

The K^+ channel $iK_{CA}1$ potentiates Ca^{2+} influx and degranulation in human lung mast cells

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Background: Human lung and blood-derived mast cells express a Ca^{2+} -activated K^+ channel (K_{CA}) that has electrophysiological properties resembling the intermediate conductance K_{CA} ($iK_{CA}1$). This channel is predicted to enhance IgE-dependent mast cell responses.

Objective: To confirm the identity of this channel as $iK_{CA}1$ in human lung mast cells and to examine the effect of an $iK_{CA}1$ opener, 1-ethyl-2-benzimidazolinone (1-EBIO), on Ca^{2+} influx and degranulation after IgE-dependent activation.

Methods: $iK_{CA}1$ expression was examined by using RT-PCR. Ion currents were measured by using the patch clamp technique in human peripheral blood-derived mast cells, freshly isolated human lung mast cells (HLMCs), and long-term cultured HLMCs (LTHLMCs). Currents were manipulated with the specific $iK_{CA}1$ opener 1-EBIO and the $iK_{CA}1$ blockers clotrimazole and TRAM-34. Ratiometric Ca^{2+} imaging was performed on single fura-2-loaded cells, and histamine release was measured by radioenzymatic assay. **Results:** Both fresh HLMCs and LTHLMCs expressed $iK_{CA}1$ mRNA. The $iK_{CA}1$ opener 1-EBIO induced $iK_{CA}1$ currents in 89% of human peripheral blood-derived mast cells, 12% of fresh HLMCs, and 67% of LTHLMCs, which were blocked by the $iK_{CA}1$ blockers clotrimazole and TRAM-34. After cell activation with a suboptimal concentration of anti-IgE, 1-EBIO enhanced the IgE-dependent rise in cytosolic-free Ca^{2+} and potentiated IgE-dependent histamine release.

Conclusion: Opening of $iK_{CA}1$ enhances IgE-dependent Ca^{2+} influx and histamine release in HLMCs. Inhibition of $iK_{CA}1$ may provide a novel approach to the treatment of mast cell-mediated disease. (*J Allergy Clin Immunol* 2004;114:66-72.)

Key words: Mast cell, human, K^+ , ion channel, Ca^{2+} , histamine

Mast cells have many biological roles, ranging from maintenance of tissue homeostasis to deleterious activities contributing to the pathogenesis of many diseases including asthma, rheumatoid arthritis, pulmonary fibrosis, and mastocytosis.^{1,2} These latter effects presumably represent the mast cell response to an ongoing tissue

Abbreviations used

[Ca^{2+}] _i :	Cytosolic free Ca^{2+} concentration
DMEM:	Dulbecco modified Eagle medium
1-EBIO:	1-Ethyl-2-benzimidazolinone
HLMC:	Human lung mast cell
HPBDMC:	Human peripheral blood-derived mast cell
$iK_{CA}1$:	Human intermediate conductance Ca^{2+} -activated K^+ channel
K_{CA} :	Ca^{2+} -activated K^+ channel
LTHLMC:	Long-term cultured human lung mast cell
SCF:	Stem cell factor

insult with resulting tissue damage and remodeling. In these diseases, there is evidence of mast cell infiltration and/or proliferation within specific tissue compartments such as the bronchial smooth muscle in asthmatic airways,³ accompanied by the sustained release of a plethora of autacoid mediators, cytokines, and proteases.

Human mast cells express a Ca^{2+} -activated K^+ channel (K_{CA}) that is opened after IgE-dependent activation and that has electrophysiological properties suggesting that it is the intermediate conductance K_{CA} ($iK_{CA}1$).⁴ One widely proposed role for K^+ channels is to maintain a negative membrane potential during cell activation, counteracting the tendency for Ca^{2+} influx to depolarize the cell membrane. The importance of this is that a negative membrane potential should enhance Ca^{2+} influx because of the large electrical driving force for Ca^{2+} that is created and maintained, and perhaps more importantly, because store-operated Ca^{2+} channels that mediate Ca^{2+} influx after cell activation conduct larger currents at negative membrane potentials.⁵ There is evidence that $iK_{CA}1$ mediates such an effect in mitogen-activated human T cells, because attenuation of channel function by mutation or use of channel blockers reduces the activation-induced rise in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) cytokine release and cell proliferation.⁶⁻⁸ Because IgE-dependent activation of human lung mast cells (HLMCs) has an absolute requirement for the influx of Ca^{2+} from the extracellular fluid, the HLMC K_{CA} would be predicted to play a similar role. In support of this, we demonstrated recently that the $iK_{CA}1$ blocker charybdotoxin attenuated IgE-dependent histamine release from HLMCs.⁴

In the current study, we have confirmed the expression of the $iK_{CA}1$ channel in human mast cells and tested the hypothesis that opening of this channel enhances

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Received for publication February 2, 2004; revised April 1, 2004; accepted for publication April 5, 2004.

