Infection of host macrophages by the protozoan *Leishmania* has multiple effects that might contribute to intracellular survival, including inhibition of both induction of inflammatory mediators (6, 10, 27, 28) and expression of class I and class II major histocompatibility complex genes (25, 26), impairment of the oxidative burst (3, 5, 10, 22), and inhibition of apoptosis (19). Isolations of protein kinase C (PKC) have been implicated in these pathways (11, 13, 24, 30), and a number of PKC-mediated functions, including chemotaxis (10) and c-fos expression (7), have been observed to be inhibited in *Leishmania*-infected cells. Recent studies have attributed at least some of these activities to the major surface lipophosphoglycan (LPG) (8–10, 18). A systematic study of the PKC isoforms in murine macrophages that might be the targets of *L. major* infection has not been performed.

The effects of *Leishmania major* infection on PKC-dependent induction of c-fos and tumor necrosis factor alpha (TNF-α) gene expression were investigated by using bone marrow-derived macrophages (BMM) prepared from BALB/c mice as described previously (9). BMM monolayers (4 × 10⁶ cells) were left uninfected or were infected with *L. major* metacyclic promastigotes for 16 h by using 20 parasites/cell. After the monolayers were washed, cellular PKC was activated for 30 min with 100 nM phorbol 12-myristate 12-acetate (PMA). Total RNA was extracted, reverse transcribed, and used as a template for a semiquantitative PCR assay in the presence of twofold serial dilutions of the cytokine polyclonal plasmid pORS, as described previously (28). After being standardized to the levels of the constitutively expressed hypoxanthine guanine phosphoribosyltransferase (HPRT) gene, transcripts for c-fos and the TNF-α gene were reduced two- to fourfold in infected cells compared to uninfected cells (Fig. 1). Thus, as with *Leishmania donovani* (7), *L. major* inhibits PKC-mediated gene expression in macrophages.

To assess the specific PKC isoforms that might be affected by infection, monolayers were lysed and analyzed for the presence of PKC isoforms by Western blotting after sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (Fig. 2). Two members of the classical PKC isoform family, PKCa and PKCb, migrated at the appropriate 76-kDa molecular sizes. One of the novel PKCs, PKCδ, and one of the atypical PKCs, PKCe, were also identified. Antisera against PKCe recognized a doublet of proteins at 70 and 76-kDa, whereas antisera against PKCe recognized a doublet of proteins at 60 and 67 kDa. Recognition could be specifically blocked by incubation with the respective peptides against which the antibodies had been produced (Fig. 2). No other PKC isoforms, including the classical PKCγ and the novel members PKCc, PKCd, PKCe, and PKCf (which is also known as PKD), were revealed with the reagents used (data not shown). Polyclonal antisera against some of the isoforms reacted with *L. major*-derived proteins after Western blotting of promastigote lysates (2 × 10⁷ cells), although these were readily distinguished from the macrophage proteins (data not shown).

