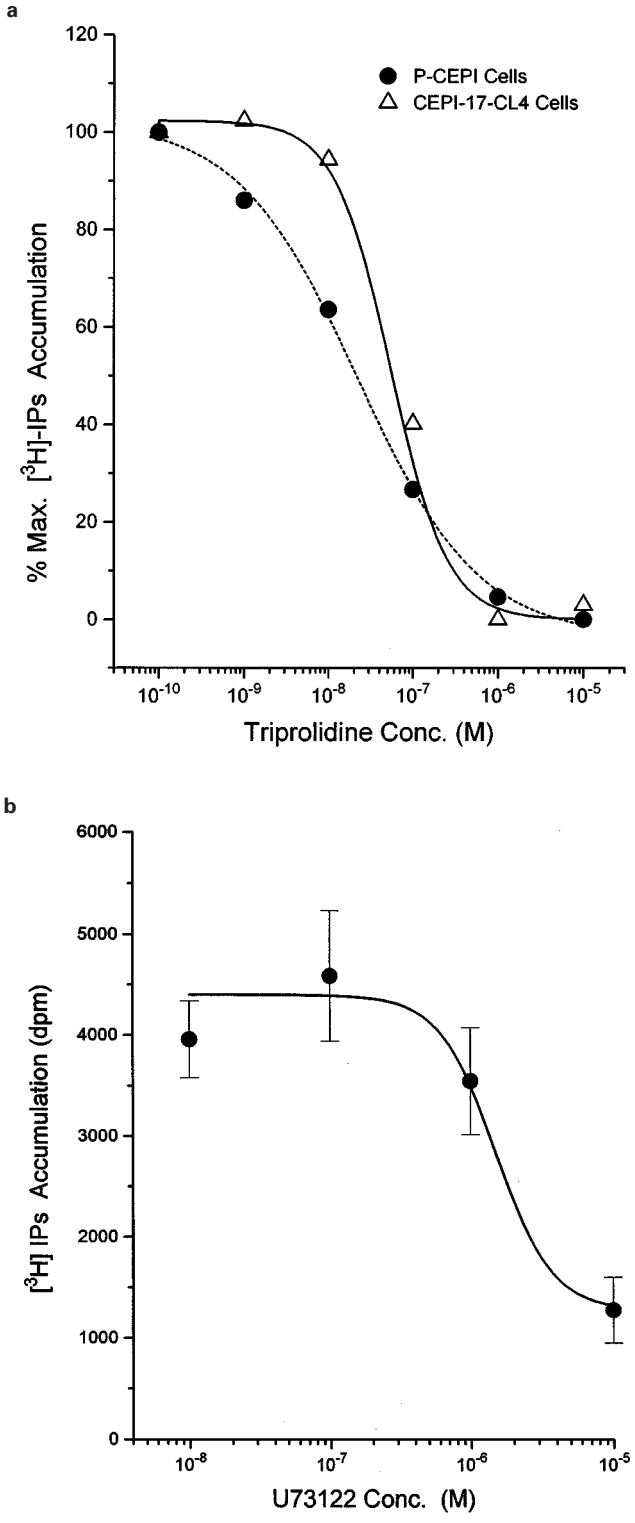

Figure 3 Effects of triprolidine and the phospholipase C inhibitor, U73122, on PI turnover in CEPI cells. (a) shows the concentration-dependent inhibition of histamine-induced PI turnover in P-CEPI (●) and CEPI-17-CL4 (△) cells. (b) shows the effects of U73122. The cells were pre-treated with various concentrations of triprolidine or U73122 prior to stimulation with 100 μM histamine to induce the accumulation of [^{3}H]-IPs. Data are from a representative experiment for each cell type using triprolidine but the composite numerical data obtained from four such experiments are shown in the Results section. Data (d.p.m.) are mean from three experiments for U73122; vertical lines show s.e.mean.