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0091-6749/\$30.00

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doi:10.1016/j.jaci.2004.04.005

IgE-dependent Ca^{2+} influx and histamine release. To achieve this, we have used patch clamp recording to study the electrophysiology of the channel in human peripheral blood-derived mast cells (HPBDMCs) and long-term cultured human lung mast cells (LTHLMCs) compared with freshly isolated HLMCs, and we have examined Ca^{2+} responses and histamine release in LTHLMCs in the presence of the iK_{CA1} opener 1-ethyl-2-benzimidazolinone (1-EBIO).⁹

METHODS

Reagents

The following were purchased: stem cell factor (SCF), IL-6, IL-10 (R&D, Abingdon, United Kingdom [UK]); 1-EBIO, clotrimazole, goat polyclonal antihuman IgE (Sigma, Poole, Dorset, UK); human myeloma IgE (Calbiochem-Novabiochem, Nottingham, UK); mouse IgG₁ mAb YB5B8 (anti-CD117; Cambridge Bioscience, Cambridge, UK); sheep antimouse IgG₁ Dynabeads (Dyna, Wirral, UK); histamine, S-adenosyl-L-[methyl-³H] methionine (Amersham Life Science, Little Chalfont, Bucks, UK); Dulbecco modified Eagle medium (DMEM)/Glutamax/Hepes, antibiotic/antimycotic solution, nonessential amino acids, and FCS (Life Technologies, Paisley, UK). Rat kidney histamine methyl transferase was a generous gift from Dr S. Harper (AstraZeneca R&D Charnwood, Loughborough, UK). TRAM-34 was a generous gift from Dr Heike Wulff (University of California Irvine).

Human mast cell purification and culture

All human subjects gave written informed consent, and the study was approved by the Leicestershire Research Ethics Committee. HLMCs were dispersed and purified from macroscopically normal lung obtained within 1 hour of resection for lung cancer by using immunomagnetic affinity selection as described previously.¹⁰ Final mast cell purity was >99% and viability >97%. HLMC were cultured in DMEM/Glutamax/Hepes containing antibiotic/antimycotic solution, nonessential amino acids, 10% FCS, 100 ng/mL SCF, 50 ng/mL IL-6, and 10 ng/mL IL-10 for as long as 10 weeks, as described previously.¹¹ Cells were sensitized with human myeloma IgE 2.5 µg/mL as required. Cells were used either fresh (within 1 week of purification) or after long-term culture (>4 weeks after purification).

Mast cells were grown from progenitors in adult peripheral blood, as previously described.⁴

Mast cell viability, monitored by exclusion of trypan blue, was >97% in all experiments.

RT-PCR for iK_{CA1}

Total RNA was isolated by using RNeasy mini-spin columns (Qiagen Ltd, West Sussex, UK) according to the manufacturer's instructions. DNA contamination was removed by using DNase I (Invitrogen, Paisley, UK), and RT reactions using the Sensiscript RT kit (Qiagen Ltd) were performed according to the manufacturer's instructions by using 50 ng RNA and random hexamers (Invitrogen) as primers. Controls for which the RT enzyme was omitted were also included. One tenth of the RT reaction was then added to a PCR reaction by using Taq MasterMix (Qiagen Ltd) and run for 35 cycles. Half the PCR reaction was then subject to agarose gel electrophoresis and stained with SybrGold (Molecular Probes, Eugene, Ore) and viewed on a Dark Reader (Clare Chemical Research, Dolores, Colo). The iK_{CA1} PCR primers were as follows: forward, TGCTGTCCCTGGCCATGC; reverse, GTGGCATTAA-CAGCCTGCC. The expected product size was 250 bp.

Electrophysiology

The whole-cell variant of the patch clamp technique¹² was used as described by us previously.^{4,11} The pipette solution contained KCl, 140 mmol/L; MgCl₂, 2 mmol/L; HEPES, 10 mmol/L; Na⁺-ATP 2 mmol/L, and guanosine triphosphate, 0.1 mmol/L; pH 7.3. The standard external solution contained NaCl, 140 mmol/L; KCl, 5 mmol/L; CaCl₂, 2 mmol/L; MgCl₂, 1 mmol/L; and HEPES, 10 mmol/L; pH 7.3. For recording, mast cells were placed in 35-mm dishes containing standard external solution.

Whole-cell currents were recorded by using an Axoclamp 200A amplifier (Axon Instruments, Foster City, Calif) and currents usually evoked by applying voltage commands to a range of potentials in 10-mV steps from a holding potential of -20 mV. The currents were digitized (sampled at a frequency of 10 kHz) and analyzed as described previously.^{4,11} Experiments were performed at 27°C, with temperature controlled by a Peltier device, and a perfusion system (Automate Scientific, San Francisco, Calif) was used to allow solution changes. Drugs were added directly to the recording chamber.

Ca^{2+} imaging

Changes in $[\text{Ca}^{2+}]_i$ were monitored fluorometrically by use of the Ca^{2+} -sensitive probe fura-2. Cells were loaded with Fura-2 (Molecular Probes) by incubation in normal physiological saline solution (130 mmol/L NaCl, 5.6 mmol/L KCl, 1 mmol/L MgCl₂, 2 mmol/L CaCl₂, 11 mmol/L glucose, 10 mmol/L HEPES, pH 7.4 with NaOH) containing 2 µmol/L fura-2-acetoxymethyl ester for 30 minutes at room temperature, then washed for 30 minutes. A coverslip with attached cells was mounted in a perfusion chamber at 37°C. The single-cell recording system has been described previously.¹¹ Data acquisition occurred at a rate of 1 dual-wavelength image every 6 seconds as the 340/380 ratio. This was converted to $[\text{Ca}^{2+}]_i$ by using a commercially available calibration kit (Molecular Probes). Drugs were added to the dish as required.

