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Inhibition of the antigen-induced activation of RBL-2H3 cells by cetiedil and some of its analogues

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Abstract

Our previous studies on rat basophilic leukaemia (RBL-2H3) cells suggested that IK_{Ca} channels similar to those in red blood cells (RBC) may be involved in the antigen-induced β-hexosaminidase release. Since cetiedil blocks these channels in both cell types, we studied the inhibition by a selection of the synthetic analogues of cetiedil (UCL compounds) of antigen-induced β-hexosaminidase release and $^{86}Rb^{+}$-efflux from RBL-2H3 cells. We tested the (+)- and (−)-enantiomers of cetiedil (UCL 1348 and UCL 1349), the more lipophilic triphenylacetic acid derivatives (UCL 1495 and UCL 1617) and (9-benzyl-fluoren)-9-yl derivatives (UCL 1608 and UCL 1710). They all inhibited antigen-induced β-hexosaminidase release and $^{86}Rb^{+}$-efflux. Their relative potency in inhibiting antigen-induced β-hexosaminidase release was UCL 1608>1710>1617>1348>1349>1495, with IC_{50} values of 9.6 ± 0.6, 14.4 ± 2.2, 23.4 ± 1.4, 29.8 ± 1.1, 77.5 ± 11.8 and 104.6 ± 14.7 (μM), respectively. These IC_{50}s suggest some dissimilarity between IK_{Ca} in RBL-2H3 cells and RBC. Lipophilicity and potency were well correlated in RBC, but not in RBL-2H3 cells.

Keywords: RBL-2H3 cell; Mast cell; Mediator release; K^{+} channels; Cetiedil

1. Introduction

The rat basophilic leukaemia cell line (RBL-2H3) is a mucosal-type mast cell line which has been widely used to investigate mechanisms of immediate hypersensitivity reactions. Like other mast cells, RBL-2H3 cells have a high affinity receptor for IgE (FcεRI) on their surface (Fewtrell and Metzger,1980; Sutton and Gould, 1993). Cross-linking of the IgE-bound to FcεRI receptors results in activation of these cells and initiates a biochemical cascade leading to cell degranulation and mediator release (Beaven et al., 1987; Oliver et al., 1988; Razin et al., 1995; Metcalfe et al., 1997; Field et al., 1999; Cissel and Beaven, 2000; Way et al., 2000; Liu et al., 2001).

The antigen response is Ca^{2+}-dependent, unlike the degranulation by the so-called 'basic secretagogues', such as compound 48/80 and the wasp venom mastoparan (Pinxteren et al., 2000). Extracellular Ca^{2+}, $[Ca^{2+}]_o$, and Ca^{2+} influx are essential for the optimal response of rat peritoneal mast cells (Foreman et al., 1977; Foreman, 1981) and RBL2H3 cells (Beaven et al., 1984) to antigen stimulation. Studies by Beaven et al. (1984) suggested that in a population of RBL-2H3 cells, antigen stimulation gives a biphasic increase in intracellular calcium, $[Ca^{2+}]_i$. The early, transient phase predominantly reflects Ca^{2+} release from internal stores, whereas the secondary, plateau phase reflects Ca^{2+} influx into the cells. The observed antigen-induced increase in $[Ca^{2+}]_i$ in individual RBL-2H3 cells is quite different, as it is oscillatory and asynchronous, with a varied profile of oscillation frequency, amplitude and shape. Removal of $[Ca^{2+}]_o$ at any time during the antigen challenge reduces both the frequency and amplitude, and changes spike shape (Narenjkar et al., 1999). The Ca^{2+} oscillation, which seems to enhance the efficiency of the transduction from antigen stimulation to mediator release, is probably due to periodic Ca^{2+} release from internal Ca^{2+} stores, coupled to Ca^{2+} influx.

Depletion of internal Ca^{2+} stores by IP_3 activates the calcium release-activated calcium (CRAC) channels (Penner et al., 1993; Hoth, 1996; Parekh et al., 1997; Fierro and Parekh, 1999, 2000; Glitsch et al., 2002). Calcium perme-

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ability through these channels depends on the membrane potential, so that membrane hyperpolarization promotes the Ca^{2+} current, whereas depolarization inhibits the current (Penner et al., 1988; Matthews et al., 1989).

It has been reported that quinidine and Ba^{2+}, nonspecific K^+ channel blockers, inhibit antigen-induced serotonin secretion in RBL-2H3 cells (Labrecque et al., 1989). Quinidine seems to block the antigen-induced K^+ channel activation, since it inhibits repolarization, secretion and $^{86}Rb^+$-efflux stimulated by antigen (Labrecque et al., 1991). By contrast, the inhibitory effects of Ba^{2+} might be due to a direct effect on the CRAC channel or on Ca^{2+} influx, rather than a consequence of blocking K^+ channels in RBL-2H3 cells. Since charybdotoxin and cetiedil [2-cyclohexyl-2-(3-thienyl)ethanoic acid 2-(hexahydro-1*H*-azepin-1-yl)ethyl ester], but not iberiotoxin, margatoxin, tetraethylammonium or apamin, are able to inhibit the antigen-induced response in RBL-2H3 cells (Narenjkar et al., this issue), in this study the effects of several cetiedil-related compounds (UCL compounds; Roxburgh et al., 2001) on antigen-induced secretion and $^{86}Rb^+$-efflux from these cells were tested and their relative potency in inhibiting these responses compared to that observed in the inhibition of potassium permeability in rabbit red blood cells.

2. Methods

2.1. Cell culture

RBL-2H3 cells were maintained in monolayer culture in minimal essential medium (S-MEM) supplemented by 10% foetal calf serum, 2 mM glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37 °C in a humidified atmosphere with 95% air–5% CO_2. The cells were passively sensitised to the dinitrophenyl (DNP) chemical group (DNP-specific mouse monoclonal IgE antibody 0.5 μg ml^{-1}) overnight. For β-hexosaminidase release measurement, cells were seeded in 96-well tissue culture plates (3×10^4 cells in 100 μl S-MEM/well) (Gomperts and Tatham, 1992; Wan et al., 2001). For $^{86}Rb^+$ efflux experiments, cells were washed with phosphate-buffered saline and were then harvested by treatment with Sigma's ethylenediaminetetra-acetic acid 0.02% solution for 15 min at 37 °C. The cell suspension was centrifuged at $225 \times g$ for 3 min and the cell pellet was then resuspended in fresh supplemented S-MEM medium at $\sim 4 \times 10^6$ cell ml^{-1}.

