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Inhibition of the antigen-induced activation of RBL-2H3 cells by charybdotoxin and cetiedil

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Abstract

Quinidine and Ba^{2+}, non-selective K^+-channel blockers, have previously been shown to inhibit antigen-induced mediator (β-hexosaminidase) release from RBL-2H3 cells, a mucosal-type mast cell line. We therefore used selective blockers of Ca^{2+}-activated and other K^+ channels to determine if there was a role for these channels in antigen-induced mediator release. Charybdotoxin and cetiedil dose-dependently inhibited β-hexosaminidase release with IC_{50} values of 133 nM and 84 μM, respectively. Charybdotoxin also inhibited the repolarization phase of the antigen-induced biphasic change in the membrane potential (IC_{50} 84 nM), antigen-stimulated $^{86}Rb^+$-efflux and increase in free intracellular calcium, $[Ca^{2+}]_i$. Iberiotoxin, margatoxin, apamin and tetraethylammonium had no effect on β-hexosaminidase release. These results suggest that K^+ conductances play a significant role in mediator release from RBL-2H3, that these conductances are of the intermediate conductance Ca^{2+}-activated K^+ channel (IK_{Ca}) type, and that they are somewhat similar to those which have been described in red blood cells, though they are much less sensitive to clotrimazole.

Keywords: RBL-2H3 cell; Mediator secretion; K^+ channel blocker; Charybdotoxin; Cetiedil

1. Introduction

The activation of RBL-2H3 cells has been extensively studied, largely because mast cells and basophils are considered as prime targets for therapeutic intervention in allergic, inflammatory diseases such as asthma. In immunoglobulin E (IgE)-sensitized tissue mast cells, blood basophil leucocytes and the mucosal-type mast cell line, RBL-2H3, crosslinking by antigen of the IgE antibodies occupying the tetrameric ($\alpha\beta\gamma_2$) FcεRI high affinity receptor for IgE, causes the aggregation of these receptors (Ishizaka and Ishizaka, 1975; Fewtrell and Metzger, 1980; Kinet, 1999). This results in the recruitment and activation of cytosolic tyrosine kinases Syk and Lyn which in turn activate various signal transduction pathways: phosphorylation and activation of enzymes, including phospholipases C and D isoforms, phosphoinositol-3 kinase and protein kinase C (PKC), and of guanine-nucleotide exchange factors (such as vav), and mobilization of stored and extracellular Ca^{2+}. These processes lead to the secretion of histamine and other inflammatory mediators (Beaven and Cunha-Melo, 1988; Razin et al., 1995; Metcalfe et al., 1997; Field et al., 1999; Cissel and Beaven, 2000; Way et al., 2000; Oliver et al., 2000).

The antigen stimulation also leads to the activation of cytoskeleton dynamics (which include relocation of vesicle fusion proteins t-SNARE {target soluble NSF-attachment protein receptor} SNAP 23 and rearrangement of actin, Guo et al., 1998). Phospholipase C activation generates inositol(1,4,5)-trisphosphate (IP_3), which releases Ca^{2+} from internal stores and raises its level in the cytosol, and diacylglycerol, which activates protein kinase C (PKC). Ca^{2+}-dependent exocytosis involves an isoform of synaptotagmin (probably synaptotagmin III) which is expressed in mast cells and which is a potential Ca^{2+} sensor (Baram et al., 1998, 1999; Pinxteren et al., 2000).

An alternative pathway in granule exocytosis, which has been implicated in a number of inflammatory conditions, and which is also initiated by the 'so-called' basic secretagogues (natural, e.g. the wasp venom mastoparan, or synthetic, e.g. compound 48/80), involves a direct interaction with heterotrimeric GTP-binding proteins, which in turn

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leads to activation of phospholipase D. Mastparan differs from antigen in selectively activating phospholipase D2 and in acting independently of certain other factors (e.g. Ca^{2+} and PKC) which are involved in the antigen activation (Chahdi et al., 2003).

Optimal secretory response of RBL-2H3 cells to antigen (inflammatory mediator secretion) requires a sustained, though oscillatory rise in intracellular free cytosolic Ca^{2+}, $[Ca^{2+}]_i$ (Narenjkar et al., 1999). This rise in $[Ca^{2+}]_i$ is dependent on not only release from internal stores but also a sustained Ca^{2+} influx (Foreman et al., 1977; Foreman, 1981; Beaven et al., 1984). The transmembrane influx of Ca^{2+} is activated by the depletion of the internal Ca^{2+} store via the opening of "Ca^{2+} Release Activated Calcium channels" (CRAC channels, causing capacitance Ca^{2+} entry) (Penner et al., 1993; Parekh et al., 1997; Fierro and Parekh, 1999, 2000; Glitsch et al., 2002). It has been reported that membrane hyperpolarization promotes Ca^{2+} influx through these channels, whereas depolarization reduces Ca^{2+} influx (Penner et al., 1988; Matthews et al., 1989). This is consistent with the inhibition of antigen-induced Ca^{2+} influx and secretion in RBL-2H3 cells depolarized in a high K^+ solution (Mohr and Fewtrell, 1987a, 1987b). An additional mechanism in antigen-stimulated Ca^{2+} influx occurs via phosphoinositide 3-kinase and its conversion of phosphoinositol(4,5)-bisphosphate to phosphoinositol(3,4,5)-trisphosphate, which directly stimulates a Ca^{2+} transport system in plasma membrances (Ching et al., 2001). Inositol(1,3,4,5) tetrakisphosphate (IP_4) has been implicated in mediating Ca^{2+} entry in many non-excitable cells but questions remain about its second messenger role in FcεRI-mediated Ca^{2+} influx and its relationship to Ca^{2+}-release activated Ca^{2+} channels (Ching et al., 2001).