HLMC activation

For analysis of histamine release, 1×10^4 mast cells were warmed to 37°C in triplicate in 50 µL of DMEM. 1-EBIO or dimethyl sulfoxide control at 4 times the final concentration in 25 µL was added just before addition of 25 µL medium containing 4 times the final concentration of goat polyclonal antihuman IgE (1:1000 final dilution, which gives maximal histamine release; 1:30,000 final dilution, which gives submaximal histamine release). After 30 minutes of incubation at 37°C, the cells were centrifuged at 250g for 4 minutes, the supernatant decanted, and control cell pellets lysed in sterile deionized water for measurement of total histamine content, as described previously.¹⁰ The final concentration of dimethyl sulfoxide in all wells was 0.1%.

Data presentation and statistical analysis

IgE-dependent histamine release is expressed as net release (total minus background) as a percentage of cell total. Data are expressed as means $\pm$ SEMs unless otherwise stated. Differences between groups of data were explored by using the Student paired or unpaired *t* test (2-tailed) as appropriate. Frequency of channel expression under different culture conditions was compared between groups by using the χ^2 test. A *P* value of <.05 was considered statistically significant.

RESULTS

Human lung mast cells express iK_{CA1} mRNA

Human lung mast cells and HPBDMCs express a K_{CA} , which opens in a proportion of cells in response to IgE-dependent activation. Electrophysiological properties of

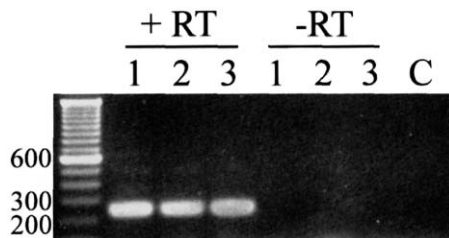


FIG 1. RT-PCR expression of iK_{CA1} mRNA in freshly isolated HLMCs (lane 1) and LTHLMCs (lanes 2 and 3). In lanes 4 to 6, the RT enzyme was omitted. In lane C, the iK_{CA1} PCR primers were omitted.

this K_{CA} suggest that it is the intermediate conductance K^+ channel iK_{CA1} . Using RT-PCR, both freshly isolated HLMCs and LTHLMCs expressed iK_{CA1} mRNA, suggesting the mast cell K_{CA} is indeed iK_{CA1} (Fig 1).

iK_{CA1} opener 1-EBIO opens the human mast cell K_{CA} with preferential expression in HPBDMCs and LTHLMCs

The iK_{CA1} opener 1-EBIO opens iK_{CA1} with a half-maximal value of approximately 30 $\mu\text{mol/L}$ for heterologously expressed iK_{CA1} , with a maximal effect at approximately 300 $\mu\text{mol/L}$.⁹ The effects of 1-EBIO are relatively specific for iK_{CA1} , opening it by enhancing its sensitivity to $[\text{Ca}^{2+}]_i$.⁹ Thus, at 100 $\mu\text{mol/L}$ EBIO, maximal K^+ currents are achieved in the presence of 100 nmol/L free Ca^{2+} , which is below the resting $[\text{Ca}^{2+}]_i$ of most cell types.

Small iK_{CA1} -like currents were present in 12/174 (6.9%) LTHLMCs and 5/23 (21.7%) HPBDMCs at rest, but no resting freshly isolated HLMCs. In HLMCs and HPBDMCs, addition of 1-EBIO 100 $\mu\text{mol/L}$ activated a K^+ current with properties identical to those seen previously after IgE-dependent activation⁴ (Fig 2, A and B). Thus, the current appeared within 90 seconds of adding 1-EBIO to the bath and reached a plateau within 30 seconds. The current appeared immediately as voltage steps were applied, did not decay during a 100-ms pulse (Fig 2, C), and demonstrated inward rectification from membrane potentials positive to approximately +40 mV (Fig 2, A and B). Interestingly, this response to EBIO was seen in only 2/17 (11.8%) freshly isolated HLMCs compared with 28/42 (66.7%) LTHLMCs ($P < .001$) and 16/18 (88.9%) HPBDMCs ($P < .001$). In these responding HLMCs, LTHLMCs, and HPBDMCs, current at +40 mV increased from 18.1 ± 11.1 to 74.1 ± 58.7 pA, 9.4 ± 1.2 to 61.5 ± 11.1 pA, and 10.7 ± 1.6 to 98.0 ± 19.8 pA, respectively. This was accompanied by a negative shift in membrane potential from 0.0 ± 0.0 to -38.5 ± 16.5 mV (fresh HLMCs), -9.2 ± 2.5 to -42.6 ± 3.4 mV (LTHLMCs), and -13.3 ± 2.7 to -32.6 ± 4.1 mV (HPBDMCs), in keeping with the opening of a dominant K^+ conductance. This current was carried predominantly by K^+ ions, as demonstrated by a positive shift in reversal potential from -59.5 ± 7.0 to -4.2 ± 4.1 mV, and an increase in inward current at -120 mV from 48.1 ± 21.0 to 326.6 ± 75 pA, on switching

from 5 mmol/L external K^+ to 140 mmol/L external K^+ ($n = 3$). Under these conditions, the current was observed to exhibit weak inward rectification (Fig 3, A).

