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Membrane Potential Modulates Release of Tumor Necrosis Factor in Lipopolysaccharide-stimulated Mouse Macrophages

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Lipopolysaccharide (LPS)-mediated synthesis of macrophage gene products such as tumor necrosis factor (TNF) is controlled by different signaling pathways. We investigated intracellular free Ca^{2+} (Ca^{2+}_{ic}) and the membrane potential as early cellular responses to LPS and their role in the synthesis and release of TNF. In peritoneal macrophages and in the RAW 269 mouse macrophage cell line, LPS and its biologically active moiety lipid A stimulated TNF synthesis but exerted no significant effects on these early cellular responses using Fura-2/Indo-1 to measure Ca^{2+}_{ic} and bis-oxonol, as well as the patch-clamp technique to monitor membrane potential. In contrast, the platelet-activating factor transiently induced both an increase in Ca^{2+}_{ic} and cell membrane depolarization but no significant TNF release. Increased extracellular K^+ concentrations or K^+-channel blockers, such as quinine, tetraethylammonium, or barium chloride, inhibited the LPS-stimulated release of TNFα, as well as the accumulation of cell-associated TNFα as found by enzyme-linked immunosorbent assay analysis, but did not inhibit TNFα mRNA accumulation. Concentrations of quinine (>125 μM) or of enhanced extracellular K^+ (25–85 mM) required to inhibit TNF production both significantly depolarized macrophages. These results indicate a lack of ion transport activities as early cellular responses of macrophages to LPS but suggest an important regulatory role of the membrane potential on the posttranscriptional synthesis and release of TNF in macrophages.

INTRODUCTION

The synthesis of many secretory molecules is known as a consequence of macrophage stimulation with lipopolysaccharide (LPS). However, cellular mechanisms involved in LPS-mediated macrophage activation still are not fully understood.

Binding of lipid A, the biologically active moiety of LPS, to macrophage proteins, as well as binding of LPS complexed with a serum-binding protein to the monocyte differentiation antigen CD14 (Hampton *et al.*, 1988; Schumann *et al.*, 1990; Wrigth *et al.*, 1990), activation of GTP-binding proteins (Jakway and DeFranco, 1986), and phosphatidyl-inositide hydrolysis (Wightman and Reatz, 1984; Prpic *et al.*, 1987), has been reported as early consequences of macrophage interaction with LPS. A role of Ca^{2+} in LPS-stimulated tumor necrosis factor (TNF) release is still controversial (Newton, 1987; Prpic *et al.*, 1987), whereas the platelet-activating factor (PAF) has been shown to induce an increase in intracellular free calcium (Ca^{2+}_{ic}) (Randriamampita and Trautmann, 1989). Different signaling pathways may control macrophage genes stimulated with LPS (Introna *et al.*, 1987; Tannenbaum *et al.*, 1988). Multiple kB type enhancers are involved in the transcriptional activation of the mouse TNF gene, but additional factors are also necessary for its expression (Shakhov *et al.*, 1990). In response to LPS, transcription of the TNF gene is accelerated 3-fold, and as TNF protein secretion increases ~10 000-fold, much of the regulation has to occur posttranscriptionally (Beutler, unpublished data). Human TNF is derived from a precursor that contains 76 additional amino acids attached to the N-terminus of the mature protein and lacks a signal peptide sequence characteristic for secretory proteins (Junming and Vilcek, 1987).

The membrane potential is discussed to affect secretory processes (Gelfand *et al.*, 1987; Mohr and Fewtrell,

1987), and a delicate regulation of the membrane potential by a large number of different K^+ channels has been demonstrated (for review, see Cook, 1988). K^+ channels and the membrane potential are clearly shown to be involved in the regulation of immune cells: antibodies against the T-cell receptor open ligand-gated channels (MacDougall *et al.*, 1988), expression of K^+ channels is upregulated because of T-cell differentiation, and K^+ channels modulate multiple T-cell functions (Chandy *et al.*, 1984, 1986; McKinnon and Ceredig, 1986; LeFever and Liepins, 1990; for review, see Lewis and Cahalan, 1988). The specific K^+-channel inhibitor, charybdotoxin, inhibits proliferation and IL-2 production in human peripheral blood lymphocytes (Price *et al.*, 1989), and K^+-channel blockers, like tetraethylammonium (TEA) and 4-aminopyridine, inhibit T-lymphocyte proliferation (Schell *et al.*, 1987). T-cell mitogens (Tsien *et al.*, 1982; Gelfand *et al.*, 1987) cause changes in T-cell membrane potentials, and membrane depolarization selectively inhibits receptor-operated calcium channels in T lymphocytes (Sarkadi *et al.*, 1990). In B lymphocytes, functional K^+ channels have been shown to be upregulated due to mitogen stimulation (Choquet and Korn, 1988).

This study investigated $Ca^{2+}{}_{ic}$ and the membrane potential as early macrophage responses to LPS and, in particular, the role of the membrane potential in LPS-stimulated TNF synthesis and release. The membrane potential of macrophages was measured by the patch-clamp technique, as well as by the fluorescent dye bis-oxonol. With both techniques, we observed a significant depolarization of macrophage membrane potential by quinine and increased extracellular K^+ concentrations correlating with a pronounced decrease of the LPS-stimulated release of TNF bioactivity, as well as of cell-associated TNFα, whereas TNFα mRNA accumulation was not effected.

MATERIALS AND METHODS

Reagents and Solutions

LPS from *Salmonella abortus equi* prepared by phenol water extraction, apamin, ionomycin, valinomycin, and quinine was bought from Sigma (St. Louis, MO). A recombinant rat γ-IFN and human TNFα were purchased from Genzyme (Boston, MA) and Dulbecco's modified Eagle's serum (DMEM) and fetal calf serum (FCS) from GIBCO (Grand Island, NY). Fura-2/AM, Indo- 1/AM, bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA)/AM, bis-oxonol (DiSBAC2(3)), gramicidin, and ouabain were purchased from Molecular Probes (Junction City, OR). PAF was from Boehringer Mannheim (Mannheim, Germany) and charybdotoxin from Rec. Res. Chem. (Baltimore, MD). Stock solutions of Fura-2/AM, Indo-1/AM, Bapta/AM, bis-oxonol, and ouabain were prepared with dimethyl sulfoxide (DMSO). PAF, ionomycin, and gramicidin were dissolved in ethanol. Cells were never exposed to >1% ethanol or 0.1% DMSO.

