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Lipopolysaccharide Induction of Outward Potassium Current Expression in Human Monocyte-Derived Macrophages: Lack of Correlation with Secretion

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Summary. Although an outwardly rectifying K^+ conductance ($I_{K,A}$) is prominently expressed in human alveolar macrophages, the expression of this conductance in human monocyte-derived macrophages (HMDMs) is rare. We have analyzed the induction of the expression of $I_{K,A}$ in voltage-clamped, in vitro differentiated HMDMs by a number of stimuli which produce either priming or activation of macrophages. Cultures were stimulated with lipopolysaccharide (LPS, 2 μg/ml), interleukin 2 (IL-2, 100 U/ml), or combinations of LPS and either recombinant interferon-gamma (γ-IFN, 10 U/ml), phorbol myristate acetate (PMA, 0.01 or 1 μg/ml) and platelet activating factor (PAF, 20 ng/ml) for periods of up to 24 hr. Treatment of the cells with either LPS or IL-2 greatly enhanced the frequency of current expression. Treatment with either PMA or γ-IFN alone did not induce current expression; treatment of the cells with a combination of LPS and either PMA, γ-IFN, or PAF did not enhance current expression over that observed with LPS alone. The expression of the outwardly rectifying K^+ current was observed in 36% (n = 321) of the cells for cultures treated with LPS and 33% (n = 55) of the cells for cultures treated with IL-2. The inactivating outward K^+ current was absent in cells which were not treated with either LPS or IL-2. The kinetics of current activation and inactivation appeared identical to that previously described for the transient-inactivating outward current of the human alveolar macrophage. Cycloheximide (1 μg/ml), an inhibitor of protein synthesis, completely suppressed LPS-induced current expression. No correlation was found between peak current amplitude and cell size in LPS-activated cells expressing the outwardly rectifying K^+ current, indicating that current density was not held constant from cell to cell. The coupling of ion channel expression and secretion in individual HMDMs was studied using the reverse hemolytic plaque assay. Although an enhancement of K^+ current expression was observed following either LPS or IL-2 treatment, a quantitatively similar and uniform increase in the percentage of either IL-1 or lysozyme-secreting cells was not observed. The frequency of current expression in cells identified as secreting tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1), or lysozyme was the same or decreased over that observed for nonsecreting cells. Thus, LPS treatment increases the number of K^+ channels on HMDM membranes; however, K^+ channel expression alone was not sufficient to give rise to enhanced secretion in LPS-activated macrophages. Enhanced K^+ channel expression appears to be a part of the primary activation signal. K^+-channel activation would hyperpolarize the membrane potential, potentially providing the driving force for calcium entry through voltage-independent pathways activated by the subsequent binding of soluble substances to membrane surface receptors, the secondary signal linked to secretion.

Key Words macrophage · potassium channels · lipopolysaccharide · secretion · activation · interleukin 2 · tumor necrosis factor · interleukin 1

Introduction

Of the three types of outward K^+ currents activated upon depolarization in voltage-clamped human monocyte-derived macrophages (HMDMs), the outwardly rectifying, fast-inactivating, 4-AP sensitive component of the outward current ($I_{K,A}$) is the most infrequent. It is absent throughout differentiation in cells maintained in adherent culture (Gallin & McKinney, 1988; Nelson, Jow & Jow, 1990*a*). There is a dramatic increase in the frequency of $I_{K,A}$ current expression in the human alveolar macrophage over that observed in the HMDM (Nelson, Jow & Popovich, 1990*b*). Based on the enhanced expression of $I_{K,A}$ in the tissue macrophage over that observed in the in vitro differentiated macrophage, we sought to determine whether the expression of the current could be induced in the in vitro differentiated cell by stimuli known to activate or prime macrophages. The notion that macrophages change their membrane electrophysiological properties depending on their state of functional activation had been suggested in the studies of Ypey and Clapham (1984) on the murine peritoneal macrophage.

It has been proposed that macrophage activation occurs as a two-step process (Adams & Hamilton, 1987; Hamilton & Adams, 1987). Cells enter a "primed" state in response to an initial signal (e.g., lymphokines or LPS) which is followed by a response to a subsequent or triggering signal (e.g., immune complexes, LPS, microorganisms, high concentrations of lymphokines, opsonized particles,

or tumor cells) mediated through ligation of surface receptors. The cascade of events which are initiated as a result of receptor ligation includes (i) increased production of the cyclic nucleotides cAMP and cGMP; (ii) hydrolysis of polyphosphoinositides leading to the generation of inositol 1,4,5, trisphosphate (Ins1,4,5P_3) and the resultant elevation of intracellular Ca^{2+} levels, generation of diacylglycerol, and the subsequent activation of protein kinase C; as well as (iii) the direct or indirect opening of plasma membrane ion channels leading to changes in intracellular concentrations of Na^+ or Ca^{2+} which may be linked to alterations in intracellular pH.

Upon exposure to LPS, macrophages acquire full competence for tumor cytolysis and enhanced secretion (Morrison & Rudbach, 1981; Zacharchuk et al., 1983; Fuhlbrigge et al., 1987; Hamilton & Adams, 1987; Becker, Devlin & Haskill, 1989; Strieter et al., 1989; Cavaillon, Fitting & Haeffner-Cavaillon, 1990). Among the earliest responses of macrophages to treatment with LPS is the synthesis of new proteins thought to endow the cells with competence for a particular function (Hamilton et al., 1986).

Human monocytes respond to IL-2 with induction of IL-1 mRNA (Kovacs et al.,1989), development of tumoricidal activity (Malkovsky et al., 1987; Wahl et al., 1987), and enhancement of superoxide anion generation (Wahl et al., 1987). Espinoza-Delgado and co-workers (1990) have recently reported the constitutive expression of the p70–75 IL-2 receptor subtype on fresh resting human monocytes. The p75 receptor subtype binds IL-2 with an intermediate affinity ($K_D \approx 10$ nM).