2.2. β-Hexosaminidase release measurement

Measurement of β-hexosaminidase release was carried out according to the methods of Gomperts and Tatham (1992) and Lillie and Gomperts (1992). Cells were initially washed twice with Tyrode's buffer containing 0.05% bovine serum albumin, preincubated with the same buffer for 5 min at 37 °C and then challenged with antigen (dinotrophenyl-human serum albumin conjugate, DNP-HSA) for another 15 min. To estimate the total amount of β-hexosaminidase within each well, a lysing solution of Tyrode's buffer containing 1% Triton X-100 was also added to six wells (in a 96-well plate) and the β-hexosaminidase content of these wells was determined.

At the end of the experiment, 50 μl of the supernatant from each well was incubated with 50 μl of substrate solution (4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide, 1 mM) at 37 °C for 90 to 120 min. The enzyme reaction was terminated by the addition of 300 μl of 0.2 M Tris buffer (pH = 11). The fluorescent product (4-methylumbelliferone) was measured by spectrofluorometery on an automatic microtiter plate reader at the excitation wavelength of 365 and emission wavelength of 450 nm. β-Hexosaminidase release was expressed as a percentage of the total average cell content of β-hexosaminidase (determined in six wells). Spontaneous β-hexosaminidase release (2–8%), determined in the absence of antigen, was subtracted from the antigen-stimulated release and final results were expressed as the net percentage release.

2.3. $^{86}Rb^+$ efflux measurement

The cell suspension (4×10^6 cells ml^{-1}) was loaded with $^{86}Rb^+$ (3 μCi/ml) at 37 °C in a shaking incubator for 2 h. 0.5-ml aliquots of this cell suspension were then centrifuged ($225 \times g$ for 3 min), resuspended in 2 ml Tyrode–0.05% bovine serum albumin buffer and then incubated for 8 min at 37 °C prior to antigen stimulation. $^{86}Rb^+$ was measured in 100 μl samples taken every 2 min over a 24-min period. The basal rate of $^{86}Rb^+$-efflux was determined during the 8 min prior to application of antigen (time −8 to 0 min). Antigen-stimulated changes in the rate of $^{86}Rb^+$-efflux was determined during 16 min after antigen addition (time 0 to 16 min). The cells were removed by centrifugal sedimentation (at 16,000 × *g* for 10 s) through 200 μl phthalate oil (60% dibutyl phthalate and 40 % [phthalic acid bis(2-ethylhexyl) ester]) using an Eppendorf microfuge. To determine the $^{86}Rb^+$ content of the supernatant, 50 μl was transferred into scintillation vials containing 5 ml of distilled water and the $^{86}Rb^+$ β-emission measured in a liquid scintillation counter. The initial $^{86}Rb^+$ content of the cells was determined in a parallel experiment where cells were lysed using 5 ml perchloric acid 0.4 M.

Basal $^{86}Rb^+$ efflux rate (E_b min^{-1}) at time −8 to 0 min and in the absence of antigen in general was noted to be linear and the rate constant (min^{-1}) was calculated from the slope of the linear fit of the following equation:

$$k = \frac{\ln[(Q - \Delta Q)/Q]}{t}$$

where k is the rate constant of $^{86}Rb^+$-efflux, t is time (min), Q is the initial $^{86}Rb^+$ content, and ΔQ is the difference in cpm in consecutive samples. The rate constants of stimulated $^{86}Rb^+$-efflux (E_s) in the presence of antigen (time 0 to 16 min) showed an overall increase but did not follow a single

exponential. They were calculated from the difference between values of consecutive samples, divided by the time interval between the samples, which is 2 min. Two peaks were observed (up to four times the basal rate), an early one at time 0–2 min and a late one at 10–12 min. For simplicity, averages of these rate constants were used (Table 3). The net antigen-stimulated rate(s) (E_{sn}) of $^{86}Rb^{+}$-efflux was determined by subtracting E_b. The basal rate constant was 0.0149 ± 0.001 min^{-1} (1.49 % of Q, which gives ca. 28,000 counts per min) whilst the average (E_s) for 10 ng/ml DNP-HSA was 0.0273 ± 0.001291 min^{-1} (E_{sn}, 0.0124), i.e., the average $^{86}Rb^{+}$-efflux rate was increased by 83% following the addition of that concentration of antigen, which is maximal.

To present the data graphically, data points from the linear regression fit of the basal $^{86}Rb^{+}$-efflux were subtracted from the corresponding values for the cumulative efflux after stimulation (from time 0 to 16 min) (Fig. 7).

2.4. Materials

4-Methylumbelliferyl *N*-acetyl-β-D-glucosaminide, bovine serum albumin, dibutyl phthalate, dinitrophenyl-human albumin conjugate (DNP-HSA), DNP-specific monoclonal mouse IgE, L-glutamine 200 mM solution, penicillin–streptomycin solution, dibutyl phthalate, phthalic acid bis(2-ethylhexyl) ester, Trizma® base (tris[hydroxymethyl]aminomethane), and trypsin-ethylenediaminetetraacetic acid solution were supplied by Sigma (Poole, UK); ^{86}Rubidium (as RbCl) by New England Nuclear (Hounslow, UK); foetal calf serum, minimum essential media medium (S-MEM), phosphate buffered saline without Ca^{2+} and Mg^{2+} by Gibco (Paisley, UK); and Triton X-100 by British Drug Houses Chemicals (Lutterworth). The UCL compounds were synthesised in the Chemistry Department, University College London (Roxburgh et al., 2001). The purity of the UCL compounds, assessed by HPLC, was >99% except UCL 1495 whose purity was 97.7%. The chemical formulae and properties of these compounds are summarized in Fig. 1 and Table 1, respectively.

