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Polyamines enhance calcium mobilization in fMet-Leu-Phe-stimulated phagocytes

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Spermidine and putrescine (50 μM–1 mM), found in exudates from infection sites, significantly enhance fMet-Leu-Phe-induced Ca^{2+} mobilization in differentiated HL-60 cells and polymorphonuclear leukocytes (PMNs) by delaying the return to basal cytosolic Ca^{2+} levels. This enhancement by polyamines is associated with inhibition of Ca^{2+} efflux across the plasma membrane. In parallel with their effects on Ca^{2+} signaling, polyamines also significantly prolong the kinetics of fMet-Leu-Phe-induced protein kinase C translocation. Thus, polyamines may play a novel role in modulating regulatory events in phagocytes.

Polyamine; Calcium mobilization; Protein kinase C; Chemotactic peptide; Phagocytic leukocyte

1. INTRODUCTION

Polymorphonuclear leukocytes (PMNs) are multifunctional phagocytes which detect, migrate toward, and ingest invading microorganisms, killing them by the production of oxygen radicals and release of granules containing microbicidal agents. Their complex functional repretoire is modulated by ligand binding to a variety of surface receptors. Activation by the chemotactic peptide *N*-formyl-Met-Leu-Phe (fMLP) induces the formation of diacylglycerol, which activates protein kinase C (PKC), and inositol 1,4,5-trisphosphate (IP_3), which releases intracellular Ca^{2+} stores [1,2]. These signaling pathways interact with each other to initiate or enhance a wide range of PMN activities, including superoxide production, degranulation, and regulation of intracellular pH (pH_i) [1–4]. Activation of PKC in the PMN is preceded by its Ca^{2+}-dependent translocation from the cytosol to the plasma membrane, which can be monitored by changes in [^{3}H]phorbol 12,13-dibutyrate (PDBu) binding to membrane-associated PKC in intact phagocytes [5,6]. A model for synergistic stimulation of PMNs has been proposed in which increased cytosolic Ca^{2+} causes reversible association of PKC with the plasma membrane, thereby priming the enzyme for activation by diacylglycerol or phorbol esters [7].

Polyamines are widely distributed organic cations which play an essential role in supporting the growth and differentiation of prokaryotic and eukaryotic cells (reviewed in [8–10]). Putrescine, cadaverine, and spermidine occur at concentrations of up to 1 mM in exudates from inflamed tissues and bacterial infection sites [11–13]. Exogenous polyamines have previously been shown to enhance superoxide production by fMLP-stimulated human PMNs [14]. Although the mechanism is unclear, this effect is dependent on the presence of extracellular Ca^{2+}. In the present study, we have examined the effect of polyamines on fMLP-stimulated Ca^{2+} mobilization in phagocytes. The results demonstrate that polyamines enhance fMLP-induced Ca^{2+} mobilization and thereby enhance Ca^{2+}-dependent PKC translocation to the plasma membrane.

Abbreviations: fMLP, *N*-formylmethyionyl-leucylphenylalanine; fura-2/AM, fura-2 acetomethoxy ester; IP_3, inositol 1,4,5-trisphosphate; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; PMN, polymorphonuclear leukocyte.

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2. EXPERIMENTAL

2.1. *Materials*

Fura-2/AM was purchased from Molecular Probes. [^{14}C]putrescine and [^{14}C]spermidine were obtained from Amersham. [^{3}H]PDBu and [^{45}Ca] were purchased from DuPont-NEN. PDBu, ionomycin, fMLP, and all polyamines were obtained from Sigma.

2.2. *Methods*

HL-60 cells (ATCC) were maintained in culture and differentiated to PMN-like cells by culturing in the presence of 1.3% dimethyl sulfoxide for 7 days [15]. Human PMNs were isolated from healthy donors as previously described [16]. Assays were conducted at 37°C with cells suspended at 2×10^6/ml in 1.9 mM KH_2PO_4, 1.1 mM Na_2HPO_4, 5 mM KCl, 147 mM NaCl, 5.5 mM glucose, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ (pH 7.4).

Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was determined with fura-2. Cells were loaded by incubation with 0.5 μM fura-2/AM for 30 min at 37°C, washed, and resuspended. $[Ca^{2+}]_i$ was monitored with a Perkin-Elmer LS-5B spectrofluorometer (excitation 340 nm, emission 505 nm) and calculated with a correction for extracellular fura-2 as described by Grynkiewicz et al. [17]. [^{14}C]polyamine uptake was assayed as de-

scribed by Kakinuma et al. [18]. The apparent K_m and V_{max} of polyamine uptake were derived from Lineweaver-Burk analysis of initial uptake rates. Protein concentrations were determined by the Bradford method [19]. ^{45}Ca efflux from intact cells was assayed as described by Wong et al. [20]. PKC translocation was monitored by assaying changes in steady-state [^{3}H]PDBu binding to intact cells as described by Dougherty and Niedel [5]. Specific [^{3}H]PDBu binding was estimated by subtracting nonspecific binding (determined in the presence of 20 μM unlabeled PDBu) from total [^{3}H]PDBu binding.

3. RESULTS AND DISCUSSION

The effect of exogenous polyamines on fMLP-stimulated Ca^{2+} mobilization in differentiated HL-60 cells is shown in Fig. 1, which portrays the time course of $[Ca^{2+}]_i$ change after a 4-min preincubation in the presence and absence of 200 μM spermidine. Upon stimulation by fMLP, $[Ca^{2+}]_i$ increased rapidly from a resting level of 96 nM to approximately 500 nM and remained above basal levels for more than 4 min. Previous studies have shown that the rapid initial phase of Ca^{2+} elevation is due to release of intracellular Ca^{2+} stores by IP_3, while the sustained phase is associated with influx of extracellular Ca^{2+} [1]. Addition of spermidine alone had no direct effect on fura-2 fluorescence or resting intracellular Ca^{2+} levels (data not shown). However, preincubation with spermidine markedly lengthened the sustained phase of Ca^{2+} mobilization by fMLP. To compare mean $[Ca^{2+}]_i$ in the presence and absence of spermidine, we integrated both traces from 0–5.5 min after addition of fMLP. In the presence of 200 μM spermidine, Ca^{2+} mobilization was increased to 140 ± 0.33% of control (mean ± S.E., significant at P=0.0001). This effect was confirmed with human PMNs (143 ± 3.3% increase relative to control, P=0.006, data not shown).

Fig. 2 portrays the dose dependence of Ca^{2+} mobilization enhancement by polyamines. Spermidine increased fMLP-induced Ca^{2+} mobilization to 124% of control at a concentration of 50 μM and 140% of control at 200 μM. Putrescine and cadaverine were less effective. Putrescine increased Ca^{2+} mobilization to a maximum of 134% of control at 1 mM, while cadaverine increased

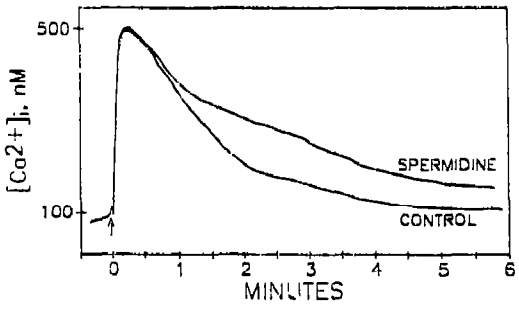

Fig. 1. The effect of spermidine on Ca^{2+} mobilization in fMLP-stimulated HL-60 cells. Cells were loaded with fura-2 and preincubated for 4 min at 37°C in the presence or absence of 200 μM spermidine. The arrow indicates the addition of 100 nM fMLP. The data are representative of at least 3 experiments.