Discussion

We recently reported in an abstract (Sharif *et al*., 1997b) the presence of histamine receptors coupled to PLC in human

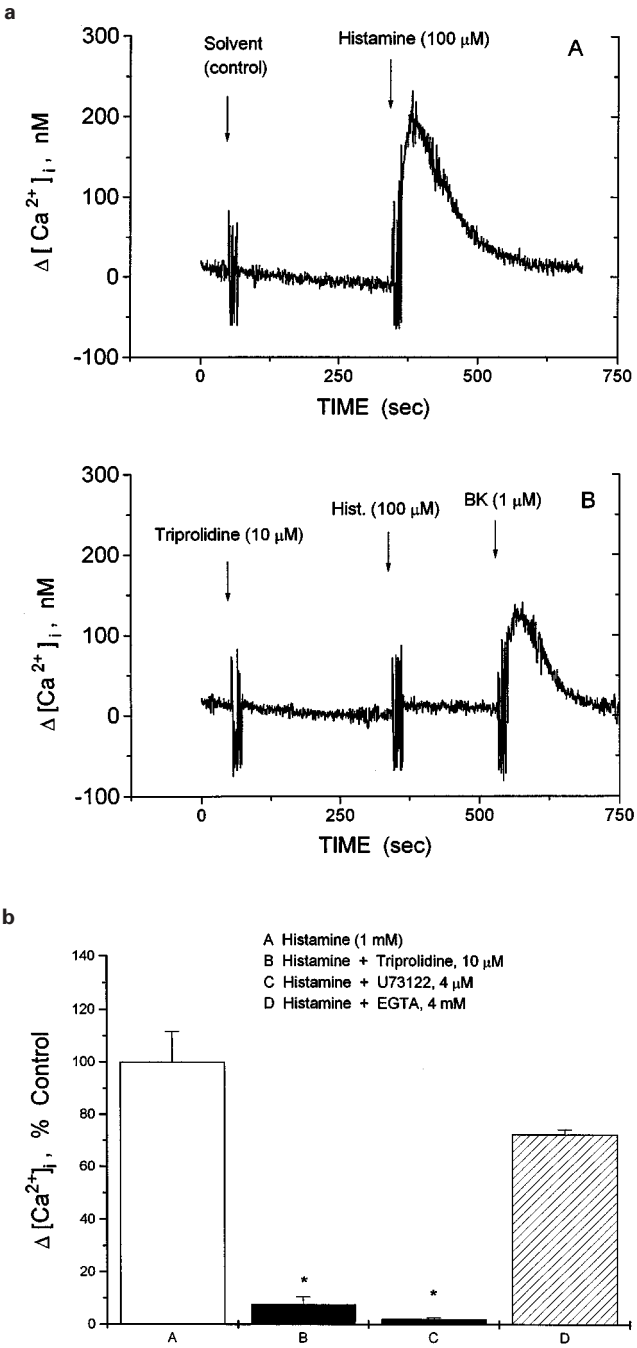

Figure 4 Effects of various agents on $[Ca^{2+}]_i$ mobilization in CEPI-17-CL4 cells. Cells were pre-treated with the test agent for 5 min prior to the addition of histamine. Representative traces of the $[Ca^{2+}]_i$ mobilization signals (a) induced by histamine (panel A) are shown. Effects of the H_1-receptor histamine antagonist, triprolidine, on histamine (100 μM)- and bradykinin (BK; 1 μM)-induced Ca^{2+}-responses are shown in panel B (a). In (b) the effects of triprolidine, U73122 and EGTA on histamine-induced $[Ca^{2+}]_i$ mobilization in CEPI-17-CL4 cells are shown. *$P<0.05$ relative to the histamine-induced response by ANOVA with a post-test.