This K^+ current persisted for as long as 45 minutes of recording in the whole-cell configuration, with minimal rundown during this period. When recording cells that had been activated with 1-EBIO for as long as 30 minutes before analysis, the same current was present immediately on achieving the whole-cell configuration in 6/8 LTHLMCs (mean outward current at +40 mV, 65.0 ± 30.8 ; mean membrane potential, -49.9 ± 6.1 mV), indicating that this current did not develop as an artefact of patch clamp recording.

iK_{CA1} blockers clotrimazole and TRAM-34 block the 1-EBIO-induced current

In 6 cells expressing the K_{CA} current after addition of 1-EBIO, clotrimazole, a blocker of iK_{CA1} and inhibitor of cytochrome P450,¹³⁻¹⁵ produced an irreversible depolarization in 5 mmol/L external K^+ at a concentration of 1 $\mu\text{mol/L}$. Thus reversal potential shifted from -43.8 ± 6.0 to -1.0 ± 5.7 mV ($P = .001$; Fig 3, B). This was matched by a decrease in current from 41.6 ± 7.2 to 8.5 ± 3.3 pA at +40 mV ($P = .001$). TRAM-34, a derivative of clotrimazole and a highly specific blocker of iK_{CA1} , also produced dose-dependent block of the 1-EBIO-induced iK_{CA1} current, with a half-maximal inhibitory concentration of approximately 20 nmol/L, as reported previously¹⁶ (Fig 3, C).

Taken together, our data confirm that HLMCs and HPBDMCs express the intermediate conductance channel iK_{CA1} .

Cytosolic free Ca^{2+} concentration in HLMCs

Baseline $[\text{Ca}^{2+}]_i$ was significantly higher in LTHLMCs (224.5 ± 8.0 nmol/L; $n = 158$) than freshly isolated HLMCs (163.2 ± 11.2 nmol/L; $n = 51$; $P < .0001$). All freshly isolated HLMCs tested exhibited a rise in $[\text{Ca}^{2+}]_i$ in response to IgE-dependent activation using a concentration of anti-IgE (1:1000 dilution) optimal for histamine release (mean peak increase, 294 ± 19 nmol/L; $n = 47$ cells from 4 donors; $P < .0001$). All LTHLMCs also responded to a 1:1000 dilution of anti-IgE (mean peak increase, 390.5 ± 25.0 nmol/L; $n = 15$ cells from 3 donors; $P < .0001$), and 93% responded to a suboptimal concentration of anti-IgE (1:30,000 dilution; mean peak increase, 149.0 ± 7.4 nmol/L; $n = 107$; $P < .0001$). The rise in $[\text{Ca}^{2+}]_i$ in LTHLMCs with 1:1000 anti-IgE was significantly higher than with 1:30,000 anti-IgE ($P < .0001$) and was significantly higher than that in fresh HLMCs ($P = .004$). The Ca^{2+} response in fresh HLMCs and LTHLMCs was relatively smooth in that the wide oscillations that characterize basophil activation were absent (Fig 4).¹⁷

1-EBIO enhances IgE-dependent Ca^{2+} influx in cultured HLMCs

Having confirmed the expression of iK_{CA1} in these experiments, we next examined the direct effects of iK_{CA1}

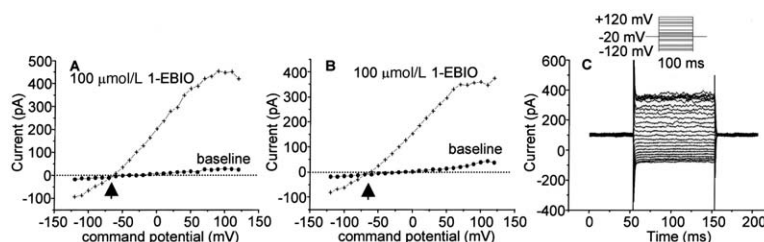


FIG 2. Representative whole-cell electrophysiological recordings of electrical currents in individual LTHLMCs and HPBDMCs. **A** and **B**, Current-voltage curves from HPBDMCs and LTHLMCs, respectively, at rest and 2 minutes after addition of 1-EBIO 100 $\mu\text{mol/L}$. Addition of 1-EBIO to the bath activates a current with a negative reversal potential (arrow). **C**, Raw current from the same cell as in **B** after 1-EBIO. The voltage protocol as described in the text is shown in the inset.

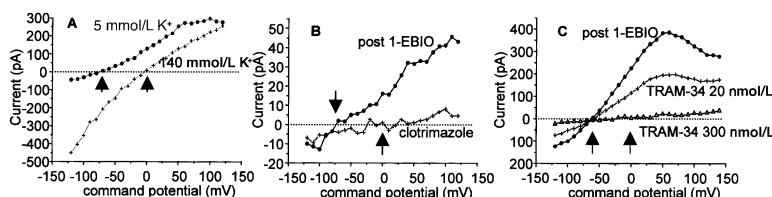


FIG 3. Representative whole-cell electrophysiological recording of electrical currents in individual LTHLMCs. **A**, A LTHLMC after 1-EBIO recorded in 5 mmol/L and 140 mmol/L external K^+ . Reversal potential shifts from -70 mV to 0 mV (arrows) on switching from 5 to 140 mmol/L external K^+ . **B**, Block of the 1-EBIO-induced current by clotrimazole 1 $\mu\text{mol/L}$. Note the positive shift in reversal potential (arrows). **C**, Block of the 1-EBIO-induced current by TRAM-34 at 20 and 300 $\mu\text{mol/L}$. Note the positive shift in reversal potential (arrows).