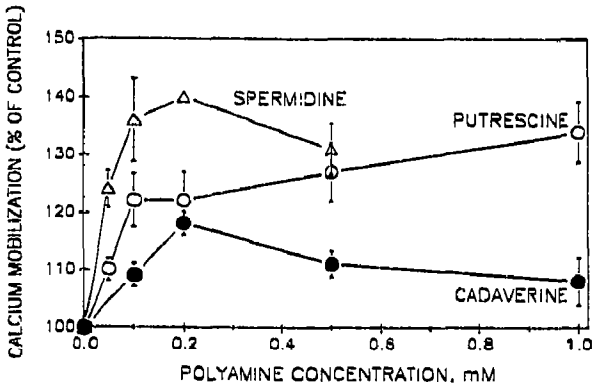

Fig. 2. The dose dependence of Ca^{2+} mobilization enhancement by polyamines. The time course of Ca^{2+} mobilization was monitored in HL-60 cells in the presence and absence of polyamines as shown in Fig. 1A. $[Ca^{2+}]_i$ was integrated from 0 to 5.5 min after the addition of 100 nM fMLP. Integrals obtained in the presence of polyamines were ratioed to control integrals obtained in the absence of polyamines. Results are presented as percent of control ± S.E.M.

Ca^{2+} mobilization by a maximum of 118% at 200 μM. With the exception of 1 mM cadaverine, all polyamine concentrations tested had a statistically significant effect on Ca^{2+} mobilization compared to controls ($P \leq 0.05$). Interestingly, polyamines occur at these concentrations in exudates from inflamed tissues and bacterial infection sites [11–13]. In human periodontal disease, for example, mean putrescine, cadaverine and spermidine concentrations of approximately 900 μM, 600 μM, and 200 μM, respectively, have been detected in inflammatory exudates, and tend to increase with the severity of disease [11,12].

To ascertain whether polyamines could potentially modulate Ca^{2+} mobilization by action at an intracellular site, we examined the extent of cell [^{14}C]polyamine uptake. Similar to other types of eukaryotic cells, HL-60 cells were found to internalize polyamines in a time-dependent manner. The kinetics of putrescine and spermidine uptake by unstimulated HL-60 cells were characterized by apparent K_m values of 5.1 μM and 1.7 μM, respectively, and apparent V_{max} values of 137 and 180 pmol/min/mg, respectively (data not shown). Stimulation by fMLP did not enhance polyamine uptake. To begin to assess the mechanism by which polyamines enhance Ca^{2+} mobilization, we examined the effect of introducing spermidine (200 μM) at various times after fMLP stimulation (Fig. 3). Whether introduced at the time of stimulation (upper traces) or 1 min after stimulation (middle traces), spermidine noticeably potentiated the Ca^{2+} signal. The magnitude of this effect decreased when spermidine was introduced 2 min after stimulation, and decreased even further when spermidine was introduced 3 min after stimulation (bottom traces). Spermidine had no apparent effect on intracellular Ca^{2+} levels when added 4 min after stimulation (not shown). Interestingly, spermidine had a nearly instantaneous effect on the kinetics of Ca^{2+} mobilization

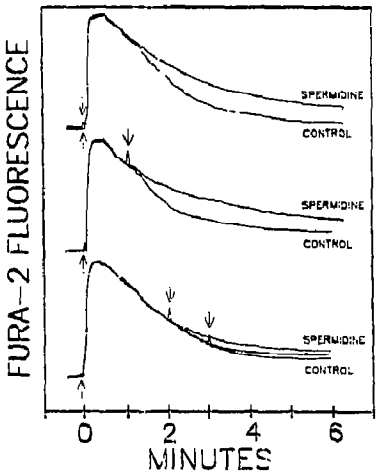

Fig. 3. The effect of spermidine on Ca^{2+} mobilization when added at various times after stimulation by fMLP. Assays were conducted with HL-60 cells under the conditions described in Fig. 1A, except that 200 μM spermidine was added where indicated by the downward-pointing arrows (0 min in the upper panel, 1 min in the middle panel, 2 and 3 min in the lower panel). Upward-pointing arrows indicate the addition of 100 nM fMLP at 0 min.