Cells

Resident or thioglycollate-elicited peritoneal exudate cells were collected from C3H/HeHan mice by peritoneal lavage, washed, resuspended in DMEM medium, and placed in culture plates. Adherent cells were determined to be >95% pure macrophages. Peritoneal macrophages were maintained under adherent culture conditions for ≥16 h before LPS stimulation. Alternatively, the airway resistance (RAW) 264 mouse macrophage cell line was cultured in monolayers using DMEM supplemented with 10% FCS. All culture media were controlled using the Limulus amoebocyte lysate assay showing LPS concentrations <15 pg/ml. Cell viability was checked using trypan blue exclusion.

TNF and Prostaglandin E_2 (PGE_2) Assay

Assays for TNF were performed as reported earlier (Haslberger *et al.*, 1988). Briefly, mouse peritoneal macrophages primed with mouse γIFN (500 U/ml) for 16 h or RAW cells were placed in culture plates at a final concentration of 1×10^6 cells/ml and allowed to adhere for 4 h. Alternatively, suspended macrophages were held under constant stirring. Subsequently, cells were washed and stimulated with LPS at the indicated concentrations for 5 h. Inhibitors were given 10 min before LPS. Each experiment was performed in triplicate. Conditioned media were assayed in duplicate for TNF by adding serial dilutions of supernatants to confluent cultures of L929 cells in the presence of 4 μg/ml actinomycin D. The plates were then stained with Giemsa, quantitated using a Titertek-multiscan, and TNF activity was defined as the amount required to lyse 50% target cells. In each experiment, three samples were assayed for TNF activity in duplicate, and the mean dilution found to lyse 50% target cells was calculated. Comparison with a TNFα standard curve yielded TNF activities (U/ml) as shown in Figures 1 and 4. Alternatively, a commercially available mouse TNFα enzyme-linked immunosorbent assay (ELISA) kit (Genzyme) was used, and TNFα is expressed in pg/ml (mean ± SD) (see Figure 7). Cell-associated TNFα was determined in 100 000 g supernatants after three washes of the stimulated cells with phosphate-buffered saline, lyses of cells by three freeze-thaw cycles, and sonification. PGE_2 was determined using a ^{125}J radioimmunoassay (New England Nuclear, Vienna, Austria).

$Ca^{2+}{}_{ic}$ Measurement

Thioglycollate-elicited mouse peritoneal exudate macrophages or RAW cells were suspended in Hank's medium supplemented with 5% FCS using a cell scraper and loaded with Fura-2/AM (5 μM) or Indo-1 (5 μM) at 37°C for 20 min (Grynkiewicz *et al.*, 1985). Similarly, macrophages were loaded with BAPTA-AM (20 μM). The Ca^{2+}-fluorescence indicators and BAPTA seem not to interfere with TNF production because loaded peritoneal macrophages were still responsive to LPS. Thereafter, cells were washed, adjusted to 2×10^6 cells/ml in Hank's medium, and 3-ml cell suspension was added to quartz cuvettes and maintained at 37°C in a thermostated cuvette holder under constant stirring with a small magnetic bar. Fluorescence was measured using a Perkin Elmer (Norwalk, CT) LS-5 fluorescence spectrometer. Excitation and emission wave length were 339 and 505 nm for Fura-2 and 330 and 400 nm for Indo-1, respectively. $Ca^{2+}{}_{ic}$ is expressed as relative fluorescence units.

Measurements of Membrane Potentials Using Bis-Oxonol (DiSBAC2(3))

RAW cells were suspended in Hank's medium and added to cuvettes as described above. Bis-oxonol (Mohr and Fewtrell, 1987; Fasolato *et al.*, 1988) was added to the cells in a final concentration of 100 nM. The dye was allowed to equilibrate with the cells for 5–10 min before the experiment was started. Bis-oxonol-loaded macrophages showed a diminished TNF release (measured after 5 h) obviously resulting from cell toxicity as confirmed by trypan blue exclusion tests. However, cell viability was not significantly decreased for ≥30 min, which is far above the time periods required to measure macrophage depolarization by KCl, quinine, or PAF. Fluorescence was excited at 540 nm, and emission was monitored at 580 nm. Addition of gramicidin (25

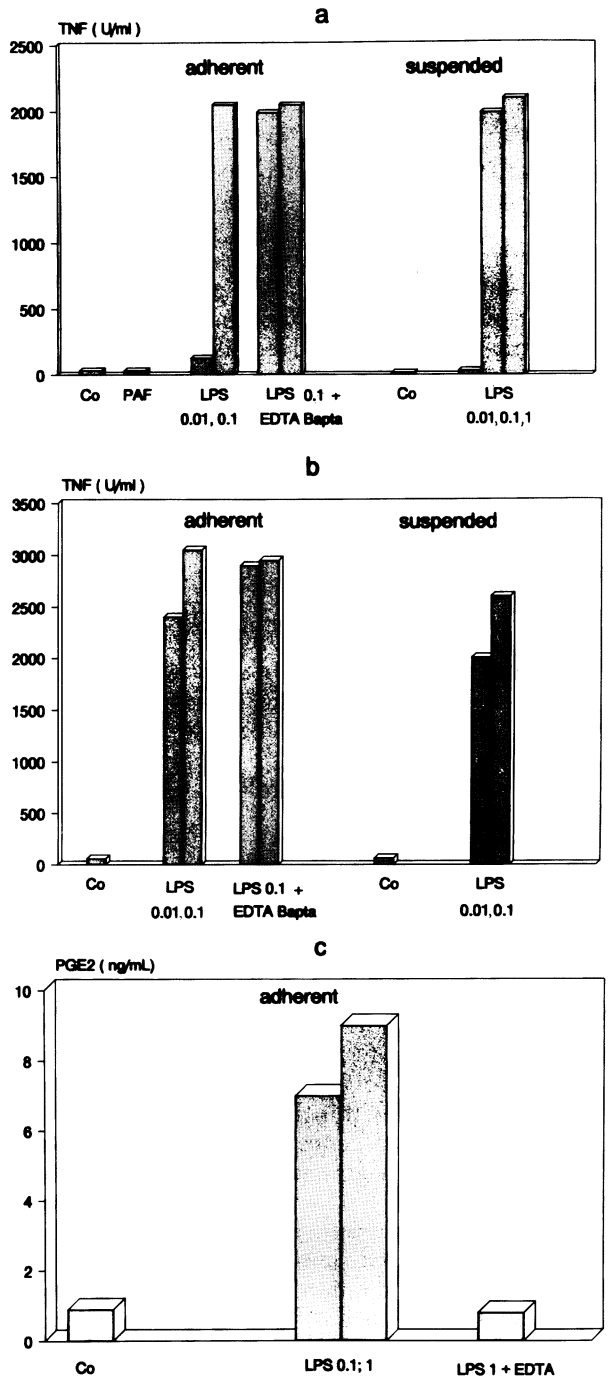

Figure 1. TNF and PGE_2 release from macrophages. LPS (0.01, 0.1, and 1 μg/ml) stimulated the release of TNF from adherent as well as suspended IFN primed peritoneal mouse macrophages (a) and RAW cells (b) as determined by the L929 lysis assay. PAF (200 nM) did not stimulate TNF release (a). Addition of EDTA (2 mM) to the extracellular medium did not significantly affect TNF release in peritoneal (a) or RAW (b) macrophages. TNF response of BAPTA-loaded adherent macrophages was also not changed in either cell type (a and b). In contrast, PGE_2 synthesis of LPS (1 μg/ml) -stimulated peritoneal macrophages was virtually abolished on chelation of extracellular Ca^{2+} by 2 mM EDTA (c). Data shown represent typical results obtained in a series of at least five independent experiments.

