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Human Macrophages Contain a Stretch-sensitive Potassium Channel that is Activated by Adherence and Cytokines

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Abstract. A variety of stimuli, including cytokines and adhesion to surfaces and matrix proteins, can regulate macrophage function, in part through changes in Ca^{2+}-dependent second messengers. While fluctuation in intracellular Ca^{2+} is an important modulator of cellular activation, little attention has been paid to the roles of other ions whose cytoplasmic concentrations can be rapidly regulated by ion channels. To examine the role of ion channels in macrophage function, we undertook patch clamp studies of human culture-derived macrophages grown under serum-free conditions. The major ionic current in these cells was carried by an outwardly rectifying K^+ channel, which had a single-channel conductance of 229 pS in symmetrical K^+-rich solution and macroscopic whole-cell conductance of 9.8 nS. These channels opened infrequently in resting cells but were activated immediately by (i) adhesion of mobile cells onto a substrate, (ii) stretch applied to isolated membrane patches in Ca^{2+}-free buffers, (iii) intracellular Ca^{2+} (EC_{50} of 0.4 μM), and (iv) the cytokine IL-2. Furthermore, barium and 4-aminopyridine, blockers of this channel, altered the organization and structure of the cytoskeletal proteins actin, tubulin and vimentin. These cytoskeletal changes were associated with reversible alteration to the morphology of the cells. Thus, we have identified an outwardly rectifying K^+ channel that appeared to be involved in cytokine and adherence-mediated macrophage activation, and in the maintenance of cytoskeletal integrity and cell shape.

Key words: Potassium channel — Patch clamp — Cytoskeleton — Cytokine — Mechanosensitivity

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Introduction

Macrophages are migratory phagocytic cells which have an important role in immune and inflammatory responses, largely through their capacity to secrete bioactive molecules. This function is regulated by a variety of external stimuli, including the matrix to which they adhere and the binding of cytokines to cell surface receptors. Some of the responses to these external stimuli are mediated through changes in calcium-dependent second messenger pathways, which may contain phosphorylated nucleotides or proteins (Gordon, Kerchav & Chung, 1988). Relatively little attention has been paid to the roles of the other major intracellular cations in these cells, however ions such as potassium and sodium are capable of modifying cytoskeletal proteins or metabolic pathways through direct protein interaction (Southwick, Tatsumi & Stossel, 1982; Toney, Hohenester, Cowan & Jansonius, 1993; Miller, 1993).

Macrophage activation involves an alteration in cell morphology, a rearrangement of the cytoskeleton, a release of cytokines and secretory products, an upregulation of cell surface antigens, and an increase in cell functions including pinocytosis, chemotaxis, respiratory burst, microbicidal and tumoricidal activities. Three types of ion channels have been identified in the plasma membrane of human monocytoid cells (Gallin, 1991), namely four different potassium channels, a nonselective cation channel, and three different chloride channels. In macrophages, the type of channel expressed varies with the culture conditions and age of the cells (Gallin & McKinney, 1988), chemotaxis (Gallin & Gallin, 1977), or Fc receptor activation or phagocytosis (Young, Unkeless & Cohn, 1985), and some authors have suggested that the expression of particular ion channels may be markers of cell activation (Nelson, Jow & Jow, 1992). There are at present, however, no reports of ion channel

opening directly and immediately associated with macrophage activation, and indeed Kanno & Takishima, 1990 reported that the frequency of ion channel opening did not change with macrophage activation induced by interferon gamma, interferon alpha or phorbol ester (PMA).

The precursor of the macrophage, the monocyte, first adheres to vascular endothelium then migrates into the extravascular space by forcing its way through the narrow junction between endothelial cells and then into the interstitial spaces. We hypothesized therefore that tension developed in the plasma membrane during this process may activate mechanosensitive ion channel activity, as has been previously suggested in other cells (McKinney & Gallin, 1990), and that this process might, in turn, provide an activation signal for the macrophage. Further, we hypothesized that these channels may also participate in cell activation mediated by receptor-ligand interaction (e.g., by cytokines).

We demonstrate here that the major ion current in human culture-derived macrophages is carried by an outwardly rectifying K^+ channel which is mechanosensitive (MS). This channel is activated by adhesion of mobile cells or by tension applied as suction to the cell membrane. It is also opened by the cytokine interleukin-2 (IL-2). Furthermore, $BaCl_2$ and 4-aminopyridine, which block this channel, both cause marked alterations in the major structural proteins of these cells consistent with a central role for this MS channel in the process of macrophage activation.

Materials and Methods

Preparation of Macrophages

Monocytes were isolated and cultured in a modified serum-free medium according to a published method (Bennett et al., 1992). Briefly, buffy-coat leukocyte concentrates from normal donors were diluted 1:2 with phosphate-buffered saline (PBS) containing 4 U/ml preservative-free heparin (Fisons Pty Ltd, Australia) and centrifuged over Ficoll-Paque (Pharmacia, Australia) to isolate peripheral blood mononuclear cells (PBMC). PBMC were washed three times in PBS and resuspended in serum-free medium which consisted of Iscove's modification of Eagle's medium (Gibco BRL, Melbourne, Australia) supplemented with L-glutamine (4 mM, CSL, Victoria, Australia), bovine serum albumin (BSA) (2 mg/ml. RIA grade, Fraction V, Sigma, #7888), sodium selenite (5 ng/ml, Sigma, #S1382), soy bean lecithin (0.1 mg/ml, ICN Biomedicals, #102147), insulin (5 mg/ml, Boehringer-Mannheim, Germany) and transferrin (5 mg/ml, Boehringer-Mannheim, Germany). There was no detectable level of endotoxin present in any culture media, as assessed by the Limulus amoebocyte lysate test (Woods Hole, MA).

The mononuclear cells were seeded at a density of approximately 4.5×10^5 cells/cm^2 in untreated plastic petri dishes (35 mm to 90 mm, Bunzel, Australia) in a final volume of 2 ml/10 cm^2. They were allowed to adhere to these dishes for 90 min at 37°C in 5% CO_2 and the nonadherent cells (mainly lymphocytes) were removed by two vigorous washes with PBS. The following day the adherent cells were washed twice with PBS, resulting in macrophage cultures of greater than 95% purity as determined by nonspecific esterase staining. Cultures were maintained in a serum-free medium with 99% cell viability, assessed by Trypan Blue exclusion, until used at day seven. Cells grown in this way replicate and produce healthy nonadherent macrophages (Bennett et al., 1992), which were harvested for experiments by collecting small (0.2 ml) aliquots of the culture supernatant. For acute electrophysiological experiments which required cells maintained in the nonadherent state, the aliquot was placed in a 35 mm plastic tissue culture dish that had been coated with a thin anti-adherent layer of 0.8% agarose (Sigma, #A6306) (Bennett et al., 1992).