opening on $[\text{Ca}^{2+}]_i$ in fura-2-loaded HLMCs by using 1-EBIO as the iK_{CA1} opener. If the hypothesis were true, 1-EBIO should have increased $[\text{Ca}^{2+}]_i$ and subsequent histamine release as a result of iK_{CA1} opening, leading to membrane hyperpolarization. Because channel expression assessed by patch clamping appeared more frequent in LTHLMCs the effects of 1-EBIO on Ca^{2+} influx and histamine release were studied in these cells. The 3 Ca^{2+} values recorded before addition of 1-EBIO were averaged and compared with the 3 Ca^{2+} values recorded 2 minutes after addition of 1-EBIO.

Interestingly, addition of 1-EBIO 100 $\mu\text{mol/L}$ to resting LTHLMCs produced an acute and sustained increase in $[\text{Ca}^{2+}]_i$ within 90 seconds in 33/41 (80.1%) of cells tested (mean increase, 93.3 ± 9.7 nmol/L; $n = 41$ cells from 4 donors; $P < .0001$; Fig 4, **A** and **B**). This rise in $[\text{Ca}^{2+}]_i$ was attenuated by preincubation of the cells with charybdotoxin 100 nmol/L (mean increase, 36.0 ± 8.7 nmol/L; $n = 21$ cells from 2 donors; $P < .0001$), indicating that the rise in Ca^{2+} occurred indirectly through the opening of iK_{CA1} channels.

We then studied the effects of 1-EBIO after IgE-dependent activation. After LTHLMC cell activation using an optimal concentration of anti-IgE in terms of histamine release (1:1000 dilution), addition of 1-EBIO 100 $\mu\text{mol/L}$ produced no further increase in $[\text{Ca}^{2+}]_i$ (mean change, -27.7 ± 3.6 nmol/L; $n = 10$ cells from 3 donors; $P = .0002$; Fig 4, **C**). In contrast, after cell activation with a suboptimal concentration of anti-IgE (1:30,000 dilution), addition of 1-EBIO potentiated the Ca^{2+} response in

78/107 (72.9%) cells (mean increase, 65.1 ± 12.2 nmol/L; $P < .0001$; $n = 107$ cells from 6 donors; Fig 4, **D**, **E**, and **F**). The increase in $[\text{Ca}^{2+}]_i$ induced by 1-EBIO after 1:30,000 anti-IgE was significantly different from the change after 1:1000 anti-IgE ($P < .0001$).

1-EBIO enhances IgE-dependent histamine release in cultured HLMCs

In LTHLMCs, addition of 1-EBIO alone had absolutely no effect on background histamine release in spite of its ability to increase intracellular Ca^{2+} concentrations (Fig 5, **A**). When LTHLMCs were activated with an optimal concentration of anti-IgE for histamine release (1:1000 dilution), 1-EBIO had no effect on histamine release (Fig 5, **B**), consistent with the observation that it did not increase Ca^{2+} influx under these conditions. In contrast, when LTHLMCs were activated with a suboptimal concentration of anti-IgE (1:30,000 dilution), 1-EBIO produced a dose-dependent increase in histamine release from $6.1\% \pm 2.14\%$ of cell total at baseline to $10.9\% \pm 3.0\%$ at 1-EBIO 300 $\mu\text{mol/L}$ ($n = 6$; $P = .045$; Fig 5, **C**).

Correlation between IgE-dependent change in $[\text{Ca}^{2+}]_i$ and histamine release

The aim of this study was not to perform a detailed mathematical analysis of HLMC Ca^{2+} responses, so we did not have paired histamine release results and Ca^{2+} data for the batches of HLMCs studied. Nevertheless, when plotting the mean data for net IgE-dependent histamine release against change in $[\text{Ca}^{2+}]_i$ for LTHLMCs activated

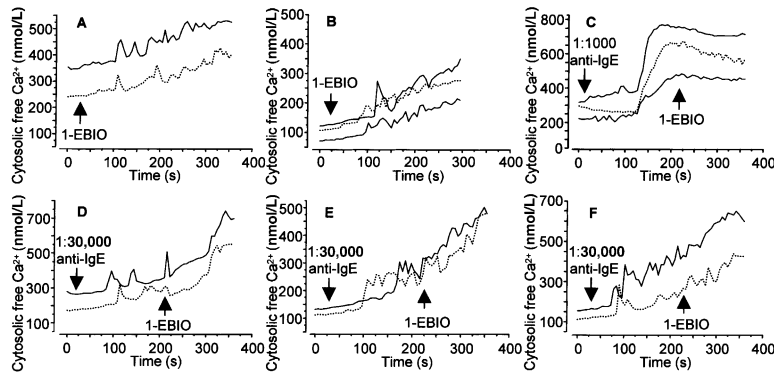


FIG 4. Representative changes in $[Ca^{2+}]_i$ in individual cells. **A** and **B**, LTHLMCs from 2 different donors during addition of 1-EBIO 100 $\mu\text{mol/L}$. **C**, Three LTHLMCs from different donors during addition of anti-IgE 1:1000 and then 1-EBIO 100 $\mu\text{mol/L}$. **D** to **F**, LTHLMCs from 3 different donors during addition of anti-IgE 1:30,000 and then 1-EBIO 100 $\mu\text{mol/L}$.