In our studies, treatment of HMDMs with either LPS or IL-2 was accompanied by an increase in the frequency of $I_{K,A}$ current expression, the magnitude of which depended upon the age of the cells in culture. Thus, the outwardly rectifying K^+ channel appears to be among the class of proteins whose expression is enhanced in the presence of LPS and IL-2. Although the outwardly rectifying K^+ channel has been characterized in a number of phagocytic leukocytes, its link to the functional state of the cell is less well understood (for review *see* Gallin & McKinney, 1990). Therefore, we employed the reverse hemolytic plaque assay (RHPA) to study the correlation between $I_{K,A}$ current expression and secretion in LPS-treated HMDMs. This assay which utilizes immunologically triggered complement-induced red blood cell lysis in the vicinity of individual secretory cells has been used to detect heterogeneity among mononuclear phagocytes in their secretion of lysozyme as well as cytokines (Smith & Hammarström, 1982; Lewis et al., 1989; Lewis et al., 1990*a*,*b*). Secretory cells form plaques representing red blood cell lysis when incubated in a monolayer with protein A-coated ovine erythrocytes in the presence of specific antiserum and complement. Individual HMDMs identified as either lysozyme, tumor necrosis factor-α (TNF-α), or interleukin 1 (IL-1) secreting cells did not show $I_{K,A}$ current expression that was enhanced over that observed in nonsecretory cells from the same culture. In addition, LPS did not significantly increase the number of either the IL-1 or lysozyme plaque-forming cells over that observed in controls.

Our results indicate that the LPS-induced enhancement of $I_{K,A}$ in HMDMs is a function of age in culture. Although, $I_{K,A}$ expression does not appear to be tightly linked to lysozyme or cytokine secretion evoked by LPS stimulation alone, it could provide a unique signal transduction step in the early stages of the activation response. Enhanced K^+ channel expression and subsequent activation would provide for membrane hyperpolarization required to support calcium entry through voltage-independent pathways activated following the binding of soluble substances, e.g., immunoglobulin fragments, complement, N-formylated peptides, and lymphokines to macrophage surface receptors. Therefore, LPS-induced expression of $I_{K,A}$ could play an important role in the regulation of membrane potential and secretion in response to a subsequent or triggering signal.

Materials and Methods

The procedures for isolating the HMDMs and the voltage-clamp techniques employed have been previously described (Nelson et al., 1990*a*). The standard pipette (internal) solution contained (in mM): 140 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 11 EGTA, and 10 HEPES/KOH; pH 7.2. The value of the free-Ca^{2+} concentration was calculated to be 38 nM. Using the calcium-sensitive dye, Fura-2, we measured the free Ca^{2+} in the intrapipette solution to be approximately 42 nM. The bath solution contained (in mM): 140 NaCl, 5.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES/NaOH; pH 7.4. Activating and priming stimuli were added to the cultures without a media change unless otherwise indicated. Macrophage activation and priming was induced in the presence of the following stimuli: LPS (List Biological Laboratories, Campbell, CA), recombinant human γ-IFN (Collaborative Research, Bedford, MA), PAF (Sigma, St. Louis, MO), PMA (Sigma, St. Louis, MO), and recombinant human IL-2 (Boehringer Mannheim, Indianapolis, IN). Cycloheximide which was used to inhibit protein synthesis was obtained from Sigma (St. Louis, MO).

Reverse Hemolytic Plaque Assay

The reverse hemolytic plaque assay (RHPA) was performed as previously described for the detection of prolactin-secreting cells in a mixed culture of adenohypophyseal cells (Neill & Frawley, 1983) as well as for the detection of lysozyme- and cytokine-

secreting human peripheral blood monocytes (Lewis et al., 1989), with minor modifications. In brief, HMDMs differentiated in suspension culture for varying periods of time (*see* Nelson et al., 1990*a*) were suspended in serum-free RPMI-1640 (1×10^6/ml) and mixed thoroughly with an equal volume of 15% ovine erythrocytes (*o*RBCs) previously coupled to *Staphylococcus* protein A with chromium chloride hexahydrate. Ovine erythrocytes were prepared fresh every two days in order to avoid the possibility of nonspecific lysis. The cell mixture was aliquoted directly onto the central portion of poly-L-lysine coated tissue culture dishes and allowed to adhere for 1 hr in a humidified atmosphere of 5% CO_2/95% air at 37°C. Excess unattached cells were removed by washing each dish with prewarmed RPMI-1640 supplemented with 1% penicillin/streptomycin to leave a confluent monolayer of cells attached to the dish. The assay was initiated with exposure of the cells to a solution of RPMI-1640 containing a dilution of rabbit antiserum raised against either recombinant human IL-1 (1 : 100, Genzyme, Boston, MA), human lysozyme (1 : 10, Accurate, Westbury, NY), or recombinant TNF-α (1 : 100, Genzyme) for 1 hr. Cells were washed with RPMI-0.1% BSA to remove excess antiserum/antibody not bound to the *o*RBCs. Secretion was induced by the addition of 25 μl of either LPS (2 μg/ml) or IL-2 (100 U/ml) containing media to the confluent layer of cells. The solution volume was kept at a minimum in order to avoid dilution of the secreted antigen. Evaporation of the small solution volume was avoided by wrapping dishes in dampened gauze and loosely enclosing them in aluminum foil during the incubation period. In general, plaques were developed 4 hr later (using HMDMs on the day of isolation up through the fourth day of differentiation in culture) by infusion of guinea pig complement (1 : 50, Gibco, Grand Island, NY) in RPMI for 10–20 min. In the IL-1 secretion studies, plaque development was determined after approximately 8 hr (the rate/magnitude of LPS-induced IL-1 secretion was lower than either lysozyme or TNF). As soon as plaques formed, the complement-containing solution was removed as extended exposure of the cells to complement seemed to compromise cell viability. Plaque formation was not observed in the absence of complement or antiserum. However, if cells were incubated with protein A coated *o*RBCs for periods longer than 16 hr, nonspecific plaques were frequently observed, and therefore, incubation times were kept below 8–10 hr. In an alternative procedure, the protein A coupled *o*RBCs were allowed to attach to the culture dish in the incubator for 1 hr. Following attachment, the unattached *o*RBCs were then removed by washing and the attached cells incubated overnight in RPMI. The HMDM cell suspension was plated on top of the *o*RBC monolayer the next day. The HMDMs were allowed to adhere for an hour before exposure to the antiserum-containing solutions. Plaque formation in drug and nondrug-containing solutions was determined in blind experiments. The plaque-forming *versus* nonplaque-forming cells were counted in a representative field at a total magnification of 100×.

Experiments were performed at room temperature (21–23°C). Summary data are expressed as means ± SEM with the number of experiments in parentheses.