2.5. Solutions

For β-hexosaminidase release experiments, modified Tyrode's solution was used. It contained 137 mM NaCl, 5

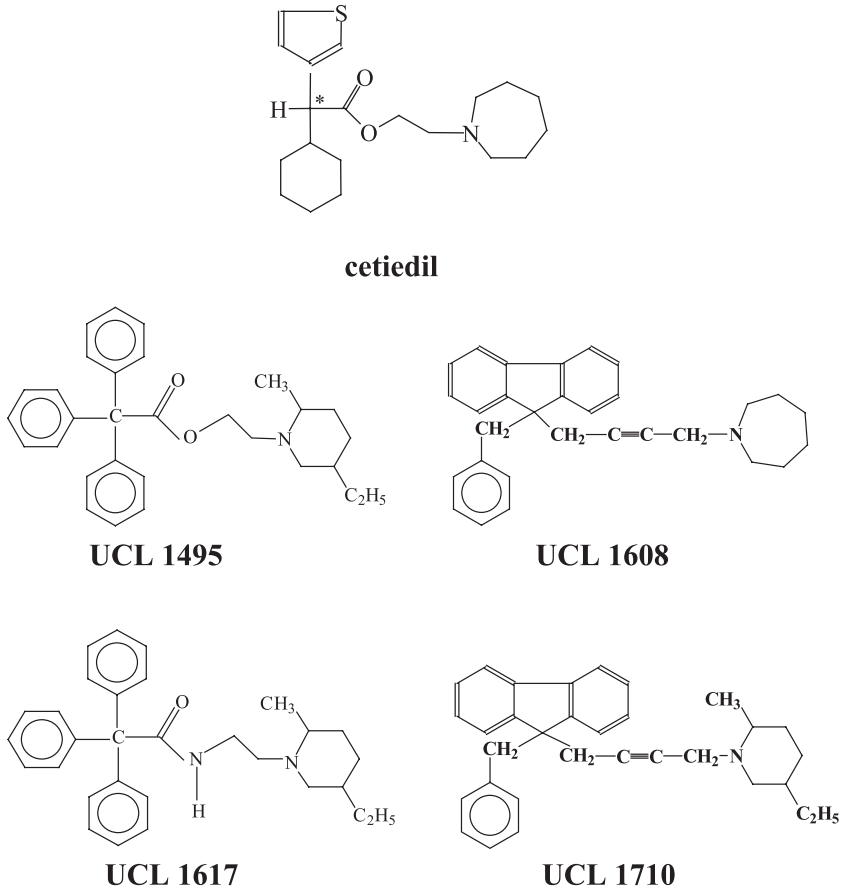

Fig. 1. Molecular structures of cetiedil and the related UCL compounds. The chiral carbon has been shown by *. To increase the lipophilicity of the molecule the α carbon of acetic acid was substituted with either triphenyl (left) to give UCL 1495 and 1617 which are α-triphenylacetic acid derivatives, but are different in having esteratic or amide bonds, or replaced by (9-benzyl-fluoren)-9-yl (right) to give UCL 1710 and 1608 which differ in the N-ring (homopiperidine in UCL 1608 and 2-methyl-5-ethyl-piperidine in UCL 1710).

Table 1
Structural substitutions, salt form, chemical formulae and properties of cetiedil and its analogues (UCL compounds)

Compound	α-Substitution	N-ring[a]	Salt form	Formula	HPLC purity	Log P[b]
(+)-cetiedil	original	HP	trifluoroacetate	$C_{20}H_{31}NO_2S$, CF_3CO_2	99.9 %	6.59
(−)-cetiedil	original	HP	trifluoroacetate	$C_{20}H_{31}NO_2S$, CF_3CO_2	97.4 %	6.59
UCL 1495	triphenyl	P	oxalate	$C_{30}H_{35}NO_2$, $C_2H_2O_4$	97.7 %	8.18
UCL 1608[c]	9-benzyl-fluoren-9-yl	HP	oxalate	$C_{30}H_{31}N$, 0.85 $C_2H_2O_4$	99.1 %	9.10
UCL 1617	triphenyl	P	base	$C_{30}H_{36}N_2O$	99.8 %	5.35
UCL 1710	9-benzyl-fluoren-9-yl	P	oxalate	$C_{32}H_{35}N$, 1.1 $C_2H_2O_4$	99.5 %	10.14

[a] HP, homopiperidine; P, piperidine.
[b] Log P (predicted octanol/water partition coefficient for the unprotonated amine) calculated as indicated in Table 3 of Mannhold et al. (1998).
[c] Described in Roxburgh et al. (2001).

mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES, 0.4 mM NaH_2PO_4 and 0.05 % bovine serum albumin, adjusted to pH 7.4 with NaOH (1 M). Bovine serum albumin (0.05 %) was added to prevent adsorptive loss of the antigen DNP-HSA.

For the β-hexosaminidase release assay, the substrate solution was 1 mM 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide dissolved in 0.2 M citrate buffer containing 0.01% Triton X-100 and adjusted to pH 4.5 with concentrated HCl (10.14 M). The substrate was dissolved in dry dimethylsulphoxide and then transferred into the citrate buffer.

The enantiomers of cetiedil and UCL 1495 were dissolved in water to give stock concentrations of 1 mM. Stock solutions of the other UCL compounds (10 mM) were in dimethylsulphoxide. Dilutions were made in the appropriate buffer.