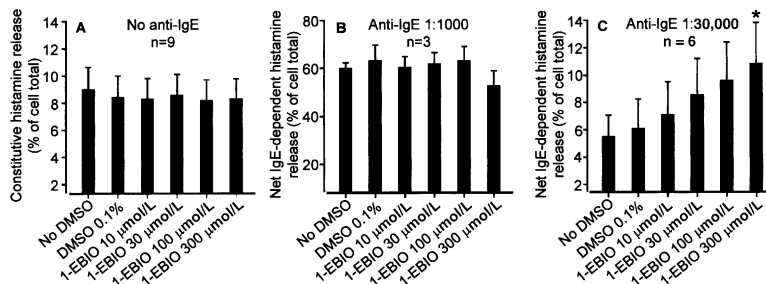


FIG 5. Histamine release expressed as a percentage of cell total from **(A)** LTHLMCs incubated with increasing concentrations of 1-EBIO alone, and **(B** and **C)** LTHLMC activated with 1:1000 (maximal) and 1:30,000 (submaximal) anti-IgE, respectively, in the presence of increasing concentrations of 1-EBIO.

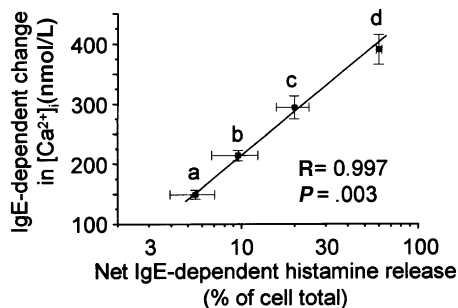


FIG 6. Plot of mean $\pm$ SEM IgE-dependent change in $[Ca^{2+}]_i$ versus net IgE-dependent histamine release in HLMCs. Point **a**, LTHLMCs 1:30,000 anti-IgE; **b**, LTHLMCs 1:30,000 anti-IgE + 1-EBIO 100 $\mu\text{mol/L}$; **c**, fresh HLMCs 1:1000 anti-IgE; and **d**, LTHLMCs 1:1000 anti-IgE.

with 1:30,000 anti-IgE, 1:30,000 anti-IgE + 1-EBIO 100 $\mu\text{mol/L}$, and 1:1000 anti-IgE, together with fresh HLMCs 1:1000 anti-IgE, there was a remarkably tight correlation (Fig 6). This finding suggests that for a given increase in $[Ca^{2+}]_i$, there is an exponential increase in histamine release, and it supports the interpretation that the increase in IgE-dependent histamine release induced by 1-EBIO is a consequence of its effect on $[Ca^{2+}]_i$.

DISCUSSION

We demonstrated previously that both HPBDMCs and freshly isolated HLMCs express a K_{CA} with electrophysiological features, suggesting it is iK_{CA1} .⁴ In the current study, we demonstrate that HLMCs express iK_{CA1} mRNA, and that the typical iK_{CA1} current develops after exposure of the cells to the iK_{CA1} opener 1-EBIO. Furthermore, this current is blocked by the specific iK_{CA1} blocker TRAM-34, providing further evidence that it is iK_{CA1} that is expressed in human mast cells.

We have made several novel and important observations regarding the role of iK_{CA1} in HLMC stimulus-secretion coupling. First, rather than using iK_{CA1} blockade to demonstrate an attenuated Ca^{2+} response as described by others previously,⁶⁻⁸ we have demonstrated directly that opening iK_{CA1} by using the iK_{CA1} opener 1-EBIO increases $[Ca^{2+}]_i$ after cell activation. This finding lends further support to the hypothesis that opening of K^+ channels in leukocytes enhances and maintains Ca^{2+} influx from the extracellular fluid. With specific reference to HLMCs, we have shown that this increase in $[Ca^{2+}]_i$ induced by 1-EBIO is accompanied by enhanced histamine release. This finding is consistent with our previous work demonstrating that

iK_{CA1} blockade with charybdotoxin attenuates IgE-dependent histamine release,⁴ so it is clear that one role for iK_{CA1} in HLMCs is to potentiate the effects of immunologic activation. This is entirely consistent with both the proposed hypothesis and the role described for iK_{CA1} in T cells, which is to enhance both Ca²⁺ influx and mediator release during immunologic responses.⁶⁻⁸

Blocking iK_{CA1} with charybdotoxin attenuates histamine release after maximal cell activation with anti-IgE, suggesting that iK_{CA1} contributes to the overall magnitude of the secretory response,⁴ but under the same conditions, 1-EBIO does not enhance the rise in [Ca²⁺]_i or secretion. However, we would not expect it to do so. This is because with the marked rise in [Ca²⁺]_i that occurs with maximal cell activation, most iK_{CA1} channels will be open, and an iK_{CA1} current as small as 25 pA at +40 mV gives a large negative shift in cell membrane potential (Fig 3, B). Thus opening of any further iK_{CA1} channels by 1-EBIO in cells already maximally activated is unlikely to have any effect on the already hyperpolarized cell membrane potential. In addition, it should be noted that this magnitude of acute cell activation is probably never achieved naturally *in vivo* except perhaps during anaphylaxis. Certainly, in chronic ongoing asthma, mast cell activation is far less aggressive, with piecemeal degranulation evident on electron microscopy rather than the anaphylactic degranulation that occurs with maximal cell activation,¹⁸ and significantly lower concentrations of mediators present in bronchoalveolar lavage fluid compared with those after allergen challenge.¹⁹