Results

The expression of voltage-activated transient outward K^+ current ($I_{K,A}$) was studied in cultured HMDMs. A comparison of a family of whole-cell currents elicited upon step depolarizations is shown in Fig. 1*A* and *B* for two cells of the same age in culture. The expression of $I_{K,A}$ (as shown in Fig. 1*B*) was detectable only in cells exposed to the activating stimuli LPS (2 μg/ml) or IL-2 (100 U/ml). In LPS- or IL-2 treated cells, inactivating outward K^+ currents were elicited in response to depolarizing voltage steps positive to −40 mV (as seen in Fig. 1*C*). Current activation was half-maximal at a membrane potential of -19.7 ± 1.3 mV ($n = 5$) as determined from Boltzmann fits to the peak chord conductance-voltage relationship (Fig. 1*C* inset and Fig. 2). The time constant of $I_{K,A}$ inactivation was voltage dependent, decreasing with increasing depolarization. LPS-induced $I_{K,A}$ exhibited steady-state inactivation which was apparent at holding potentials more positive than −60 mV; the inactivating outward currents were completely absent at holding potentials more positive than −10 mV.

The voltage-dependent availability and peak conductance *versus* voltage relationships were overlapping on the voltage axis (*see* Fig. 2). The overlap is consistent with a true window region of $I_{K,A}$ channel activity which would give rise to steady-state current activation at hyperpolarized potentials near rest (the average zero current potential in LPS-activated cells expressing $I_{K,A}$was -44.6 ± 1.54 mV ($n = 36$)). In the experiment depicted in Fig. 2, there was a 20-mV separation between the midpoints ($V_{1/2}$ values for activation and inactivation were −20.6 and −41.3 mV, respectively). Steady-state $I_{K,A}$ current in the window region of channel activation would serve to maintain a hyperpolarized resting potential. In two experiments on LPS-activated cells expressing $I_{K,A}$, treatment with the $I_{K,A}$ channel blocker, 4-AP (0.4 and 4 mM), produced a depolarization in the zero current potential of 1.9 and 27 mV, respectively. In addition to the hyperpolarizing effects on membrane potential at rest, transient depolarizations in response to a secondary stimulus would be aborted by the hyperpolarizing effect of $I_{K,A}$ current activation.

The kinetics and pharmacology of the current induced in the presence of LPS appeared identical to the transient-inactivating outward K^+ current which has been previously described for the human alveolar macrophage (HAM; Nelson et al., 1990*b*). A comparison of the kinetics of activation and inactivation for the HAM and the LPS-activated HMDM is given in Table 1. As can be seen in Fig. 1*D*, there was no clear relationship between the amplitude of the transient outward current activated at +10 mV (a potential range in which possible contamination from other outward currents is minimized, *see* Nelson et al., 1990*a*) and cell capacitance which is proportional to membrane surface area. Thus, in HMDMs $I_{K,A}$ current expression and membrane area

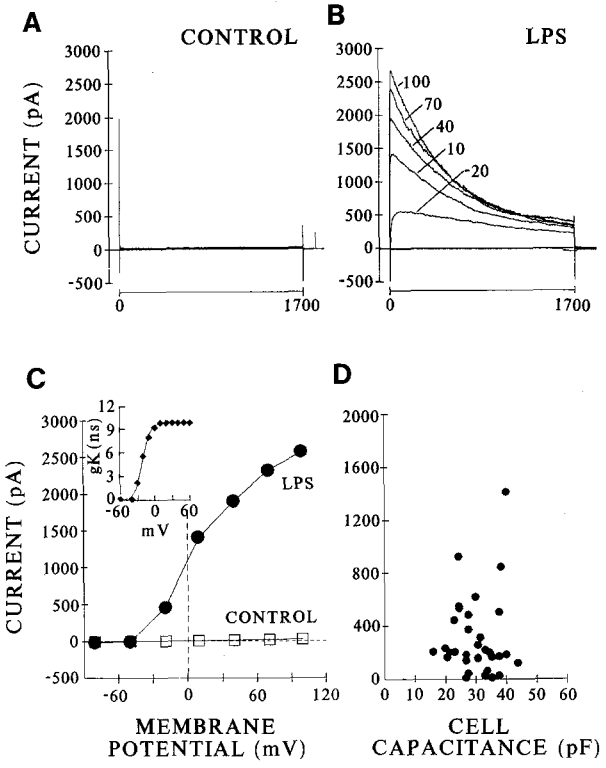

Fig. 1. Representative whole-cell currents from control and LPS-treated HMDMs. Whole-cell currents were elicited from a holding potential of −60 mV in response to step changes to the following potentials (in mV): −110, −80, −50, −20, 10, 40, 70, and 100. The interval between pulses was 40 sec to allow for complete recovery from inactivation; pulse length was 1.7 sec. Data were filtered at 500 Hz and sampled at 1 kHz. Records were obtained from two separate cells from the same donor following four days of growth and differentiation in adherent culture. The input capacitance for the cells in *A* and *B* was 35 and 40 pF, respectively. (*A*) Representative current recording from an untreated cell. (*B*) Representative current recording from a cell treated for 8 hr with LPS (2 μg/ml). Note the activation of the prominent time-dependent outward current, $I_{K.A}$. (*C*) Current-voltage relationship for the currents in *B*. The threshold for current activation was approximately −40 mV. *Inset:* Conductance-voltage relationship for $I_{K.A}$ as determined in a third LPS-activated cell. A current reversal potential of −82 mV (as determined from tail current experiments) was used to determine the peak conductance *versus* voltage curve. The continuous line through the data points represents the best fit of the peak conductance to a Boltzmann distribution of the form $g_{(K)} = g_{(K.max)}/(1 + e^{(V-V_{1/2})/k})$ where $V_{1/2}$ is the voltage at the midpoint of the conductance curve and k gives the steepness of the voltage dependence. In this experiment $V_{1/2} = -20.6$ and $k = -7.2$. (*D*) Peak $I_{K.A}$ amplitude as a function of cell capacitance. Peak current was determined for a step depolarization to +10 mV from a holding potential of −60 mV where contamination by other outward currents would be minimized (*see* Nelson et al., 1990*a*). Capacitance was measured by integrating the current during a 5- to 10-mV voltage step and subtracting a baseline established about 20 msec after the step as determined from a nonlinear fit to the data. Capacitance data was obtained from a total of 33 cells which had been maintained in culture for 2–4 days and exposed to LPS for a period of 4 to 29.5 hr (average LPS exposure time was 12.8 ± 1.7 hr).