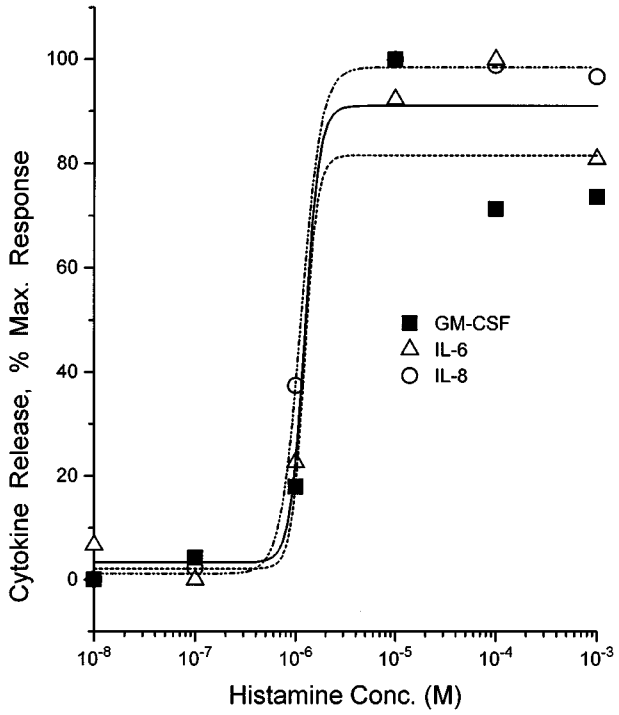

Figure 5 Effects of histamine on cytokine release from P-CEPI cells. Effects of various concentrations of histamine (10 nM – 1 mM) on the release of IL-8, IL-6 and GM-CSF are shown. Similar results were obtained with CEPI-17-CL4 cells (data not shown). Data are mean from cells obtained from a representative of four human donors. IL-1 (10 ng ml^{-1}) significantly ($P<0.05$ as compared to the basal level by ANOVA with a post-test) stimulated the release of IL-8, IL-6, GM-CSF and TNF-α from these cells (see Results).

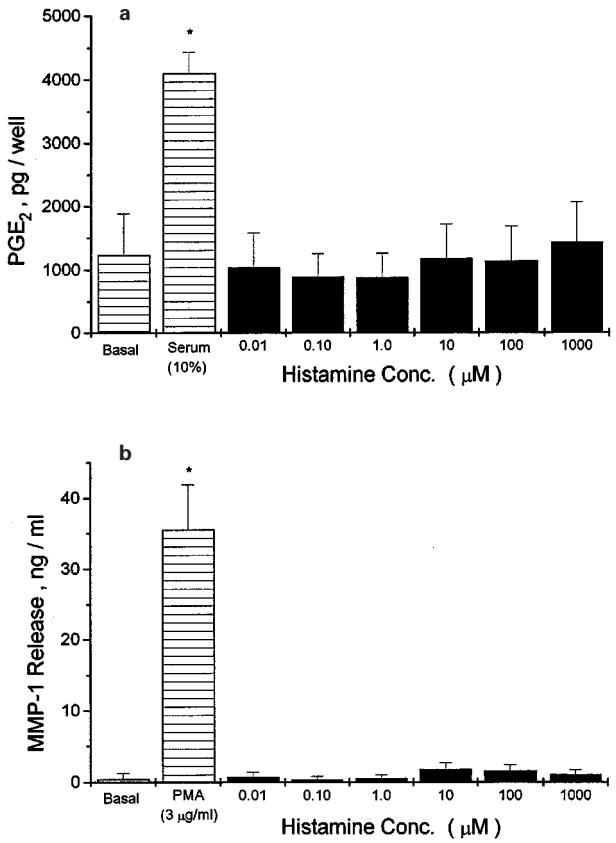

Figure 6 Effects of histamine and foetal bovine serum on PGE_2 release (a) and effects of histamine and phorbol ester on MMP-1 release (b) from CEPI cells. Data are mean from 3 – 4 experiments/donors using histamine (0.01 – 1000 μM) or serum (10%) or PMA (3 μg ml^{-1}) to stimulate the cells; vertical lines show s.e.mean. *$P<0.05$ as compared to the basal level by ANOVA with a post-test.

