Kv1.3 potassium channels in human alveolar macrophages

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VOLTAGE-GATED K⁺ CHANNELS (Kv channels) are rapidly activating, potassium-selective channels expressed by both excitable and nonexcitable cells. All Kv channel subunits have six transmembrane domains (S1–S6), including a voltage sensor (S4) and a pore-forming region. Kv1.1 was the first mammalian Kv channel cloned and expressed (6, 38), and seven further subgroups (Kv1–6, Kv8, Kv9) were subsequently identified. Although Kv channel gene expression and function in excitable membranes have been well documented, less is known regarding the functional role of Kv channels expressed by cells of myeloid origin, particularly macrophages and platelets.

Kv1.3 is the Kv channel subtype found in cells of myeloid cell lineage including T and B lymphocytes, platelets, and megakaryocytes. In T cells, Kv1.3 is reported to be a primary regulator of cell activation, maintaining the resting membrane potential and counteringbalancing potassium influx necessary to maintain Ca²⁺ influx (3, 24). Blockade of Kv1.3 attenuates the production of proinflammatory cytokines and proliferation in vitro (18, 22, 33). Additionally, Kv1.3 channels are considered to be an important therapeutic target for autoimmune disease such as multiple sclerosis, where selective Kv1.3 channel block slows the progression in experimental models of the disease (9).

Less is known regarding the expression of Kv channels in monocytes and macrophages. Voltage-gated potassium currents resembling Kv1.3 channels have been recorded from human blood monocyte-derived macrophages, mammalian macrophages, microglia, and monocyte-derived cell lines (10, 12, 13, 19, 20, 26, 28, 29). In hippocampal microglia, toxins selective for Kv channels prevent proliferation in vitro (20). Additionally, macrophage Kv channel expression is highly variable according to the state of cell activation. Cytokines, Toll receptor activation, and phorbol esters all upregulate the expression of Kv channels (27, 36). Kv channel expression has been shown to be strongly upregulated in resident alveolar macrophages compared with blood monocyte-derived macrophages (29), but little more is known regarding the functional role of the Kv channels in macrophages.

In this study we sought to identify the molecular identity and functional role of the Kv channel subtype expressed in primary macrophages of the human lung. Human alveolar macrophages reside within both the alveolar spaces and the lung parenchyma. They play a major role in the host defense system of the respiratory tract, mediating the release of proinflammatory (IL-1 and TNF-α) and anti-inflammatory cytokines (IL-10) in response to invading pathogens. Kv channel expression has previously been documented by Nelson et al. (29), demonstrating high expression of an outwardly rectifying K⁺-selective conductance compared with blood monocyte-derived macrophages. In this study we used the whole cell patch clamp recording technique to pharmacologically characterize Kv currents and RT-PCR to examine expression of Kv channel mRNA. Furthermore, we investigated the possible functional consequences of channel activation using measurements of membrane potential, proinflammatory cytokine release, and a phagocytosis assay. Preliminary results have appeared in abstract form (25).

MATERIALS AND METHODS

Materials. Zymosan A (Saccharomyces cerevisiae) bioparticles labeled with BODIPY FL (excitation 505 nm/emission 513 nm) and the corresponding opsonizing agent (rabbit

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anti-zymosan IgG) were obtained from Molecular Probes (Eugene, OR). Goat F(ab')2 anti-rabbit-IgG R-phycocerythrin conjugate was from Caltag Laboratories (Burlingame, CA). Margatoxin (Sigma-Aldrich, Gillingham, Dorset, UK) was prepared as a stock solution of 1 mM in BSA containing Tris-buffered saline and diluted from 1:1,000 to 1:10,000 in normal physiological saline. CP-339,818 (Tocris, Bristol, UK) was reconstituted in normal physiological saline. Cell culture reagents were from Gibco (Invitrogen, Paisley, UK), and all other compounds were from Sigma-Aldrich.