μM) shown to depolarize completely cells (Levitt, 1990) is reflected by an increase in fluorescence. Membrane potential is expressed as relative fluorescence units.

Preparation of RNA and Northern Blot Hybridization

Thioglycollate-elicited peritoneal exudate cells from C3H/HeHan mice were plated on culture plates (3×10^7 cell/dish) for 4 h, washed, and the resulting macrophage-monolayers were incubated with the different reagents in DMEM medium. At the indicated times, accumulation of TNFα mRNA was measured. Total cellular RNA was extracted by guanidinium isothiocyanate extraction followed by cesium chloride density gradient centrifugation (Chirgwin *et al.*, 1979; Maniatis *et al.*, 1982). Twenty micrograms of RNA were run on 1% formaldehyde agarose gels and transferred to hybond N filters (Amersham, Arlington Heights, IL) in a northern capillary transfer using 20× SSC as buffer. Filters were then baked and prehybridized for 4 h. Hybridization with a TNFα end-labeled oligonucleotide probe was done at 60°C. The 26-mer oligonucleotide probe (5′ > CCA CCC ATG TGC TCC TCA CCC ACA CC < 3′) was synthesized on solid support using standard cyano-ethyl-phosphamidite chemistry. Hybridization with an actin control probe labeled with ^{32}P-dATP by random priming was done at 45°C. Filters were washed at 45° or 60°C and autoradiographed with an intensifying screen at −70°C.

Electrophysiological Experiments

RAW macrophages were bathed in a standard extracellular solution consisting of (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)/Na, pH 7.4. Patch-clamp experiments were performed at room temperature using a L/M-EPC5 amplifier. The current-clamp mode was employed to measure membrane potentials (Hamill *et al.*, 1981). In the current-clamp recordings, membrane capacitance was routinely checked to control whole-cell configuration. Soft glass pipettes with a resistance of 1.5–3 MΩ were used. The pipette solution contained (in mM): 140 KCl, 2 NaCl, 1 $MgCl_2$, 0.655 $CaCl_2$ (corresponding to a free Ca^{2+} concentration of 10^{-7} M), 1 EGTA, 10 HEPES/K, pH = 7.2. The recording chamber was perfused at a flow rate of 1 ml/min.

RESULTS

TNF Release is Independent of Ca^{2+}

LPS (0.1 and 1 μg/ml) stimulated the release of TNF from γ-IFN-treated peritoneal mouse macrophages (Figure 1a). Adherent macrophages responded to a similar extent as suspended cells. PAF up to a concentration of 200 nM did not induce TNF release (Figure 1a). In accordance with previous results (Newton, 1987), LPS-stimulated release of TNF was not inhibited by chelation of extracellular Ca^{2+} with 2 mM EDTA (Figure 1a). In contrast, the release of PGE_2 was almost completely abolished by the addition of EDTA to the extracellular medium (Figure 1c). The involvement of Ca^{2+} in TNF response was further investigated using Bapta-loaded peritoneal macrophages. Intracellular Bapta might prevent increases in Ca^{2+}_{ic} of LPS-stimulated macrophages. The TNF response was not, however, effected on intracellular application of BAPTA (Figure 1a).

Alternatively, the RAW 264 mouse macrophage cell line was employed to complement results obtained with peritoneal macrophages. TNF response from LPS-stim-