P-CEPI cells and showed the immortalized cells to continue to express these receptors. The aims of the present studies were to use radioligand binding and second messenger assays to pharmacologically characterize these receptors on the primary and immortalized CEPI cells, and then to endeavour to associate these characteristics with possible functional coupling of the receptors to the release of some pro-inflammatory mediators (e.g. cytokines and prostaglandin E_2) since histamine causes symptoms of allergic conjunctivitis on the ocular surface (Berdy *et al*., 1991; Abelson & Schaefer, 1993).

In the current studies, [^{3}H]-pyrilamine bound to washed CEPI-17-CL4 cell homogenates with nanomolar affinity and to a finite number of saturable binding sites (Figure 1a) which exceeded those for [^{3}H]-BK (Wiernas *et al*., 1998). Since [^{3}H]-pyrilamine is an H_1-selective histamine antagonist radioligand (Hill, 1990), our data suggested that the receptors present on these cells were of the H_1-subtype. This was confirmed further by the finding that both triprolidine and pyrilamine (both being H_1-selective; Sharif *et al*., 1994) potently competed for [^{3}H]-pyrilamine binding (nanomolar affinities) while the H_2- and H_3-selective antagonists (ranitidine and clobenpropit) were weak inhibitors of [^{3}H]-pyrilamine binding (Figure 1b). These data compared well with the ligand binding characteristics of the rodent brain H_1-receptors (Hill, 1990; Sharif *et al*., 1994; 1996b) and those in other cells/tissues (Hill, 1990) and helped pharmacologically define the histamine receptor binding sites on CEPI cells as the H_1-subtype binding sites.

Histamine stimulated PI turnover and intracellular Ca^{2+} ($[Ca^{2+}]_i$) mobilization in the CEPI cells in a concentration-dependent manner with a micromolar potency (Figure 2a). These observations compared well with those previously reported for human conjunctival epithelial cells (Sharif *et al*., 1996a), human trabecular meshwork cells and human corneal fibroblasts (Sharif *et al*., 1994; 1997c) and smooth muscle cells (DDT1MF-2 cell-line) (Dickenson & Hill, 1991). Once again, since the histamine-induced PI turnover and $[Ca^{2+}]_i$ mobilization in CEPI cells were potently blocked by triprolidine but weakly influenced by cimetidine and clobenpropit (Figures 2b and 3a), these functional responses appeared to be mediated by the H_1-receptor subtype, this being in agreement with the radioligand binding studies on CEPI-17-CL4 cells discussed above and also correlating well with the functional studies on human conjunctival epithelial cells (Sharif *et al*., 1996a; 1997b). In addition, the histamine-induced $[Ca^{2+}]_i$ mobilization in these cells was specifically antagonized by triprolidine, which was without any significant effect on the BK-induced response (Figure 4a).

The fact that the PLC inhibitor, U73122, effectively blocked both the PI turnover and $[Ca^{2+}]_i$ mobilization in CEPI cells (Figures 3b and 4b) suggested that the product(s) of the inositol lipid hydrolysis were directly involved in mobilizing $[Ca^{2+}]_i$ in these cells as noted previously in Swiss 3T3 cells (Griffin *et al*., 1997), rat vascular smooth muscle cells (A7r5) (Griffin *et al*., 1998), platelets and neutrophils (Bleasdale *et al*., 1990) and other cell-types (Berridge & Irvine, 1984). The histamine-induced $[Ca^{2+}]_i$ mobilization in CEPI-17-CL4 cells appeared to be primarily derived from intracellular stores since pre-treatment of the cells with the Ca^{2+}-chelator, EGTA, only partially reduced the Ca^{2+}-signal (Figure 4b). These results

suggest that the histamine-induced functional responses in the CEPI cells depend largely on the PLC-induced generation of inositol trisphosphate (IP_3) to release $[Ca^{2+}]_i$ from the cellular endoplasmic reticulum, and also perhaps on the diacylglycerol (DAG) which is concomitantly produced from PI hydrolysis (Berridge & Irvine, 1984). However, these and other roles of DAG in the CEPI cells remain to be investigated.