Electrophysiological Recording

In some experiments, cells adherent to the culture dishes were used to record single ion channel activity in the inside-out and cell-attached configurations (Hamill et al., 1981). In other experiments, the whole-cell and cell-attached configurations were used with nonadherent macrophages maintained as indicated above. Single-channel and whole-cell currents were recorded at room temperature (23°C).

In all experiments, the mechanical pressure on the cell during seal formation was minimized by only applying gentle suction (<10 mm Hg) for a brief period, and then allowing the "giga-seal" to form over a period of 5–10 min with no further suction. This is important in studying MS channels since their properties may be artifactually altered by excessive membrane-cytoskeleton disruption (McBride & Hamill, 1993; Small & Morris, 1994). Patch pipettes with a tip-opening between 0.9 and 1.5 μm (Martin & Cook, 1990) were fabricated from thin-walled borosilicate glass (Vitrex Microhematocrit Tubes, Modulohm I/S, Denmark). The impedance of these pipettes, with the standard bath and pipette solutions as described, was 3–7 MΩ. The channel currents were amplified and filtered at 1 kHz (−3 dB point) using an Axopatch 1B amplified (Axon Instruments) and sampled on-line by a microcomputer (IBM 486 compatible) using commercial software and associated A/D hardware (pClamp 5.5.1/Labmaster TL-1-125, Axon Instruments and Scientific Solutions). The single-channel open probability and kinetics were determined according to published methods (Martin et al., 1994). Briefly, open probability was calculated from the areas of Gaussian curves fitted to amplitude histograms compiled from 2-min channel records. Channel kinetics were determined from distributions of dwell times, compiled from recordings containing only one channel, using the 50% amplitude threshold criterion. The input impedance of the cells was estimated by the least-squares fit of currents recorded between −50 and +30 mV where all the K^+ was replaced by Cs^+ in the pipette. The cell capacitance was measured by integrating the capacitative current trace recorded at a potential of −10 mV.

The solution used for superfusion of the cells contained (mM): NaCl (130), KCl (4.8), $MgCl_2$ (1.2), NaH_2PO_4 (1.2), HEPES (N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) (10), glucose (12.5), $CaCl_2$ (1.0), and Bovine Albumin (0.5 mg/ml, fraction V, Sigma, #A7888) (pH = 7.4, with NaOH). The various channel blocking drugs, including tetraethylammonium$^+$ (TEA), 4-aminopyridine (4-AP), $BaCl_2$, N-phenylanthranilic acid (diphenylamine-2-carboxylic acid, DPC), verapamil, or nifedipine were added to this solution. The pipette solution contained (in mM): KCl (140), $MgCl_2$ (1.2), EGTA (5), and HEPES (10) (pH = 7.2, with KOH). A Ca^{2+}-EGTA buffering system (Findlay, Dunne & Petersen, 1985) was used to control free Ca^{2+}. Thus, to maintain a free Ca^{2+} concentration of nominally $<10^{-9}$ M we employed Ca^{2+}-free solutions and added EGTA (5 mM). For a nominal Ca^{2+} concentration of 1.5×10^{-7} M we added 0.97 μM Ca^{2+} and 1.92 μM EGTA. For a nominal Ca^{2+} concentration of 10^{-6} M we added 1.54 μM Ca^{2+} and 1.73 μM EGTA. Cytokines were dissolved in Hanks Balanced Salt Solution and stored as stock solutions at −20°C until

used. They were added directly to the extracellular superfusion solution to achieve a final concentration of 50 U/ml (interleukin-2, IL-2), 50 U/ml (interleukin-6, IL-6), and 10 ng/ml (transforming growth factor-β, TGF-β). In some experiments, cells were pretreated for 18 hr with interferon-γ (IFN-γ) at a concentration of 1000 U/ml of culture medium.

Ion currents are referred to the trans-patch potential (V_m). For cell-attached patches, this was determined from the pipette potential (V_p), cell resting potential (E_m) and the liquid junction potential (E_L) between the bath and pipette solutions. E_L was calculated using commercial software (Barry, 1994), and was typically around 4 mV. Thus

$$V_m = (E_m - V_p) + E_L \quad (1)$$

For inside-out patches, the V_m was only determined by V_p and E_L, with $E_m = 0$ in equation (1). This was also the case for whole-cell recordings except that the sign of V_p and E_L altered, with

$$V_m = V_p - E_L \quad (2)$$

Effect of Channel Blockers on Cytoskeletal Structure and Morphology

The morphology of viable cells was also monitored by phase-contrast microscopy. In these experiments, nonadherent macrophages were allowed to adhere in the presence or absence of either $BaCl_2$ (5 mM), 4-aminopyridine (1 mM), or verapamil (1 mM). The culture dishes were maintained in a humidified incubator at 37°C with 5% CO_2 and observed and photographed periodically for up to 24 hr. At the end of this period, the medium containing the drugs was replaced by fresh drug-free medium and the cells were observed for a further 24 hr.

Nonadherent macrophages were randomly assigned to no treatment or treatment with $BaCl_2$ (5 mM), 4-aminopyridine (1 mM), or verapamil (1 mM). After 24 hr cells were fixed for 30 min with 3.5% formaldehyde in phosphate-buffered saline (PBS) and 0.1% Triton X-100. Cytoplasmic actin was analyzed by incubating the cells for 30 min at 4°C with a 1:100 dilution in PBS of FITC labeled phalloidin (Sigma, #P5282). Cytoplasmic tubulin and vimentin were detected by incubating the cells for 30 min at 4°C in the dark with monoclonal antibodies to alpha tubulin (1:400 in PBS) or vimentin (1:50 in PBS) (Amersham, Australia), followed by two PBS washes. These antibodies were visualized by incubating the slides for 30 min with FITC conjugated rabbit anti-mouse Ig antibody (Silenus, Australia) at 1:40 dilution in PBS. Slides were washed twice in PBS and mounted with 1 mg/mL para-phenylenediamine (Sigma, #P6001) in 90% glycerol/PBS.

The structure and distribution of actin, tubulin and vimentin in the cells were visualized by confocal laser scanning microscopy (Sarastro 2000, Molecular Dynamics, Sunnyvale, CA), with a plan apochromat 60×/1.40 NA oil immersion lens and an argon-ion class II laser. Optical sections (usually at 0.3 μm intervals) through FITC-labeled cells were captured using a 50 μm fixed pinhole, with excitation at 488 nm, a 510 nm beam splitter and a 510 nm barrier filter. Image processing was performed using a Silicon Graphics Personal Iris 4D 35 workstation. The effect of the channel blockers on the morphology of the cells was assessed, using the cells stained for tubulin, by measuring the cell volume and the "footprint" area. The cell volume was determined by integrating the area of successive optical sections taken through the cell, starting at its base, using z-sectioning. The "footprint" area was measured from the optical section closest to the surface of the culture dish.