RBL-2H3 cells have a resting membrane potential close to the equilibrium potential of K^+, variously reported as −94 mV (Kanner and Metzger, 1983), −70 mV (Ikeda and Weight, 1984), −90 mV (Lindau and Fernandez, 1986). The latter authors, however, were unable to detect significant changes in the ionic conductances in either RBL-2H3 cells or rat peritoneal mast cells following antigenic stimulation. Subsequently, indirect evidence of antigen-induced changes in ion conductances came from work in which the potential-sensitive fluorescent probe bis-oxonol was used. This work showed that antigen stimulation results in depolarization followed by a repolarization of the membrane (Kanner and Metzger, 1983; Mohr and Fewtrell, 1987a,b; Labrecque et al., 1989, 1991). It is generally believed that the membrane depolarization by antigen is primarily due to Ca^{2+} influx, while repolarization is mediated by an outward K^+ current (Labrecque et al., 1991). Since secretion, membrane repolarization and antigen-induced $^{86}Rb^+$-efflux can be inhibited by quinidine, a K^+ channel blocker, it has been suggested that K^+ conductances may be involved (Labrecque et al., 1989, 1991). Direct evidence for the involvement of K^+ conductances in the immunological stimulation of RBL-2H3 cells came from electrophysiological studies. The outward K^+ current thus activated was blocked by Ba^{2+}, Cs^+, tetraethylammonium, quinidine and K^+-free bath solution, while the chloride transport blocker 4,4′ -diisothiocyanato-stilbene-2,2′ -disulfonic acid (DIDS) had no effect (Gericke et al., 1995). However, the four K^+ channel blockers among these: Ba^{2+}, Cs^+, tetraethylammonium and quinidine are non-selective in their action. We, therefore, undertook a study of the effect of selective K^+ channel blockers on antigen-induced mediator secretion, on changes in the membrane potential, particularly repolarization (after initial depolarization, see above), $^{86}Rb^+$-efflux and increase in the intracellular free calcium concentration $[Ca^{2+}]_i$ in RBL-2H3 cells to try to identify the K^+ conductance involved.

2. Materials and 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 sensitized to the dinitrophenyl (DNP) chemical group (DNP-specific mouse monoclonal IgE antibody 0.5 μg ml^{-1}) overnight (Gomperts and Tatham, 1992; Wan et al., 2001).

2.2. Measurement of β-hexosaminidase release

Passively sensitized RBL-2H3 cells in 24-well culture plates were harvested by treatment with 0.02% ethylenediaminetetraacetic acid in a calcium- and magnesium-free Tyrode's buffer (no trypsin to avoid proteolysis of IgE antibody bound to its cell receptors). Cells in a 2-ml suspension (10^6 cells ml^{-1}) were initially washed twice with 0.05% bovine serum albumin-supplemented Tyrode's buffer, then preincubated with the same buffer for 5 min at 37 °C and finally challenged with antigen dinitrophenyl–human serum albumin conjugate for another 15 min. To assess the effect of inhibitors, they were frequently tested against more than one concentration of antigen (most frequently 1 ng ml^{-1} and 10 ng ml^{-1}) to ensure that the antigen response was submaximal. Triplicate samples were used for each treatment in all experiments. Measurement of β-hexosaminidase release was carried out according to the method of Professor B.D. Gomperts (Gomperts and Tatham, 1992; Lillie and Gomperts, 1992). The concentration of the released enzyme was determined by spectrofluorometric analysis of a fluorescent enzyme product (4-methylumbelliferone). The proportion released (%) and the % inhibition of the net % release with antigen (R_{an}, after subtracting the spontaneous % release), in the presence of an inhibitor (R_{ani}), given by the sum $(R_{an} - R_{ani})/R_{an} \times 100$, were calculated.

2.3. Measurement of membrane potential

Changes in the membrane potential were monitored by using a potential-sensitive fluorescent dye, bis-oxonol (Mohr and Fewtrell, 1987a,b; Labrecque et al., 1989). Briefly, passively sensitized RBL-2H3 cells in 24-well culture plates were harvested by treatment with 0.02% ethylenediaminetetraacetic acid in a calcium- and magnesium-free Tyrode's buffer. Cells in a 2-ml suspension (10^6 cells ml^{-1}) were washed with Tyrode's buffer containing 0.05% gelatin. After the second wash, the cell pellet was resuspended in the same buffer and transferred into a quartz cuvette, and maintained at 37 °C with constant stirring. After a 5-min preincubation, bis-oxonol was added to the cells to give a final concentration of 50 nM and this was allowed to equilibrate until the fluorescence reached a constant value (2–3 min). The fluorescence increased five to six times when the dye was added to the cell suspension. The cells were then treated with drug and/or antigen dinitrophenyl–human serum albumin conjugate for another 15 min.

Continuous recordings were performed on a LS-5 Perkin-Elmer spectrofluorimeter operating in ratio mode (excitation wavelength 540 nm and emission 580 nm). Antigen stimulation of these cells results in a biphasic change in membrane potential, an early depolarization which is then followed by a partial repolarization (Labrecque et al., 1989, 1991). The degree of repolarization was determined as a fraction of the initial depolarization (F_R = fluorescent intensity at the steady state of repolarization phase/peak fluorescent intensity during the depolarization phase). All the other results are plotted as a percentage of the depolarizing response induced by gramicidin (1 μg ml^{-1}) determined at the end of the experiment.

2.4. Measurement of $^{86}Rb^+$ efflux

The method used (Narenjkar et al., 1999) was based on that of Labrecque et al. (1991) in which $^{86}Rb^+$ (as RbCl) was used as a marker for K^+. Passively sensitized RBL-2H3 cells in 24-well culture plates were harvested by treatment with ethylenediaminetetraacetic acid 0.02 % in calcium- and magnesium-free Tyrode's buffer. The cells were spun down at 225 *g* for 3 min and then resuspended in fresh S-MEM medium at 4×10^6 cells ml^{-1}, before being incubated with $^{86}Rb^+$ (3 μCi ml^{-1}) at 37 °C for 2 h, with shaking. Aliquots of 0.5 ml of the isotope-loaded cell suspension were then centrifuged and the cell pellets resuspended in 2 ml modified (with 0.05% bovine serum albumin) Tyrode's buffer. The cell suspension was incubated at 37 °C for a control period of 8 min (time: −8 to 0 min) before the addition of antigen, and 16 min thereafter (time: 0–16 min). During those periods, 100 μl aliquots of the cell suspension was taken every 2 min and added to 200 μl phthalate oil (60% dibutyl phthalate and 40% phthalic acid bis(2-ethylhexyl) ester). The sample tubes were spun at 16,000 *g* for 10 s and the $^{86}Rb^+$ β-emission in 50 μl of the supernatant was measured using a liquid scintillation counter. The initial $^{86}Rb^+$ content of the cell aliquots (Q) was determined in a parallel set of samples where cells were lysed using 5 ml perchloric acid 0.4 M. The results were normalized by expressing as a proportion of the initial $^{86}Rb^+$ cell content (giving approximately 28,000 counts per min) both the counts lost from cells at different time points (ΔQ, Fig. 4A) and those left in the cells ($Q - \Delta Q$). The normalized counts remaining in cells, $[(Q - \Delta Q)/Q]$, were plotted against time and the rate constants of $^{86}Rb^+$ loss (efflux) were calculated from the slopes of this plot (Fig. 4B). 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:

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

where k is the rate constant of $^{86}Rb^+$-efflux, t is the duration of the collection period (2 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 (0 to 16 min), which did not follow a single exponential, were calculated from the difference between values of consecutive samples, divided by the time interval between the samples, which is 2 min. The net antigen-stimulated rate(s) (E_{sn}) of $^{86}Rb^+$-efflux was determined by subtracting E_b from those values (Fig. 4C).

