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Macrophage Protein Kinase C: Its Role in Modulating Membrane Microviscosity and Superoxide in Leishmanial Infection

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Pretreatment of macrophages with PMA, an agonist of PKC, showed diverse effects on degradation and survival of two virulent strains of *Leishmania donovani* promastigotes. Treatment of macrophages with PMA for 45 min at 37°C generated significant amounts of superoxide anions and reduced the parasite burden of macrophages by up to 48 and 43% when AG83 and GE-1 strains were used for infection. Staurosporine, an inhibitor of PKC, inhibited PMA-dependent killing of the parasites, while tyrphostin AG 126, an inhibitor of protein tyrosine kinase, showed very little effect. Depletion of PKC by prolonged incubation with PMA drastically reduced the superoxide anion generation and increased the uptake and multiplication of the parasites. Finally, to understand the mechanism of higher uptake of the parasites by PKC-depleted macrophages, membrane microviscosity was measured by fluorescence depolarization. Membrane microviscosity was found to be approximately 40% lower in PKC-depleted macrophages than in normal macrophages, indicating the role of membrane fluidity in the infection process. Together, these data suggest PKC activation, superoxide generation, and membrane fluidity are essential factors in the efficient regulation of leishmanial infection.

Key words: *Leishmania donovani*, macrophages, membrane microviscosity, protein kinase C, superoxide.

Leishmania, the causative agent of leishmaniasis, resides and proliferates within the hostile environment of phagolysosomes (*1*) of host macrophages. One of the primary microbicidal activities of phagolysosomes is the oxidative burst, an event that is very critical to host defense. The parasite may alter this pathway to survive. Activation of the oxidative burst and generation of superoxide (O_2^-) in phagocytes are directly dependent on the protein kinase C (PKC) system (*2*). Although LPG, a cell-surface molecule of *Leishmania donovani,* inhibited PKC-dependent events such as chemotactic locomotion in human monocytes (*3*), *c-fos* gene expression in murine macrophages (*4*), and attenuated PMA-stimulated oxygen consumption (*5*), nothing is known about the kinetics of activation and desensitization of PKC in macrophages, particularly their role in alteration of membrane fluidity and their consequences on uptake and further infection.

PKC is a well-characterized example of calcium- and phospholipid-dependent cellular kinases, which play a pivotal role in the regulation of a variety of cell functions (*6*). In cells, these kinases are translocated and activated in response to stimulation by hormones or phorbol esters (*7*). PMA, a well-known phorbol ester, has been shown to activate (*8*) and deplete (*9*) PKC from cells depending upon the incubation period. Stimulation of cells with PMA increases intracellular concentrations of diacylglycerol, which induces translocation of PKC from cytosol to the plasma membrane of the cells. Activation of PKC results in phosphorylation of several proteins in different cell systems including phagocytes, which in turn activates NADPH oxidase (*11*). The functional end points of this signal transduction cascade in phagocytic cells are superoxide generation (*12*).

Because the initial attachment of *Leishmania* promastigotes to macrophages is receptor-mediated, the expression of the receptor may play a vital role in the interaction of parasites with macrophages. In erythrocytes and macrophages, the expression of receptors has been shown to be significantly altered by changes in the membrane microviscosity (*13, 14*). As lipid-protein association is one of the major parameters which controls membrane macroviscosity, it is presumed that depletion of PKC from macrophages may also change the membrane microviscosity and thus affect the interaction with the parasites.

The present study was undertaken to investigate the relationship between generation of superoxide and activation or desensitization of PKC in cultured macrophages. The role of membrane microviscosity of PKC-desensitized macrophages in the initial uptake of the parasites and the key role of superoxide in the multiplication of the parasites were also studied.