Unless otherwise stated, all data are reported as mean ± SEM with the number of separate cells/patches indicated in parentheses.

Results

The resting cell potential (E_m) in the cells studied was −24.1 ± 3.1 mV in 27 nonadherent and −40.7 ± 5.2 mV in 14 adherent cells. This difference was statistically significant ($P = 0.01$). The input impedance was 2.1 ± 0.9 GΩ (n = 4) for the nonadherent cells, and the cell capacitance was 126 ± 18 pF (n = 5).

Ion Channels in Resting Macrophages (Nonadherent Cells)

All 27 nonadherent macrophages studied showed strongly outward rectifying whole-cell currents which activated at trans-patch potentials (V_m) positive to 10 mV (Fig. 1*a*). The chord conductance of this current was 10.6 ± 1.7 nS (V_m range + 70 mV to + 120 mV). Twenty of the 27 cells also showed a small inward current at V_m negative to −90 mV (Fig. 1*b*), which had a chord conductance of 0.7 ± 0.2 nS (V_m range −80 mV to −150 mV). This latter channel will not be discussed further in this report.

Substitution of Cs^+ for K^+ in the pipette solution reduced the outward conductance by 92%, resulting in a residual current of 0.8 ± 0.1 nS (n = 4), and suggesting that this outwardly rectifying current is carried mainly by K^+ (hence K_{OR}). This K_{OR} current was blocked by 4-aminopyridine (4-AP, 1 mM) and partially inhibited by $BaCl_2$ (5 mM). K_{OR} was not inhibited by tetraethylammonium$^+$ (TEA, up to 20 mM), verapamil (1 mM) or nifedipine (50 μM) (Fig. 2).

The residual outwardly rectifying current (8% of total) was carried by nonselective cation channels, and not Cl^- channels, since its magnitude was not affected by complete substitution of the extracellular Cl^- with an impermeant anion ($C_2H_3O_2^-$, acetate), and its reversal potential was not affected by substituting Cs^+ or Na^+ for K^+. The chord conductance for this nonselective current was largest for K^+, followed by Cs^+ and least for Na^+. The conductance was 42% less in a Ca^{2+}-free solution (0.48 nS) compared to the control solution (1.14 nS) that contained 1 mM Ca^{2+}, suggesting that this nonselective cation channel may be modulated by extracellular Ca^{2+} (Fig. 3).

Single Channel Studies of K_{OR}

We studied the single channels underlying the dominant K_{OR} current in cell-attached and inside-out membrane patches from both adherent and nonadherent cells. In inside-out patches from adherent cells studied in symmetrical solution (150 mM KCl; Ca^{2+}-free), the single-channel conductance was 229 ± 13 pS (n = 4). When considered with the previous data from the whole-cell recordings, the reversal potential of 0 mV for the inside-

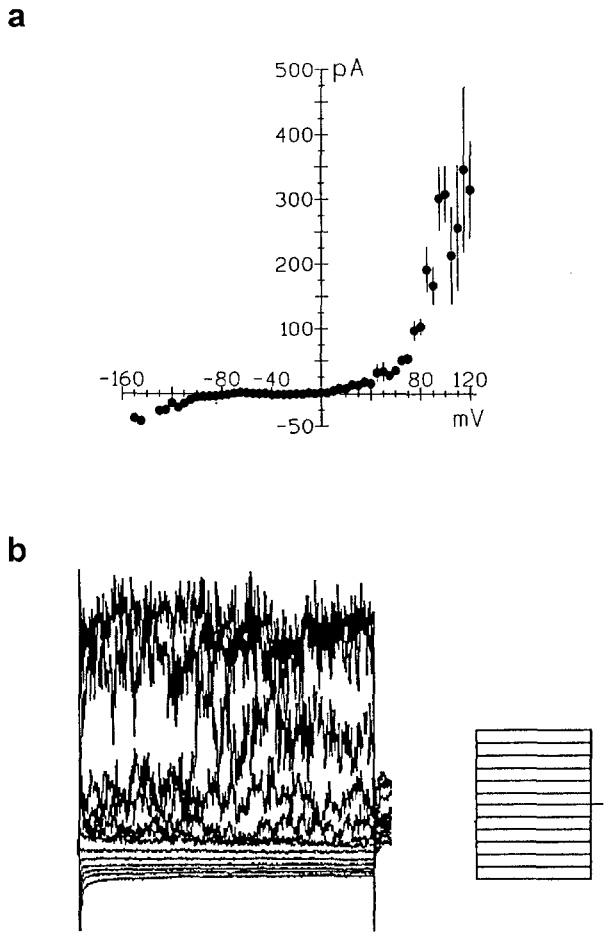

Fig. 1. Macroscopic ion currents in nonadherent macrophages with normal ion gradients, K^+-rich pipette and Na^+-rich bath. (*a*) Steady-state whole cell current-voltage relation for a sample of 27 cells. The vertical lines represent SEM where this is larger than the symbol. An ohmic leak that reversed near the resting membrane potential (−19.9 ± 3.1 mV), corresponding to an average impedance of 1.4 ± 0.5 GΩ, was subtracted from these data. The large outwardly rectifying current activated at potentials more positive than +10 mV and the small inward current activated at potentials more negative than −80 mV. (*b*) Recording of macroscopic ion currents from a non-adherent macrophage. The currents were elicited by 750 msec voltage steps in the range from −120 to +120 mV, from a holding potential of −30 mV (protocol inset). Note that there is essentially no current recorded at negative potentials, with the outwardly rectifying whole-cell current activating after 0 mV (step 7). Scale: 100 msec, 100 pA.

out patch recordings suggests that the channel was K^+-selective. With a Ca^{2+}-free solution (K^+-rich) bathing the cytosolic face of inside-out patches, the distribution of channel open times was best-fit with a single exponential function, with a mean open-time of 1.0 ± 0.2 msec (n = 4) and the distribution of closed times was best-fit with a double exponential function, with mean closed times of 4.6 ± 0.3 msec (n = 4) and 52.8 ± 4.5 msec. The open probability of the channel in untreated adherent macrophages was 0.16 ± 0.03 (n = 13). The open probability of the channel recorded from macrophages in suspension (nonadherent) was less than 10% of that seen in adherent cells (open probability = 0.01 ± 0.01; n = 8; $P < 0.001$).