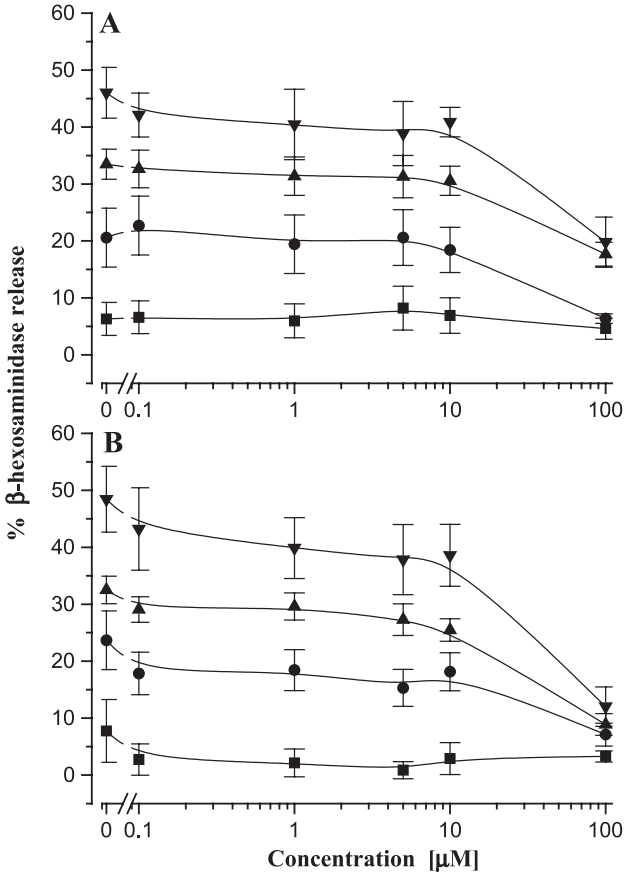

Fig. 2. Inhibitory effect of UCL 1348 and 1349 on antigen-stimulated β-hexosaminidase release. (A) UCL 1349, (B) UCL 1348. After 5 min of preincubation at 37 °C, UCL compounds were added at different concentrations and after 15 min cells were then challenged with antigen (for 15 min). Each point on the curves represents the mean ± S.E.M. of % net β-hexosaminidase release in four experiments. Spontaneous release was 6.5 ± 0.5% and was not altered by 100 μM of either compound. ■ 0.01, ● 0.1, ▲ 1, ▼ 10 ng/ml DNP-HSA.

2.6. Data analysis

All values are presented as mean ± S.E.M. for the number of experiments (with triplicate samples for each data point, in each experiment) carried out and cited in the legend for each graph or table. Statistical comparison of results was carried out by using Student's paired *t*-test or Mann–Whitney *U*-test (where appropriate).

2.6.1. Determination of IC_{50}

Concentration–inhibition curves were fitted using the Hill equation. This was done with the aid of the least squares fitting tool provided in Origin (v. 5; OriginLab, previously Microcal). The data were weighted by the reciprocal of the variance for each data point. The maximal inhibition was usually constrained to 100%. The program provided esti-

Table 2
Blocking effect of cetiedil and UCL compounds on IK_{Ca} channels in rabbit erythrocytes and on antigen-induced β-hexosaminidase release from RBL-2H3 cells

Compound	IC_{50}, IK_{Ca} blocking 0.1 mM $[K^+]_o$	IC_{50}, IK_{Ca} blocking 5.4 mM $[K^+]_o$[a]	IC_{50}, RBL-2H3 secretion
Cetiedil	26 ± 1 μM	100 μM	84.3 ± 10.5 μM
UCL 1348	25 ± 0.2 μM	59 μM	29.8 ± 1.1 μM
UCL 1349	24 ± 1.4 μM	54 μM	77.5 ± 11.8 μM
UCL 1495	1.2 ± 0.08 μM	30 μM	104.6 ± 14.7 μM
UCL 1617	6.6 ± 0.52 μM	n.d.	23.4 ± 1.4 μM
UCL 1710	0.3 ± 0.03 μM	3 μM	14.4 ± 2.2 μM
UCL 1608	1.5 ± 0.1 μM	n.d.	9.6 ± 0.6 μM

n.d.: not done.
[a] Incomplete data.

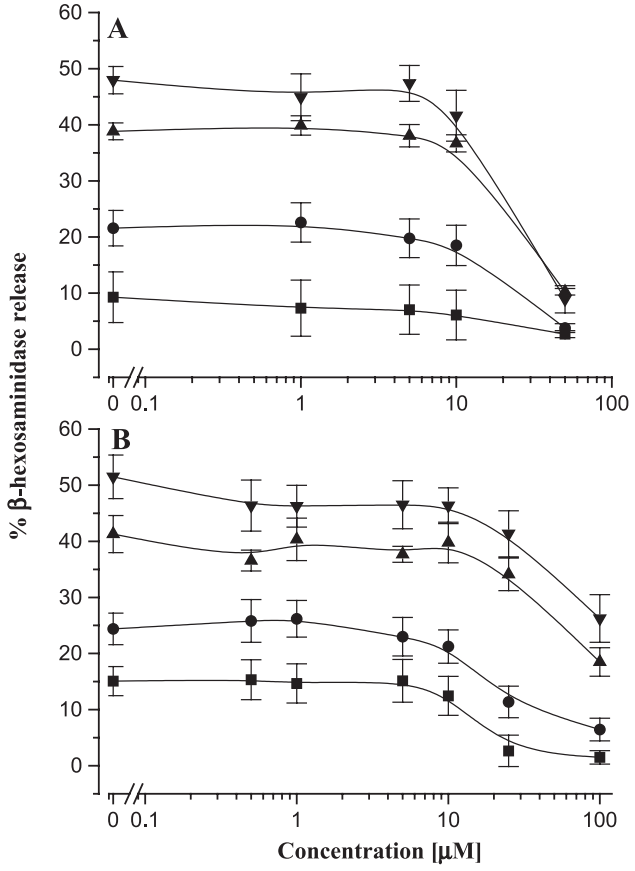

Fig. 3. Inhibitory effect of UCL 1617 and 1495 on antigen-stimulated β-hexosaminidase release. (A) UCL 1617, (B) UCL 1495. After 5 min of preincubation at 37 °C, UCL compounds were added at different concentrations and after 15 min cells were then challenged with antigen (for 15 min). Each point on the above curves represents the mean ± S.E.M. of % net β-hexosaminidase release in four experiments. Spontaneous release was 7.2 ± 0.5% and was not affected by 100 μM of either compound. ■ 0.01, ● 0.1, ▲ 1, ▼ 10 ng/ml DNP-HSA.

mates of K and n_H, where K is the IC_{50} and n_H is the Hill coefficient.

3. Results

3.1. Inhibitory effects of cetiedil and its analogues (UCL compounds) on antigen-stimulated β-hexosaminidase release

3.1.1. Cetiedil enantiomers

Since cetiedil has an asymmetrical carbon atom, its enantiomers can be used to investigate the importance of the stereochemistry of the molecule in relation to the inhibitory effect on antigen-induced β-hexosaminidase release from RBL-2H3 cells. The optical enantiomers of cetiedil, (+)-cetiedil (UCL 1348) and (−)-cetiedil (UCL 1349) were tested, using four different antigen concentrations (0.01, 0.1, 1 and 10 ng ml^{-1} DNP-HSA) (Fig. 2). Although the compounds did not show any significant effect up to 10 μM, they inhibited antigen-induced β-hexosaminidase release at 100 μM. When tested against an antigen concentration of 10 ng ml^{-1}, the calculated IC_{50} was 29.8 ± 1.1 and 77.5 ± 11.8 μM for UCL 1348 and UCL 1349, respectively (Fig. 5). Thus, the (+)-enantiomer was 2.6 times more potent than the (−)-enantiomer against antigen-triggered secretion in RBL-2H3 cells.