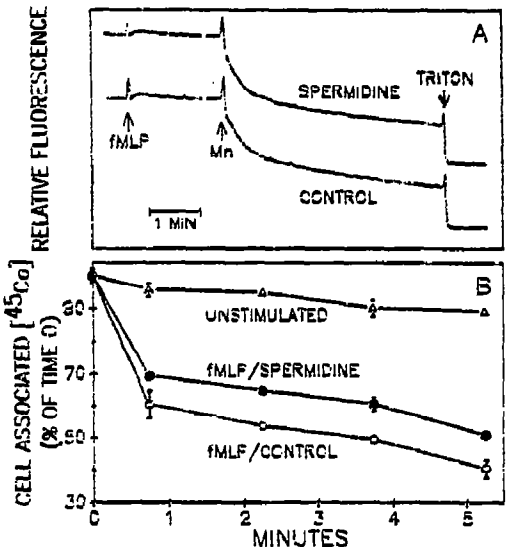

Fig. 4. The effect of spermidine on Ca^{2+} channel opening and Ca^{2+} efflux from HL-60 cells. Panel A. Cells were loaded with fura-2, washed, and resuspended in buffer containing 1 μM Ca^{2+} just prior to assay in the presence and absence of spermidine (200 μM). In these experiments, fura-2 was excited at the Ca^{2+}-insensitive wavelength of 360 nm. Arrows indicate the addition of fMLP (100 nM), Mn^{2+} (100 μM), and Triton X-100 (to 0.1% final). The traces are representative of 3 experiments. Panel B. The effect of spermidine on ^{45}Ca efflux from fMLP-stimulated HL-60 cells. Cells were loaded with ^{45}Ca, washed, and resuspended with or without 200 μM spermidine. In the bottom plots, the cells were stimulated with fMLP at time 0. At the indicated times, aliquots of cell suspension were withdrawn and pelleted by centrifugation. Radioactivity associated with the cell pellet was counted and expressed as a percentage of prestimulation levels. The results are presented as mean ± S.E.M.

when added 1–3 min after stimulation, suggesting that extensive internalization of polyamines is not required to produce this effect. Moreover, the data suggest that polyamines modulate events that follow fMLP receptor occupancy and release of intracellular Ca^{2+} stores. This could potentially involve enhancement of extracellular Ca^{2+} influx through plasma membrane Ca^{2+} channels or inhibition of Ca^{2+} efflux via plasma membrane Ca^{2+}-ATPases.

Ca^{2+} influx through plasma membrane channels can be indirectly monitored by following the entry of extracellular Mn^{2+}, which profoundly quenches intracellular fura-2 fluorescence [21]. We used this appraoch to evaluate the effect of polyamines on Ca^{2+} channel opening during the sustained phase of Ca^{2+} mobilization (Fig. 4A). Spermidine (200 μM) had no apparent effect on the rate or extent of fura-2 quench produced when Mn^{2+} was added to the cell suspension 2 min after stimulation by fMLP. Similarly, there were no apparent differences in fura-2 quench attributable to spermidine when Mn^{2+} was added at other time points from 0 to 3 min after fMLP stimulation (not shown). Thus, these studies provide no evidence that polyamines enhance Ca^{2+} influx by potentiating Ca^{2+} channel opening. We therefore examined the effect of polyamines on Ca^{2+} efflux from fMLP-stimulated HL-60 cells that had been passively loaded with ^{45}Ca (Fig. 4B). When compared to the control, ^{45}Ca efflux was inhibited in the presence of spermidine (200 μM). This inhibition of efflux was significant from 2.25 to 5.25 min after cell stimulation by fMLP ($P<0.01$). The results suggest that polyamines could inhibit the plasma membrane Ca^{2+}-ATPase and thereby delay the return to resting cytosolic Ca^{2+} levels. By inhibiting Ca^{2+} efflux, polyamines disrupt the equilibrium between Ca^{2+} influx and efflux across the plasma membrane, which is a major determinant of $[Ca^{2+}]_i$ during the sustained phase of intracellular Ca^{2+} elevation [1]. Polyamines have previously been shown to inhibit other types of membrane-bound ATPases (reviewed in [22]).