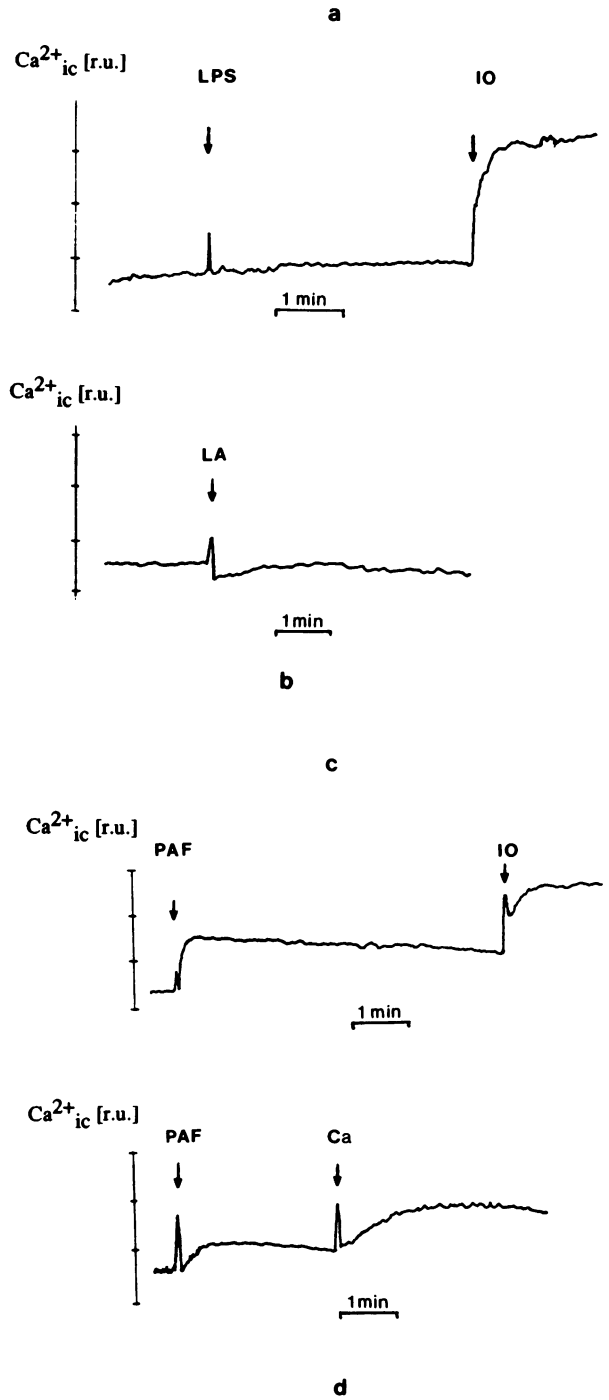

Figure 2. Ca^{2+}_{ic} responses of peritoneal mouse macrophages. Ca^{2+}_{ic} concentrations (rel. fluorescence units, [Ca^{2+}_{ic} r.u.]) were recorded in a LS5 spectrometer using Fura-2 or Indo-1. LPS or lipid A (LA) did not induce significant alterations of the Ca^{2+}_{ic} within 10 min using peritoneal macrophages (a, 5 μg/ml LPS; b, 2 μg/ml LA). Ionomycin (IO, 2 μM) reproducibly increased Ca^{2+}_{ic} to saturating fluorescence levels (see a and c, n = 4). PAF (c, 1 nM) induced an increase in Ca^{2+}_{ic}. Depletion of extracellular Ca^{2+} by 2 mM EDTA reduced the Ca^{2+}_{ic} response due to 10 nM PAF followed by an enhanced Ca^{2+} influx after recalcification to 2 mM Ca^{2+} (d). Data shown represent typical results from at least three independent experiments.

ulated RAW macrophages exhibited similar characteristics (Figure 1b) compared with γ-IFN primed peritoneal macrophages, i.e., TNF production and its independence of Ca^{2+}, even though RAW cells were somewhat more sensitive to LPS showing TNF release already at 0.01 μg/ml LPS (compare a and b). Thus, cellular signaling in response to LPS is apparently similar in both types of macrophage cells.

A lack of effect on TNF response either on removal of extracellular Ca^{2+} or chelation of intracellular Ca^{2+} in either type of macrophage suggests that Ca^{2+}_{ic} changes might not be involved as an early signal in LPS-stimulated macrophages. This hypothesis was directly tested by measurements of Ca^{2+}_{ic}.

LPS or Lipid A Exhibits No Effect on Ca^{2+}_{ic}

Macrophage cells were loaded with Fura-2 or, in more recent experiments, with Indo-1. Peritoneal mouse macrophages showed no significant increase in Ca^{2+}_{ic} in response to stimulation by LPS or lipid A in concentrations between 1 and 5 μg/ml (Figure 2, a and b). RAW cells behaved similarly. In contrast, PAF (10 pM–10 nM) induced significant Ca^{2+} responses in either macrophage (see Figure 2c). Removal of extracellular Ca^{2+} led to a reduction in Ca^{2+}_{ic} responses to PAF followed by an enhanced Ca^{2+} influx after recalcification (Figure 2d).

LPS or Lipid A Exhibits No Effect on Macrophage Membrane Potential

Bis-oxonol was used to measure plasma membrane potential because the negative charge of this dye may prevent significant accumulation in mitochondria (Mohr and Fewtrell, 1987). LPS or Lipid A did not induce a significant alteration of the bis-oxonol fluorescence in RAW macrophages within 5 min (Figure 3, a and b). The increase in fluorescence by a subsequent depolarization of the cells by gramicidin was also not affected by LPS or Lipid A.

Alternatively, membrane potential of RAW cells was monitored by the patch-clamp technique employing the current-clamp configuration. The resting level of Ca^{2+}_{ic} of macrophages has been reported to be around 10^{-7} M (Randriamampita and Trautmann, 1987). Therefore, an intracellular solution containing 10^{-7} M free Ca^{2+} was used to mimic physiological conditions. Under this condition, RAW macrophages exhibited a resting potential of −55±12 mV (mean ± SD, n = 39). In accordance with the results of bis-oxonol measurements, LPS (5–10 μg/ml) exerted no significant effect on membrane potential within a 5-min recording period (n = 5) (see below Figure 6a). In control experiments, membrane potential remained stable for ≥10 min.

In contrast, PAF dose dependently and transiently depolarized RAW cells as detected by bis-oxonol mea-

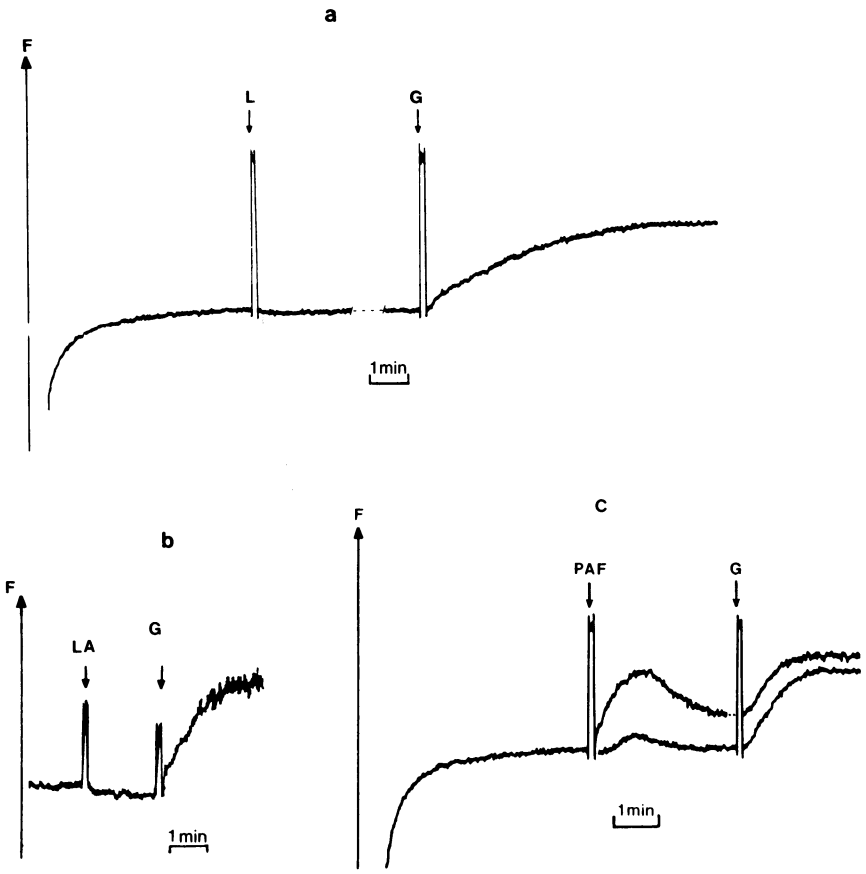

Figure 3. Membrane potential of stimulated RAW macrophages. Membrane potentials were monitored using bis-oxonol (DiSBAC2 (3), 100 nM) fluorescence. LPS (a, 5 μg/ml) and lipid A (b, 2 μg/ml LA) did not change the membrane potential of RAW macrophages within 6 min. PAF (c, 200 nM, 1 μM) induced a dose-dependent, incomplete, and transient depolarization of the cells. In each experiment, Gramicidin (G) further depolarized the cells as reflected by an increase in fluorescence. Membrane potential is expressed in relative fluorescence units (F). Data shown represent typical results from at least three independent experiments.

surements (Figure 3c). Depolarization was incomplete because gramicidin further enhanced fluorescence.