Modulation of K_{OR} by Membrane Stretch and Cell Adherence

To observe the effects of cell adhesion on the activity of K_{OR}, we formed giga-seals on nonadherent cells and then gently apposed them to a glass coverslip on the bottom of the culture dish. Contact with the coverslip was confirmed by a slight increase in the cell diameter, and the giga-seals did not deteriorate during this procedure. The open probability of the K_{OR} channel increased dramatically about 10 sec after contact, remained very high for approximately 20 sec, and then decreased (Fig. 5). The macrophage remained firmly attached to the coverslip. This was confirmed at the conclusion of the experiments by withdrawing the pipette, leaving the cell attached to the coverslip. As noted above, the channel activity of cells in the chronically adherent state was significantly higher than cells in suspension. It was not, however, as high as the levels of channel activity seen during the 20–30-sec period immediately following contact, when channel open probabilities exceeding 0.5 were commonly seen.

Figure 5(*b,c*) shows the effect of repeated application and release of suction in the pipette solution. This resulted in repetitive cycles of instantaneous activation and deactivation of this channel. This observation was reproducible in both cell-attached and inside-out patches, and was observed over a range of pressures from −20 to −110 mm Hg. The fact that mechanically-triggered activation of the channels was seen in excised patches bathed in Ca^{2+}-free medium containing 5 mM EGTA suggests that neither Ca^{2+} nor other second messengers are involved.

Modulation of K_{OR} by Cytokines and Calcium

We studied the effects of the cytokines IL-2 (50 U/ml), IL-6 (50 U/ml) and TGF-β (10 ng/ml) on the K_{OR} channels in cell-attached patches under various conditions. None of these agents had any effect on the single-channel open probability, when applied to macrophages in suspension. Essentially the same was true of adherent cells that had been in culture for one week, with the exception that IL-2 produced a marked (>500%) increase in K_{OR} activity in one out of four cells to which it was applied. No significant changes was seen in the remaining three cells exposed to IL-2, nor in any cell exposed to IL-6 or TGF-β.

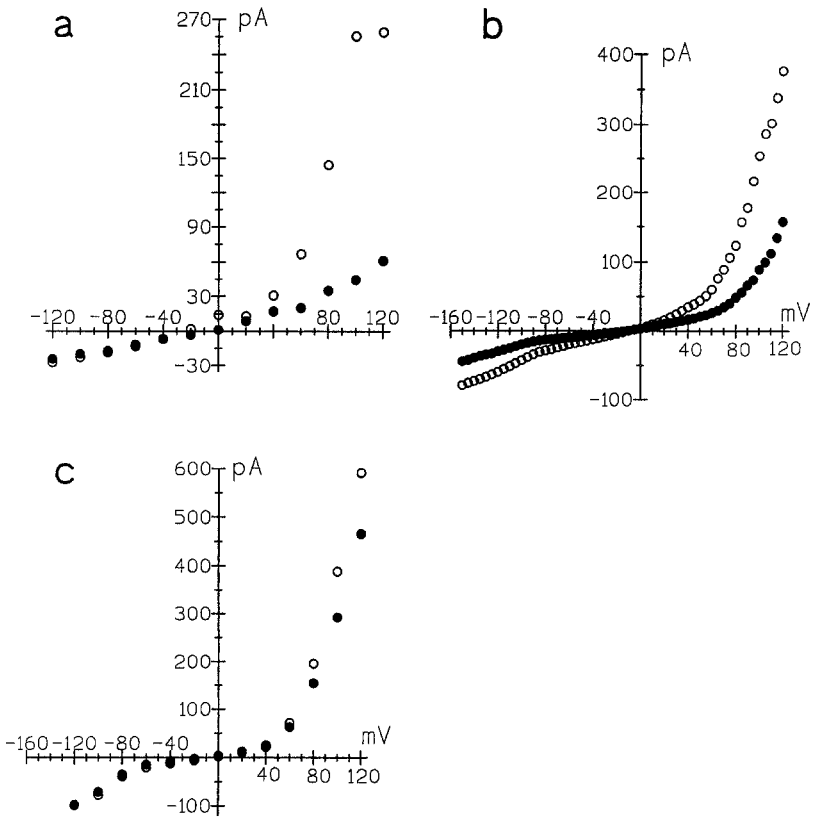

Fig. 2. Effect of blocking agents on the macroscopic currents in typical cells. In each panel, the open circles (○) represent control and the closed circles (●) represent the blocking agent. (*a*) 4-AP (1 mM) blocked the outward current completely, leaving only the ohmic component. (*b*) $BaCl_2$ (5 mM) partially inhibited both the inward and outward currents. (*c*) TEA (20 m*M*) did not affect the inward or outward currents. A similar lack of effect was observed with verapamil (1 mM) and nifedipine (50 μM).

We repeated the above experiments following pretreatment of the cells for 18 hr with IFN-γ (1000 U/ml). We again saw no effect of any of these cytokines on K_{OR} activity in nonadherent cells, but noted marked activation of the channel within 10–15 seconds following exposure to IL-2 in the adherent cells (Figs. 5 and 6). A trend in the same direction was seen with IL-6, with marked increases in activity (>300%) observed in three out of four cells on a similar time-scale and no effect in the remaining cell ($P = 0.09$ overall; Fig. 6). TGF-β, on the other hand, had no apparent effect on channel activity under any of the conditions studied. Calcium, applied to the cytosolic surface of the membrane (isolated inside-out patches), also activated K_{OR} (Fig. 7). This was strongly concentration-dependent over the range of $<10^{-9}$ M to 10^{-6} M, with an EC_{50} of 4.2×10^{-7} M.

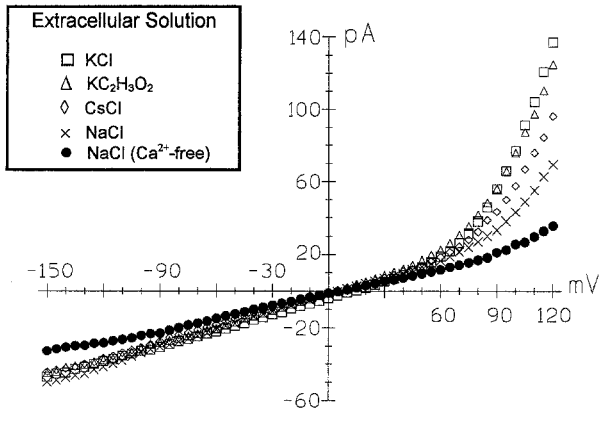

Fig. 3. Whole-cell ion currents to demonstrate the small nonselective cation current present in the macrophages. Steady-state current-voltage relation of a representative cell with a Cs^+-rich pipette and a Na^+-rich bath. Note that the currents are markedly reduced in amplitude, indicating that the major outward K^+ current was blocked by intracellular Cs^+. Inset shows the extracellular solution that corresponds to the symbols. The amplitude of the remaining outward current was unaffected by substituting extracellular KCl (□) by $KC_2H_3O_2$ (△). The conductance of this residual current was reduced by replacing extracellular KCl by CsCl (◊), or by NaCl (X). The residual current was reduced following removal of Ca^{2+} from the bath solution (●).