MATERIALS AND METHODS

Reagents—RPMI 1640 with L-glutamine, M199, and FCS were from GIBCO Laboratories, USA. Phorbol-12-myri-

Abbreviations: PMA, phorbol myristate acetate; PKC, protein kinase C; LPG, lipophosphoglycan; DPH, diphenyl hexatriene; KRPG, Kreps Ringer phosphate glucose; HEPES, *N*-2-hydroxyethyl piperazine-*N*-2-ethyl sulphonic acid; O_2^-, superoxide anion; TG, thioglycollate.

state-13-acetate (PMA), staurosporine, phosphatidyl serine (PS), β-mercaptoethanol (ME), ethylene glycol tetraacetic acid (EGTA), phenyl methyl sulphonyl fluoride (PMSF), and histone III were purchased from Sigma Chemical (St. Louis, MO). Tyrphostin AG126 was a generous gift from Prof. A. Levitzki (Dept. of Biological Chemistry, The Hebrew Univ. of Jerusalem). Diphenyl hexatriene (DPH) and all other reagents used were of analytical grade.

Peritoneal Macrophages—Thioglycollate (TG)-elicited macrophages were isolated by peritoneal lavage from female Swiss albino mice. Four days after thioglycollate injection, macrophages were isolated by the method of Russell *et al.* (*15*) from the peritoneal cavity, by washing with 3–4 ml of RPMI-1640 medium supplemented with 25 mM HEPES, 100 μg/ml streptomycin, 100 μg/ml penicillin, and 20% of FCS. The macrophages were plated onto 18-mm coverslips and allowed to adhere at 37°C for 2 h. Nonadherent cells were separated from macrophages by extensive washing of the coverslips with PBS. Cells obtained this way were ~85% viable as judged by trypan blue exclusion test.

Parasites—*L. donovani* AG83 (MHOM/IN/1983/AG83) and GE-1 (MHOM/IN/89/GE-1) strains were maintained in female BALB/c mice. Parasites were recovered from the spleen of infected mice by adding spleens to culture medium M199 supplemented with 20% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cultures were kept for 5 d at 22°C to obtain promastigotes, which were then washed and used for infection of macrophages.

Infection of Macrophages—Peritoneal macrophages were incubated with promastigotes for 2 h at 37°C at a 10:1 parasite-to-cell ratio. Noningested parasites were removed by three washes with warm PBS. Infection levels were determined by microscopic examination of Giemsa-stained coverslips.

Activation and Depletion of PKC in Macrophages—Macrophages were treated with 100 ng/ml or 1 μg/ml of PMA at 37°C for 45 min for activation of PKC (*8*). For depletion of PKC, cells were treated with 100 ng/ml PMA for 24 h, washed three times, and resuspended in complete medium. This treatment specifically depletes PKC activity in different systems (*9*).

Preparation of Membrane Fractions for PKC Assay—Cells from the TG-elicited mice macrophages were obtained after incubation for 2 h at 37°C, then washed with PBS. After removing the nonadherent cells, the macrophages were stimulated for different times with PMA (100 ng/ml), then sonicated in buffer containing 0.25 mM sucrose, 5 mM EGTA, HEPES 20 mM, Leu-pep 50 μg/ml, PMSF 0.2 mM, and 2 mM β-mercaptoethanol.

The sonicate was centrifuged at 100,000 ×*g* for 30 min. The pellet was resuspended in buffer supplemented with 1% (v/v) Triton-X 100 and centrifuged at 100,000 ×*g* for 30 min. The detergent-treated supernatant fraction, *i.e.*, the membrane fraction was assayed for PKC activity as described (*10*) using histone III as protein substrate.

Briefly, PKC activity was measured as the incorporation of ^{32}P from ^{32}P ATP into histone III at 37°C in the presence of 10 mM $MgCl_2$, 50 μM ATP (containing approx. 6×10^5 cpm ^{32}P), 40 μg histone, 0.4 mM EGTA, 0.2 mM PMSF, 10 mM 2ME, 0.01% Triton X-100, 1 mM $CaCl_2$, 20 μg/ml PS, and 2 μg/ml Diolein.

Samples were assayed in duplicate in both the presence and absence of added Ca^{2+}, PS, and diolein. PKC activity was determined by subtracting from the maximal apparent activity the amount of ^{32}P incorporated into histone in the absence of essential cofactors, and expressed as percentage (%) ^{32}P incorporation/mg of protein.