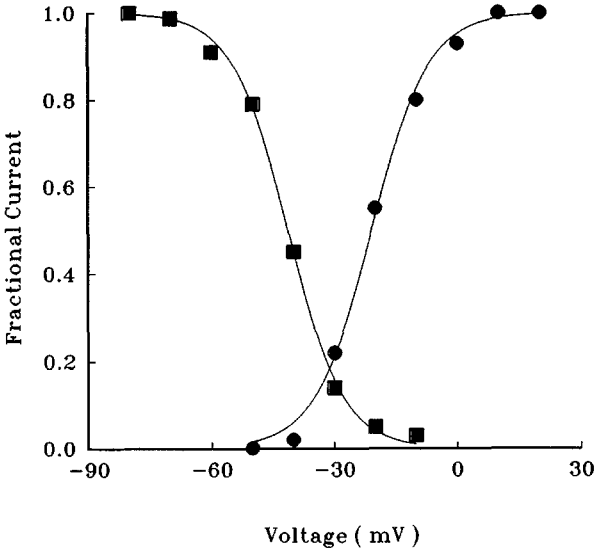

Fig. 2. Steady-state inactivation and conductance-voltage relationship. Steady-state inactivation (filled squares) of LPS-induced $I_{K.A}$ current as a function of membrane potential. The conductance *versus* voltage relationship (filled circles) was determined in the same cell. The continuous lines drawn through the data points represent the best fit of the steady-state inactivation and peak conductance *versus* voltage curves with a single Boltzmann isotherm of the form given in the legend to Fig. 1.

appear to vary independently of one another in a manner similar to that observed for the HAM (Nelson et al., 1990*b*).

Increase in Cell Capacitance in the Presence of LPS

LPS did, however, produce an increase in cell capacitance over that observed for non LPS-treated cells. Membrane capacitance was measured in cells from eight separate isolations maintained in culture for one to four days. Capacitance in control cells increased from 7.9 ± 0.5 pF (n = 11) at day 1 following attachment to 25.6 ± 2.5 pF (n = 10) at day 4. This is to be compared to an average capacitance of 9.6 ± 0.7 pF (n = 10) at day 1 to 36.8 ± 1.6 pF (n = 17) at day 4 for cells of the same age in culture treated with LPS for 4–29 hr. The difference between the two groups was determined to be significant at the $P < 0.0001$ level using analysis of variance. Longer LPS-incubation periods were not associated with further increases in cell capacitance.

Enhanced Expression of $I_{K.A}$ is a Function of the Activation Stimulus

The expression of $I_{K.A}$ was never detected in adherence-purified cells which were not exposed to exoge-

Table 1. Voltage-dependent pharmacology of time-dependent outward current ($I_{K,A}$) in alveolar and LPS-activated HMDMs

	Alveolar macrophage[a]	LPS-activated HMDM
Gating		
$V_{1/2}$ activation[b]	−17.7 ± 6.0 mV (n = 3)	−19.7 ± 1.3 mV (n = 5)
Cumulative inactivation[c]	Yes	Yes
Current inactivation[d] τ (+70 mV)	561 ± 77 msec (n = 10)	535 ± 35 msec (n = 6)
Recovery from inactivation[e] τ (+10 mV)	32.6 sec	32.0 ± 4 sec (n = 3)
Midpoint steady-state inactivation	−44.2 mV (n = 2)	−41.5 ± 1.0 mV (n = 4)
Current Inhibition		
4-AP (0.4 to 4 mM)	97.3% (n = 2)	81.9 ± 5.2% (n = 4)
Ba^{2+} (4 mM)	ND	56% (n = 2)

[a] Data from Nelson et al., 1990*b*.

[b] $V_{1/2}$ activation is the membrane potential at which half of the current is activated as determined from Boltzmann fits to peak conductance *versus* voltage curves.

[c] Cumulative inactivation was determined to be present if there was a progressive decrease in peak current amplitude during 1.7-sec pulses to +10 mV applied at a frequency of 1 Hz.

[d] The time constant describing current inactivation was determined by fitting single-exponential curves to the current decays at +70 mV.

[e] The time course of recovery from inactivation was determined using a paired-pulse protocol. Pairs of identical pulses to +10 mV, 3.2 sec in duration, separated by various intervals were applied from a holding potential of −60 mV. The ratio of the peak current during the second pulse to that of the first pulse was plotted as a function of the interval between pulses and fitted to a single exponential.

[f] The voltage dependence of steady-state inactivation was determined by varying the holding potential and recording the peak current at a constant test potential of +20 mV. The data points were fitted with a Boltzmann function, and the midpoint determined from the fit.

ND indicates that the experiments were not performed.

nous LPS. The expression of $I_{K,A}$ was detected in 36% of the cells (n = 321) which had been treated with LPS for time periods between 4 and 24 hr throughout development in culture. A similar response (33%, n = 55) was obtained if the cells were treated with the cytokine IL-2 (100 U/ml) for 4–17 hr. A somewhat smaller percentage of cells (19%, n = 16) demonstrated current expression when treated with PAF (20 ng/ml) for a 24-hr period. Neither the phorbol ester, PMA, (1.0 μg/ml) nor the cytokine γ-IFN (10 U/ml) enhanced current expression over that observed in control cells. Treatment of the cells with LPS in combination with either PMA, γ-IFN, PAF, or γ-IFN plus PAF did not enhance current expression over that obtained with LPS alone. A summary of these results is given in Table 2.

Expression of $I_{K,A}$ is Inhibited by Cycloheximide

To determine whether the enhanced current expression was dependent upon protein synthesis or whether the LPS induction of current activation was simply due to an unmasking of channels present in the membrane, cells were treated with the protein synthesis inhibitor cycloheximide. Cycloheximide (100 μM) has been shown to inhibit greater than 90%

Table 2. Summary of experiments demonstrating induction of $I_{K,A}$ current expression following exposure of HMDMs to a number of stimuli known to induce priming and activation in macrophages, resulting in enhancement of secretory processes as well as tumoricidal function

Group	Treatment	Percentage of cells expressing $I_{K,A}$ current	
		No LPS	LPS (2 μg/ml)
1	Control media	0 (n = 602)	36 (n = 321)
2	PMA (1.0 μg/ml)	0 (n = 5)	32 (n = 70)
3	PMA (0.01 μg/ml)	—	32 (n = 19)
4	γ-IFN (10 U/ml)	0 (n = 18)	30 (n = 138)
5	PAF (20 ng/ml)	—	19 (n = 16)
6	PAF + γ-IFN	—	35 (n = 20)
7	IL-2 (100 U/ml)	33 (n = 55)	—