Human CEPI (P-CEPI and CEPI-17-CL4) cells express mRNAs for pro-inflammatory cytokines (Sharif *et al*., 1997b). Therefore, it was noteworthy that histamine concentration-dependently stimulated the release of IL-8, IL-6 and GM-CSF, without significantly stimulating TNF-α release (Figure 5) with potencies matching histamine's effects on PI turnover and $[Ca^{2+}]_i$ mobilization. This indicated some degree of specificity of the histamine effect even within the cytokine family. Notably, however, the positive control agent (IL-1α) tested alongside with histamine was a potent secretagogue of all four cytokines in these cells. These findings corroborate other reports on the ability of human CEPI cells/corneal tissue to secrete cytokines in response to various stimuli (Wakefield & Lloyd, 1992; Cubitt *et al*., 1993; Faquet *et al*., 1997) and also during the development of the pathology of pseudophakic bullous keratopathy (Rosenbaum *et al*., 1995). The cytokine release induced by histamine in the CEPI cells was specifically related to histamine since BK was not a cytokine secretagogue (Wiernas *et al*., 1998). However, even though the positive control agents (serum and PMA) potently stimulated the release of PGE_2 and collagenase-1 (MMP-1) from CEPI cells (Figure 6), respectively, histamine over a wide concentration range failed to stimulate PGE_2 or MMP-1 secretion from these cells. This is in contrast to the stimulatory effects of platelet-activating factor (PAF) on these pro-inflammatory mediator release from rabbit CEPI cells as measured from rabbit corneas *in vitro* (Hurst *et al*., 1995; Tao *et al*., 1995; Bazan *et al*., 1997).

In conclusion, our studies have shown the presence of high-affinity [^{3}H]-pyrilamine binding sites on human CEPI cells which represent the pharmacologically responsive H_1-receptors mediating PI hydrolysis, leading to the generation of IPs, which in turn stimulate $[Ca^{2+}]_i$ mobilization predominantly from intracellular stores. The consequence of H_1-receptor activation in the human CEPI cells appears to be the stimulation of cytokine release indicating a possible link to the involvement of corneal epithelial cells in causing and/or amplifying the allergic/inflammatory conditions associated with the human ocular surface. Collectively, our results have shown that the CEPI cells are fully responsive to the mast cell mediator histamine with the ability to potentially further amplify the allergic inflammation by elaborating pro-inflammatory cytokines in response to histamine.

Additionally, CEPI cells *in vivo* could be recruited by the conjunctival mast cells and epithelial cells to initiate/propagate the inflammation on the ocular surface. In terms of possible therapeutic implications, these results suggest that treatment with antihistamines such as emedastine (Emedine™; Sharif *et al*., 1994; 1997c; Yanni *et al*., 1994) and/or compounds like olopatadine (Patanol™; Sharif *et al*., 1996b; Yanni *et al*., 1997) which possess antihistaminic and antiallergic properties to combat allergic conjunctivitis would potentially offer additional benefits by also inhibiting potential secretion of proinflammatory cytokines from the corneal epithelial cells. Therefore, the CEPI cells in culture offer means to study ocular inflammatory mechanisms and they could also be useful in the drug discovery process to develop new generation of drugs to treat allergic inflammatory diseases of the human ocular surface.

The Alcon internal reviewers are thanked for critically reading the manuscript and for providing helpful comments. Dr S.D. Dimitrijevich and colleagues (University of North Texas, Health Sciences Center, UNTHSC, Fort Worth, Texas) are thanked for providing primary cell cultures from tissue provided by Alcon. The human donors and their families who so generously donated their eyes for science and made the current studies possible are gratefully acknowledged.

References

ABELSON, M.B. & SCHAEFER, K. (1993). Conjunctivitis of allergic origin: immunologic mechanisms and current approaches to therapy. *Survey Ophthalmol.,* **38,** 115–132.

ALLANSMITH, M.R. & ROSS, R.N. (1988). Ocular Allergy. *Clin. Allergy,* **18,** 1–13.