2.5. Measurement of intracellular free calcium concentration $[Ca^{2+}]_i$ in single cells

The indo-1 method used in this study was as previously described by Robbins et al. (1993), Trouslard et al. (1993) and Narenjkar et al. (1999). Cells (0.5 ml suspension containing $\sim 2.5 \times 10^4$ cells) were placed directly into the 'calcium recording chamber' which consisted of a glass ring (diameter 16 mm; height 3 mm) attached to a microscope slide coverslip. Cells were loaded with the acetoxymethyl ester form of the Ca^{2+} indicator indo-1 (indo-1 AM) 1 μM for 30–45 min at 37 °C) (Grynkiewicz et al., 1985). The recording chamber was continuously perfused with modified Tyrode's buffer (8–12 ml min^{-1} at 37 °C) and various treatments (antigen, drugs) were applied by addition to the Tyrode solution. The dual emission of indo-1 was monitored using photomultiplier tubes at 408 and 480 nm and the ratio 408:480 output was computer digitized at 2.5 Hz using a Digidata 1200 and 'pClamp 6.0' software (Axon Instruments). The estimation of $[Ca^{2+}]_i$ was undertaken, on-line, after subjecting the data to the algorithm of Grynkiewicz et al. (1985). The measured constants used in this equation were obtained in a separate set of experiments carried out using a whole-cell intracellular calibration procedure with

intracellular solutions of known Ca^{2+} concentrations (Molecular Probes).

The antigen-induced response was quantified by the integration of the calcium responses (Narenjkar et al., 1999), i.e. the measurement of the area under the antigen-induced $\Delta[Ca^{2+}]_i$/time curve (AUC).

2.6. Assessment of cell viability

Cell viability was checked in the various procedures described above, to ensure the validity of the results. A dye-exclusion method was used, mixing equal volumes of Trypan Blue solution (0.25% w/v in phosphate-buffered saline) and of cell suspension before cell counting in a Neubauer haemocytometer. Percent viability was determined from the proportion of cells remaining unstained. Values of 95–98% were obtained.

2.7. Statistical analysis

All values are presented throughout this study as mean± standard error of the mean (S.E.M) for the number of experiments carried out and cited under each graph or table. The points on the graphs or figures in the tables are means. The number of experiments (n) is noted and the vertical bars represent the S.E.M. With the exception of the intracellular-free calcium concentration $[Ca^{2+}]_i$, statistical significance (P) was determined by the Student's paired t-test or analysis of variance as appropriate. For $[Ca^{2+}]_i$ (area under the curve, AUC), the Mann–Whitney test was used. Values of $P<0.05$ are considered significant. Denotation by asterisks *, **, *** represent significance of $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

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

2.8. Drugs and materials

4-Methylumbelliferyl *N*-acetyl-β-D-glucosaminide, 9-aminoacridine, apamin, bovine serum albumin, CsCl, dibutyl phthalate, dinitrophenyl human serum albumin (DNP-HSA), DNP-specific monoclonal mouse IgE, iberiotoxin, L-glutamine 200 mM solution, margatoxin, penicillin–streptomycin solution, phthalic acid bis(2-ethylhexyl) ester, tetraethylammonium, Trizma® base (tris[hydroxymethyl] aminomethane), Indo-1AM, gelatin, gramicidin and trypsin–ethylenediaminetetraacetic acid solution were supplied by Sigma (Poole, UK); ^{86}Rubidium (as RbCl) by New England Nuclear (Hounslow, UK); bis-oxonol by Molecular Probes (Cambridge); foetal calf serum, minimum essential medium, phosphate buffered saline without Ca^{2+} and Mg^{2+} by Gibco (Paisley, UK); and Triton X-100 by BDH Chemicals, Lutterworth. Additionally, samples of cetiedil (the (±)-2-hexahydro-1*H*-azepin-1-yl) ethyl ester of 2-cyclohexyl-2-(3-thienyl) ethanoic acid) (Innothéra, Arceuil, France), charybdotoxin (from Dr. P.N. Strong), dequalinium (decamethylene bis-4-aminoquinaldinium, generous gift from the Laboratories of Applied Biology, London, UK) and clotrimazole (Sigma), were generously donated by Prof. D.H. Jenkinson (Department of Pharmacology, University College London).

2.9. Buffer solutions

For β-hexosaminidase release experiments, modified Tyrode's solution was used. It contained 137 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES (*N*-2-hydroxyethyl piperazine-*N*′′-2-ethane sulphonic acid), 0.4 mM NaH_2PO_4 and 0.05% bovine serum albumin, adjusted to pH 7.4 with NaOH (1M). Bovine serum albumin (0.5 mg ml^{-1}) was added to prevent adsorptive loss of the minute quantities of antigen, except for membrane potential change measurement experiments in which 0.5 mg ml^{-1} gelatin was added instead.

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 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.

3. Results

3.1. Effect of K^+ channel blockers on antigen stimulation of β-hexosaminidase release

Antigen concentration and the response it elicited, whether maximal or submaximal, usually but not invariably had an influence on the degree of inhibition and the IC_{50} values of various inhibitors. Antigen-induced of β-hexosaminidase release was always submaximal with 1 ng ml^{-1} of the antigen dinitrophenyl–human serum albumin conjugate, and most of the comparisons of the relative potency of various inhibitors were carried out at that antigen concentration (Table 1).