Taken together, these data indicate that LPS exhibits no effect on Ca^{2+}_{ic} and on membrane potential as early responses of macrophage activation.

Inhibition of TNF Release by Potassium Channel Blockers and By Increasing Extracellular K^+ Concentrations

The K^+-channel blockers TEA, barium chloride ($BaCl_2$), and quinine dose dependently inhibited the LPS-induced TNF release from peritoneal macrophages (Figure 4a). Almost identical results were obtained in RAW cells (Figure 4b). Effective concentrations are similar to those reported to block potassium channels (Kakuta *et al.*, 1988). None of these compounds, however, act specifically on one particular type of K^+ channel. The more specific K^+-channel blockers, apamin (50–100 nM) and charybdotoxin (10–100 nM), reported to block Ca^{2+}-activated K^+ channels (Blatz and Magleby, 1987), failed to inhibit TNF release in peritoneal macrophages (Figure 4a).

As K^+ channels are known to be involved in maintaining the resting membrane potential, we tested for the effect of progressive cell depolarization on TNF release. Stimulation of macrophages by LPS was performed at increasing concentrations of extracellular K^+ (25, 45, or 85 mM KCl). A dose-dependent inhibition of TNF release was found in peritoneal macrophages and in RAW cells (Figure 4, a and b; see below Figure 7a). Inhibition of the TNF response could already be seen at 25 mM K^+. This may be explained by the fact that an increase in extracellular K^+ concentrations might effect all kinds of K^+ channels, thus being most potent in cell depolarization.

Reversibility of the quinine- or KCl-induced inhibition of TNF release was demonstrated by a restimulation of RAW cells in standard DMEM medium after a 3-h incubation period in media containing quinine (500 μM) or elevated extracellular K^+ (85 mM) (Figure 4c). Application of gramicidin (25 μM), which was shown to depolarize macrophages (see Figure 3), led also to an inhibition of TNF release in LPS-stimulated peritoneal macrophages (Figure 4a). A similar inhibition of TNF release was found in the presence of valinomycin (5 μM), which might hyperpolarize macrophages toward the K^+-equilibrium potential of about −80 mV. However, cytotoxic effects due to prolonged exposure (5 h) of macrophages to the ionophores used cannot be excluded.

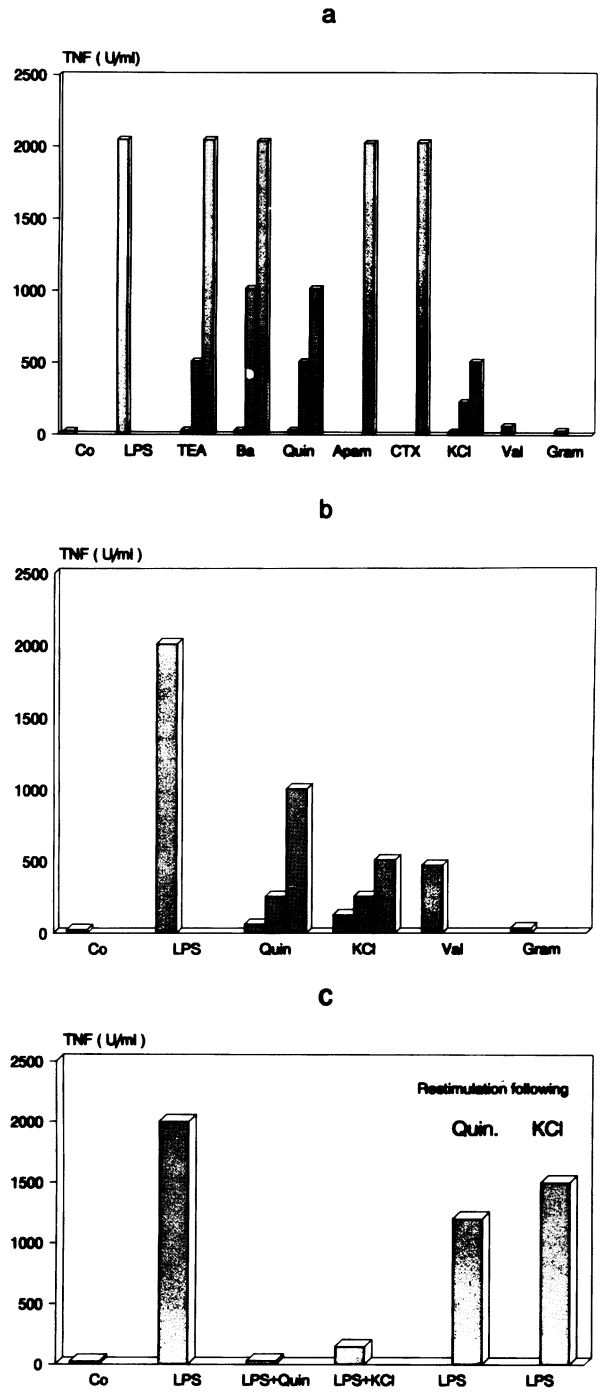

Figure 4. Inhibition of TNF release from LPS-stimulated macrophages. In peritoneal macrophages (a) the LPS (0.1 μg/ml)-stimulated release of TNF was inhibited dose dependently by TEA (60, 40, 20 mM), $BaCl_2$ (Ba; 20, 10, 1 mM), and quinine (Quin; 500, 250, 125 μM). Apamin (Apam; 100 nM) and charybdotoxin (CTX, 100 nM) failed to inhibit TNF release. Additionally, elevated concentrations of K^+ (85, 45, or 25 mM) and the ionophores valinomycin (Val; 5 μM) and gramicidin (Gram; 25 μM) inhibited TNF release (a). Similar results were obtained using RAW macrophages (b). Quinine (500 μM)- and KCl (85 mM)-induced inhibitions of LPS-stimulated TNF release in both macrophage cells were reversible as shown by TNF release from LPS (0.1 μg/ml)- restimulated RAW cells after removal of inhibitors (c). Data shown represent typical results of three to five independent experiments.