ARAKI-SASAKI, K., OHSAKI, Y., SASABE, T., HAYASHI, K., WATANABE, H., TANO, Y. & HANDA, H. (1995). An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest. Ophthalmol. Vis. Sci.,* **36,** 614–621.

BAZAN, H.E.P., TAO, Y., DECOSTER, M.A. & BAZAN, N.G. (1997). Platelet-activating factor induces cyclooxygenase-2 gene expression in corneal epithelium. *Invest. Ophthalmol. Vis. Sci.,* **38,** 2492–2501.

BEDARD, M., MCCLURE, C.D., SCHILLER, N.L., FRANCOEUR, C., CANTIN, A. & DENIS, M. (1993). Release of interleukin-8, interleukin-6 and colony stimulating factors by upper airway epithelial cells: implication for cystic fibrosis. *Am. J. Cell. Mol. Biol.,* **9,** 455–462.

BENEZRA, D., GRIFFIN, B.W., MAFTZIR, G., SHARIF, N.A. & CLARK, A.F. (1997). Topical formulations of novel angiostatic steroids inhibit rabbit corneal neovascularization. *Invest. Ophthalmol. Vis. Sci.,* **38,** 1954–1962.

BERDY, G., ABELSON, M.B., GEORGE, M.A., SMITH, L.M. & GIOVANONI, R.L. (1991). Allergic conjunctivitis: a survey of new anti-histamines. *J. Ocular Pharmacol.,* **7,** 313–324.

BERRIDGE, M.J., DOWNES, C.P. & HANLEY, M.R. (1982). Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary gland. *Biochem. J.,* **206,** 587–595.

BERRIDGE, M.J. & IRVINE, R.F. (1984). Inositol triphosphate, a novel second messenger in cellular signal transduction. *Nature,* **312,** 315–321.

BLEASDALE, J.E., THAKUR, N.R., GREMBAN, R.S., BUNDY, G.L., FITZPATRICK, F.A., SMITH, R.J. & BUNTING, S. (1990). Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. *J. Pharmacol. Expt. Ther.,* **255,** 756–768.

BOWEN, W.P. & JERMAN, I. (1995). Nonlinear regression using speadsheets. *Trends, Pharmacol. Sci.,* **16,** 413–417.)

CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.,* **22,** 3099–3108.

CUBITT, C.L., TANG, Q., MONTERIO, C.A., LAUSCH, R.N. & OAKES, J.E. (1993). IL-8 gene expression in cultures of human corneal epithelial cells and keratocytes. *Invest. Ophthalmol. Vis. Sci.,* **34,** 3199–3206.

DICKENSON, J.M. & HILL, S.J. (1991). Histamine-stimulated increases in intracellular calcium in the smooth muscle cell line, DDT1MF-2. *Biochem. Pharmacol.,* **42,** 1545–1550.

ECKMANN, L., KAGNOFF, M.F. & FIERER, J. (1993). Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. *Infect. Immunol.,* **61,** 4569–4574.

FAQUET, B., BUIATTI-TCHENG, M., TROMVOUKIS, Y., OFFORD, E.A. & LECLAIRE, J. (1997). Cytokine production after treatment with surfactants in a human corneal epithelial cell line. *Invest. Ophthalmol. Vis. Sci.,* **38,** (suppl): Abst. 4046.

GAMACHE, D.A., DIMITRIJEVICH, S.D., WEIMER, L.K., LANG, L., GRAFF, G. & YANNI, J.M. (1997). Secretion of proinflammatory cytokines by human conjunctival epithelial cells. *Ocular Immunol. Inflamm.,* **5,** 117–128.

GRIFFIN, B.W., WILLIAMS, G.W., CRIDER, J.Y. & SHARIF, N.A. (1997). FP prostaglandin receptors mediating inositol phosphates generation and calcium mobilization in Swiss 3T3 cells: a pharmacological study. *J. Pharmacol. Expt. Ther.,* **281,** 845–854.