Isolation of pure macrophage population. Macrophocibly normal lung tissue derived from lung resections from patients undergoing surgery for carcinoma were used in this study. All patients provided informed consent. Lung tissue was stripped of pleura and chopped finely with scissors in sterile phosphate-buffered saline (PBS). The chopped tissue was washed with PBS over 100-μm-diameter-pore nylon mesh, and the lung macrophages were collected in the wash. The macrophage-rich suspension was spun (1,200 rpm for 5 min), and the pellet was resuspended in penicillin (1,000 U/ml) and streptomycin (100 μg/ml) containing DMEM-PS. Macrophages were distinguished from myofibroblasts on the basis of adherence to tissue culture plastic, and resulting adherent cells were washed with sterile PBS to remove nonadherent cells. The remaining adherent cells (macrophages and myofibroblasts) were stored in DMEM-PS. Adherent cells were removed by scraping. For single cell studies the cells were pelleted (1,200 rpm for 4 min), resuspended in serum-free DMEM, and plated directly onto glass coverslips.

For extraction of total RNA, a pure macrophage population was isolated from contaminating cells (primarily myofibroblasts) by fluorescence-activated cell sorting (FACS). Cells that adhered to tissue culture flasks were incubated in serum-free DMEM for 2 h at 37°C. We prepared zymosan-BODIPY conjugate by incubating equal amounts of zymosan-BODIPY conjugate with opsonizing reagent for 1 h at 37°C. The zymosan-BODIPY conjugate with opsonizing reagent was then pelleted by centrifugation (1,000 g, 5 min). The pellet was washed three times in cold PBS and resuspended in 300 μl of cold PBS. One hundred milliliters of this suspension were added to 15 ml of cold serum-free DMEM and allowed to adhere to cells at 4°C for 30 min, before being incubated for 1 h at 37°C to allow phagocytosis to take place. Cells were then washed in cold PBS and treated with 1:1,000 diluted goat F(ab')2 anti-rabbit IgG (heavy and light chain) R-phycocerythrin conjugate for 30 min at 4°C. A fluorescence-activated cell sorter (Beckman Coulter, High Wycombe, Buckinghamshire, UK) was then used to separate those cells with the green fluorescence of the ingested BODIPY-zymosan complex from cells with the red fluorescence of surface-aderent IgG.

RT-PCR. Total RNA from FACS-sorted macrophages was extracted with TRIzol (Life Technologies, Invitrogen). The RNA samples were treated with DNase I (Ambion, Huntingdon, UK) to remove any chromosomal DNA contamination. cDNA was made from the total RNA with Superscript II RT kit (Life Technologies) with oligo(dT) primers as recommended. As a control, duplicate RNA samples were incubated under identical conditions but without RT. PCR was conducted using pre aliquoted PCR master mix (ABgene; Epsom, Surrey, UK) in a thermocycler (Hybaid, Ashford, Middlesex, UK). The PCR cycle conditions were 94°C for 2 min, then 35 cycles of 94°C/30 s, 55°C/30 s, and 72°C/60 s, followed by one 72°C/5-min step. PCR products were separated by agarose gel (1%) electrophoresis. cDNA prepared from human brain RNA (Invitrogen) was used for positive controls to test primers.

Electrophysiological recording. Standard whole cell patch clamp recordings were made 12–72 h following plating of macrophages onto glass coverslips with a HEKA EPC10 amplifier and on-line compensation for series resistance and capacitance. All currents were analyzed using Pulsefit (HEKA Electronik). Patch pipettes (filled resistance 5–10 MΩ) were pulled from borosilicate glass and filled with solution containing (in mM): 150 KCl, 10 HEPES, 10 glucose, 2 CaCl2, and 1 MgCl2 brought to pH 7.3 with KOH. Normal external saline was (in mM): 145 NaCl, 5 KCl, 10 HEPES, 12 glucose, 2 CaCl2, and 1 MgCl2 buffered to pH 7.2 with KOH. Normal external saline was replaced with increasing concentrations of NaCl. The osmolarities of all solutions ranged from 300 to 310 mOsmol/l. All recordings were performed at ambient room temperature (20–22°C).