Effects of K_{OR} Channel Blockade on Cytoskeletal Proteins and Cell Morphology

Under phase contrast microscopy, adherent macrophages treated with blockers of K_{OR} ($BaCl_2$ and 4-AP) became noticeably rounder within 20 min, and this effect was maximal at 6 hr. The cells remained viable and adherent during this time. The rounding-up started to reverse immediately on removal of $BaCl_2$ or 4-AP, and was fully reversed after 3 hr with the cells remaining adherent and viable until observations were ceased at 24 hr following removal of the drug. The Ca^{2+} channel blocking drug verapamil, which we had previously demonstrated to have no effect on the whole-cell current, had no effect on cell morphology. This morphological change suggested

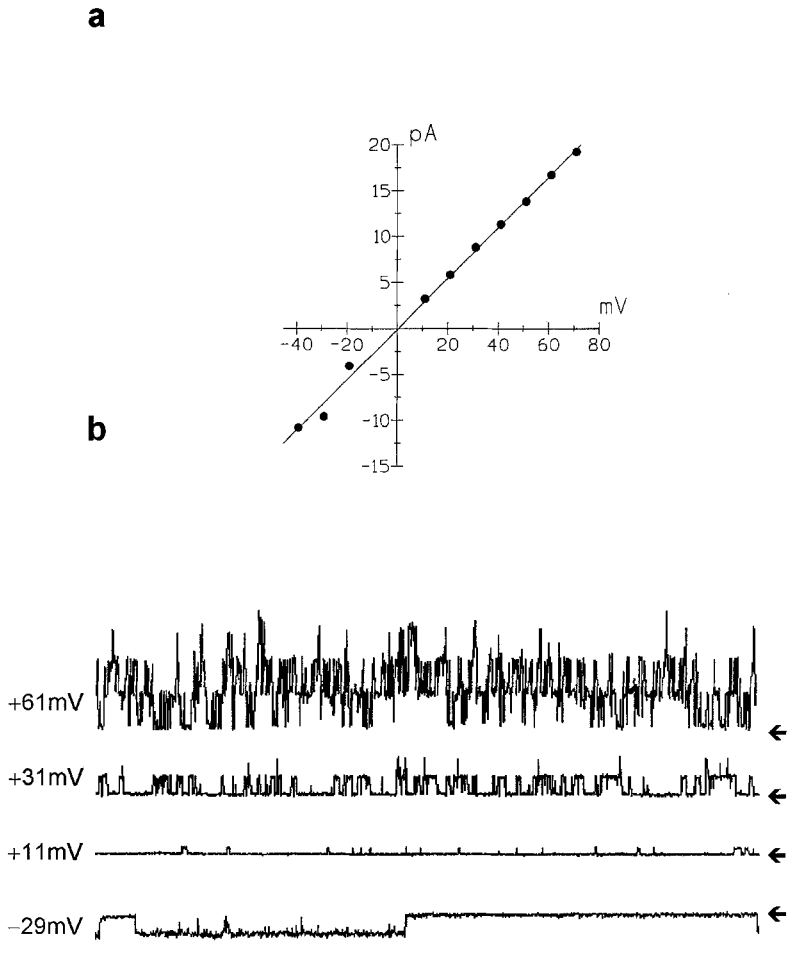

Fig. 4. Single channel K_{OR} currents in macrophages. (*a*) Representative steady-state current-voltage relation from an inside-out patch with symmetrical K^+-rich (140 mM) KCl solutions. The single-channel conductance for this patch was 276 pS. The abscissa represents the trans-patch membrane potential (V_m) and outward current is positive. The reversal potential is near 0 mV, which indicates that the channels were K^+-selective. (*b*) Single-channel currents for this representative cell. The transmembrane potential is indicated beside each trace. Upward deflections indicate an outward current from the cell. The baseline level, where there is no current flowing through the channel, is indicated in each trace by the solid arrow. Scale: 200 ms, 20 pA.

a possible linkage between the plasma membrane K^+ channels and the cytoskeleton, and we studied it further using confocal microscopy.

Figure 8 illustrates the distribution of actin, tubulin and vimentin in adherent macrophages treated with $BaCl_2$ (5 mM), 4-AP (1 mM), verapamil (1 mM) or in control medium. In the untreated cells, actin is distributed around the entire cell close to the plasma membrane. However, there were many focal concentrations of actin at the surface of the cells apposed to the culture dish. Few, if any, stress fibers were observed in the cells. Both vimentin and tubulin were distributed in a filamentous pattern throughout the cytosol. The tubulin and vimentin filaments radiate from a central point adjacent to the nucleus out toward the plasma membrane. The average volume of control cells was 4803 ± 479 μm^3 (n = 10), and their footprint area was 717 ± 92 μm^2.

$BaCl_2$ altered the distribution of actin so that it was spread more evenly throughout the cytosol, and the focal concentrations were no longer present. The distribution of tubulin and vimentin was also altered markedly (Fig. 8), with the filaments becoming disorganized and collapsing into aggregations. In addition, vacuoles formed in the cytosol, and the footprint area of the cells was significantly smaller (314 ± 38 μm^2, n = 10, $P < 0.01$) while the cell volume did not alter significantly (3652 ± 377 μm^3; n = 10; p = NS). Thus, the cells became rounder and taller.

Treatment of the cells with 4-AP resulted in changes to actin, tubulin and vimentin similar to those observed with $BaCl_2$ (Fig. 8). The footprint area of the treated cells was 503 ± 71 μm^2 (n = 10; p = NS) and the cell volume remained unchanged at 4777 ± 357 μm^3 (n = 10, p = NS). The rounding up was not statistically significant, although our observations using phase-contrast microscopy suggested that the cells tended to become rounded up while remaining adherent.

Verapamil had no effect on the distribution of actin, tubulin or vimentin (Fig. 8), nor on the footprint area (746 ± 104 μm^2, n = 10; p = NS) and cell volume (5855 ± 658 μm^3; n = 10; p = NS).