Charybdotoxin, a blocker of the high-conductance Ca^{2+}-activated K^+ channel (BK_{Ca}), intermediate conductance Ca^{2+}-activated K^+ channel (IK_{Ca}) and the voltage-sensitive $K_{V1.3}$ channel (Bokvist et al., 1990; Strong et al., 1989; Garcia et al., 1995; Lewis and Cahalan, 1988) inhibited the antigen-induced β-hexosaminidase release in a dose-dependent manner with an IC_{50} of 61 ± 4 and 133 ± 52 nM for the

Table 1
The K^+ channel modulators tested, their [a]IC_{50} on K^+ channels in other tissues and the measured IC_{50} on antigen-stimulated β-hexosaminidase release in RBL-2H3 ([b]1 ng ml^{-1} antigen, dinitrophenyl–human serum albumin, [c]10 ng ml^{-1})

Name	Type of channel	IC_{50}[a]	Inhibitory effect on RBL secretion IC_{50}	References[d]
Quinidine	most	15–400 μM	45 μM[b] 83 μM[c]	Cook and Haylett (1985), Lew and Ferreira (1978), Sakuta et al. (1992)
$BaCl_2$	IR K^+ channels OR K^+ channels	100 μM 2–20 mM	6 mM[b,c]	McCloskey and Cahalan (1990)
CsCl	IR K channel OR K^+ channels	0.5 mM 20 mM	~ 20 mM[b]	Lewis et al. (1991), Piguet and North (1992), Gericke et al. (1995)
Apamin	SK_{Ca} channels	1–3 nM	NS at 1 μM[b]	Bourque and Brown (1987), Dunn (1994)
Charybdotoxin	BK_{Ca} channels IK_{Ca} channels	1–30 nM 30 nM	61 ± 4 nM[b] 133 ± 52 nM[c]	Schneider et al. (1989), O'Grady et al. (1991), Tas et al. (1988)
Tetraethyl–ammonium	several	0.3–10 mM	NS at 20 mM[b]	Cook and Haylett (1985), Davies et al. (1989), Stanfield (1983)
Cetiedil	IK_{Ca} channels	25–150 μM	84 ± 10 μM[c]	Christophersen and Vestergaard-Bogind (1985)
Iberiotoxin	BK_{Ca}	1–3 nM	NS at 100 nM[b]	Candia et al. (1992)
Margatoxin	$K_{V1.3}$	<1 nM	NS at 100 nM[b]	Garcia et al. (1995)
Dequalinium	SK_{Ca} channels	1–1.5 μM	NS at 50 μM[b]	Castle et al. (1993), Dunn (1994)
Clotrimazole	IK_{Ca} channels	1–24 nM	~ 70 μM	Alvarez et al. (1992)
9-Aminoacridine	SK_{Ca} and K_{ATP}	70 μM	NS at 60 μM[b]	Cook and Haylett (1985), Cook and Hales (1984)

NS—No significant effect. [d]K^+ channel modulators and their IC_{50} in other tissues.

stimulation by 1 and 10 ng ml^{-1} dinitrophenyl–human serum albumin conjugate, respectively (Fig. 1 and Table 1). In contrast, apamin, a selective blocker of the small-conductance Ca^{2+}-activated K^+ channel (SK_{Ca}) channel; iberiotoxin, a selective blocker of BK_{Ca} channel and margatoxin, a selective blocker of $K_{V1.3}$, did not affect antigen-induced β-hexosaminidase secretion (Table 1).

Cetiedil, a blocker of IK_{Ca}, volume-sensitive and G-protein-regulated K^+ channels (Berkowitz and Orringer, 1981, 1984; Sarkadi et al., 1984, 1985; Jones et al., 1994) inhibited dose-dependently the β-hexosaminidase release induced by antigen. The IC_{50} was 84 ± 10 μM when the cells were stimulated by 10 ng ml^{-1} dinitrophenyl–human serum albumin conjugate (Fig. 1).

Clotrimazole, an IK_{Ca} channel blocker in red blood cells with an IC_{50} of 0.1 μM (Alvarez et al., 1992) inhibited antigen-induced secretion from RBL-2H3 cells but at much higher concentrations: At an antigen (diphenyl-human serum albumin conjugate) concentration of 1 ng ml^{-1}, the IC_{20} was 25 μM and the IC_{50} ~70 μM, an accurate figure could not be obtained because of the compound's low solubility.

The SK_{Ca} channel blockers, dequalinium (Castle et al., 1993), apamin (Bourque and Brown, 1987; Dunn, 1994) and 9-aminoacridine (Cook and Haylett, 1985) had no effect on the antigen-induced secretion (Table 1).

Tetraethylammonium which blocks most classes of Ca^{2+}-activated K^+ channels, e.g. SK_{Ca}, BK_{Ca} and IK_{Ca} channels as well as K_V and the ATP-sensitive K^+ channel (K_{ATP}) (Cook and Haylett, 1985; Davies et al., 1989; Stanfield, 1983) did not affect antigen-induced β-hexosaminidase release at concentrations of up to 20 mM (Table 1). Cesium, a nonspecific K^+ channel blocker, had a little effect at concentrations below 10 mM, but at 20 mM, it produced 50% inhibition when tested against 1 ng ml^{-1} dinitrophenyl–human serum albumin conjugate (Table 1).

3.2. Effect of charybdotoxin and cetiedil on the antigen-induced membrane potential changes

Antigen stimulation in RBL-2H3 cells results in a rapid depolarization, peaking at 1 min, which is then followed by a slow repolarization (Fig. 2A) which reaches a plateau by 8 min, where the repolarization remains incomplete (~ 75% of the depolarization). Gramicidin was usually added at 9 min to produce another more marked and complete depolarization which was used in the calibration of the changes in the membrane potential with antigen and drug treatment. In nominally calcium-free solution, the depolarizing phase was slowed while repolarization was markedly inhibited (Fig. 2B), suggesting that both depolarization and repolarization are, in part, mediated by a calcium-sensitive conductance mechanism (Labrecque et al., 1991). This suggestion that repolarization was calcium-sensitive was supported by the inhibition (albeit partial, 35%) of antigen-induced $^{86}Rb^+$-efflux in the absence of extracellular calcium (in the buffer). However, under these conditions, there was almost complete (95%) inhibition of