ELISA assay for secretion of IL-1β. Adherent alveolar macrophages were plated into a 24-well plate and treated for 4 h with endotoxin lipopolysaccharide (LPS, 10 μg/ml). Cells were washed twice with extracellular saline; then control solution (2°C (4-benzoyl)ATP (BzATP, 200 μM), or BzATP plus margatoxin (1 nM)) was applied for 30 min. The solution was then collected and directly assayed for the presence of IL-1β. Sandwich ELISA plates were generated using antibodies obtained from Endogen (Loughborough, UK).

RESULTS

Expression of Kv channel mRNA. To extract total RNA from lung macrophages it was necessary to isolate a population of macrophages uncontaminated by blood cells, fibroblasts, and cell debris. We initially screened a range of antibodies targeted against CD antigens (CD11c, CD45, CD14) and tested for expression using flow cytometry. This approach was unsuccessful due to highly variable expression of CD antigens between lung samples. We therefore designed the FACS-based double-labeling protocol described in MATERIALS AND METHODS to extract macrophages from other cells on the basis of specific Fc receptor-mediated phagocytic potential. From 37 lung tissue samples, 23.8 ± 1.6% of the adherent cell population was sorted as functional macrophages. Table 1 lists the primers used for the 14 Kv channels tested; Fig. 1 illustrates the result that the only detectable transcript was Kv1.3. All the other primer sets amplified products of the expected size from human brain mRNA.

Whole cell recordings. Mononuclear lung macrophages were distinguished from myofibroblasts on the basis of their size (15–25 μm diameter), ruffled plasma membrane, and abundant phagocytic granules easily visualized by engulfed environmental carbon particles that were often apparent. Internal carbon particles suggest that the macrophages are of alveolar origin rather than from the interstitial population. Depolarization from −100 to −40 mV or greater activated an outward current (Fig. 2) in >95% cells. The rate of activation increased steeply with depolarization, and at potentials greater than −20 mV the current began to inactivate within several tens of milliseconds (Fig. 2A). Cell capacitance was 32.6 ± 2.3 pF (n = 23), and the density of the outward current recorded at 0 mV varied from 1.4 to 65 pA/pF (mean 15.2 ± 3.4 pA/pF,

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Table 1. Primers used for RT-PCR

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<th>Channel</th>
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<td>5' CGATCGACCAACACACCAAGGT 3'</td>
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<td></td>
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<td></td>
<td>5' GGAATATGGTTGGAAGCTCA 3'</td>
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<td>Kv1.3</td>
<td>XM052282</td>
<td>5' GAGCTGCCACGCTGTCCTCTC 3'</td>
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<td>5' GCACCTCTGACCAATATCTGACG 3'</td>
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<td>XM066330</td>
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<tr>
<td></td>
<td></td>
<td>5' CAGAGAATCTTGGCGTTCAGGAG 3'</td>
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Kv, voltage-gated K⁺.

of the outward current (at 20 mV); with 1 μM, 31 ± 9% (n = 5) of the initial current remained. The voltage range over which outward currents begin to activate in macrophages (~40 to ~30 mV, Fig. 2A) is close to the resting potential (~44 mV, Ref. 27). We therefore asked whether Kv1.3 contributed to the resting potential of the cells. The zero-current (resting) potential in our experiments was −34 ± 1.1 mV (n = 7). Margatoxin induced a slow depolarization of the membrane potential to −4.6 ± 1.1 mV (n = 5, Fig. 3C).

**Phagocytosis assay.** We used margatoxin to test the effect of blocking Kv1.3 channel activity on Fc receptor-mediated phagocytosis. Phagocytic cells were detected using fluorescently conjugated IgG-coated zymosan particles. The percentage of macrophages in the total cell population was counted by flow cytometry. In control conditions, 37 ± 7.6% (n = 3) of cells were sorted as having ingested zymosan particles. Margatoxin (1 nM) added at the same time had no effect on this proportion (35 ± 8.3%, n = 3).