Discussion

The K_{OR} channel we describe in this study has very similar characteristics to that described in human macrophages by Gallin (1984), Gallin & McKinney (1988)

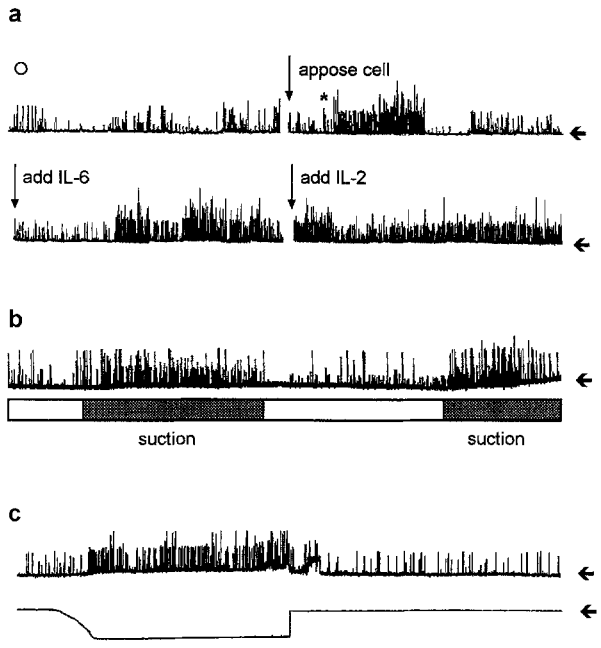

Fig. 5. Activation of K_{OR} channels by cytokines, cell adhesion and direct membrane stretch. In all panels the K_{OR} channel opening is shown as an upward deflection from the baseline (←, zero-current). Scale: 5 s, 10 pA or 100 mm Hg (panel *c*). (*a*) A typical experiment, where there were infrequent channel openings when the cell was suspended in the recording solution (○). The channels were more active (*) about 10 sec after the cell had been apposed to a coverslip in the recording chamber and began to adhere to the coverslip. This enhanced channel activity decreased after about 30 sec. Addition of IL-6 (50 U/ml) further augmented the activity of K_{OR} channels, after a slight delay of about 12 sec. The channels remained active following addition of IL-2 (50 U/ml). (*b*) The effect of direct membrane stretch on K_{OR} in a cell-attached patch. The channel became more active in response to suction (−108 mm Hg) applied via the pipette (shaded sections of the horizontal bar). This effect was reversible on release of the suction. (*c*) Effect of direct membrane stretch on K_{OR} in an inside-out patch in the absence of Ca^{2+}. Channels became more active in response to suction applied via the pipette. The lower trace is the output of a pressure transducer which recorded the suction (downward deflection) simultaneously with the current recording. Both the pipette and bath solutions contained EGTA (10 mM) with no added Ca^{2+}.

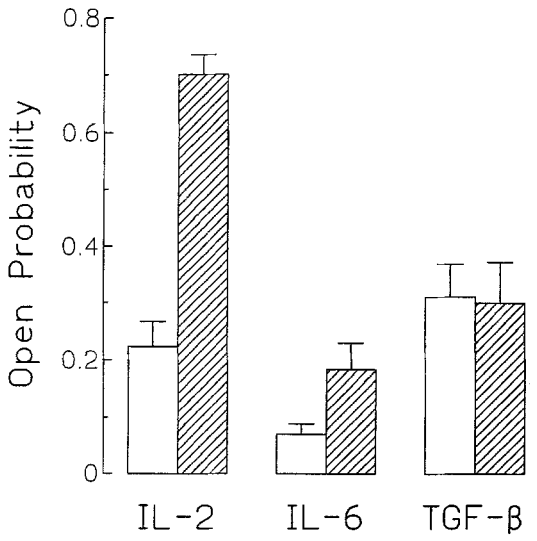

Fig. 6. Summary of the effect of cytokines on the open probability of K_{OR} channels in cell-attached patches from adherent macrophages. The data are averaged from 4 cells for each cytokine. For each cytokine the hollow bars represent the control condition for pretreatment with IFN-γ (1000 U/ml) for 18 hr. The hatched bars represent the open probability following addition of the cytokine. The vertical bars are the SEM. There was no statistically significant difference among these conditions, apart from the effect of IL-2 ($P < 0.001$). The effect of IL-6 on IFN-γ pretreated cells was not quite significant, with $P = 0.09$. However, IL-6 was effective in increasing the channel open probability in the experiment shown in Fig. 4.

or Nelson, Jow & Jow (1990). All of these channels share a similar voltage and calcium dependence and their single-channel conductances in symmetrical solutions are the same. To our knowledge, however, the mechanosensitivity of this channel and the apparent association between cell activation and channel open probability have not been previously reported in macrophages, although a large conductance Ca^{2+}-activated K^+ channel in mesenteric artery smooth muscle cells has recently been shown to be activated by stretch (Dopico et al., 1994).

Significance of Mechanosensitivity of K_{OR}

The adherence and migration of the monocyte through the vascular endothelium is a very important step that marks its transition from monocyte to inflammatory macrophage. While adherence is known to activate macrophages so that their response to stimuli is enhanced (Cohen, Ryan & Root, 1981), the underlying mechanisms and biological relevance are unclear. We have modelled this process in vitro with adherence of human macrophages to glass coverslips and plastic culture dishes. The open probability of the K_{OR} channel increased markedly and immediately when the macrophage was apposed to a glass coverslip. Channel activity fell somewhat during chronic adherence, but remained approximately tenfold greater than in nonadherent cells, presumably accounting for the increased resting membrane potential measured in this state. McKinney & Gallin (1990) reported a similar increase in the potassium conductance of cultured murine macrophages soon after adherence, and a corresponding increase in the membrane potential. The activation of mechanosensitive channels in cell-attached patches can result from two very different factors. They may be responding to cytoplasmic chemicals or they may be being controlled directly or indirectly by forces generated by contractile elements in the cytoskeleton that perturb the plasma membrane. We suggest that the increase in K_{OR} activity following adherence is due to direct mechanical perturbation of the plasma membrane, since suction applied to the membrane in both cell-attached and cell-free excised