The submaximal activation protocol we have used, which releases much less histamine, is likely to be of more physiological relevance. Under these circumstances, the IgE-dependent rise in [Ca²⁺]_i is much smaller, and fewer iK_{CA1} channels are likely to be open, with the result that cell membrane potential will be relatively depolarized. It is under these conditions of submaximal activation that perhaps the most important implication of iK_{CA1} expression in HLMCs becomes apparent. Here we can demonstrate that 1-EBIO increases [Ca²⁺]_i and potentiates histamine release in the same concentration range that opens the iK_{CA1} channel during patch clamp recording.⁹ Thus, for net histamine release of approximately 6% with 1:30,000 anti-IgE, this was almost doubled to 11% in the presence of 1-EBIO. This is probably an underestimate of its effect, however, because approximately 70% of LTHLMC cells express the channel as determined by electrophysiology and their response to 1-EBIO in terms of [Ca²⁺]_i. The important point is that a molecule that opens iK_{CA1} but does not itself induce histamine release significantly potentiates the mast cell secretory response to a small immunologic stimulus. This raises the interesting possibility that other molecules that potentiate HLMC secretion but do not themselves induce secretion, such as adenosine, might operate through the enhanced opening of iK_{CA1}.

Expression of iK_{CA1} is clearly increased in HLMCs after culture with SCF, which not only is an essential growth factor for these cells but also initiates degranula-

tion and cytokine production.²⁰ iK_{CA1} currents were induced by 1-EBIO in approximately 12% of freshly isolated HLMCs in this study and in approximately 30% of freshly isolated cells after IgE-dependent activation in our previous study.⁴ Because there is heterogeneity of mast cells even within the same tissue, the presence of iK_{CA1} may be very important for the function of the normal, freshly isolated HLMCs that do express this channel. However, HLMCs from normal subjects are relatively quiescent compared with those from their asthmatic counterparts, which demonstrate increased constitutive and IgE-dependent mediator release.^{21,22} Interestingly, these same features become apparent in HLMCs once they have been in culture with SCF, IL-6, and IL-10, and because expression of these cytokines is increased in asthmatic airways,^{23,24} it could be argued that the long-term cultured cells used in our experiments are more representative of those seen in asthma. Certainly, mitogenic activation of T cells leads to increased iK_{CA1} expression and increases the number of iK_{CA1} channels in the plasma membrane from approximately 10 to 500 channels per cell.⁷ It will be interesting in the future to examine the effects of activation with anti-IgE and SCF on transcriptional regulation of iK_{CA1} in HLMCs, and whether this channel demonstrates increased expression in disease states such as asthma in which the cells are continuously activated.² However, at present, it is not possible to isolate mast cells from the bronchoalveolar lavage of human subjects in sufficient purity or quantity for molecular and electrophysiological studies.

Interestingly, 1-EBIO alone produced a rapid increase in baseline [Ca²⁺]_i, which may have arisen from increased Ca²⁺ conductance through Ca²⁺ or nonselective cation channels that are open at rest. This is plausible, because our cells are cultured in 100 ng/mL SCF, which itself activates the HLMCs for histamine release and which requires Ca²⁺ influx.²⁰ This effect of 1-EBIO on [Ca²⁺]_i was attenuated by the iK_{CA1} blocker charybdotoxin, indicating that it occurred as an indirect effect of K⁺ channel opening. It also seems likely that this effect of 1-EBIO alone on [Ca²⁺]_i is mediated through the same channels as those activated by anti-IgE, because 1-EBIO did not potentiate the rise in [Ca²⁺]_i after maximal activation of LTHLMCs. Although 1-EBIO alone increased [Ca²⁺]_i, it did not release histamine on its own, suggesting that activation of other signaling pathways is also necessary for degranulation. However, from the correlation of change in [Ca²⁺]_i and net histamine release shown in Fig 6, the 90-nmol/L increase in [Ca²⁺]_i induced by 1-EBIO alone would be expected to produce only as much as 3% net histamine release, and it is possible that there is a threshold change required for secretion to occur. Nevertheless, this correlation presented in Fig 6 does support the interpretation that the enhanced IgE-dependent Ca²⁺ influx and histamine release induced by 1-EBIO are causally related.

In summary, we have provided definitive evidence that HLMCs express the intermediate conductance K⁺ channel iK_{CA1}. We show that opening of this channel enhances

Ca²⁺ influx and histamine release in response to subtle immunologic challenge, whereas previous data indicate that it also contributes to the overall secretory response after maximal cell activation.⁴ Inhibition of iK_{CA1} activity may therefore prove to be a useful approach for the attenuation of mast cell hypersecretion associated with many diverse diseases.