Membranes of PKC-Depleted Macrophages and Determination of Microviscosity by Fluorescence Depolarization—Membranes of normal and PKC-depleted macrophages were isolated from other organelles by different centrifugation according to Werb *et al.* (*16*) and purified by discontinuous sucrose density gradient centrifugation. Fluidity of the macrophage membranes was determined by fluorescence depolarization measurement using DPH as the probe (*13*). In short, DPH solution (2 mM) in THF was injected with rapid stirring into 1,000 volumes of PBS at room temperature. It was stirred for 2 h, then examined for negligible fluorescence. In experiments, both normal and PKC-depleted macrophage membranes equivalent to 100 μg of cell protein were incubated in 2 ml of PBS containing 1 μM DPH preparation for 2–4 h at 37°C with occasional stirring. Fluorescence intensity was measured in a fluorescence polarization spectrometer (Perkin-Elmer, SL-8) at room temperature with an excitation wavelength of 365 nm and an emission wavelength of 430 nm, and fluorescence anisotropy was calculated using the equation

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp}),$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities oriented, respectively, parallel and perpendicular to the direction of polarization of the exciting light. The microviscosity parameters $[(r_o/r) - 1]^{-1}$ were calculated in each case, knowing the fluorescence aniosotropy r and the maximal limiting fluorescence anisotropy r_o, which for DPH was observed to have an experimental value of 0.362 (*17*). All measurements were performed within 24 h after incubation.

Measurement of Superoxide of Activated and PKC-Depleted Macrophages—Superoxide anion generation by macrophages was determined at 37°C as previously described (*18*). Briefly, adherent cells on coverslips were washed thoroughly with PBS and placed in 35-mm diameter Petri dishes containing 1 ml of the reaction mixture. The reaction mixture contained 80 μM ferricytochrome *c* and 100 ng/ml PMA in phosphate buffer (pH 7.4) consisting of 137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8.06 mM Na_2HPO_4, 0.8 mM $CaCl_2$, 0.4 mM $MgCl_2$, and 5.5 mM glucose, with or without 50 μg/ml superoxide dismutase (SOD). After incubation for 60 min at 37°C, O_2^- release was determined as SOD-inhibitable reduction of ferricytochrome *c* at 550 nm.

RESULTS

Analysis of PMA-Pretreated Macrophage Uptake or Killing of Promastigotes of L. donovani AG83 and GE-1 Strains—To evaluate the role of PMA-pretreated macrophages in the process of infection by the parasites, we used TG-elicited peritoneal macrophages treated with PMA (100 ng/ml) for 45 min, 4 h, or 24 h. We examined the uptake by incubating the promastigote form of the parasites (strain AG83 or GE-1) with macrophage monolayers pretreated with PMA for 4 or 24 h at 37°C. Parasite uptake was assayed by light microscopy using Giemsa-stained slides (Table Ia). Uptake was found to be significantly higher for both the strains with macrophages pretreated for 4 or 24 h; approximately 30–40% more parasite uptake was found in

24-h PMA-pretreated macrophages than in the nontreated macrophage control. In contrast, when macrophages were preincubated for 45 min with PMA, leishmanicidal activity of macrophages was observed, and approximately 40–50% parasites were killed by PMA-activated macrophages (Table Ib). It is known that phorbol esters stimulate macropinocytosis and solute flow through macrophages (*19*), and here we show that they have diverse effects on leishmania infection.