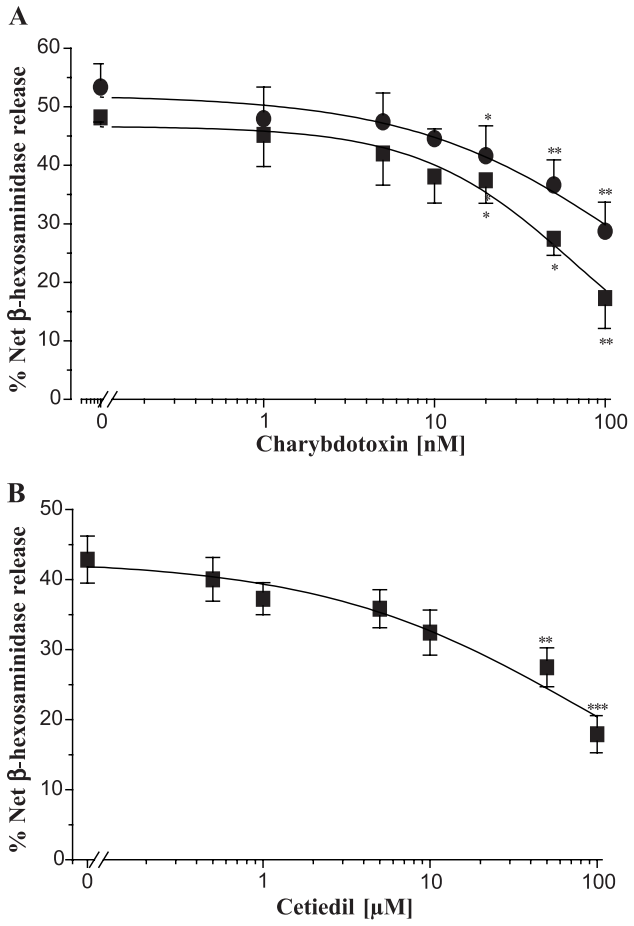

Fig. 1. Inhibitory effect of charybdotoxin (A) and cetiedil (B) on antigen-stimulated β-hexosaminidase release. Each point on the curves represents the mean ± S.E.M of %net β-hexosaminidase release in six experiments. Data have been fitted to the Hill equation and with B_{max} constrained to the maximum net release for each curve. Hill coefficient (n_H) was 0.84 ± 0.05 (A) and 0.54 ± 0.04 (B). ■ 1, ● 10 ng ml^{-1} antigen (dinitrophenyl–human serum albumin conjugate). Student's paired *t*-test *$P<0.05$ and **$P<0.01$, ***$P<0.001$.

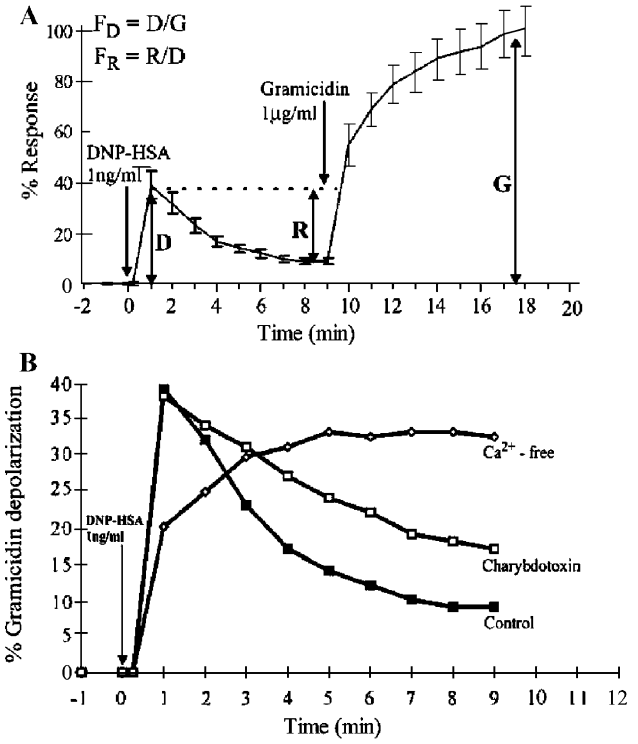

Fig. 2. (A) Antigen-induced changes in the membrane potential of RBL-2H3 cells (10^6 cells ml^{-1}), measured by the bis-oxonol method and expressed as a % of the gramicidin-induced depolarization (*G*) at the end of each experiment. F_RD/G = antigen-induced depolarization and F_RR/D = the repolarization that follows. Antigen (1 ng ml^{-1} dinitrophenyl–human serum albumin) was added at time 0 min. (B) Changes in the membrane potential induced by the addition of 1 ng ml^{-1} dinitrophenyl–human serum albumin (added at time 0 min) and effect of incubation in a Ca^{2+}-free solution or with charybdotoxin (100 nM added at time −8 min). The data shown were obtained from a typical single experiment. Six other experiments gave similar results.

mediator (β-hexosaminidase and histamine) release (results not shown). The antigen-induced depolarization obtained in the absence of extracellular calcium was abolished when sodium in the Tyrode solution was substituted with either choline or *N*-methyl-D-glucosamine (data not shown).

Charybdotoxin had no effect on the resting membrane potential of RBL-2H3 cells even at 100 nM. However, it was capable of partially inhibiting the repolarization phase of the antigen-induced depolarization with an IC_{50} of 84 ± 7 nM (Fig. 3). The charybdotoxin inhibition of the potassium conductance was confirmed in the $^{86}Rb^+$-efflux experiments (Fig. 4).

Attempts were made to study the effect of cetiedil on the antigen-induced membrane potential changes, but interpretation of the results was made difficult by the fluorescence interference with bis-oxonol. Cetiedil by itself at concentrations >1 μM (which is well below the IC_{50} for inhibition of β-hexosaminidase release) caused a

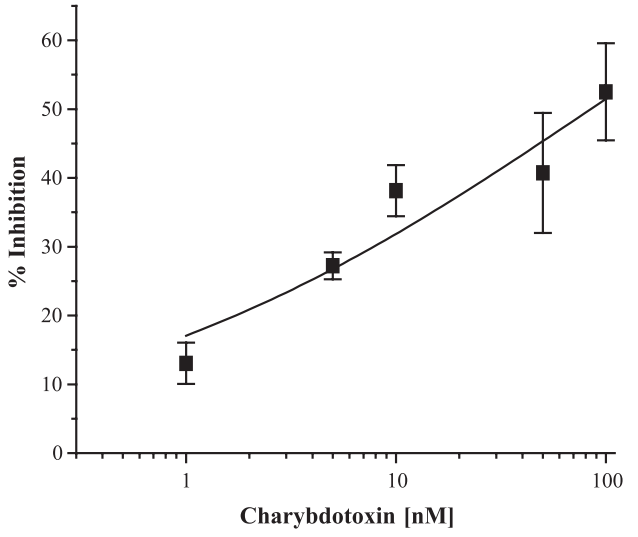

Fig. 3. Dose–response curve of the inhibition by charybdotoxin of the repolarization phase of the antigen-induced change in the membrane potential. Data presented are mean ± S.E.M of %inhibition from four experiments. Data have been fitted to the Hill equation with B_{max} constrained to 100%. Hill coefficient (n_H) was 0.36 ± 0.01.