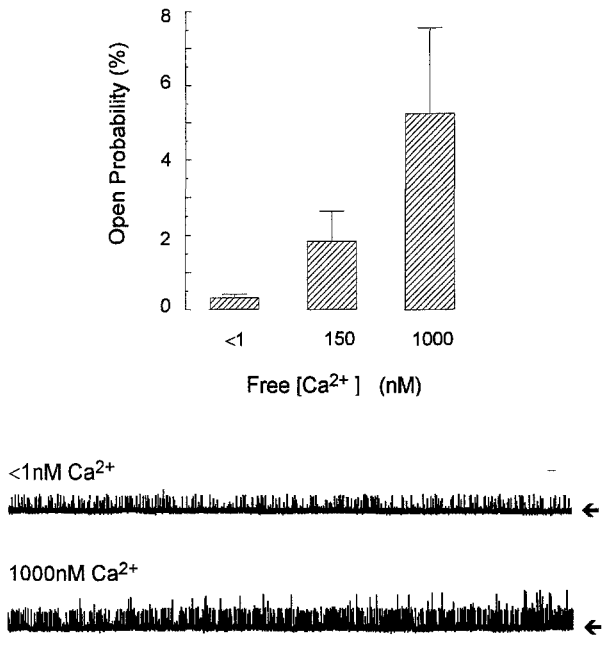

Fig. 7. (*a*) Activation of the K_{OR} channels by intracellular Ca^{2+} in three cells. The EC_{50} from these measurements was 420 nM. (*b*) Representative recordings of the effect of <1 nM and 1000 nM intracellular Ca^{2+} on the activity of the K_{OR} channels. Upward deflections indicate an outward current from the cell. The baseline level, where there is no current flowing through the channel, is indicated in each trace by the solid arrow. Scale: 2.5 sec, 10 pA.

patches directly opened the channel in calcium-free medium containing EGTA.

The application of K_{OR} blockers, 4-AP and $BaCl_2$, led to alterations in the morphology and cytoskeleton in the macrophages. Macrophages that were previously adherent, with many processes, became rounded and lost most of these processes following treatment with the channel blockers. The K_{OR} channel blockers caused the collapse of the intermediate filament and microtubular organization, which led to the appearance of amorphous aggregates in the cytoplasm. Both 4-AP and $BaCl_2$ caused very similar changes in the cytoskeletal proteins. These effects were rapidly reversible on withdrawal of the potassium channel blockers, with the cells remaining adherent and viable until observations were ceased 24 hr following removal of either drug. These morphological and cytoskeletal changes were not observed with verapamil, a calcium channel blocker. Since we did not record any calcium channels and a charged ion such as Ba^{2+} does not normally diffuse through the plasma membrane, it is unlikely that Ba^{2+} ions entered the cell. Taken together, our observations suggest that the cytoskeletal changes associated with 4-AP and $BaCl_2$ were due to K_{OR} blockade, rather than some other intracellular effect.

Kruskal & Maxfield (1987) reported that after macrophages had attached to a substrate, there was an increase in intracellular Ca^{2+} that always preceded spreading of the cells. This rise in intracellular Ca^{2+} appeared to be due to release from intracellular pools, since removal of extracellular Ca^{2+} did not alter the magnitude of the rise nor the subsequent cell spreading. This suggested that there was another signal that occurred after the attachment of the macrophages but before the release of Ca^{2+} intracellularly. We propose that this other signal is due to alteration of the intracellular K^+ concentration and the cytoskeleton, which would result from the direct mechanosensitive opening of the K_{OR} channel following the attachment of the macrophages. This hypothesis is supported by recent reports that a reduction in intracellular K^+ stimulates the production of interleukin-1β (Perregaux & Gabel, 1994; Walev et al., 1995). The opening of the mechanosensitive K_{OR} channel in the plasma membrane might represent a priming stimulus to activate the macrophage, since physical forces associated with adherence could be translated into biochemical and functional changes within the cell. The subsequent alteration in the ionic composition of the cell interior might in turn modify cellular functions such as cytoskeletal interactions (Southwick et al., 1982), DNA synthesis (Amigorena et al., 1990), maturation of interleukin-1β (Perregaux & Gabel, 1994), gene transcription or mRNA stability and thus contribute to the activation process.

In this context, it is interesting to note that Small & Morris (1994) have recently reported a link between a mechanosensitive K^+ channel and the cytoskeleton in Lymnaea neurons, whereby stretch-activated K^+ channel mechanosensitivity in those cells increases when cortical cytoplasm is disrupted. We found that inhibiting the mechanosensitive K^+ channel led to disruption of the cytoskeleton. This suggests that changes in the activity of mechanosensitive K^+ channels may be a sufficient condition to trigger the reorganization of the cytoskeleton. In recent experiments, Wang & Ingber (1994) demonstrated that the plasma membrane was an important regulator of the cell shape, contact with extracellular matrix and permanent deformation in endothelial cells in experiments where stress was applied directly to the cells. Indeed, an active K^+ channel appears to be important to the regulatory volume decrease function in lymphocytes (Deutsch & Chen, 1993) and in PC12 pheochromocytoma cells the regulatory volume decrease is suppressed by disruption of the cytoskeletal microfilament network by cytochalasin B (Cornet, Ubl & Kolb, 1993). In addition, actin filaments have been shown to regulate the opening of plasma membrane Na^+ channels in epithelial cells (Cantiello et al., 1991).

Our studies cast no direct light on the possible mechanism by which membrane K^+ channels and intracellular structural proteins may be linked, and indeed we have not as yet performed measurements of intracellular K^+ concentrations in these cells. It is certainly possible that fluxes in intracellular K^+ concentration might produce