To strengthen the hypothesis that depolarization of macrophages modulates TNF response, we investigated the effect of K^+-channel blockers on macrophages' membrane potential.

Macrophages are Depolarized by High Extracellular K^+ and Quinine

RAW macrophages were progressively depolarized by increasing extracellular K^+ concentrations from 5 to 40, 80, 120, and 140 mM (addition of either K-aspartate or KCl) as shown by an increase in bis-oxonol fluorescence (Figure 5a). Membrane potential measurements in adherent RAW cells by the patch-clamp technique are in excellent accordance with fluorescence data obtained with suspended macrophages, showing a membrane potential of -55 ± 12 mV at the standard medium K^+ concentration of 5.8 mM K^+ and a depolarization to about -34, -15, and -2 mV at 40, 80, and 140 mM extracellular K^+, respectively. Because an important role of the Na^+/K^+-ATPase in the regulation of the membrane potential of neutrophils has been reported (Bashford and Pasternak, 1985, 1986), we measured the membrane potential after an inhibition of the Na^+/K^+-ATPase in RAW macrophages. We could, however, not detect any significant depolarization induced by the Na^+/K^+-ATPase inhibitor ouabain (Figure 5c) in these cells.

Taken together, this suggests plasma membrane permeability to K^+ as the main contribution to the regulation of the resting membrane potential in RAW cells. Similar observations have been reported for rat basophilic leukemia cells (Mohr and Fewtrell, 1987). In mouse spleen macrophages, however, a minor contribution of ~6 mV to the resting membrane potential by an electrogenic Na^+/K^+ pump has been reported (Gallin and Livengood, 1983).

The K^+-channel blocker quinine (200 and 500 μM) led to a dose-dependent depolarization of RAW macrophages as shown by an increase in the bis-oxonol fluorescence (Figure 5d). Accordingly, membrane potential measurements by the patch-clamp technique revealed a decrease in membrane potential from about -55 ± 12 mV to -12 ± 4 mV ($n = 5$) on application of 500 μM quinine (see Figure 6b). Depolarization observed in bis-oxonol experiments with suspended macrophages is significant but apparently not as large as observed in current-clamp recordings. Furthermore, TEA (80 mM) was also found to depolarize RAW macrophages to ~15 mV as measured in current-clamp recordings. Subsequent application of LPS to depolarized RAW macrophages did not lead to any changes in the membrane potential when measured by either technique.

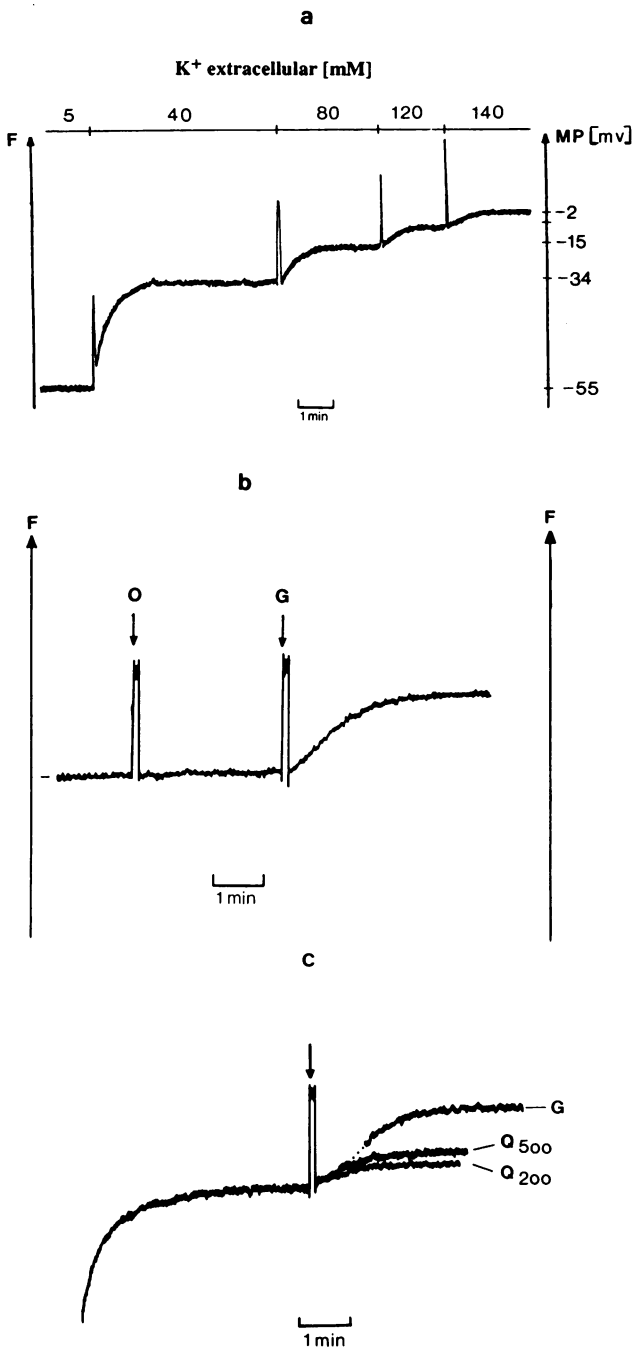

Figure 5. Elevated K^+ and quinine depolarize RAW macrophages. Increasing extracellular K^+ concentrations from 5.8 mM K^+ (basal medium) to 40, 80, 120, and 140 mM (by addition of K-aspartate) progressively depolarized RAW macrophages (a) as measured by bisoxonol fluorescence (F). Corresponding membrane potentials measured by the patch-clamp technique were −34, −15, and −2 mV at 40, 80, and 140 mM K^+, respectively, as shown at the right voltage axis (MP). The Na^+/K^+-ATPase inhibitor ouabain (O, 1 mM) exerted no effect on the membrane potential (b). Quinine (Q, 200 and 500 μM), however, led to a significant membrane depolarization, which was incomplete as compared with depolarization induced by gramicidin (c). Data shown represent typical results of three to five independent experiments.

These results clearly indicate that macrophages depolarize in response to elevated extracellular K^+ as well as in response to the K^+-channel blockers quinine and TEA.