Activation of PKC isoforms is associated with their translocation from soluble to particulate fractions within the cell. The kinetics of translocation were assessed after activation with PMA. Infected or uninfected BMM (1.5 × 10⁶ cells) were stimulated with 100 nM PMA for designated times, scraped into ice-cold homogenization buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM EGTA, 100 μM phenylmethylsulfonyl fluoride, and 5 μg each of soybean trypsin inhibitor, aprotinin, and leupeptin per ml), and centrifuged at 100,000 × g each of soybean trypsin inhibitor, apro- tinin, and leupeptin per ml), and centrifuged at 100,000 × g to create particulate and soluble fractions for analysis by Western blotting and densitometric quantitation (Fig. 3). These data can be summarized as follows. (i) PKCα translocated from the cytosol to the particulate compartment after stimulation, with a peak accumulation by 15 min before subsequent degradation. A similar distribution and translocation kinetics occurred in infected macrophages, although the disappearance of PKCα from the particulate fraction was consistently delayed compared to that in uninfected cells. (ii) PKCb was comparably distributed between cytosolic and particulate fractions in rest- ing infected and uninfected macrophages and rapidly translo- cated after stimulation with PMA in both groups of cells. Degradation of PKCb began sooner after translocation than that of PKCa, and this disappearance from the particulate fraction was even more affected by infection with *L. major* even after 30 min, by which time 80% of the particulate- associated enzyme had disappeared in uninfected cells, over 70% of the kinase remained localized to the particulate fraction in infected cells. (iii) PKCδ was predominantly in the cytosolic fraction, translocated rapidly after stimulation, and demonstrated partial recovery in the cytosol after 5 min but was subsequently maintained at a stable level. Infection had little effect on the distribution, translocation, or degradation of PKCe. (iv) PKCe was predominantly membrane associated, and its distribution was unaffected by *L. major* infection. In agreement with prior studies, PKCe did not translocate in
response to PMA (20). None of the isoforms translocated in response to attachment or infection in the absence of PMA as assessed by analyses from 5 min to 4 h after addition of organism, by which time 90% of the BMM were infected with three parasites. Because individual PKC isoforms translocate to distinct intracellular sites (15, 21), we used immunofluorescence to examine activation-induced trafficking. In resting, uninfected BMM, PKCβ was located predominantly in the cytosol and in the nucleus; infection with *L. major* did not change this localization (Fig. 4a and b). After PMA activation, PKCβ translocated to circular, filamentous structures located well within the peripheral aspects of the cells and having the appearance of cytoskeletal elements (Fig. 4c). In agreement with the Western blot experiments, translocation of PKCβ was unimpeded in infected cells (Fig. 4d). The presence of the parasites was evident because they could be readily stained with the polyclonal anti-PKCβ antiserum, presumably reflecting cross-reactivity of the antibodies with parasite antigen (Fig. 4d).

PKCβ was localized diffusely in the cytosol in resting, uninfected BMM and was concentrated in areas between the nuclei of dividing cells (Fig. 5a and b). After PMA activation, PKCβ translocated to punctate areas at the ends of the cells (Fig. 5c). Infection affected neither the distribution nor the translocation pattern (Fig. 4d).

Like PKCβ, PKCζ was present in the cytosol and nucleus in BMM, and its distribution was unaffected by either PMA stimulation, in agreement with the biochemical analysis, or infection with *L. major* (data not shown). Attempts to immunolocalize PKCα were unsatisfactory with both the monoclonal anti-PKCα antibody used in the Western blots and various polyclonal antisera raised against PKCα-specific peptides. The filamentous structures revealed by translocated PKCβ and the peripheral punctate structures revealed by translocated PKCζ suggested their association with cytoskeletal elements. Most soluble intracellular components, together with the plasma and intracellular organelle membranes, can be extracted with Triton X-100, leaving behind only stable cytoskeletal components (31). Uninfected or infected BMM (2 × 10^7 cells) were left untreated or were incubated with 10 or 100 ng of PMA per ml for 15 min, lysed in ice-cold homogenization buffer to create whole-cell lysates (crude extracts), and ultra-centrifuged (100,000 × g) to create soluble cytosolic extracts. The membrane pellets were resuspended for 60 min at 4°C in homogenization buffer containing 1% (vol/vol) Triton X-100 and then recentrifuged to separate Triton-soluble and -insoluble constituents. The crude extracts, soluble cytosolic extracts, Triton-soluble membranes, and Triton-stable cytoskeleton were analyzed (10^5 cell equivalents) by Western blotting to localize PKCβ and PKCζ (Fig. 6). Activation with PMA resulted in the dose-dependent translocation of PKC from a predominantly cytosolic compartment to a Triton-insoluble compartment, consistent with movement to a cytoskeletal-associated form. Comparable results were obtained with these isoforms, and infection of BMM with *L. major* did not affect translocation (Fig. 6).