Activation and Depletion of PKC and Intracellular Fate of Leishmania—It has been shown that PMA, an agonist of PKC, activates or depletes PKC in different cell systems (*8, 9*) depending upon the time of treatment. The results of time-dependent PKC assay on normal and infected macrophage membranes are shown in Table II. Pretreatment with PMA for the shorter time (45 min) clearly promoted the activation of PKC, whereas longer (24 h) preincubation with PMA led to its depletion. To test whether *Leishmania* killing or uptake by PMA-pretreated macrophages is PKC-mediated, two approaches were taken. (i) Macrophages were incubated with inhibitors of PKC or tyrosine kinase. Inhibitors of kinases were used because it was thought that the killing of the parasite might be due to protein kinase–mediated O_2^- generation, and also because it is known that, in addition to the PKC-dependent phosphorylation of serine and threonine residues in specific proteins, the respiratory burst is accompanied by the phosphorylation of some proteins at tyrosine residues (*11*). Pretreatment of macrophages with staurosporine (0.1 μM, 10 min, 37°C), an inhibitor of PKC (*32, 33*), protected the parasites significantly, whereas tyrphostin AG126, a well-known inhibitor of protein tyrosine kinase (*20*) in macrophages, showed very little effect. Staurosporine protected the GE-1 parasite significantly (approximately 85% of control parasites survived in staurosporine-treated macrophages) in PMA-activated (45 min treated) macrophages compared to control PMA-activated macrophages (Table III). (ii) PKC was depleted from macrophages by treatment with 100 ng/ml of PMA for 24 h (*9*) as described before. The depleted macrophages were then challenged with either AG83 or GE-1 parasites. Table I indicates that 42 ± 2.5 and 20 ± 6.5% more uptake was found for AG83 and GE-1 strains. Collectively, these results indicated that PMA pretreatment of macrophages contributed significantly towards the leishmanicidal activity or uptake of the parasites *via* a PKC-dependent mechanism.

Depletion of PKC Affects the Membrane Fluidity/Microviscosity of Macrophages—To examine whether membrane fluidity or microviscosity interferes with the parasite uptake by PKC-depleted macrophages, we isolated membranes from both normal and PKC-depleted macrophages. Membranes were incubated with the DPH probe at 37°C for 2 h, then fluorescence was measured and microviscosity calculated (*21*). As shown in Table IV, microviscosity of PKC-depleted macrophage membranes was found to be 0.7 ± 0.05 (n = 3), whereas that of normal was 1.16 ± 0.10, indicating that membrane of PKC-depleted macrophages was approximately 40% more fluid and thus possibly more permeable. The effects of variation in macrophage membrane microviscosity on the attachment and internalization of *L. donovani* promastigotes was previously reported from this laboratory (*21*). In that report, we demonstrated that macrophage membrane fluidity or microviscosity significantly influences the attachment and internalization of parasites, and this influence may be causally related to the expression of receptors on the macrophage surface. The increase in microviscosity (or decrease in fluidity) helps in receptor expression and, thus, the attachment of parasites.

TABLE I. **Effect of PMA-pretreatment of mouse peritoneal macrophages on the (a) uptake and (b) killing of *L. donovani* AG-83 and GE-1 promastigotes.**

(a) Uptake

Pretreatment[a]	*Leishmania*/100 macrophages, 2 h after infection[b]		% uptake	
	AG-83	GE-1	AG-83	GE-1
None	196 ± 6	301 ± 12		
PMA (4 h)	321 ± 7	328 ± 7	119	109
PMA (24 h)	277 ± 2	364 ± 6	142	121

(b) Killing

Pretreatment[a]	*Leishmania*/100 macrophages, 2 h after infection[b]		% killing	
	AG-83	GE-1	AG-83	GE-1
None	194 ± 6	301 ± 12		
PMA (45 min)	121 ± 6	171 ± 7	48	43

[a]Untreated or PMA-pretreated (for indicated times) mouse peritoneal macrophages were incubated for 2 h with AG-83 or GE-1 parasites in complete medium, washed, cultured for another 2 h, washed, fixed, and stained as described in "MATERIALS AND METHODS." [b]These values represent the means ± SD of three separate experiments performed with duplicate samples.