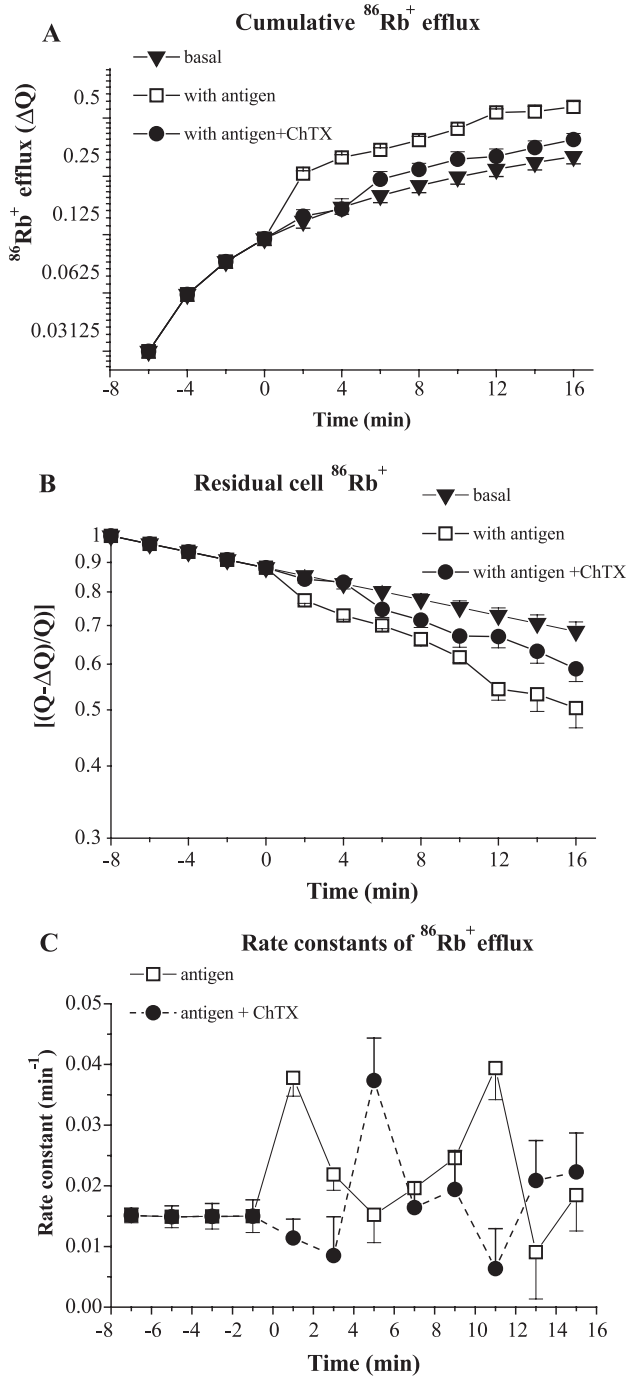

Fig. 4. Effect of charybdotoxin on the antigen-stimulated $^{86}Rb^+$ loss (efflux) from $^{86}Rb^+$-loaded RBL-2H3 cells (total $^{86}Rb^+$ load in each cell aliquot, Q giving ca. 28,000 counts per min). Cells were incubated in modified Tyrode's buffer only (▼, basal efflux), with antigen (□, 10 ng ml^{-1} dinitrophenyl–human serum albumin conjugate, added at time 0 min) or with charybdotoxin 100 nM added at time −8 min plus 10 ng ml^{-1} antigen added at time 0 min (●). $n=5$. (A) Time-course of the normalized cumulative $^{86}Rb^+$ efflux (ΔQ). (B) $^{86}Rb^+$ remaining in cells, expressed as a proportion of the total cell load of $^{86}Rb^+$ [$(Q-\Delta Q)/Q$]. (C) Rate constants, k (min^{-1}) of $^{86}Rb^+$-efflux.

concentration-dependent increase in the bis-oxonol fluorescence, which may be misinterpreted as membrane depolarization.

3.3. Effect of charybdotoxin and cetiedil on $^{86}Rb^+$-efflux

Fig. 4A and B shows the time-course of the cumulative loss (efflux) of $^{86}Rb^+$ and the proportion left in the cells, respectively, in control samples (basal efflux), in the presence of antigen and in the presence of antigen as well as charybdotoxin (100 nM). It can be seen that $^{86}Rb^+$ loss (efflux from cells) increases with incubation time and that it was accelerated in antigen presence (stimulated efflux). This acceleration of $^{86}Rb^+$-efflux was significantly reduced by the addition of 100 nM charybdotoxin ($P<0.001$, one-way analysis of variance). Apart from the overall increase in the rate of $^{86}Rb^+$-efflux during the period of incubation with antigen, there were marked variations within that period

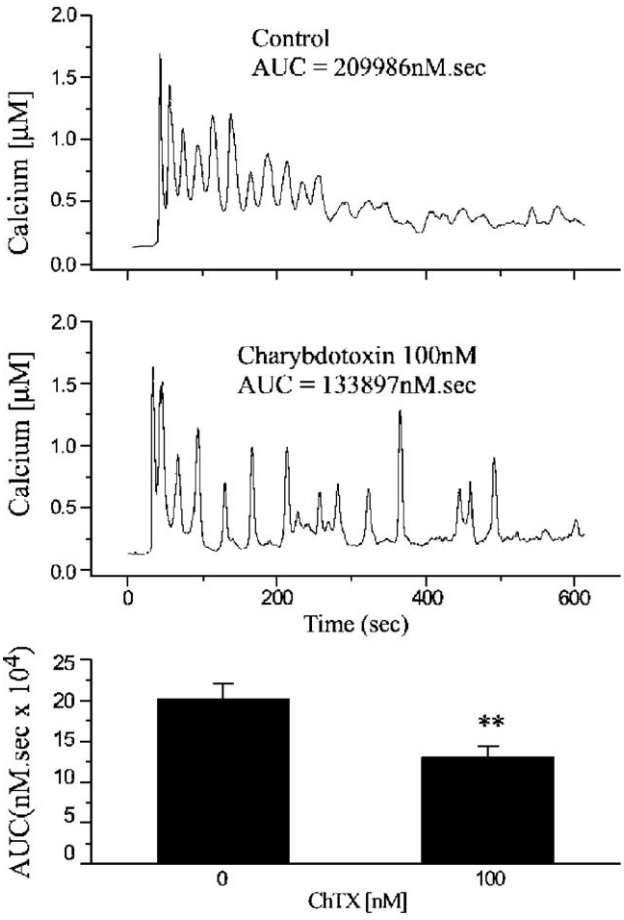

Fig. 5. Effect of charybdotoxin on the antigen-induced intracellular free Ca^{2+} concentration in single cells. Antigen (10 ng ml^{-1} dinitrophenyl–human serum albumin) was added at time 0. The upper and middle traces show typical examples (single cells) of the antigen-induced intracellular calcium oscillations in the absence and presence of charybdotoxin (100 nM added at time −8 min). The lower graph shows the accumulated data from 46 cells ($n=34$ for control and $n=12$ for charybdotoxin). The area under the curve (AUC) is for 10 min and background is subtracted. **$P<0.01$ (Mann–Whitney test).