Cetiedil and the UCL compounds are more potent in blocking erythrocyte K^+ loss at low extracellular K^+ concentration. They have lower IC_{50} at 0.1 mM $[K^+]_o$ than at 5.4 mM $[K^+]_o$ (see Table 2) (Benton, 1995; Roxburgh et al., 1996). Since at low $[K^+]_o$ the antigen-induced secretion was already markedly inhibited, it was not considered feasible to elucidate the effect of cetiedil in solutions containing different concentrations of K^+.

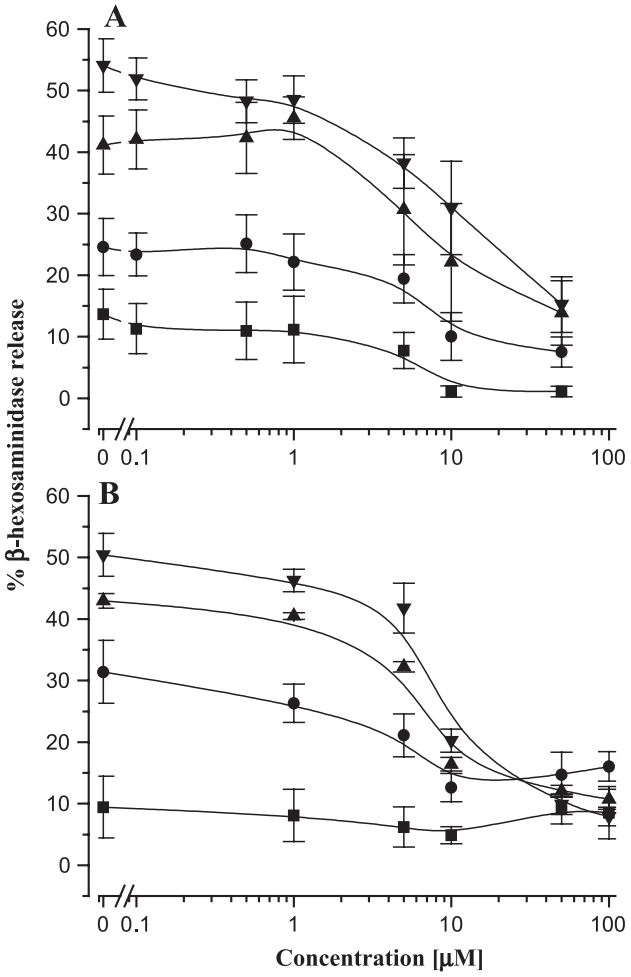

Fig. 4. Inhibitory effect of UCL 1710 and 1608 on the antigen-stimulated β-hexosaminidase release. (A) UCL 1710, (B) UCL 1608. After 5 min of preincubation at 37 °C, UCL compounds were added at different concentrations and after 15 min cells were then challenged with antigen (for 15 min). Each point on the above curves represents the mean ± S.E.M. of % net β-hexosaminidase release in four experiments. Spontaneous release was 5.2 ± 0.3% and was not affected by 100 μM of either compound. ■ 0.01, ● 0.1, ▲ 1, ▼ 10 ng/ml DNP-HSA.

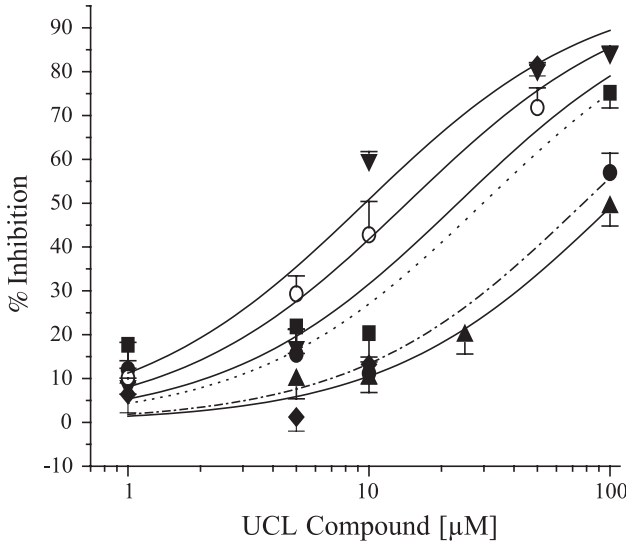

Fig. 5. Concentration–response curves of the UCL compounds for the inhibition of antigen-stimulated β-hexosaminidase release. After 5 min of preincubation at 37 °C, UCL compounds were added at different concentrations and after 15 min cells were then challenged with antigen (10 ng/ml DNP-HSA for 15 min). Each point on the curves represents the mean ± S.E.M. of % net β-hexosaminidase release in four experiments. Data have been fitted to the Hill equation and with B_{max} constrained to 100 %. Hill coefficient (n_H) was 0.91 ± 0.01. Spontaneous and antigen-induced β-hexosaminidase releases were 6.6 ± 0.3% and 49.7 ± 1.2%, respectively. ■ UCL 1348 (- - - curve), ● UCL 1349 (– · – curve), ▲ UCL1495, ▼ UCL 1608, ○ UCL 1710, ♦ UCL 1617.

3.1.2. Triphenylacetic acid ester derivatives (UCL 1495 and UCL 1617)

Increasing lipophilicity by nonpolar substituents in any part of the cetiedil molecule causes an increase in activity for the block of the IK_{Ca} channels in red blood cells (Benton, 1995; Roxburgh et al., 2001). UCL 1495 is the 2-[*N*-(2-methyl-5-ethyl piperidino)-ethyl] ester of triphenylacetic acid with a higher lipophilicity than cetiedil itself. It was reported to be 20 times more potent than cetiedil in blocking erythrocyte IK_{Ca} channels (Benton, 1995). UCL 1617 is an amide analogue of UCL 1495. UCL 1617 has a lower lipophilicity than UCL 1495 and is six times less potent on IK_{Ca} channel blocking in red blood cells (Benton, 1995).