TABLE II. **Effect of PMA on specific activity of PKC in macrophage membrane protein.**[a]

Treatment	Histone phosphorylation (% ^{32}P incorporation/mg of protein) in macrophage membrane fractions		
	Activated (% ^{32}P incorporation/mg of protein)	Nonactivated (% ^{32}P incorporation/mg protein)	Actual ^{32}P incorporation
Control	1.53	1.15	0.38
PMA (45 min)	1.88	1.31	0.57
PMA (45 min) + *L. donovani* (AG-83)	1.61	1.14	0.47
PMA (24 h)	0.92	0.75	0.17
PMA (24 h) + *L. donovani* (AG-83)	0.78	0.69	0.09

[a]Macrophages were incubated with or without *L. donovani* (AG-83) (for 2 h in a cell: parasite ratio of 1:10), with or without pretreatment with PMA (100 ng/ml). PKC activities were assayed in membrane fractions.

TABLE III. **Inhibitory effect of staurosporine or tyrphostin AG 126 on PMA-induced killing of *L. donovani* GE-1 parasites.**

Treatment[a]	*Leishmania*/100 macrophages[b] 2 h after infection
None	303 ± 15.2
PMA	171 ± 7
Staurosporine + PMA	260 ± 8.02
Tyrphostin AG 126 + PMA	194 ± 6.55

[a]Staurosporine (0.1 μM for 10 min) or tyrphostin AG 126 (20 μM for 2 h) was added prior to the addition of PMA (100 ng/ml for 45 min). Macrophages were then infected for 2 h and killing of the parasites was assessed by light microscopy. [b]All values are means ± SD of duplicate samples.

TABLE IV. **Determination of membrane microviscosity of macrophages[a] after prolonged treatment with PMA (PKC-depleted).**

Membrane	Microviscosity parameters $[(r_o/r) - 1]^{-1}$[b]
(i) Normal macrophages	1.16 ± 0.098
(ii) PKC-depleted macrophages	0.7 ± 0.045

[a]All values are means ± SD (n = 3). [b]The microviscosity parameter was calculated from fluorescence depolarization measurement using 1,3,5-diphenyl hexatriene as fluorescent probe.

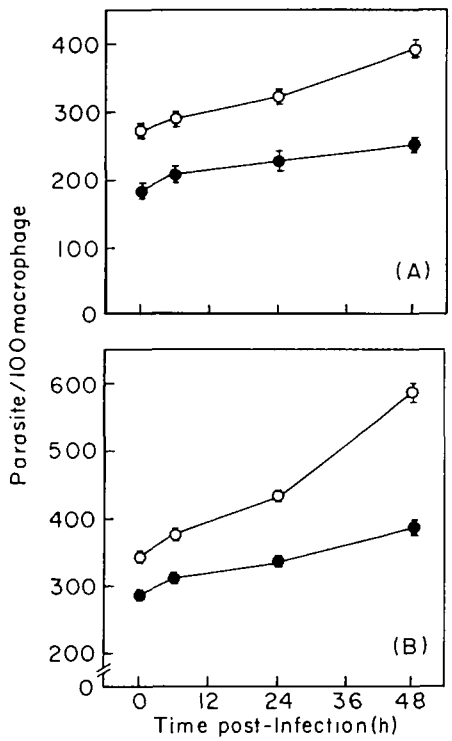

Fig. 1. **Intracellular multiplication of AG-83 or GE-1 parasites in PKC-depleted macrophages.** Untreated (closed symbols) and PMA-pretreated (open symbols) macrophages were infected with parasites as described in "MATERIALS AND METHODS," and parasites survival was assessed at the indicated times. A, AG-83 promastigotes; B, GE-1 promastigotes. Each point represents the mean ± SD of triplicate samples.

On the contrary, an increase in fluidity (or decrease in microviscosity) does not increase parasite attachment, because of poor receptor expression, but expedites the process of compartmentalization or invagination, which is a prerequisite for internalization of parasites or endocytosis. In close agreement with the previous report, we show here that PKC-depleted macrophages internalize more parasites due to increased fluidity of their membranes. Previous studies with artificial and natural membranes have demonstrated the importance of lipid-protein association in modulating the distribution of membrane proteins, membrane-fusion, and membrane permeability (*22*).