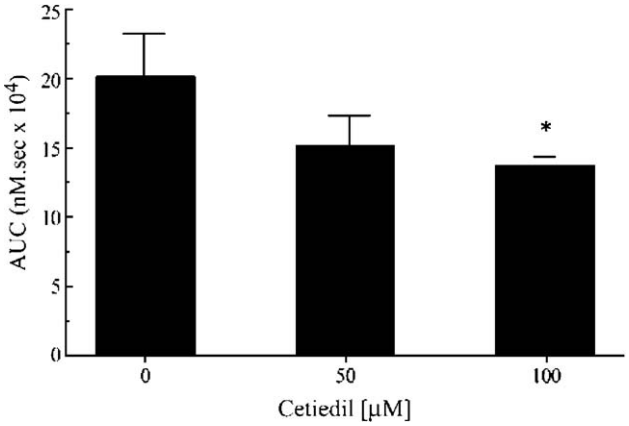

Fig. 6. Effect of cetiedil on the antigen-induced Ca^{2+} response. $n=34$ for control and $n=9$ for each concentration of cetiedil. *$P<0.05$ (Mann–whitney test).

(Fig. 4C). In the first 2 min (time 0–2 min) after the addition of 10 ng ml^{-1} antigen (dinitrophenyl–human serum albumin conjugate), ^{86}Rb-efflux rate was sharply increased from a basal rate (E_b) of 0.0156 ± 0.001 min^{-1} (i.e. spontaneous $^{86}Rb^+$ loss occurred at a rate of $1.56 \pm 0.1\%$ per min in time -8 to 0 min) to 0.0648 min^{-1} (E_s), i.e. four-fold. A second major peak (E_s 0.06321 min^{-1}) was noted in the time-period 10–12 min and was followed by drop in efflux rate to near basal level by the end of the 16-min incubation with antigen. When 100 nM charybdotoxin was added at $t=-8$ min (before antigen) neither the early nor the late peak was seen. Instead, there was a single peak in the time period 4–6 min. Average net increase in ^{86}Rb-efflux rate induced by antigen stimulation, E_{sn} (with 10 ng ml^{-1} dinitrophenyl–human serum albumin conjugate), was reduced from 0.0124 ± 0.001 to 0.0063 ± 0.002 min^{-1} ($P<0.01$) by the addition of 100 nM charybdotoxin and 0.0033 ± 0.001 min^{-1} by 100 μM cetiedil.

3.4. Effect of charybdotoxin and cetiedil on the antigen-induced intracellular Ca^{2+} response

The area under the curve (AUC) of the antigen-induced $[Ca^{2+}]_i$ response was significantly reduced by the addition of charybdotoxin (100 nM) (Fig. 5). The examples of $[Ca^{2+}]_i$ measurement in Fig. 5 show that charybdotoxin decreases the initial frequency of the antigen-induced oscillations and reduces the amplitude of the late plateau phase (>300 s). Cetiedil at a concentration of 50 μM had no significant effect on the calculated AUC of the antigen-induced oscillatory rise in $[Ca^{2+}]_i$, but at 100 μM, it significantly reduced the AUC ($P<0.05$, Fig. 6).

4. Discussion

It has been established that a sustained increase in $[Ca^{2+}]_i$ aided at least partly by a maintained electrochemical driving force for Ca^{2+} influx, is required for optimal antigen-induced mediator release from RBL-2H3 cells, a tumour analogue of mucosal mast cells. We have shown that the repolarization that follows the antigen-induced depolarization requires extracellular calcium and that it is inhibited by the potassium channel blocker charybdotoxin. Antigen-stimulated K^+-efflux, which is also calcium-dependent (results not shown), is also significantly reduced by charybdotoxin (Fig. 4). These results are in accord with the previous suggestion that in RBL-2H3 cells, a calcium sensitive potassium channel may perhaps be involved in the antigen-induced mediator release mechanism. There is no evidence in the literature that charybdotoxin has cell interactions other than those with the Ca^{2+}-activated K^+ channels (intermediate, IK, or large conductance, BK), that it penetrates the cell membrane, or that it has intracellular actions. Despite the lack of K^+ selectivity of cetiedil, quindine, Ba^{2+} or Cs^+, their inhibitory effect on antigen-induced β-hexosaminidase secretion from RBL-2H3 cells (Table 1) further supports the notion of possible involvement of potassium channels in mediator secretion. However, K^+ channel involvement in the activation of other types of mast cells is uncertain.

Mast cells show heterogeneity in phenotype and function, even in the same species (Metcalfe et al., 1997). In rodents, there are two major phenotypes, mucosal mast cells and connective tissue mast cells. Antigen-activated ion currents vary between different rodent mast cell phenotypes. Until recently, no potassium channel had been detected in rat peritoneal mast cells, which are classified as connective tissue mast cells (Lindau and Fernandez, 1986; Penner et al., 1988) although they were expressed in the less mature bone-marrow-derived mast cells (McCloskey and Qian, 1994). Hill et al. (1996) obtained higher rates of expression of K^+ channel activity in both bone marrow mast cells and peritoneal mast cells in the rat by using the amphotericin-B-perforated patch technique and by temperature control.

In humans, where the classification of mast cells into mucosal mast cells and connective tissue mast cells cannot be applied, a whole spectrum of mast cell types based on enzyme and proteoglycan composition is apparent. Recently, Duffy et al. (2001) have described the electrophysiological characterization for the first time of ion currents in purified human lung mast cells and human peripheral blood-derived mast cells. Both mast cell types differ fundamentally from RBL2H3 cells in having a resting membrane potential of around 0 mV. Following IgE-mediated activation, these mast cells develop a Ca^{2+}-activated K^+ current exhibiting weak inward rectification, which polarizes the cell to around -40 mV and a smaller outwardly rectifying Ca^{2+}-independent $Cl-$ conductance. The K^+ current was reversibly blocked by charbdotoxin. However, charybdotoxin did not consistently attenuate histamine or leukotriene C_4 release. Therefore, Duffy et al. (2001) concluded that the K^+ current was not essential for the release of these mediators, but may possibly enhance the release process.