In summary, we have established the inhibitory effect of *L. major* on PKC-mediated activation of BMM. As with *L. donovani* (7), c-fos gene expression was impaired in infected cells after stimulation with PMA. Additionally, TNF-α gene expression, previously shown to be reduced after incubation of infected cells with lipopolysaccharide (6), was linked to impairment of PKC-mediated signalling. Further, we identified four PKC isoforms—PKCα, PKCζ, PKCβ, and PKCζ—in BMM and characterized their translocation after PMA activation in the presence or absence of *L. major* infection. We could discern no differences in the amounts or distributions of these enzymes after infection, nor did infection affect the initial translocation in the cell. Although the associations of PKCζ and PKCβ with membranes occurred with similar kinetics, the subsequent disappearance from the particulate fraction was substantially delayed in infected macrophages compared to that in uninfected macrophages. The ability of LPG to inhibit membrane-associated PKCζ in an in vitro assay using unila-
mellar vesicles may be relevant to this finding (12). LPG was inhibitory even when applied to the opposite side of the monolayer to which PKC was bound, even though the amounts of bound enzyme remained unaffected (12). Prior studies using *L. donovani* have been conflicting; attenuation of PMA-induced translocation (22), a lack of substantial effects on translocation (9), and attenuated activity despite normal translocation (3) have been found with different assays. However, none of these studies examined specific PKC isoforms. The prolonged association of PKC*α* and PKC*β* found in this study may ensure inhibition of the membrane-associated enzymes. As shown here and elsewhere (16), a number of isoforms of PKC exist in macrophages. These may differ among discrete macrophage populations (11, 16, 24, 32, 33), and infection may differentially affect specific isoforms.

As shown here, PKC*β* and PKC*δ* translocated to compartments associated with the macrophage cytoskeleton. The regulation of membrane-cytoskeleton interactions constitutes an important aspect of PKC signalling (14). The cytoskeletal proteins that are the major targets of PKC isoforms, however, can

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**FIG. 3.** Translocation of PKC isoforms in uninfected and *Leishmania*-infected macrophages. BMM monolayers were left uninfected (+ *L. major*) or were infected with *L. major* metacyclic promastigotes (+ *L. major*) (29) before activation with 100 nM PMA. After the designated times, cytosolic and particulate fractions were prepared and analyzed by Western blotting for PKC*α*, PKC*β*, PKC*δ*, and PKC*ζ* as described in the legend to Fig. 2. The numbers on the sides of the gels are molecular masses, in kilodaltons. The densities of translocated PKC*α* (graph in panel A) and PKC*β* (graph in panel B) were assessed with ImageQuant version 3.3 (Molecular Dynamics, Sunnyvale, Calif.), and induction was calculated by comparing each density with that of the control in the particulate fraction. Closed circles, *L. major*-infected BMM; open circles, uninfected BMM.
be cell specific. PKC\(\beta\) associated with newly formed focal adhesion points in 3T3 fibroblasts (2) and with vimentin in HL60 cells which have differentiated into monocytes/macrophages (23). The punctate structures seen in BMM with activated PKC\(\beta\) resembled actin dots described as adhesion structures in close contact with the substratum in murine peritoneal macrophages (17). Recently, PKC\(\beta\) was reported to specifically bind to and be activated by F-actin (4). Additional studies will be needed to correlate PKC-mediated cellular events with individual PKC isoforms and to analyze cytoskeletal proteins as potential enzyme substrates. As shown here, infection with Leishmania affects only a portion of the PKC isoforms present in phagocytic cells, although the functional consequences remain to be determined.

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REFERENCES
9. Fujihara, M., N. Connolly, N. Ito, and T. Suzuki. 1994. Properties of protein potential enzyme substrates. As shown here, infection with Leishmania affects only a portion of the PKC isoforms present in phagocytic cells, although the functional consequences remain to be determined.

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kinase C isoforms (βII, ε, and ζ) in a macrophage cell line (J774) and their roles in LPS-induced nitric oxide production. J. Immunol. 152:1898–1906.


