Primed Neutrophils May Be "Primed" to Augmented Effector Function, eg, superoxide (O_2^-) Production in the Respiratory Burst, Upon a Second Stimulation with a Variety of Soluble Agonists Including Formylated Methionyl-Leucyl-Phenylalanine (FMLP) and Phorbol Myristate Acetate (PMA). At priming concentrations of FMLP (5 x 10^-8 mol/L) that did not initiate O_2^- generation, two metabolic activities were noted: (1) approximately a threefold increase in the baseline intracellular calcium (Ca^{2+}) level, that was not dependent on extracellular Ca^{2+}, and (2) a rapid rise in intracellular pH that was blocked by 5-(N,N-dimethyl) amiloride (DA), that had no effect on the Ca^{2+} response to priming. Furthermore, there were no significant increases in inositol metabolites in cells primed and stimulated with FMLP compared with cells receiving the stimulating dose of FMLP alone and pretreatment with pertussis toxin (PT) (before the addition of the priming -5 x 10^{-9} mol/L dose of FMLP), whereas abolishing the response to FMLP during the second stage of stimulation, had (1) no effect on FMLP-primed cells subsequently stimulated with PMA, and (2) only partially ablated the rise in Ca^{2+}, initiated with FMLP. That FMLP priming involved distinctive processes to those of the well characterized FMLP-coupled Ca^{2+}-dependent activation cascade was shown by the full priming effect attained in a Ca^{2+}-free buffer, which did not sustain an O_2^- response to a second-stage FMLP stimulation, but sustained a primed response to PMA. These data demonstrate that FMLP primes human neutrophils by a Ca^{2+}-independent and PT-insensitive pathway, offering a functional model for studying heterogeneous FMLP receptor-coupled reactions.

**HUMAN NEUTROPHIL** stimulation may be modulated by preincubation of cells with low concentrations of various agonists that do not evoke observed end-functions of stimulation (ie, chemotaxis, respiratory burst); yet when these same cells are then exposed to an agonist at concentrations that normally evoke cell responses, these functions are augmented relative to untreated cells. This phenomenon is referred to as priming and has been studied using a broad variety of soluble stimulants, eg, chemotactic formylpeptides, endotoxin, 1-oleoyl-2-acetyl-glycerol (OAG), and platelet activating factor. The mechanism for priming is not clearly defined for any agonist. Although the primed response to formylated methionyl-leucyl-phenylalanine (FMLP) has been thought to depend on an upregulation of its receptor, this is not explained by priming with leukotriene B_4 (LTB_4) and lipopolysaccharide (LPS). The role of an upregulated protein kinase C (PK-C) also has been invoked, but again under certain conditions do not exhibit either a translocation of PK-C to the membrane or an increase in its activity. We have examined the mechanisms of FMLP priming in regard to defining Ca^{2+} changes in "primed" resting and stimulated cells. Depending on the priming agent, the rise of intracellular Ca^{2+} (Ca^{2+}) may be either depressed, augmented, or bypassed altogether. The nature then of the role of mobilized intracellular Ca^{2+} is problematic and served as the focus of these studies.

In this report, we present data that demonstrate the quantitative rise in cytosolic-free Ca^{2+} in FMLP-stimulated neutrophils that closely correlates with O_2^- generation, as seen with ionomycin-primed neutrophils; there is, however, no significant rise in inositol phosphate metabolites in cells primed and stimulated compared with cells receiving the stimulating dose of FMLP alone. Our data also show increased influx of [45Ca] into neutrophils following primed stimulation. Priming with FMLP is not pertussis toxin (PT)-sensitive, in that O_2^- response to stimulating concentrations of FMLP is abolished, but a normal primed response is observed with phorbol myristate acetate (PMA). These data then serve to illustrate the mechanism of priming by FMLP through a PT-insensitive pathway that may serve as a useful functional assessment of FMLP receptor heterogeneity, which has recently been appreciated as coupled to unique G proteins, of which a low molecular weight species (approximately 22 to 26 Kd) is insensitive to ribosylation by PT.

**METHODS**

Reagents. The following reagents were used in this study: FMLP, PMA, superoxide dismutase (SOD), ferricytochrome-c, and bovine serum albumin (BSA; Sigma Chemical Co, St Louis, MO); Ficoll-Hypaque, dextran, and citrate (Pharmacia Fine Chemicals, Piscataway, NJ); Fura-2 AM penta acetoxyethyl ester (Cal Biochem, La Jolla, CA); [H]-inositol-1,4,5-triphosphate, D-[inositol-2-3H (N)] (4.0 Ci/mmol/L), [H]-inositol 1-4 bisphosphate, D-[inositol-2-3H (N)] (0.1 Ci/mmol/L), (CaCl_2) (1.5 Ci/mmol/L), ([H]-inositol specific activity 12.8 Ci/mmol/L) (New England Nuclear, Boston, MA); H-7 (Seikagaku America, St Petersburg, FL); PF (List Biological Labs Inc, Campbell, CA); silicone oil (George Mann & Co, Providence, RI); and organic solvents (Fisher Scientific, Fairlawn, NJ).