Cells were treated for time periods between 4 and 24 hr with either γ-IFN (10 U/ml), LPS (2 μg/ml), PMA (1 μg/ml), or IL-2 (100 U/ml) alone, or a combination of LPS and either (i) γ-IFN, (ii) PMA (0.01 or 1 μg/ml), (iii) PAF (20 ng/ml) or (iv) γ-IFN and PAF. Note that LPS alone was as effective in inducing current expression when compared to combinations of activating stimuli. IL-2 was as effective as LPS in enhancing current expression. Currents were activated using the voltage-pulse protocol described in Fig. 1. Current identification was made on the basis of (i) the presence of time-dependent current inactivation in the depolarizing voltage range, (ii) the threshold of current activation at approximately −40 mV, and (iii) the presence of steady-state inactivation.

Table 3. Effect of cycloheximide (CHX) on LPS-induced $I_{K,A}$ current expression in HMDMs

Treatment	Percentage of cells expressing $I_{K,A}$ current	Input resistance (GΩ)
LPS	81.5 (n = 27)	2.7 ± 0.4 (n = 19)
LPS + CHX	0 (n = 23)	7.0 ± 1.3 (n = 15)

Cells were exposed to CHX (1 μg/ml) and LPS (2 μg/ml) simultaneously. Cells were exposed either to LPS or LPS plus cycloheximide for 4–8 hr at which point current activation/expression was determined. Experiments were carried out on 3–7-day-old cells (from three separate isolations) which were maximally responsive to LPS with respect to enhancement of current expression. Input resistance was determined over the voltage range of −80 to −50 mV on 2–3-day-old cells from the same donor.

of ^{3}H-leucine incorporation into the murine macrophage, J774 (McKinney & Gallin, 1990). HMDMs were exposed to cycloheximide (3.6 μM [1 μg/ml]) and LPS simultaneously. As can be seen in Table 3, cycloheximide abolished current expression in LPS-treated cells. Because prolonged treatment of cells with cycloheximide is likely to decrease viability, current expression was not tested for periods beyond 8 hr. Experiments were carried out on 3–7-day-old cells in which current expression was detectable during the first 8 hr of LPS exposure (*vide infra*). The input resistance of cells treated with cycloheximide did not differ significantly over that observed for control cells (2.7 ± 0.4 GΩ (n = 19) for control cells and 7.0 ± 1.3 GΩ (n = 15) for cells treated with cycloheximide) and was equivalent to input resistances previously reported for HMDMs over the same period of differentiation (Nelson et al., 1990*a*). Thus, cell viability remained constant over the time period of 8 hr in the presence of cycloheximide.

Magnitude and Time Course of $I_{K,A}$ Expression is a Function of Culture Age

The time course of $I_{K,A}$ current expression in HMDMs following LPS treatment was a function of the time following the introduction of the freshly isolated cells into adherent or suspension culture. In general, 29% (n = 38, from three donors) of the cells treated with LPS on the day of isolation began to show current expression at 2 hr. This is in contrast to experiments performed on cells at later times in culture in which current expression was not detected until at least 4 hr following exposure to LPS. Based on the observation that the early cells demonstrated a somewhat higher percentage of $I_{K,A}$ expression with a faster time course, we examined the magnitude and time course of LPS-induced current expression in two separate donors. A summary of these experiments is given in Fig. 3*A* and *B*. In 2–4-day-old cells $I_{K,A}$ activation was first detected at 4 hr; the percentage of cells expressing current increased with a variable time course which appeared to be dependent upon the donor. In 6–9-day-old cells, current expression was not detected until about 6–7 hr and, in general, the percentage of cells expressing current was reduced over that observed for the 2–4-day-old cells. If, however, the media was changed in the 7–9-day-old cells immediately prior to the addition of LPS to the culture both the time course and magnitude of current expression shifted to values obtained for the younger cells. Data is given for a single donor but a similar effect was observed in two other donors. It appears then that cells at later times in culture are less able to respond to LPS as an activation stimulus possibly due to depletion of important serum factors. Thus, as the summary data given in Table 2 detailing the percentage of current expression as a function of activation stimulus is an average value over time in culture it is likely to represent an underestimate of current expression for cells at early times in culture.

Correlation of Secretion with Current Expression

Macrophages which have undergone activation exhibit an enhanced rate of secretion for a wide variety of molecular products including enzymes, coagulation factors, arachidonic acid metabolites, cytokines, and reactive oxygen intermediate (for a review *see* Adams & Hamilton, 1988). Secretory heterogeneity has been demonstrated in HMDMs (Lewis et al., 1989), and therefore, we investigated whether the heterogeneity in current expression which we observed in the LPS-activated cells could be related to the secretory state. We used the RHPA which unambiguously identifies single secretory cells in combination with the voltage-clamp determination of current expression to determine whether the enhanced expression of $I_{K,A}$ could be correlated with secretion.

Figure 4*A* and *B* illustrates plaque formation due to lysozyme release from LPS-stimulated HMDMs. The secretion of lysozyme, IL-1, and TNF-α was determined in separate experiments. Current recordings were made on equal numbers of secretory as well as nonsecretory cells for each culture. Recordings were made on a range of secretory HMDMs with different cell diameters which formed plaques of different diameters. Figure 4*C* summarizes correlative data pooled from 37 separate assays, demon-