As shown in Fig. 3 and Table 2, both UCL 1495 and 1617 inhibited antigen-stimulated β-hexosaminidase release from RBL-2H3 cells. When tested against an antigen concentration of 10 ng ml^{-1}, the values of IC_{50} for these compounds were 104 ± 15 and 23 ± 1 μM for UCL 1495 and 1617, respectively. Since increasing lipophilicity caused a decrease instead of an increase in the inhibitory effect of UCL 1495, these data are not consistent with those obtained from erythrocyte K^+ loss experiments. However, the higher potency of UCL 1617 may suggest that the ester linkage does not contribute significantly to the inhibitory effect on the secretion from RBL-2H3 cells.

3.1.3. (9-Benzyl-fluoren)-9-yl derivatives (UCL 1608 and UCL 1710)

The next compound tested was UCL 1608, in which the ester function is replaced by ethyne, and the triphenylmethyl group is replaced by (9-benzyl-fluoren)-9-yl (Fig. 1). Compared with UCL 1617, this compound has a higher lipophilicity and a lower IC_{50} (1.5 μM) for causing block of IK_{Ca} channels in red blood cells. In view of these results, it was suggested that the ester linkage is not necessary for channel blocking activity (Benton, 1995).

UCL 1710, which combines features of UCL 1495 and UCL 1608, has a higher lipophilicity and a lower IC_{50} (0.31 μM) than UCL 1608 for inhibition of the erythrocyte K^+ loss induced by Ca^{2+} ionophore (Table 2) (Benton, 1995). Fig. 4 shows the inhibitory effect of UCL 1608 and UCL 1710 on antigen-induced β-hexosaminidase release. They were the two most potent of the tested UCL compounds: at an antigen concentration of 10 ng ml^{-1} their IC_{50} values were 9.6 ± 0.6 and 14.4 ± 2.2 μM, respectively (Table 2 and Figs. 5 and 6).

3.2. Inhibitory effect of the cetiedil-related compounds (UCL compounds) on antigen-stimulated $^{86}Rb^+$-efflux

Since cetiedil and some related compounds ('UCL compounds') inhibit antigen-induced β-hexosaminidase release,

Table 3
Effect of UCL compounds on the average rate (min^{-1}, expressed as a fraction of the total $^{86}Rb^+$ cell load) of spontaneous and antigen-stimulated $^{86}Rb^+$-efflux (time 0–16 min)

Condition	Spontaneous $^{86}Rb^+$-efflux	Stimulated $^{86}Rb^+$-efflux	Net stimulated $^{86}Rb^+$-efflux	% Inhibition at	
				min 4	min 16
Control (buffer)	0.0149 ± 0.001	0.0273 ± 0.002	0.0124 ± 0.002	–	–
UCL 1348 (100 μM)	0.0143 ± 0.002	0.0176 ± 0.002	0.0033 ± 0.001[a]	72.4%	73.2%
UCL 1349 (100 μM)	0.0129 ± 0.002	0.0156 ± 0.002	0.0027 ± 0.002[b]	48.5%	78.2%
UCL 1495 (100 μM)	0.0146 ± 0.0009	0.0228 ± 0.001	0.0082 ± 0.0004[a]	100%	33.8%
UCL 1617 (50 μM)	0.0131 ± 0.001	0.0177 ± 0.001	0.0046 ± 0.001[b]	100%	63.0%
UCL 1710 (50 μM)	0.0134 ± 0.0005	0.0135 ± 0.0007	0.0001 ± 0.0003[c]	100%	99.2%
UCL 1608 (10 μM)	0.0137 ± 0.0002	0.0141 ± 0.0005	0.0004 ± 0.0007[b]	64.6%	96.8%

$^{86}Rb^+$-loaded cells (each aliquot giving ca. 28,000 counts per min) were incubated in modified Tyrode's buffer with or without the UCL compounds at time − 8 min. Samples were taken every 2 min. Antigen (DNP-HSA 10 ng/ml) was added 8 min later and sampling was continued for another 16 min. Data are presented as the mean ± S.E.M. of rate constant (min^{-1}) from three to five experiments. (Student's paired *t*-test, [a] $p < 0.05$, [b] < 0.01, [c] < 0.001).

it was interesting to assess their effects on the stimulated $^{86}Rb^{+}$-efflux. As shown in Table 3, the UCL compounds had no significant effect on basal $^{86}Rb^{+}$-efflux. However, all compounds at a concentration near to their IC_{50} (on antigen-mediated secretion) inhibited antigen-induced $^{86}Rb^{+}$-efflux (Table 3 and Fig. 7). Some of the UCL compounds (UCL 1495 and UCL 1617) have a greater inhibitory effect on the early phase of antigen-stimulated $^{86}Rb^{+}$-efflux (Table 3) and are, therefore, similar to charybdotoxin in that respect. Unfortunately, because of insufficient supply of the UCL compounds, it was not feasible to carry out enough experiments to establish dose–response curves.

4. Discussion

In this study, the effects of some cetiedil analogues, synthesised in the Dept. of Chemistry at University College London (UCL compounds), on antigen-induced secretion and $^{86}Rb^{+}$-efflux from RBL-2H3 cells (a tumour analogue of mucosal mast cells) were investigated. The inhibition of antigen-induced β-hexosaminidase release by charybdotoxin and cetiedil (but not by tetraethylammonium, iberiotoxin or margatoxin) suggested that the K^{+}-channels involved in the antigen activation of RBL-2H3 cells may resemble the IK_{Ca} channel in red blood cells (Narenjkar et al., this issue). Like charybdotoxin and cetiedil itself, the cetiedil analogues inhibit IK_{Ca} channels in red blood cells. Since charybdotoxin and cetiedil had an inhibitory effect on the stimulated secretion from RBL-2H3 cell, it was useful to assess the effect of a selection of cetiedil analogues on antigen-induced β-hexosaminidase release. The structure–activity relationship of these compounds in blocking the IK_{Ca} channel in red bloods, as well other actions, has been reported (Roxburgh et al., 1996, 2001).