GRIFFIN, B.W., MAGNINO, P., PANG, I.-H. & SHARIF, N.A. (1998). Pharmacological characterization on an FP prostaglandin receptor on rat vascular smooth muscle cells (A7r5) coupled to phosphoinositide turnover and intracellular calcium mobilization. *J. Pharmacol. Expt. Ther.* **286,** 411–418.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{++} indicators with greatly improved fluorescent properties. *J. Biol. Chem.,* **260,** 3440–3450.

HIGORANI, M. & LIGHTMAN, S. (1995). Therapeutic options in ocular allergic disease. *Drugs,* **50,** 208–221.

HILL, S.J. (1990). Distribution, properties, and functional characteristics of three classes of histamine receptor. *Pharmacol. Rev.,* **42,** 45–83.

HURST, J.S. & BAZAN, H.E.P. (1995). Platelet-activating factor preferentially stimulates the phospholipase A_2/cycloxygenase cascade in the rabbit cornea. *Curr. Eye Res.,* **14,** 769–775.,

IRANI, A., BUTRUS, S., TABBARA, K. & SCHWARTZ, L. (1990). Human conjunctival mast cells: distribution of MC_1 and MC_{TC} in vernal conjunctivitis and giant papillary conjunctivitis. *J. Allergy Clin. Immunol.,* **86,** 34–39.

KAHN, C.R., YOUNG, E., LEE, I.H. & REHM, J.S. (1993). Human corneal epithelial primary cultures and cell lines with extended life span: *in vitro* model for ocular studies. *Invest. Ophthalmol. Vis. Sci.,* **34,** 3429–3441.

LEONARDI, A., SECCHI, A.G., BRIGGS, R. & ALLANSMITH, M.R. (1992). Conjunctival mast cells and the allergic late phase reaction. *Ophthalmic Res.,* **24,** 234–242.

MARINI, M., VITTORI, E., HOLLEMBORG, J. & MATTOLI, S. (1992). Expression of the potent inflammatory cytokines, granulocyte-macrophage-colony-stimulating factor and interleukin-6 and interleukin-8, in bronchial epithelial cells of patients with asthma. *J. Allergy Clin. Immunol.,* **89,** 1001–1009.

MCGEE, D.W., BEAGLEY, K.W., AICHER, W.K. & MCGHEE, J.R. (1992). Transforming growth factor- enhances interleukin-6 secretion by intestinal epithelial cells. *Immunology,* **77,** 7–12.

MCPHERSON, G.A. (1983a). A practical computer based approach to the analysis of radioligand binding experiments. *Computer Prog. in Biomed.,* **17,** 107–114.

MCPHERSON, G.A. (1983b). Analysis of radioligand binding experiments on a microcomputing system. *Trends Pharmacol. Sci.,* **4,** 369–370.

PROIA, A.D. (1994). Inflammation. In *Pathobiology of ocular disease*, eds. Garner A & Klintworth G.K., Chapter 2, New York: Marcel Dekker. pp. 63–99.

PROUD, D., SWEET, J., STEIN, P., SETTIPANE, R.A., KAGEY-SOBOTKA, A., FREIDLAENDER, M.H. & LICHTENSTEIN, L.M. (1990). Inflammatory mediator release on conjunctival provocation of allergic subjects with allergen. *J. Allergy Clin. Immunol.,* **85,** 896–905.

REIM, M., KOTTEK, A. & SCHERAGE, N. (1997). The cornea surface and wound healing. *Prog. Retinal & Eye Res.,* **16,** 183–225.

ROSENBAUM, J.T., PLANCK, S.R., HUANG, X-N, RIC, L. & ANSEL, J.C. (1995). Detection of mRNA for the cytokines, interleukin-1α, and interleukin-8, in corneas from patients with pseudophakic bullous keratopathy. *Invest. Ophthalmol. Vis. Sci.,* **36,** 2151–2155.

SAUDER, D.N. (1990). The role of epidermal cytokines in inflammatory skin diseases. *J. Invest. Dermatol.,* **95,** 27S–28S.