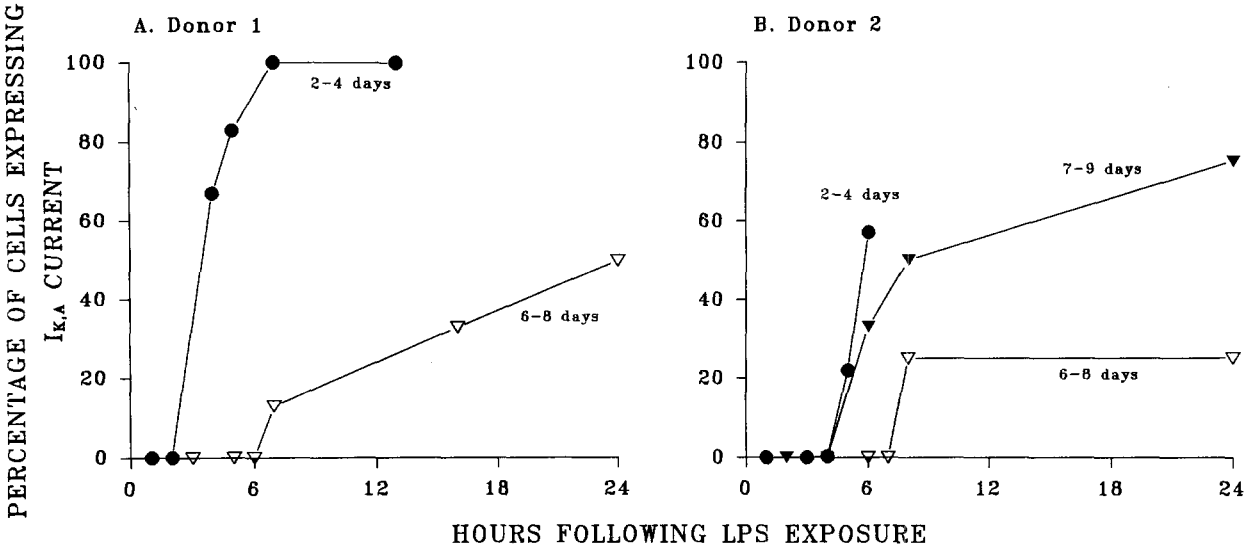

Fig. 3. Time course of LPS-induced current expression. HMDMs maintained in adherent culture for varying periods of time were exposed to LPS (2 μg/ml). The presence of $I_{K,A}$ was probed for with time following exposure of the cells to LPS. Experimental data is from two donors and plotted separately in *A* and *B*. Experimental data is pooled from three separate isolations in *A* and five isolations in *B*. Each data point in *A* and *B* represents data obtained from 3 to 13 cells. Frequency of current expression was monitored over a 24-hr period. Current expression was never observed during the first 1–3 hr following LPS exposure in cells beyond the day of isolation (*see* text). Time-dependent $I_{K,A}$ currents were first observed in cells after 4 hr of incubation in cells differentiated for 2–4 days in culture. The percentage of cells expressing time-dependent current expression was maximal in cells which had been maintained in culture for 2–4 days. In cells which had been cultured for 6–9 days, current expression was not observed until at least 7 hr following LPS treatment and the percentage of current expression was significantly decreased over that observed for the 2–4-day-old cells. If the media was changed on the cells which had been maintained in culture for 7–9 days immediately prior to adding the LPS the time course of current expression was shifted to much shorter times with an enhancement of the percentage of cells expressing current. (*A*) Donor 1: LPS current expression in 2–4-day-old cells (filled circles) and in 6–8-day-old cells (open triangles). (*B*) Donor 2: Current expression in 2- to 4-day-old cells (filled circles), in 6- to 8-day-old cells (open triangles) and in 7- to 9-day-old cells following media change (filled triangles).

strating that an enhancement of $I_{K,A}$ current expression was not preferentially observed in cells secreting either lysozyme or IL-1. A significant decrease in current expression was observed in cells actively secreting TNF-α (33.3% in nonsecretory cells, 8% in secretory cells). The frequency of current expression in the lysozyme-secreting cells (28%) was similar to that observed for nonsecretory cells in the same culture (26%). In separate assays, similar results were obtained for the IL-1 secreting cells, 42% of which expressed $I_{K,A}$ current compared to the nonsecretory cells, 35% of which showed enhanced K^+ current expression.

Frequency of Plaque Formation as a Function of Activation Stimulus

A summary of the frequency of plaque formation for four activation stimuli: LPS, LPS + PMA, IL-2, and IL-2 + PMA, is given in Table 4. Under control conditions, attachment provided a sufficient secretory stimulus but not a sufficient stimulus for current expression. The percentage of plaque-forming cells was determined for lysozyme, IL-1, and TNF. TNF secretion (plaque formation) was not observed under control conditions. Plaque formation due to lysozyme or IL-1 secretion was detectable following a 4-hr incubation of the cells with or without the addition of exogenous activation stimuli. Plaque formation was determined for HMDMs maintained in suspension culture for periods of 0 to 2 days following isolation. Trypan blue exclusion, a measure of cellular viability, was determined to be greater than 90% in similar incubation studies for all of the compounds tested. Due to the high degree of plaque-formation variability from experiment to experiment for any given stimulus, there was no significant difference in the frequency of plaque formation between activation stimuli. It should be noted that we did not attempt to quantitate plaque size as a function of activation stimulus. An enhancement of secretion could possibly have been detected as a shift in the plaque size distribution in the presence of activation stimuli. Lewis et al. (1989) have shown that simultaneous treatment of monocytes with both LPS and PMA