Apart from K_{Ca} channels, raised $[Ca^{2+}]_i$ may activate Cl^- coductances which may play a role in antigen-induced mediator secretion from rat peritoneal mast cells and RBL-2H3 cells (Lindau and Fernandez, 1986; Penner et al., 1988; Matthews et al., 1989; Romanin et al., 1991; Redrup et al., 1997). The Na^+/Ca^{2+} exchanger and Na^+/K^+-ATPase are also sensitive to $[Ca^{2+}]_i$ and may be involved in mediator release. A ouabain-sensitive Na^+/K^+-ATPase may take part in maintaining the resting membrane potential (which is much lower in rat peritoneal mast cells than in RBL-2H3 cells) and in the activation of rat peritoneal mast cells (Friis et al., 1997).

So far, several types of K^+ conductance have been described in RBL-2H3 cells. The first report of an inward rectifying (IR) K channel in RBL-2H3 cells was published in 1986 by Lindau and Fernandez. This conductance which is involved in the maintenance of the resting membrane potential, is negatively regulated by activation of a pertussis toxin-insensitive G-protein (McCloskey and Cahalan, 1990; Lewis et al., 1991) and is blocked by Ba^{2+}, Cs^+ and tetraethylammonium (Ikeda and Weight, 1984; McCloskey and Cahalan, 1990). Physiological and molecular characterization of the predominant IRK channel in RBL-2H3 has demonstrated its similarity to the cloned mouse IRK1 channel (Wischmeyer et al., 1995). The other major K conductance, thus far described in these cells, is an outwardly rectifying K conductance (K_{OR}^+) that is inhibited by quinidine and is believed to be activated by a pertussis toxin-sensitive G-protein which is functionally associated with receptors for a variety of ligands, including adenosine and ATP (McCloskey and Cahalan, 1990; Qian and McCloskey, 1993). Antigen-triggered K_{OR}^+ activation was inhibited by Ba^{2+} and nitrendipine at concentrations which were higher than those required for blocking K_{OR}^+ and the voltage-gated L-type Ca^{2+} channels, respectively. Unlike activation of K_{OR}^+ by antigen, purinergic activation is not calcium sensitive (Qian and McCloskey, 1993). Characterization of whole-cell current in rat bone marrow mast cells by patch-clamp recording showed that two K^+ channels were expressed in these cells: a constitutively active inwardly rectifying (K_{OR}^+) and a latent outwardly rectifying (K_{OR}^+) channel (McCloskey and Qian, 1994; Hill et al., 1996). The K_{OR}^+ described by Hill et al. (1996) in both rat bone marrow mast cells and rat peritoneal mast cells was reversibly blocked by the chloride channel blocker 4,4′-diisocyanato-2,2′-stilbenedisulphonate (DIDS), but not by potassium channel blockers.

Calcium-activated potassium channels are a diverse species and the pharmacology of the various channel types is still a developing field. However, utilizing the available compounds, we attempted to elucidate the subtype involved in antigen-induced mediator release from RBL-2H3 cells.

Charybdotoxin inhibited the antigen-stimulated mediator secretion dose-dependently with an IC_{50} of ~ 61 and 133 nM for the stimulation by 1 and 10 ng ml^{-1} of antigen (dinitrophenyl–human serum albumin conjugate), respectively (Fig. 1 and Table 1). This peptide can block several types of potassium channels, BK_{Ca} (IC_{50} = 3 nM) (Castle et al., 1989), IK_{Ca} (IC_{50} = 30–100 nM) (Strong et al., 1989; Beech et al., 1987), and $K_{V1.3}$ (IC_{50} = 5 nM) (Lewis and Cahalan, 1988). The failure of tetraethylammonium (20 mM), which blocks most classes of K^+ channels and iberiotoxin (100 nM), a selective inhibitor of BK_{Ca} channels (Davies et al., 1989; Stanfield, 1983) to inhibit stimulated secretion, suggests that the inhibitory effect of charybdotoxin is not via BK_{Ca} channels. Margatoxin (100 nM), a specific blocker of voltage-activated $K_{V1.3}$ channels (Garcia-Calvo et al., 1993) also did not affect the antigen-induced secretion in RBL-2H3 cells (Table 1). These results suggest that BK_{Ca} and $K_{V1.3}$ channels have a minimal role in antigen-induced secretion.

Cetiedil inhibited antigen-induced β-hexosaminidase release (IC_{50} of 84 ± 10 μM) and has been shown to be a blocker of IK_{Ca}, volume-sensitive and G-protein-regulated K^+ channels which have been found in various cells, including human red blood cells and lymphocytes (Berkowitz and Orringer, 1981, 1984; Sarkadi et al., 1984, 1985; Christophersen and Vestergaard-Bogind, 1985; Jones et al., 1994; Benton et al., 1994). Cetiedil (100 μM) also inhibited the antigen-activated $^{86}Rb^+$-efflux. Unfortunately, we were unable to assess the effects of cetiedil on membrane repolarization, as the compound was found to be fluorescent within the same spectral range as the membrane potential indicator used (bis-oxonol).

Cetiedil has been used in the palliative treatment of sickle cell disease where it has been proposed to act by inhibition of the Ca^{2+}-dependent K^+ permeability and membrane bound ATPases (Abu-Salah and Gambo, 2002), thereby preventing erythrocyte shrinkage. The drug also possesses analgesic, antispasmodic, local anaesthetic and vasodilator activities. Cetiedil is also one of several K^+ channel blockers which have been shown to inhibit the activation of thymocytes and T and B lymphocytes (Lewis and Cahalan, 1995).

SK_{Ca} channel blockers, such as apamin and dequalinium, had no effect on antigen-induced β-hexosaminidase release (Table 1). However, apamin, has been reported to produce significant inhibition of contraction of guinea-pig isolated trachea (modest inhibition) and of histamine release (~ 30% inhibition) from minced guinea-pig lung tissue, induced by submaximal antigen concentration, whereas leukotriene C_4 release was not affected (Yamauchi et al., 1994).

By elimination, our results thus far seem to indicate that IK_{Ca} type channels might be involved in antigen-induced secretion. However, clotrimazole, which has been shown to have IK_{Ca} channel blocking activity in red blood cells, had no significant effect on antigen-stimulated β-hexosaminidase release at concentrations that completely block IK_{Ca} channels (Alvarez et al., 1992). Nevertheless, charybdotoxin inhibited β-hexosaminidase release, membrane repolarization and $^{86}Rb^+$-efflux with similar IC_{50} (60–100 nM). It was also able to partially inhibit the rise in $[Ca^{2+}]_i$ (35%

reduction in AUC at 100 nM) suggesting that the same underlying conductance might be involved in each of these responses. Our evidence thus suggests that the calcium-dependent antigen-induced K^+ conductance has some similarities to the IK_{Ca} conductance found in red blood cells though its relative insensitivity to clotrimazole indicates that the channel involved differs in some regards. This remains for further study.

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