Effect of PKC-Depletion on Intracellular Multiplication of L. donovani Strains—Murray (*23*) showed that peritoneal macrophages pretreated for 2 h with PMA had reduced leishmanicidal activity, indicating a role for PKC in the regulation of leishmanial infection. Here we compared the uptake and multiplication of AG83 and GE-1 strains of *L. donovani* in PMA-pretreated peritoneal macrophages. Uptake of AG83 and GE-1 parasites/100 macrophages amounted to 231 ± 7 and 328 ± 7 in 4-h PMA-pretreated cells, compared to 194 ± 6 and 301 ± 12 in nontreated cells, whereas in 24-h PMA-pretreated cells, the figures were 277 ± 2 and 364 ± 6, respectively. Thus, approximately 20 and 40% more uptake of GE-1 and AG83 parasites was found in 24-h PMA-treated macrophages. The effect of 24-h PMA pretreatment was also determined on the multiplication of parasites. As shown in Fig. 1A, 24 h after infection, PMA-pretreated macrophages contained 40% more AG83 and 29% more GE-1 (Fig. 1B) than control, and by 48 h, PMA-pretreated macrophages contained 52% more AG83 and 53% more GE-1 than control.

TABLE V. **Production of superoxide anion by control and PMA-activated or PKC-depleted macrophages.**

Cell treatment[a]	O_2^- nmol/h/mg of protein[b]
None	87 ± 7.5
PMA (45 min)	276 ± 25.2
PMA (24 h)	47 ± 6.8

[a]Macrophage monolayers were prepared on coverslips as described in "MATERIALS AND METHODS." Triplicate sets of macrophages were untreated or incubated with PMA for 45 min or 24 h, washed, then incubated with PMA containing KRPG for assay of O_2^-. [b]Mean ± SD of triplicate determinations for each treatment group.

Superoxide of Activated and PKC-Depleted Macrophages—Because PMA activates both the respiratory burst and PKC, superoxide generation was measured both in PMA-induced and PKC-depleted macrophages. TG-elicited peritoneal macrophages were tested for superoxide anion production after incubation for 1 h with PMA in Kreb's Ringer phosphate glucose (KRPG). O_2^- production was increased by at least 3-fold compared with controls when macrophages were pretreated for 45 min with PMA (Table V). In contrast, the production of O_2^- was significantly reduced to far below the basal level in PKC-depleted macrophages. These results indicated that O_2^- might interfere with the killing and multiplication of parasites inside macrophages.

DISCUSSION

Infection of monocytes or macrophages with *Leishmania* attenuates PMA-stimulated oxygen consumption (*5*) and inhibits PKC-mediated events such as chemotactic locomotion (*3*) and *c-fos* gene expression (*4*). Although these observations indicate the involvement of PKC-mediated events in intact cells, there was no previous report on the activation and deactivation of PKC in macrophages and their role in degradation or survival of the parasites during infection. Therefore, in this study, we have investigated the effect of activation or depletion of PKC on uptake, multiplication, and degradation of two virulent strains of *L. donovani*.

It has been reported that, depending upon the time of pretreatment with PMA, macrophages activate or deplete PKC in intact cells (*8, 23*). It is known that PMA pretreatment for a longer period causes long-lasting depletion of PKC isoforms in many biological systems (*23*). In our experiment, when the cells were pretreated with PMA for 45 min, 40–45% parasites were killed; but when the cells