REFERENCES

- Bradding P, Holgate ST. Immunopathology and human mast cell cytokines. *Crit Rev Oncol Haematol* 1999;31:119-33.
- Bradding P. The role of the mast cell in asthma: a reassessment. *Curr Opin Allergy Clin Immunol* 2003;3:45-50.
- Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast cell infiltration of airway smooth muscle in asthma. *N Engl J Med* 2002;346:1699-705.
- Duffy SM, Lawley WJ, Conley EC, Bradding P. Resting and activation-dependent ion channels in human mast cells. *J Immunol* 2001;167:4261-70.
- Hoth M, Penner R. Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* 1992;355:353-6.
- Fanger CM, Rauer H, Neben AL, Miller MJ, Rauer H, Wulff H, et al. Calcium-activated potassium channels sustain calcium signaling in T lymphocytes: selective blockers and manipulated channel expression levels. *J Biol Chem* 2001;276:12249-56.
- Ghanshani S, Wulff H, Miller MJ, Rohm H, Neben A, Gutman GA, et al. Up-regulation of the IKCa1 potassium channel during T-cell activation: molecular mechanism and functional consequences. *J Biol Chem* 2000;275:37137-49.
- Jensen BS, Odum N, Jorgensen NK, Christophersen P, Olesen SP. Inhibition of T cell proliferation by selective block of Ca(2+)-activated K(+) channels. *Proc Natl Acad Sci U S A* 1999;96:10917-21.
- Pedersen KA, Schroder RL, Skaaning-Jensen B, Strobaek D, Olesen SP, Christophersen P. Activation of the human intermediate-conductance Ca(2+)-activated K(+) channel by 1-ethyl-2-benzimidazolinone is strongly Ca(2+)-dependent. *Biochim Biophys Acta* 1999;1420:231-40.
- Sanmugalingam D, Wardlaw AJ, Bradding P. Adhesion of human lung mast cells to bronchial epithelium: evidence for a novel carbohydrate-mediated mechanism. *J Leukoc Biol* 2000;68:38-46.
- Duffy SM, Lawley WJ, Kaur D, Yang W, Bradding P. Inhibition of human mast cell proliferation and survival by tamoxifen in association with ion channel modulation. *J Allergy Clin Immunol* 2003;112:970-7.
- Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch* 1981;391:85-100.
- Ishii TM, Silvia C, Hirschberg B, Bond CT, Adelman JP, Maylie J. A human intermediate conductance calcium-activated potassium channel. *Proc Natl Acad Sci U S A* 1997;94:11651-6.
- Jensen BS, Strobaek D, Christophersen P, Jorgensen TD, Hansen C, Silahatoglu A, et al. Characterization of the cloned human intermediate-conductance Ca2+-activated K+ channel. *Am J Physiol* 1998;275:C848-56.
- Khanna R, Chang MC, Joiner WJ, Kaczmarek LK, Schlichter LC. hSK4/hIK1, a calmodulin-binding KCa channel in human T lymphocytes: roles in proliferation and volume regulation. *J Biol Chem* 1999;274:14838-49.
- Wulff H, Miller MJ, Hansel W, Grissmer S, Cahalan MD, Chandy KG. Design of a potent and selective inhibitor of the intermediate-conductance Ca2+-activated K+ channel, IKCa1: a potential immunosuppressant. *Proc Natl Acad Sci U S A* 2000;97:8151-6.
- MacGlashan DJ, Guo CB. Oscillations in free cytosolic calcium during IgE-mediated stimulation distinguish human basophils from human mast cells. *J Immunol* 1991;147:2259-69.
- Djukanovic R, Lai CK, Wilson JW, Britten KM, Wilson SJ, Roche WR, et al. Bronchial mucosal manifestations of atopy: a comparison of markers of inflammation between atopic asthmatics, atopic nonasthmatics and healthy controls. *Eur Respir J* 1992;5:538-44.
- Wenzel SE, Westcott JY, Larsen GL. Bronchoalveolar lavage fluid mediator levels 5 minutes after allergen challenge in atopic subjects with asthma: relationship to the development of late asthmatic responses. *J Allergy Clin Immunol* 1991;87:540-8.
- Frenz AM, Gibbs BF, Pearce FL. The effect of recombinant stem cell factor on human skin and lung mast cells and basophil leukocytes. *Inflamm Res* 1997;46:35-9.
- Flint KC, Leung KB, Hudspeth BN, Brostoff J, Pearce FL, Johnson NM. Bronchoalveolar mast cells in extrinsic asthma: a mechanism for the initiation of antigen specific bronchoconstriction. *BMJ* 1985;291:923-6.
- Casolaro V, Galeone D, Giacommo A, Sanduzzi A, Melillo G, Marone G. Human basophil/mast cell releasability, V: functional comparisons of cells obtained from peripheral blood, lung parenchyma, and bronchoalveolar lavage in asthmatics. *Am Rev Respir Dis* 1989;139:1375-82.
- Olsson N, Rak S, Nilsson G. Demonstration of mast cell chemotactic activity in bronchoalveolar lavage fluid collected from asthmatic patients before and during pollen season. *J Allergy Clin Immunol* 2000;105:455-61.
- Walker C, Bauer W, Braun RK, Menz G, Braun P, Schwarz F, et al. Activated T cells and cytokines in bronchoalveolar lavages from patients with various lung diseases associated with eosinophilia. *Am J Respir Crit Care Med* 1994;150:1038-48.