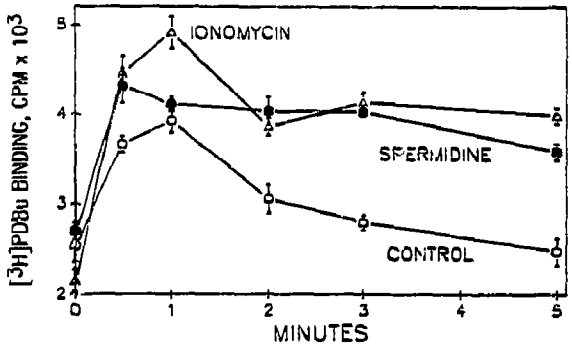

Fig. 5. The effect of spermidine on the time course of PKC translocation in HL-60 cells. Specific [^{3}H]PDBu binding to intact cells was assayed at 37°C. Cells were preincubated in the presence or absence of 200 μM spermidine for 4 min prior to stimulation with 100 nM fMLP. For comparison, the effect of stimulation with a combination of 100 nM fMLP and 100 nM ionomycin is shown. The results are presented as mean ± S.E.M.

We assessed the effect of polyamines on Ca^{2+}-dependent PKC translocation by examining changes in steady-state phorbol ester binding to intact cells. Polyamines alone had no significant effect on phorbol ester binding to unstimulated cells (data not shown). Fig. 5 shows the time course of [^{3}H]PDBu binding after stimulation with 100 nM fMLP. After preincubation in both the presence and absence of spermidine (200 μM), stimulation with fMLP upregulated [^{3}H]PDBu binding to over 150% of prestimulation levels within 1 min. In the absence of spermidine, [^{3}H]PDBu binding was downregulated to 121% of prestimulation levels after 2 min and 98% after 5 min of fMLP stimulation. In the presence of spermidine, [^{3}H]PDBu binding was downregulated to 149% after 2 min, 133% after 5 min, and eventually returned to prestimulation levels by 10 min (data not shown). This enhancement of [^{3}H]PDBu binding by spermidine from 2 to 5 min after fMLP stimulation was significant ($P<0.004$), and paralleled its effects on the time course of fMLP-induced Ca^{2+} mobilization (Fig. 1). For comparison, we monitored the kinetics of [^{3}H]PDBu binding after stimulation with a combination of 100 nM fMLP and 100 nM ionomycin (which produces a sustained $[Ca^{2+}]_i$ increase). This combination of stimuli induced a more profound upregulation of [^{3}H]PDBu binding than fMLP alone. Similar to the effects of spermidine, ionomycin caused a marked delay in the downregulation of [^{3}H]PDBu binding from 2 to 5 min after stimulation by fMLP.

Few studies have previously examined the effect of exogenous polyamines on PMN function or signal transduction. The present study has shown that naturally occurring polyamines can significantly enhance phagocyte Ca^{2+} mobilization over the range of extracellular polyamine concentrations found at bacterial infection sites, and thereby modulate certain Ca^{2+}-dependent regulatory events (e.g. PKC translocation). This effect by polyamines is associated with inhibition of Ca^{2+} efflux rather than enhanced Ca^{2+} entry through plasma membrane channels. Thus, polyamines appear to enhance Ca^{2+} mobilization by altering the equilibrium between Ca^{2+} influx and efflux across the plasma membrane, which is a primary determinant of $[Ca^{2+}]_i$ during the sustained phase of intracellular Ca^{2+} elevation [1]. However, since this study's $[Ca^{2+}]_i$ measurements were conducted with large cell populations, we cannot rule out the possibility that polyamines have more complex effects on the temporal aspects of Ca^{2+} signaling in individual cells.

The findings of this study provide a mechanism to explain how polyamines can enhance fMLP-induced oxidative function in human PMNs [14], and suggest that polyamines could enhance other Ca^{2+}-dependent aspects of PMN function (e.g. secretion of specific granules [1]). Thus, the influence of these polycations could have important implications for host defense modulation at bacterial infection sites.

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