were preincubated with staurosporine, a potent inhibitor of PKC, parasites were protected (Table III). On the other hand, AG126, an inhibitor of tyrosine kinase, had little effect on the protection. Pretreatment of macrophages with PMA for 24 h depletes PKC activity by increasing its rate of degradation (*24*). We found that the initial uptake of both AG83 and GE-1 was significantly higher in PMA-pretreated macrophages than in nontreated cells. This suggests that phorbol ester–sensitive PKC depletion helps in the ingestion of *L. donovani* promastigotes, and this result is in agreement with the previous report by Murray (*23*). During the first 48 h of infection, we found that PKC-depleted macrophages contained more parasites than normal macrophages. This enhanced growth in PMA-pretreated macrophages was evidenced further when we compared the multiplication of the parasites in normal and treated macrophages (Fig. 1). Collectively, these findings provide evidence that PKC-dependent events played a significant role on the proliferation of *L. donovani* inside macrophages. Although LPG from *L. donovani* promastigotes (*25*), and glycosylinositolphospholipids (*26*) from amastigotes were shown to be potent inhibitors of purified PKC *in vitro*, there might be a possibility that these type of molecules might down-regulate or deplete PKC activity in intact macrophages.

The initial uptake of the parasites in PKC-depleted macrophages was higher than in normal macrophages, indicating the possibility of alteration of membrane fluidity or the microviscosity of macrophages. Our present data on membrane microviscosity showed that PKC-depleted macrophages are approximately 1.6-fold more fluid than the normal macrophages. This finding clearly indicates that, whatever the exact mechanism of uptake might be, the fluidity or microviscosity of the membrane has a significant effect on the process, and this decrease in microviscosity or increase in fluidity may up- or down-regulate the expression of *Leishmania* recognition receptors. This result is in close agreement with the previous finding by Mukherjee *et al.* (*21*) that the microviscosity of the macrophage membrane is inversely correlated with the number of parasites that are internalized. Though the exact molecular mechanism of higher uptake of the parasites is not clearly understood, it has been shown that modulation of artificial and natural membranes seems to affect the activity of many membrane-bound enzymes, the distribution of membrane proteins, membrane permeability, and membrane fusion (*22*).

PMA activates both the respiratory burst and PKC in phagocytic cells (*27, 28*). The mechanism by which intracellular *Leishmania* interferes with the oxidative mechanism of host cells is unknown. To examine the relationship between PKC, O_2^- generation, and the intracellular fate of *Leishmania* parasites, we tested the O_2^- generation in PMA-induced (activated) and PKC-depleted macrophages. It is noteworthy that PMA (45 min) induced a significant amount of O_2^- generation in macrophages and killed both AG83 and GE-1 at the level of 40 and 45% respectively (Tables III and IV); but killing was inhibited when macrophages were pre-incubated with staurosporine, an inhibitor of PKC. Indeed, staurosporine inhibited the PMA-induced oxidative burst and helped in the survival of the parasites. On the other hand, depletion of PKC drastically reduced the O_2^- generation and facilitated the intracellular multiplication of the parasites. These data collectively suggest that PKC and O_2^- are the major factors in parasite degradation and multiplication inside macrophages, although a recent report found altered activation and translocation of PKC in *Leishmania*-infected human monocytes (*29*).

PKC is a key enzyme involved in the regulation and functions of many cell types *via* phosphorylation of its substrate proteins. For example, activation of neutrophils by several agents, including phorbol esters, results in phosphorylation of PKC-dependent proteins that are involved in superoxide anion production. A similar mechanism might be operative in the present study of PKC activation and O_2^- generation in macrophages, as shown by parasite killing and multiplication. It has been shown in bone marrow-derived macrophages that LPG of *L. donovani* inhibited the phosphorylation of MARCKS (myristoylated alanine-rich C kinase substrate), a well-known substrate of PKC (*30*), but whether this inhibition is related with O_2^- generation and multiplication of the parasites, remains to be determined. In this connection, it is interesting to note that in CGD (chronic granulomarous disease) neutrophils fail to express a respiratory burst due to failure in PMA-induced phosphorylation of a 48-kDa cytosolic protein (*31*).

In conclusion, our findings suggest that activation and depletion or inhibition of PKC of host macrophages regulates leishmanial infection, at least for the two virulent strains we examined. Although it is known that the depletion of PKC may alter various host functions, in the present report we show that down-regulation of membrane microviscosity and superoxide generation of PKC-depleted macrophages might facilitate the uptake and multiplication of the parasites.

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