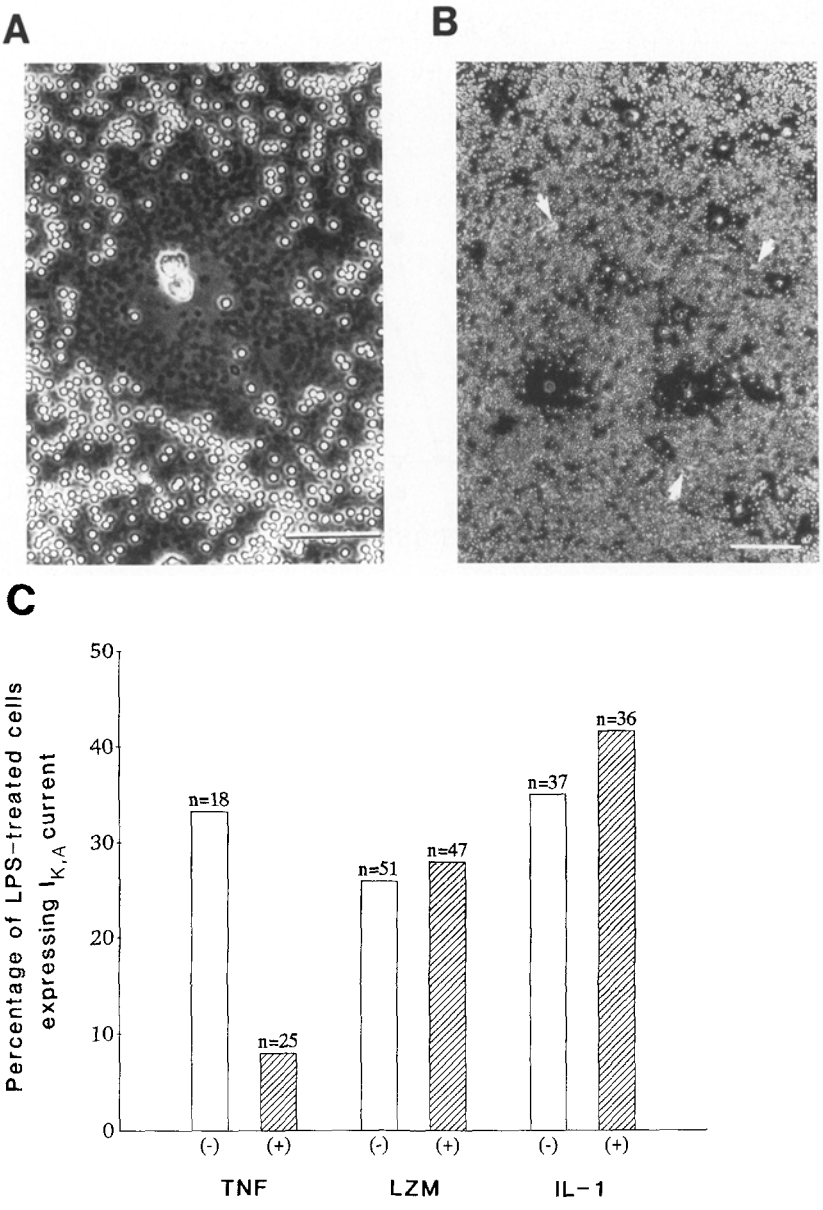

Fig. 4. Correlation between $I_{K,A}$ current expression and either TNF-α, IL-1 or lysozyme secretion in the RHPA. (*A*) Photomicrograph of a viable LPS-stimulated HMDM at the center of a lysozyme plaque. Note the *o*RBC ghosts seen in the plaque surrounding the secretory cell. Similar plaques were formed in non LPS-containing media where attachment provided the secretory stimulus. Calibration bar = 50 μm. (*B*) Low magnification photomicrograph of plaques formed around LPS-induced lysozyme-secreting cells. Note the wide variation in plaque size which has been correlated with HMDM cell size (Lewis et al., 1989). Arrows mark nonsecretory cells. Calibration bar = 100 μm. (*C*) Correlation of TNF-α, lysozyme, and IL-1 secretion with $I_{K,A}$ current activation. Whole-cell voltage-clamp recordings were made on nonplaque-forming (nonshaded bars) and plaque-forming (shaded bars) cells maintained under the same experimental conditions. The number of cells in each experimental group are indicated above each bar. Secretion of lysozyme, IL-1, and TNF-α was induced by incubation of the cells with LPS for 2–8 hr. TNF-α secreting cells demonstrated a significantly lower frequency of $I_{K,A}$ current expression than nonplaque forming cells, whereas current expression was equivalent between the lysozyme- or IL-1 secreting and nonsecreting cells. It should be noted that plaques were developed in the assay prior to the electrophysiological experiments, and therefore, may not reflect the secretory activity of the cells at the time of recording. Data is pooled from 37 separate RHPA experiments. The total number of cells in each experimental condition is given at the top of each bar. Approximately the same number of plaque-forming *versus* nonplaque forming cells were voltage clamped in each assay.

resulted in significantly larger IL-1 plaques than those formed in the absence of activation stimuli or those formed by exposure to LPS, γ-IFN, or PMA alone. In a similar set of studies on lysozyme secretion in HMDMs, Lewis et al. (1990*a*) were able to demonstrate that while the frequency of HMDMs forming lysozyme plaques is reduced in the presence of LPS, average plaque size increased.

Discussion

When HMDMs are activated using LPS or IL-2 with or without the addition of γ-IFN or PMA, the frequency of expression of the transient-inactivating outwardly rectifying, $I_{K,A}$, current is greatly enhanced. The time course as well as the magnitude of the frequency of $I_{K,A}$ expression in response to an activation stimulus is dependent upon the age of the cells in culture. Enhanced current expression is inhibited in the presence of cycloheximide, an inhibitor of protein synthesis, indicating that LPS appears to be inducing new protein synthesis and is not merely unmasking pre-expressed "silent" channels. LPS treatment of HMDMs, in addition, produces an increase in the membrane capacitance over that observed for control cells. However, no correlation was found between $I_{K,A}$ peak current and cell size, demonstrating that the expression of K^+ channels is not proportional to the increase in membrane

Table 4. Frequency (in percent) of lysozyme, IL-1, and TNF plaque-forming cells as a function activation stimulus

Plaque type	Activation stimulus				
	Control	LPS	LPS + PMA	IL-2	IL-2 + PMA
Lysozyme	37 ± 8.0 (n = 6) 359 cells	33 ± 6.0 (n = 6) 487 cells	ND	41 ± 6.0 (n = 3) 599 cells	37 ± 5.2 (n = 3) 231 cells
IL-1	16 ± 8.6 (n = 5) 425 cells	27 ± 13.9 (n = 5) 245 cells	43 ± 14.9 (n = 5) 231 cells	18 ± 3.5 (n = 3) 488 cells	20 ± 3.4 (n = 3) 514 cells
TNF	0 (n = 3)	14 ± 4.1 (n = 3) 481 cells	ND	ND	ND

Secretion data are given as the average percentage of plaque-forming or secretory cells in separate RHPA experiments (isolations) with the number of experiments given in parentheses, followed by the total number of cells, both secretory and nonsecretory, in all experiments. Total cell counts were not determined in those experiments where plaque formation was absent, i.e., TNF secretion in the absence of a secretory stimulus. Secretion of lysozyme, IL-1, or TNF was determined as a function of the following activation stimuli: LPS (2 μg/ml), IL-2 (100 U/ml), and either LPS or IL-2 plus PMA (0.1 μg/ml). The percentage of plaque-forming cells in drug- and nondrug-containing solutions was determined in blind experiments. The plaque-forming cells were counted in a representative field at a total magnification of 100×. Experiments not performed are indicated by the abbreviation ND.

surface area. Finally, the expression of K^+ current showed no apparent correlation with LPS-induced lysozyme or cytokine secretion where individual secretory cells were identified in an RHPA.