Macrophage Depolarization Inhibits LPS-Stimulated Accumulation of Cell-Associated and of Released TNFα but Exerts no Effect on TNFα mRNA or c-fos mRNA Accumulation

To elucidate the mechanisms underlying the inhibition of LPS-stimulated TNFα release by macrophage depolarization, we pursued the effects of membrane depolarization at sequential levels of TNFα production, i.e., TNFα secretion, cell-associated TNFα accumulation, and TNFα mRNA accumulation in comparison with the LPS-induced early protein c-fos

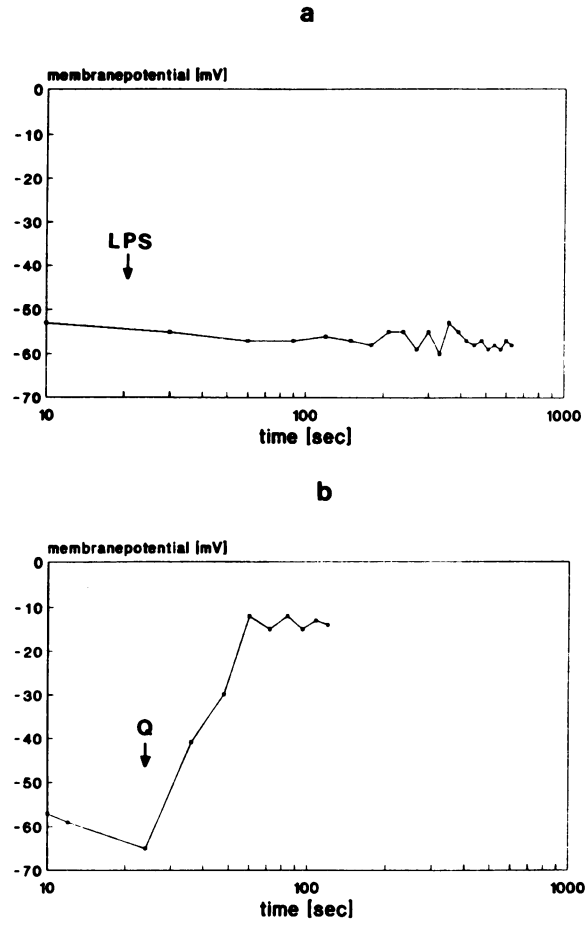

Figure 6. Patch-clamp recordings of membrane potentials of RAW macrophages. With the use of the current-clamp mode, a mean resting potential of −55 mV was determined for RAW macrophages. In control experiments, membrane potential remained stable for ≥10 min. LPS (5 μg/ml) did not affect the membrane potential within 5 min (a). Quinine (Q, 500 μM) depolarized the RAW macrophage cell from −55 to −10 mV (b). Each data represent a typical recording out of a series of five experiments.

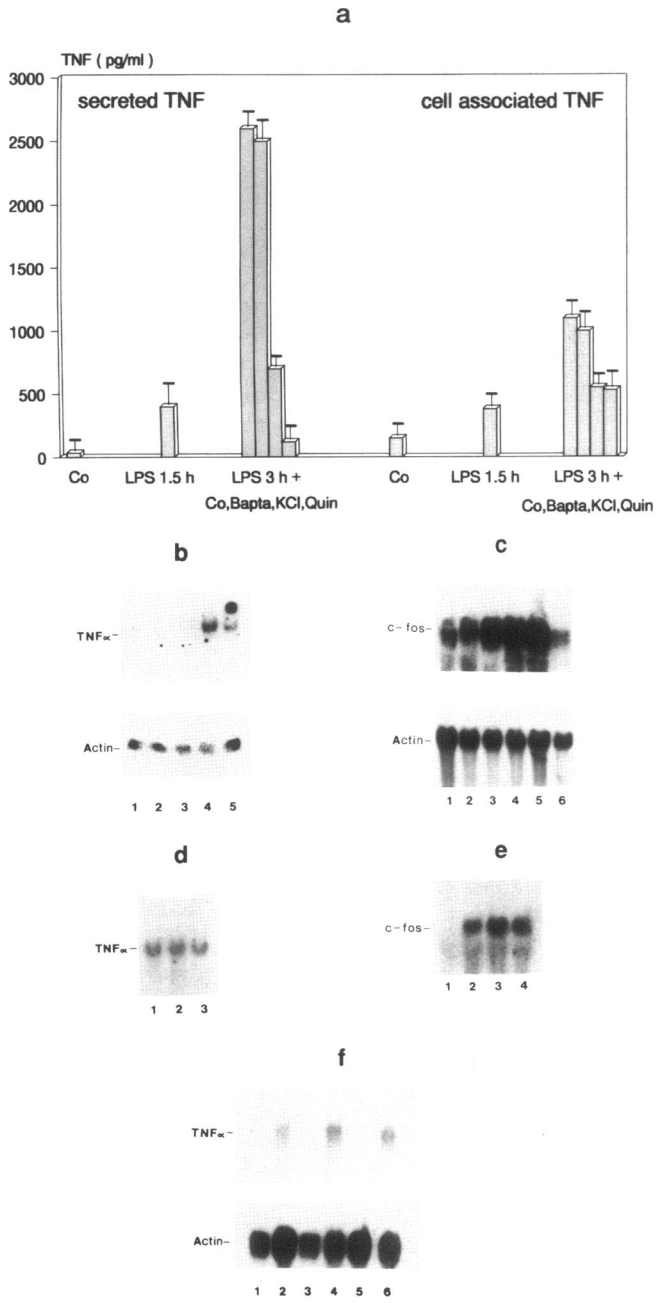

Figure 7. Effects of macrophage depolarization on TNF secretion, synthesis, and transcription. ELISA analysis in accordance with the TNF bioassay revealed an increased production of TNFα between 1.5 and 3 h after LPS (0.1 μg/ml) stimulation of RAW macrophages, as found by a separate determination of secreted and cell-associated TNFα (a). BAPTA-loaded macrophages responded to a similar extent in secreted or cell-associated TNFα. Enhanced extracellular K^+ concentrations (85 mM) and quinine (Quin, 500 μM) led to a significant inhibition of TNFα release 3 h after LPS stimulation (a). Furthermore, cell-associated TNFα measured in RAW cell lysates was also found to be reduced under these conditions (a). In contrast, mRNA levels of TNFα or c-fos remained unchanged (see b–f) in LPS (2 μg/ml)-stimulated peritoneal macrophages and RAW cells in the presence of quinine (500 μM) or elevated K^+ (85 mM). LPS stimulated a rapid increase in TNFα mRNA accumulation (b, lanes 1–5, 5, 35, 50, 90, 120 min) and a transient increase of c-fos mRNA (c, lanes 1–6, 4, 15, 25, 35, 50, 90 min) compared with β-actin controls (lower part in b and c). Expression of TNFα mRNA (d, determination 90 min after LPS) and c-fos mRNA (e, determination 50 min after LPS) was not inhibited by either addition of quinine or increased extracellular K^+ concentrations as shown in d (lanes 1, LPS; 2, LPS + quinine; 3, LPS + elevated K^+) and in e (lanes 1, control; 2, LPS; 3, LPS + quinine; 4, LPS + elevated K^+), respectively. Consistently, TNFα mRNA was not inhibited by quinine or increased extracellular K^+ concentrations in LPS-stimulated RAW cells. (f, lanes 1, control; 2, LPS; 3, control; 4, LPS + quinine; 5, control + quinine; 6, LPS + elevated K^+).

mRNA using ELISA measurements and northern blot analysis, respectively.