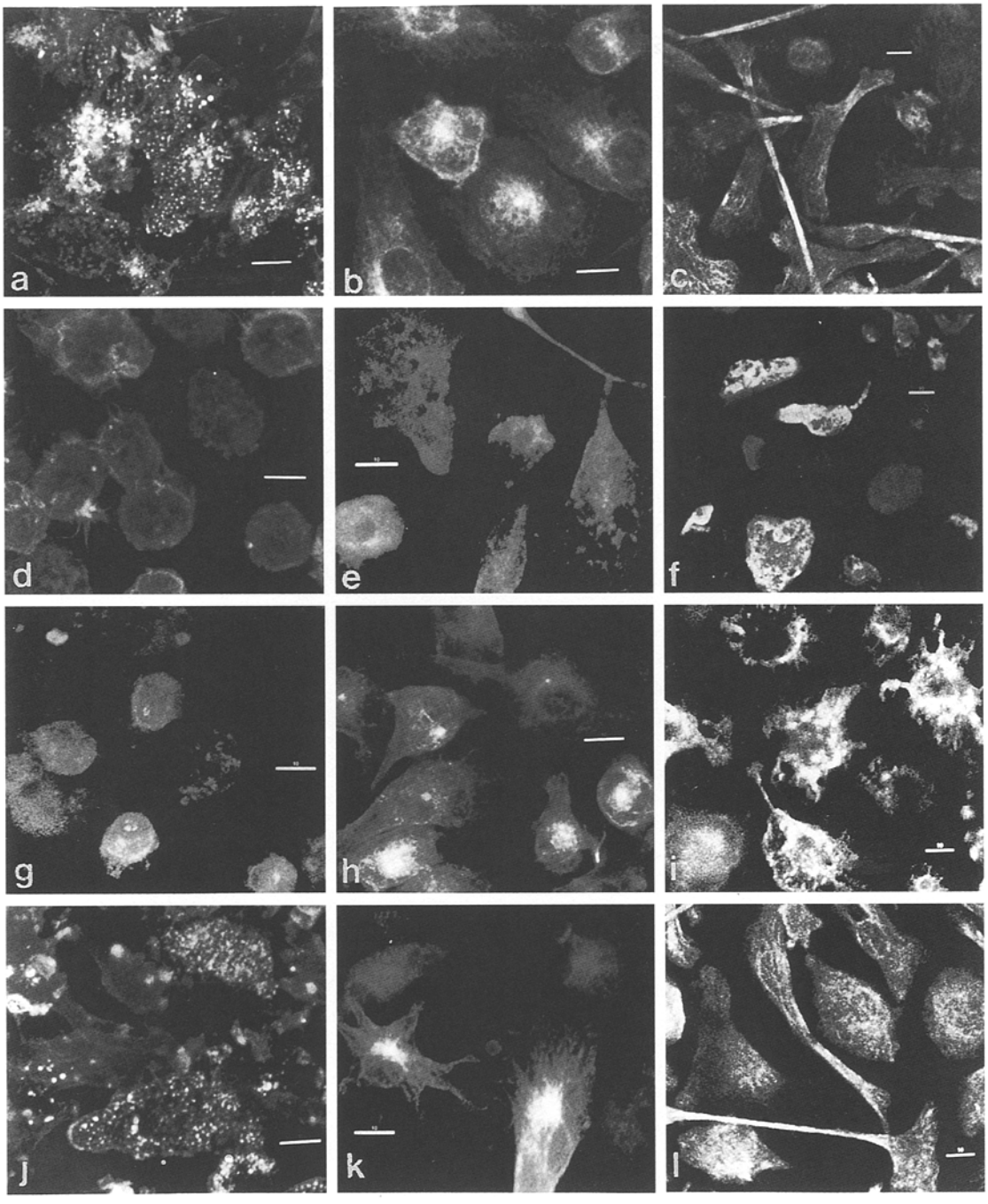

Fig. 8. Effect of channel blocking drugs on the distribution and appearance of actin, tubulin and vimentin. In all panels the horizontal scale bar represents 10 μm. Confocal optical sections were 0.3 μM. (*a–c*) The control condition, where no channel blocker was used, is shown for actin (*a*), tubulin (*b*) and vimentin (*c*). Actin is distributed around the entire cell in focal concentrations that are close to the plasma membrane that is adhering to the culture dish. Both vimentin and tubulin are distributed in a filamentous structure throughout the cytosol (*d–f*) $BaCl_2$ (5 mM) altered the distribution of actin (*d*) tubulin (*e*) and vimentin (*f*). Actin is spread more evenly throughout the cytosol, and the focal concentrations are no longer present. Tubulin and vimentin filaments became disorganized and collapsed into aggregations. The cells had shrunk and altered in overall size and shape, and had developed vacuoles. (*g–i*) 4-aminopyridine (1 mM) altered the distribution of actin (*g*), tubulin (*h*) and vimentin (*i*) in a similar way to $BaCl_2$. The cells became slightly smaller, with remnants of the cytoskeleton that had comprised the processes visible after the cells had shrunk. The cells also developed vacuoles. (*j–l*) Verapamil (1 mM) had no effect on the appearance of actin (*j*), tubulin (*k*) or vimentin (*l*), and did not alter the overall morphology of the cells.

direct effects on microtubules and actin. There are precedents for ions such as potassium and sodium modifying metabolic pathways through direct interactions with proteins. One structural basis for this has recently been demonstrated in a crystallographic analysis of the structure of dialkylglycine decarboxylase (DGD) (Toney et al., 1993; Miller, 1993), which is a potassium-requiring enzyme and is antagonized by sodium. A potassium ion is encapsulated in an oxygen cage within the DGD molecule in such a way as to stabilize the active site of the enzyme. If crystallization occurs in the absence of potassium, the cage decreases in size and will then only admit sodium. In either the absence of potassium or the presence of sodium, secondary adjustment to the position of nearby tyrosine and glutamine residues occurs resulting in the inactivation of DGD. Intracellular potassium levels might also modulate the contractile machinery of the macrophage, because the macrophage contains an actin-modulating protein (acumentin) whose activity is modified by changes in intracellular K^+ in the range from 100–200 mM (Southwick et al., 1982).

Modulation of K_{OR} by Cytokines

Further evidence of a central role for the K_{OR} channel in macrophage activation comes from the data obtained using cytokines to stimulate channel activity. In adherent cells preincubated with IFN-γ, the activating cytokine IL-2 produced clear and very dramatic increases in channel activity. A very similar effect was seen in three out of four cells exposed to IL-6 but no such effect with TGF-β. In the absence of pretreatment with IFN-γ, one out of four cells exposed to IL-2 showed marked increase in K_{OR} activity and no convincing responses were seen to IL-6. A stretch-activated K^+ channel in Aplysia sensory neurons has also been shown to be activated by the neuropeptide FMRFamide (Vandorpe et al., 1994). Nelson et al. (1992) reported that IL-2 treatment enhanced the expression of an outward (inactivating) K^+ current in 33% of human monocyte-derived macrophages. It is possible that pretreatment of macrophages with IFN-γ leads to an increased number of cell surface IL-2 receptors, thus explaining our observations. The cytokines had no effect on nonadherent cells. Since the K_{OR} channel is essentially inactivated in macrophages in suspension, we propose that it is this property of the K_{OR} channel that may be one reason why circulating monocytes are not routinely activated by circulating cytokines.

Thus, we propose that the K_{OR} channel is intimately associated with the process of macrophage activation by physical factors and cytokines. This channel remains in a nonconducting state unless the cell is attached to a substrate, thereby isolating circulating cells from activating factors in their environment within the circulation. The process of attachment, as would occur when the monocyte adheres to the vascular endothelium, directly induces channel opening and leads to biological activation of the macrophage. In our experiments, IL-2 induced channel opening only in adherent cells and had no effect on nonadherent cells. The K_{OR} channel may represent one of the mechanisms whereby physical forces exerted on the macrophage plasma membrane, as well as other classical activation triggers such as cytokines, may be translated into metabolic changes within the cell ("cell activation").

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