The UCL compounds were able to inhibit antigen-induced activation of RBL-2H3 cells (Table 2 and Figs. 2–4), and to block the antigen-stimulated $^{86}Rb^{+}$-efflux (Table 3 and Fig. 7). These findings were compatible with the hypothesis that the inhibitory effect of charybdotoxin on one hand, and by cetiedil and its analogues (the UCL compounds), on the other, on antigen-induced activation in RBL-2H3 cells, was via a common mechanism, namely, the blockade of antigen-activated K^{+}-channels (presumably IK_{Ca}). However, since cetiedil and the UCL compounds are not selective blockers of IK_{Ca} channels, the IC_{50} of charybdotoxin in RBL-2H3 cells is high, compared to other cells (133 nM, with 10 ng ml^{-1} DNP-HSA; Narenjkar et al., this issue), and the relative insensitivity of the K^{+}-channels in RBL-2H3 cells to clotrimazole and to nitrendipine, it is possible that the inhibitory effect of these compounds may be on K^{+} channels other than the IK_{Ca} subtype (or a further subtype of IK_{Ca}, distinct for that in red blood cells). The relative potencies of the cetiedil enantiomers and of UCL 1495 in inhibiting the antigen-induced secretion from RBL-2H3 cells are also in favour of such a distinction. However, UCL 1617, 1710 and 1608 are all highly potent in inhibiting secretion in RBL-2H3 cells and in blocking IK_{Ca} action in red blood cells (Table 2 and Fig. 6).

The UCL compounds also inhibited antigen-induced $^{86}Rb^{+}$-efflux in the concentration range which inhibited the stimulated secretion (Table 3). Since creating a dose–response curve for each UCL compound was not feasible, the results obtained cannot be used to determine structure–response relationships. However, it is clear that the inhibitory effects of these compounds on secretion and $^{86}Rb^{+}$-efflux are closely related. Since $^{86}Rb^{+}$-efflux rate constants during the time-course of antigen activation exhibit two peaks (one early and one late; Narenjkar et al., this issue) it was interesting to note that UCL 1495 and UCL 1617 seemed to act like charybdotoxin in that

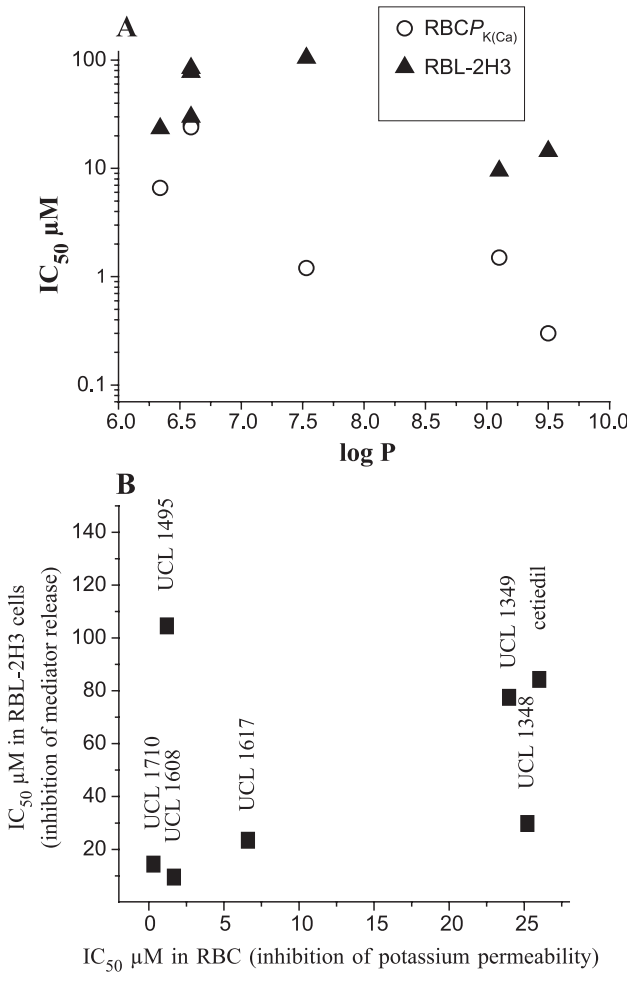

Fig. 6. (A) Comparison of the correlation between the logarithm of the octanol/water partition coefficient (log *P*) of the UCL compounds and the IC_{50} of these compounds in inhibiting the calcium-dependent K^{+} permeability ($P_{K(Ca)}$) in rabbit erythrocytes (with $[K^{+}]_o$ of 0.1 mM) and in inhibiting β-hexosaminidase release from RBL-2H3 cells (with DNP-HSA of 10 ng ml^{-1}). (B) Relationship between the IC_{50} of the UCL compounds in inhibiting the calcium-dependent K^{+} permeability ($P_{K(Ca)}$) in rabbit erythrocytes (with $[K^{+}]_o$ of 0.1 mM) and in inhibiting β-hexosaminidase release from RBL-2H3 cells (with DNP-HSA of 10 ng ml^{-1}).

they had a greater inhibitory effect on the early phase of the antigen-stimulated $^{86}Rb^{+}$-efflux (Table 3). By contrast, UCL 1710 which is one of the two most potent UCL compounds in inhibiting β-hexosaminidase release (and is the most potent among the analogues in blocking erythrocyte IK_{Ca} channel) causes inhibition of the early as well as the late phases of antigen-stimulated $^{86}Rb^{+}$-efflux (50 μM causing complete inhibition of either phase; Table 3 and Fig. 7).

In addition to the $^{86}Rb^{+}$-efflux studies, we have attempted to confirm the K^{+} channel blocking activity of the cetiedil-related compounds (UCL compounds) by investigating the effect of these compounds on the antigen-induced changes in the membrane potential, but the results (which are not shown here) were inconclusive. It was unfortunate that cetiedil and the UCL compounds showed fluorescence interference with bis-oxonol, the membrane potential-sensitive fluorescent dye (Labrecque et al., 1989) we used.

An alternative explanation for the action of the cetiedil analogues (the UCL compounds) and for the difference in the relative potencies of some of them in RBL-2H3 cells and in red blood cells is that the action in the RBL cells may not be via a K^{+}-channel blockade. Like ceteidil itself, these compounds are not only less selective than charybdotoxin in their K^{+} channel pharmacology, but they also have other actions that may not be via K^{+} channels (Roxburgh et al., 1996, 2001). They may possibly interfere with the signal transduction pathways in RBL-2H3 cell activation, leading to the inhibition of β-hexosaminidase release. However, without knowing which part of the signal transduction is involved in this action, it is difficult to deduce how would it be achieved, or how might the inhibition of $^{86}Rb^{+}$-efflux be explained.