SHARIF, N.A., CRIDER, J.Y., GRIFFIN, B.W., DAVIS, T.L. & HOWE, W.E. (1997a). Pharmacological analysis of mast cell mediator and neurotransmitter receptors coupled to adenylate cyclase and phospholipase C on immunocytochemically-defined human conjunctival epithelial cells. *J. Ocular Pharmacol. Ther.,* **13,** 321–336.

SHARIF, N.A., OFFORD, E.A., TROMVOUKIS, Y., HOWE, W.E., GRIFFIN, B.W., WIERNAS, T.K. & PFEIFER, A.M.A. (1997b). Immortalized human corneal epithelial (CEPI) cells for use in ocular inflammation and toxicity studies. *Invest. Ophthalmol Vis. Sci.,* **38,** (suppl): Abst. 3349.

SHARIF, N.A., HELLBERG, M.R. & YANNI, J.M. (1997c). Antihistamines, topical ocular. In: *Burger's Medicinal Chemistry & Drug Discovery*, Fifth edition, ed Wolff, M.E., Chapter 64, Volume 5, New York: John Wiley & Sons. pp. 255–279.

SHARIF, N.A., XU, S.X., MAGNINO, P. & PANG, I.-H. (1996a). Human conjunctival epithelial cells express H_1-histamine receptors coupled to phosphoinositide turnover and intracellular calcium mobilization: role in ocular allergic and inflammatory diseases. *Exp. Eye Res.,* **63,** 169–178.

SHARIF, N.A., XU, S.X., MILLER, S.T., GAMACHE, D.A. & YANNI, J.M. (1996b). Characterization of the ocular anti-allergic and anti-histamine effects of olopatadine (AL-4943A), a novel drug for treating ocular allergic diseases. *J. Pharmacol. Expt. Ther.,* **278,** 1251–1260.

SHARIF, N.A., XU, S.X. & YANNI, J.M. (1994). Histamine receptor-subtype affinities, selectivities, and potencies of emedastine, a novel H_1-selective antagonist, and other ocularly employed antihistamines. *Drug Dev. Res.,* **33,** 448–453.

TAO, Y., BAZAN, H.E.P. & BAZAN, N.G. (1995). Platelet-activating factor induces the expression of metalloproteinases-1 and -9 but not -2 or -3, in the corneal epithelium. *Invest. Ophthalmol. Vis. Sci.,* **36,** 345–354.

WAKEFIELD, D. & LLOYD, A. (1992). The role of cytokines in pathogenesis of inflammatory eye disease. *Cytokine,* **4,** 1–5.

WIERNAS, T.K., DAVIS, T.L., GRIFFIN, B.W. & SHARIF, N.A. (1998). Effects of bradykinin on signal transduction, cell proliferation, and cytokine, prostaglandin E_2 and collagenase-1 release from human corneal epithelial cells. *Br. J. Pharmacol.,* **123,** 1127–1137.

WIERNAS, T.K., GRIFFIN, B.W. & SHARIF, N.A. (1997). The expression of functionally-coupled B_2-bradykinin receptors in human corneal epithelial cells and their pharmacological characterization with agonist and antagonists. *Br. J. Pharmacol.,* **121,** 649–656.

YANNI, J.M., MILLER, S.T., GAMACHE, D.A., SPELLMAN, J.M., XU, S.X. & SHARIF, N.A. (1997). Comparative effects of topical ocular anti-allergy drugs on human conjunctival mast cells. *Ann. Allergy Asthma Immunol.,* **79,** 541–545.

YANNI, J.M., STEPHENS, D.J., PARNELL, D.W. & SPELLMAN, J.M. (1994). Preclinical efficacy of emedastine, a potent, selective histamine H_1 antagonist for topical ocular use. *J. Ocular Pharmacol.,* **10,** 665–675.

ZANVIT, A., BUIATTI-TCHENG, M. & LECLAIRE, J. (1997). Localization of nitric oxide synthase (NOS) in a human corneal epithelial cell line. *Invest Ophthalmol Vis Sci.,* (suppl 38): Abst., 4045.

(Received May 8, 1998
Revised August 21, 1998
Accepted September 1, 1998)