**IL-1β release.** Release of proinflammatory IL-1β was assayed by ELISA following preincubation with LPS for 12 h. Stimulation of P2X7 receptors with BzATP (200 μM for 30 min) evoked a release of IL-1β above background (control 59 ± 21 pg/ml, n = 3; BzATP 1,351 ± 39 pg/ml, n = 3; P < 0.0001; Fig. 3D). Margatoxin had no effect on the secretion of IL-1β elicited by stimulation of P2X7 receptors (1,247 ± 56 pg/ml, n = 3; P > 0.1; Fig. 3D) compared with control in the absence of toxin. Stimulation of P2X7 receptors in the presence of high extracellular potassium (150 mM) also had no effect on secretion of IL-1β.
DISCUSSION

In this study we have identified the Kv channel subtype expressed by human alveolar macrophages. From the biophysical and pharmacological properties of currents described in this study, several Kv channels are unlikely candidates (7) and Kv1.3 appears to account for these currents. The outward potassium-selective currents lack the very rapid (N-type, time constant \( \tau = 50 \) ms) inactivation of Kv1.4 and Kv4 channels, whereas Kv1.1, 1.2, 1.5, and 1.6 display no or little inactivation. The pronounced inactivation (time constant 100–400 ms) is characteristic of cloned and native Kv1.3 channels of T lymphocytes. These currents are also comparable with those previously reported for monocyte-derived cell lines, mammalian macrophages, and microglia from 6 to 10 days in culture (10, 12, 13, 19, 20, 26, 28, 29). Kv currents have most extensively been characterized in microglia, the macrophage-like cells, of the central nervous system. Margatoxin and agiotoxin-2 blocked Kv currents on days 6–10 in culture, suggesting the expression of Kv1.3 channels (20). In our study, margatoxin blocked outward \( K^+ \) currents with a potency (IC\(_{50} = 162 \) pM) comparable with that reported to block cloned and native Kv1.3 channels. Higher margatoxin concentrations (IC\(_{50} > 1 \) nM) also block Kv1.2, 1.6, and 1.7 channels (7, 14); however, the current in human alveolar macrophages differs in biophysical characteristics from those channels. Thus toxin sensitivity and inactivation kinetics are most consistent with the expression of Kv1.3 channels in human alveolar macrophages.

High expression of Kv1.3 channel was confirmed by RT-PCR from total macrophage RNA. To date, Kv1.3 channel mRNA expression has only been reported for phorbol ester-differentiated THP-1 cells (10) and culture rat microglia (20). A novel FACS-based double-labeling sort was designed to isolate a pure population of macrophages and total RNA extracted. An array of Kv channel primers were designed against 14 channel subtypes; however, only the primers for Kv1.3 yielded a product to the correct size confirming the expression of Kv1.3 channels and no other Kv channel subtype.

Of all the myeloid cells, Kv channels have been most comprehensively investigated in T lymphocytes. Kv channels are thought to be responsible for setting resting membrane potential and providing the driving...
force for calcium influx and subsequent cell activation. Indeed, blocking Kv channels in vivo proves to be anti-inflammatory and ameliorates the progression of experimental encephalitis, a chronic inflammatory autoimmune disease (2). In current clamp recordings, we found a resting membrane potential of $\approx -34$ mV, which is comparable with previous whole cell recordings from human alveolar macrophage ($-44$ mV: Ref. 28) and potential-sensitive dye measurements from rat alveolar macrophages ($-37$ mV: Ref. 5). At these potentials, Nelson et al. (29) report that steady-state inactivation was incomplete ($\approx 30\%$), suggesting that the Kv channel would respond to changes in membrane potential and contribute to the resting membrane potential of lung macrophages; this is consistent with our findings. On the other hand, macrophages have a prominent inward rectifier potassium conductance (13), and this may be the major contributor in conditions with less leakage conductance.