In accordance with data obtained with the TNF bioassay ELISA, TNFα analysis revealed an increase in both cell associated and released TNFα 1.5 and 3 h after LPS stimulation of RAW cells (Figure 7a). Buffering of intracellular Ca^{2+} changes in BAPTA-loaded macrophages did neither effect TNFα secretion nor accumulation of cell-associated TNFα. In contrast, quinine (500 μM) and enhanced extracellular K^+ concentrations (85 mM) significantly inhibited TNFα production as found for both released and cell-associated TNFα 3 h after LPS stimulation of RAW cells (Figure 7a; compare with Figure 4). Very similar results were obtained with peritoneal macrophages.

Accumulation of TNFα mRNA could already be detected 35 min after LPS stimulation of peritoneal macrophages, showing peak values at 90 min (Figure 7b, upper). Quinine (500 μM) or increased extracellular K^+-concentrations (85 mM) did not affect accumulation of TNFα mRNA assayed 90 min after LPS stimulation in peritoneal mouse macrophages (Figure 7d) or RAW cells (Figure 7f). Furthermore, quinine and enhanced extracellular K^+ exerted no effect on LPS-stimulated transient c-fos mRNA expression (Figure 7e), which showed peak values at ~50 min in peritoneal mouse macrophages (Figure 7c, upper). β-Actin controls remained unchanged on LPS-stimulation either in peritoneal macrophages (lower part in b and c) or in RAW cells in the presence and absence of quinine or elevated K^+-concentrations (lower part in f). Consistently, no effects of quinine (250–500 μM) on LPS-stimulated mRNA accumulation of TNFα-, IL-1, and IL-8, but also β-actin controls, were found in the monocyte-like MONOMAC cell line (Bevec, personal communication).

These experiments suggest that the inhibition of TNFα protein synthesis and of TNFα release by quinine or enhanced extracellular K^+ is not due to an impairment of LPS signaling pathways affecting TNFα gene transcription but rather a posttranscriptional effect.

DISCUSSION

The cellular signaling pathways of LPS in mediating activation of specific macrophage genes are still not fully

understood. We report here that neither Ca^{2+}_{ic} nor the membrane potential are effected in the macrophage response to LPS. We found, however, that the LPS-stimulated TNFα release in macrophages is highly dependent on the membrane potential of the cells. Pronounced inhibition of TNFα release was observed by increasing extracellular K^+ concentrations, resulting in macrophage depolarization. A similar inhibition of TNF release was found when LPS was applied in combination with K^+-channel blockers such as quinine, TEA, or $BaCl_2$. Patch-clamp recordings and bis-oxonol fluorescence measurements unequivocally showed that K^+-channel blockers similar to elevated extracellular K^+ concentrations caused a significant depolarization of macrophages.

Several different K^+ channels are reported to occur on macrophages, such as an inward-rectifying voltage-dependent conductance (G_{Ki}), a large calcium- and voltage-activated K^+ conductance (G_{LKCa}), a Ca^{2+}-activated inward-rectifying K^+ conductance (G_{Kh}), and a delayed rectifier K^+ conductance (Randriamampita and Trautmann, 1987; Gallin and McKinney, 1988). Because the employed K^+-channel blockers are not very specific among the various K^+ channels and the more specific inhibitors like apamin and charybdotoxin were ineffective in the inhibition of TNF synthesis, it is reasonable to assume that the main contribution to depolarization is caused by blockage of that K^+ channel, which is predominantly open at a resting membrane potential of -50 mV and at an Ca^{2+}_{ic} concentration of 10^{-7} M. The G_{Ki} and the delayed rectifier exhibit their maximum activation mainly in the hyperpolarized (below K^+-equilibrium potential -80 mV) and in the depolarized voltage range, respectively. At the calcium concentration of 10^{-7} M employed, G_{LKCa} has been found to open only at membrane potentials of +40 mV. Therefore, the main candidate for inhibition by quinine leading to depolarization is G_{Kh}, which has been shown to open at an Ca^{2+}_{ic} concentration below 1 μM and at a membrane potential of -80 to -30 mV (Gallin, 1989). Further information on the K^+ channel involved in the quinine-induced depolarization of macrophages is expected from single channel studies. Blockade of K^+ channels has also been found to inhibit cellular responses of T lymphocytes (Price *et al.*, 1989) and of rat basophilic leukemia cells (RBL) (Labrecque *et al.*, 1988).

Evidence for a regulatory role of the membrane potential in the release of mediators has been found in other nonexcitable cells. In rat basophilic leukemia cells, depolarization by increased extracellular K^+ leads to an inhibition of exocytosis (Mohr and Fewtrell, 1987). Secretion of IL-2 from T lymphocytes stimulated by PHA is also inhibited by K^+-induced depolarization (Gelfand *et al.*, 1987). In these cells, exocytosis is strongly dependent on the influx of extracellular Ca^{2+}, which is modulated by the membrane potential. In the activation of macrophages or monocytes, however, there is apparently a lack of a central role for calcium in response to LPS as demonstrated by Fura-2, Indo-1, and BAPTA experiments. In addition, membrane potential measurements by the patch-clamp technique and bis-oxonol fluorescence did not provide evidence for the induction of ion movements on macrophage activation by LPS or Lipid A as early phase responses. Depolarization of macrophages, however, was found to inhibit cell-associated TNFα protein accumulation partially and TNFα release almost completely, whereas TNFα mRNA induction by LPS was not impaired. Thus, because much of the regulation of TNF production is known to occur posttranscriptionally (Beutler, unpublished data), Ca^{2+}-independent, but membrane potential-dependent, cellular processes may be involved in the protein synthesis and secretion of TNF.

In contrast, application of PAF to macrophages led to a transient increase in Ca^{2+}_{ic} concentration and was not followed by a release of TNF. Membrane potential measurements by the fluorescent dye bis-oxonol revealed a transient membrane depolarization concomitant to the increase in Ca^{2+}_{ic} induced by PAF, which has also been reported in a recent abstract (Katnik and Nelson, 1991).

Taken together, these results indicate a lack of ion movements in the stimulation of macrophages by LPS but point to a regulatory role of the membrane potential controlled by K^+ channels in the posttranscriptional synthesis and release of proteins like TNF.

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