LPS Modulation of K^+ Channel Expression

LPS-induced enhanced outwardly rectifying K^+ channel expression has been observed for B lymphocytes entering the G1 phase of the cell cycle (Amigorena et al., 1990); LPS-activated B lymphocytes express a fivefold increase in channel density as compared to controls. However, functional K^+ channels do not appear to be essential for the transition of B lymphocytes from the G0 to the G1 phases but do appear to be critical to the proliferative response. In contrast to the enhancement of K^+ channel expression observed for the B lymphocyte and the HMDM, murine macrophage J774 cells responded to LPS treatment with an increase in membrane area but failed to express an upregulation of the inwardly rectifying K^+ channel sufficient to be detected as an increase in specific membrane conductance (McKinney & Gallin, 1990). It is notable that LPS does not induce expression of an outwardly rectifying K^+ current in J774.1 cells as shown by McKinney & Gallin (1990).

K^+ Channel Expression and Secretion

In recent years, a number of studies have been directed at determining direct experimental evidence for a causal link between changes in K^+ channel expression and the modulation of functional activity in phagocytic cells (*see* review by Gallin & McKinney, 1990). The present studies were conducted in an attempt to clarify the role of the fast-inactivating $I_{K,A}$ current in the modulation of the secretory response in HMDMs. The observation that the K^+ channel blocker, 4-AP, inhibited IL-1 production by LPS-stimulated adherent macrophages (Gupta et al., 1985) suggested that the current expression and secretion might well be correlated.

The RHPA has been used to demonstrate functional secretory heterogeneity among mononuclear phagocytes (Lewis et al., 1990*a*) as well as to visualize and quantify the secretion of lysozyme, IL-1, and type-β transforming growth factor by individual monocytes and macrophages (Lewis et al., 1989). Plaque size is proportional to the amount of secretory protein released; therefore, secretory heterogeneity can be assayed not only as the number of cells forming plaques but also as the range of plaque diameters associated with secretory cells. Plaque formation, thus, allows one to determine whether LPS-induced K^+ channel expression is restricted solely to secretory cells.

We have found that enhanced K^+ channel expression is not solely restricted to secretory cells. The frequency of channel expression was approximately equal for nonsecretory cells as for cells identified as secreting lysozyme or IL-1. The frequency of K^+ channel expression in cells secreting TNF was significantly reduced over that of the nonsecretory cells in the same culture, indicating a possible auto-

crine or paracrine effect of TNF-α on gene expression. Exogenous TNF has been shown to inhibit γ-IFN-induced F_c receptor expression directly, mimicking the inhibition produced by LPS of the γ-IFN-induced F_c receptor upregulation (Arend, Ammons & Kotzin, 1987). It is thought that the LPS inhibition of γ-IFN-enhanced F_c receptor expression on human monocytes occurs in an autocrine fashion via stimulation of TNF production.

The fact that we were unable to demonstrate a correlation between enhanced current expression and secretion in individual cells is difficult to reconcile with the earlier observations of Gupta et al. (1985), demonstrating that the K^+-channel blocker, 4-AP, inhibited IL-1 secretion in a population of LPS-activated adherent macrophages. Functional K^+ channels may well be required for normal transport of essential metabolites required to support secretion, as TEA and 4-AP have been found to inhibit uptake of thymidine and phenylalanine by cloned T cells (Schell et al., 1987). As the studies of Gupta and co-workers (1985) did not include an electrophysiological examination of the secreting cells, it is difficult to ascertain whether the inhibition of secretion was associated with a specific inhibition of $I_{K,A}$ channel activation.

K^+ Channels May Play a Role in the Regulation of the Ca^{2+} Influx Seen in the Second Stage of Macrophage Activation

In the absence of an agonist-activated, voltage-insensitive Ca^{2+} influx pathway, K^+ channel expression alone would not be expected to enhance Ca^{2+}-dependent secretion or degranulation. The K^+ channels induced during the initial priming stages of macrophage activation could provide the basis for a hyperpolarization-driven Ca^{2+} influx seen following surface receptor ligation during the second stage of activation. Hyperpolarization-driven Ca^{2+} influx has been described for secretagogue-stimulated mast cells (Matthews, Neher & Penner, 1989*a*,*b*), anti-IgM-stimulated B cells (MacDougall, Grinstein & Gelfand, 1988), and mitogen-activated leukemic T cells (Lewis & Cahalan, 1989). Our observation that an enhancement of K^+ channel expression was not accompanied by an increase in secretion as determined by an increase in the percentage of IL-1 or lysozyme plaque-forming cells following LPS or IL-2 treatment may be explained by a requirement for a secondary signal which provides a receptor-operated calcium-influx pathway. The secretion of IL-1 by both murine and human macrophages is markedly enhanced in the presence of calcium ionophores that induce a pronounced elevation in intracellular calcium, a response which is abrogated in the absence of external calcium (Suttles, Giri & Mizel, 1990). The influx of extracellular calcium that accompanies receptor-ligand interaction via the agonists zymosan and concanavalin A appears to be directly involved in the activation of macrophage phospholipase A_2, leading to the release of arachidonic acid in murine macrophages (Balsinde, Fernández & Diez, 1990). The dependence of lysozyme secretion is less well understood. Delta toxins which form pores across the plasma membrane and induce a rapid influx of calcium into human neutrophils are capable of stimulating lysozyme release only at high concentrations (Kasimir et al., 1990). In contrast, 10 μM Ca^{2+}-induced lysozyme release in streptolysin O permeabilized HL60 cells differentiated into a neutrophil-like phenotype (Trudel et al., 1990).

The fact that a basal level of secretory activity was observed for the cytokines as well as lysozyme in the absence of either a priming or triggering signal suggests that attachment may itself provide a sufficient secretory stimulus which may be independent of long-term ion-channel activation. A comparison of the basal secretory rate for IL-1 *versus* lysozyme in the absence of exogenous stimulation (16 *versus* 37% of the cells formed plaques) would suggest that not only the magnitude but also the regulation of the secretory response to a given stimulus (in this case attachment as well as LPS) may well be different for IL-1 and lysozyme.

In summary, LPS activation of HMDMs increases the number of K^+ channels on the macrophage cell membrane. In addition, LPS also induces an increase in membrane capacitance over that observed for control cells. Although K^+ channel expression appears to be independent of the secretory state of the cell in the presence of low levels of LPS, K^+-channel activation upon depolarization may be involved in the regulation of Ca^{2+} influx that occurs following exposure of the macrophage membrane to a second triggering stimulus, thereby augmenting the secretory response over that seen with LPS alone.

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