Since the effect of cetiedil on K^{+} permeability in red blood cells may be partly due to inhibition of Na^{+}, K^{+} and Ca^{2+}, Mg^{2+} ATPases (Abu-Salah and Gambo, 2002), the inhibitory effect of the cetiedil analogues on the antigen in RBL-2H3 may be due to a similar mechanism. Inhibition of calcium release-activated calcium (CRAC) channels by cetiedil analogues is a particularly interesting possibility, since this study cannot rule out such a direct effect. The antigen-induced change in membrane potential, $^{86}Rb^{+}$-efflux and the rise in $[Ca^{2+}]_i$ are dependent on each other and it is difficult to conclude from the present results whether the tested drugs have, at least in part, a direct effect on the CRAC channel. To study this, the effects of these compounds on the Ca^{2+} current through the CRAC channels (I_{CRAC}) will have to be measured directly. CRAC channels were found in RBL and in rat peritoneal mast cells (Mathes et al., 1999).

Cetiedil and its analogues may also possibly interfere with other activation-dependent ion (e.g., Cl^{-}) channels which have been identified in human and rodent mast cells

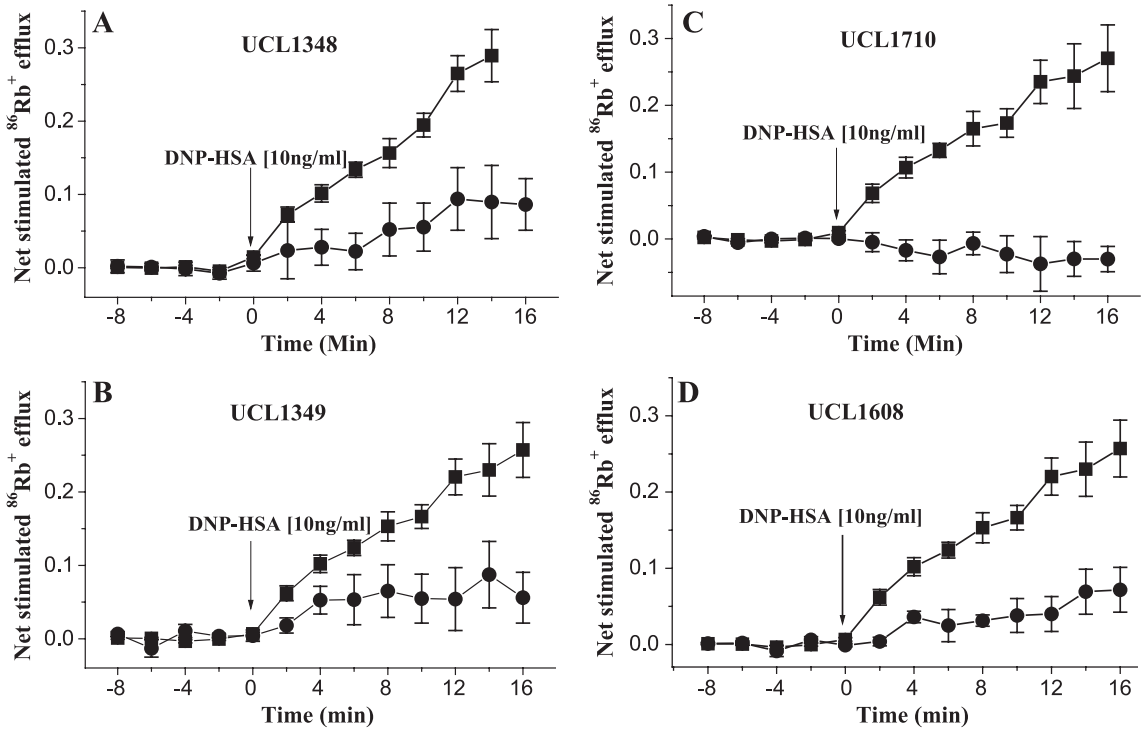

Fig. 7. Effect of UCL 1348, 100 μM (A) UCL 1349, 100 μM (B), UCL 1710 [50 μM] (C) and UCL 1608 [10 μM] (D) on the net cumulative antigen-stimulated $^{86}Rb^{+}$-efflux (after the subtraction of basal efflux in the absence of antigen), expressed as a proportion of the total isotope cell load (Δ*Q*). $^{86}Rb^{+}$-loaded cells were incubated in modified Tyrode's buffer without ■ or with the UCL compound ● at time −8 min. Samples were taken every 2 min. Antigen (DNP-HSA 10 ng/ml) was added at time 0 min and sampling was continued for another 16 min ($n = 3–4$).

(Matthews et al., 1989; Duffy et al., 2001; Bradding and Conley, 2002).

Interestingly, some of the UCL compounds were more potent than cetiedil in inhibiting RBL-2H3 cell activation by antigen. It has been shown that the cetiedil enantiomers are equipotent in blocking IK_{Ca} in rabbit red blood cells. However, (+)-cetiedil seems to be more potent (by 2.6 times) than (−)-cetiedil in inhibiting the volume-sensitive K^+ channel in liver cells (Roxburgh et al., 1996). Similarly, the (+)-enantiomer was 2.6 times more potent than the (−)-enantiomer against antigen-triggered secretion in RBL-2H3 cells (Table 2). All the UCL compounds inhibited the antigen-stimulated β-hexosaminidase release and their potency was as follows: UCL 1608>1710>1617> 1348>1349>1495 (Table 2 and Fig. 5). In contrast to what is seen in red blood cells (Benton et al., 1994), increasing lipophilicity does not seem to have a significant influence on potency in RBL-2H3 cells (Fig. 6). Thus, UCL 1617 is a more potent inhibitor of secretion (and $^{86}Rb^+$-efflux) than is the more lipophilic UCL 1495.

Mast cells are heterogeneous, exhibiting a vast phenotypic diversity both morphologically and functionally (Metcalfe et al., 1997) and the UCL compounds remain to be further tested, to see if they can inhibit the activation of other mast cell phenotyes or basophil leucocytes.

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