Increases in cytosolic calcium and sodium are concomitant with Fc receptor cross-linking and phagocytosis (11, 16, 25). Phagocytosis is the initiating phase of host defense and inflammation involving activation of phagocytic receptors, Fc, or complement, triggering a cascade of tyrosine phosphorylation, polymerization of F-actin, and contractile forces leading to internalization of particles. FcγR1 cross-linking in U-937 cells leads to opening of a calcium-activated nonselective cation channel (TRPM4: Refs. 11, 21). Additionally, FcγR-mediated phagocytosis triggers superoxide generation and leads to activation of a nonselective cation channel and membrane depolarization in peripheral mononuclear cells (16) and peritoneal macrophages (4). Recently, a current has been described in U-937 cells activated by intracellular β-NAD$^+$-free acid and extracellular hydrogen peroxide, which may account for this current (15). The activation properties of this current correspond to those reported for TRPM2 channels (34).

In this study we demonstrate that, although Kv channels contribute to the resting membrane potential and thus the driving force for cation entry, margatoxin had no obvious effect on Fc receptor-mediated phagocytosis. This suggests that activation of the nonselective cation current is a consequence of phagocytosis and Kv channel activity is not necessary to support phagocytosis. However, this FAC-s-based assay is limited in its ability to resolve total number of bioparticles per macrophage and may not detect more subtle modulation of phagocytosis.

Finally, the level of cytosolic potassium is proposed to play a critical role in the activation of caspase 1 and the release of 17-kDa IL-1β. High extracellular K$^+$

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**Fig. 3.** Margatoxin blocks outward current and depolarizes resting potential in human alveolar macrophages. A: currents evoked by depolarization from $-100$ mV to $+40$ mV (10-mV increments) before and after applying margatoxin. B: concentration-response curve for margatoxin inhibition. Values are mean for 5 cells; SE is less than symbol size. C: margatoxin depolarizes macrophage. Resting potential of cell was about $-35$ mV; application of margatoxin for 1 min brings this to close to 0 mV. D: P2X7 receptor evoked cytokine release assayed by ELISA. BzATP, 2’3’-benzoyl-(4-benzoyl)-ATP.
blocks the processing and release of 17-kDa IL-1β from microglia (32), whereas a potassium/proton ionophore (nigericin) promotes the release of 17-kDa IL-1β from mouse peritoneal macrophages and human monocytes (31, 32, 39). Knockout studies have demonstrated a central role of P2X7 receptors in the generation and release of 17-kDa IL-1β (37). Activation of P2X7 receptors, a nonselective cation channel, would lead to membrane depolarization, activation of Kv1.3 channels, and K+ efflux. Thus we investigated the consequence of Kv1.3 channel blockade on release of IL-1β. A maximal concentration of margatoxin was found to have no effect on the release of IL-1β detected by ELISA. It is clear from our studies that depolarization-evoked activation of Kv1.3 channels is not necessary for release of proinflammatory IL-1β. Interestingly, high extracellular K+ also has no effect on cytokine release, demonstrating no requirement for K+ efflux in the release mechanism.

The present experiments indicate that although Kv1.3 sets the membrane potential of human alveolar macrophages, it seems not to play a critical role either in their phagocytic or cytokine-secreting activities. Data from this investigation demonstrate that the strong expression of Kv1.3 channels in human macrophages and channel activity may be involved in other macrophage-specific activities, for example, superoxide generation or nitric oxide production. Interestingly, Kv1.3 channels have been shown to form complexes with β1-integrins and function in triggering T cell activation (23). Activation of the integrin proteins is rapid and plays a central role in enabling attachment of T cells or monocytes to the corresponding ligand on cell surfaces and extracellular matrices. Integrin receptor activation has been shown to modulate Kv channel activity in monocytes/macrophages (8, 9). It would be interesting to test the potential role of Kv channel in adhesion and chemotaxis of human alveolar macrophages.

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DISCLOSURES

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