**Neutrophil O_2 generation.** Neutrophils from healthy volunteers were isolated to greater than 95% purity as described and used within 2 hours of preparation. Efforts were made to avoid LPS contamination (neutrophils were processed using endotoxin-free water in all buffers), however, the possibility of LPS contamination...
was not totally eliminated. O$_2$- generation was measured by a discontinuous assay of the SOD-inhibitable reduction of ferricytochrome-c at 37°C as previously described. For priming, neutrophils at a concentration of $2 \times 10^9$/mL were preincubated at 37°C with $5 \times 10^{-7}$ mol/L FMLP for 10 minutes, then 5 mg/mL cytochalasin B was added, and after 5 minutes the cells were stimulated with $5 \times 10^{-7}$ mol/L FMLP or PMA (400 nmol/L) for 5 minutes, transferred to an ice bath at 4°C, centrifuged at 200 x $g$ for 5 minutes and assayed for O$_2$- generation. Controls included (1) neutrophils preincubated with buffer before stimulation with $5 \times 10^{-7}$ mol/L FMLP, (2) neutrophils primed with $5 \times 10^{-7}$ mol/L FMLP for 10 minutes but not stimulated, or (3) neutrophils unexposed to FMLP (resting neutrophils).

Neutrophils incubated with 500 ng/mL PT were processed by a modification of the procedure of Goldman et al. PT-treated and control cells at a concentration of $2 \times 10^9$/mL were incubated at 37°C in phosphate buffered saline (PBS) containing 9 mmol/L Ca$^{++}$, 2.5 mmol/L Mg$^{++}$, and 5 mmol/L glucose, pH 7.4, for 2 hours. Cells were then washed, resuspended in PBS, and assayed for O$_2$- production. The total inability to generate O$_2$- (assayed by SOD-inhibitable reduction of cytochrome-c continuously) upon stimulation with FMLP ($5 \times 10^{-7}$ mol/L) served as the criterion of effective ribosylation. Studies of primed O$_2$- generation and Ca$^{++}$, rise in response to FMLP ($5 \times 10^{-7}$ mol/L) or PMA (400 nmol/L) were then initiated.

Measurement of Ca$^{++}$, Ca$^{++}$, levels were determined using the fluorescent indicator fura-2. Isolated neutrophils were incubated with the acetoxymethyl ester of fura-2 (2.5 µmol/L) in PBS for one half hour at 37°C and resuspended in PBS at a concentration of $2 \times 10^9$/mL. Immediately before each assay, cells were again centrifuged within the assay cuvette and resuspended in fresh buffer to remove extracellular probe. Ca$^{++}$, levels were determined from readings obtained before priming cells with $5 \times 10^{-7}$ mol/L FMLP; readings were also taken after the priming dose was added, and both before and after the addition of cytochalasin-B. Cells were stimulated with $5 \times 10^{-7}$ FMLP and the peak Ca$^{++}$, levels were determined; sequential measurements were made before and every 30 seconds up to 5 minutes after addition of $5 \times 10^{-7}$ mol/L FMLP. Ca$^{++}$, determinations were made using the ratio of the peak fluorescence at excitation wavelengths of 340 to 380 nm and read on a Perkin-Elmer model LS-S spectrophuorometer (Oak Brook, IL). Emission wavelength was 510 nm. The Ca$^{++}$, concentration was calculated using a Kd of 224.$^{29}$ Two or three assays were performed for each condition, i.e., cells primed but not stimulated, stimulated without being primed, and cells that were primed and stimulated. Results were averaged and mean values were then treated as single data points in statistical calculations.

Measurement of intracellular pH. To ascertain intracellular pH (pHi), neutrophils were incubated with 6 µmol/L 2,7-bis(carboxyethyl)-5,6-carboxyfluorescein tetracetoxyethyl ester (BCECF-AM) (Molecular Probes, Junction City, OR) in buffer at 37°C for 20 minutes, centrifuged at 400 g for 4 minutes, and resuspended in fresh buffer. The fluorescent intensity (FI) of intracellular BCECF was determined at 37°C at excitation wavelengths of 506 and 455 nm and an emission wavelength of 530 nm using a Perkin-Elmer model LS-5 spectrophuorometer. The FI at excitation wavelength of 455 nm, unlike 506 nm, is relatively insensitive to pH changes, whereas both are sensitive to change in BCECF concentration.$^{29}$ The FI ratio of excitation wavelengths 506 to 455 nm then compensates for differences in probe concentration. Fluorescence intensity ratio was calibrated to pH by equilibrating the suspensions at pH 4.0 and different pH values at 37°C for 20 minutes, centrifuged at 1000 x $g$ for 5 minutes and assayed for O$_2$- generation. Where indicated, 5-(N,N-dimethyl) amiloride hydrochloride (DA [50 mmol/L]; Merck, Sharp, and Dohme, Inc, West Point, PA) was added to the cell suspension 3 minutes before the addition of FMLP.

Ca$^{++}$, efflux and influx. Efflux of Ca$^{++}$, was measured as described by Korchak et al.$^{22}$ Cells ($1 \times 10^9$/mL) were loaded with $[^{45}Ca]$ by incubation for 1 hour at 37°C in PBS to which was added 100 µCi/mL $[^{45}CaCl_2]$. After washing, cells were resuspended in buffer at 37°C. After the primed stimulation protocol (as described in the O$_2$- assay) was carried out, release of $[^{45}Ca]$ into the supernatant was measured by centrifuging cells through silicone oil for 1 minute at room temperature in an Allied microcentrifuge (Fisher Labs, Fairlawn, NJ), and measuring radioactivity in the aqueous layer.

Influx of Ca$^{++}$ into neutrophils was measured as described by Korchak et al.$^{22}$ Neutrophils at a concentration of $1 \times 10^7$ cells/mL were used in the priming protocol as described in the O$_2$- assay. $[^{45}Ca]$ was added to the cell medium just after stimulation with $5 \times 10^{-7}$ mol/L FMLP to assess the influx of Ca$^{++}$ after the priming dose of FMLP alone; $[^{45}Ca]$ was added immediately after the addition of $5 \times 10^{-9}$ mol/L FMLP in parallel experiments. The reaction was stopped by addition of ethylene glycol-bis (β-amino ethyl ether)-N, N, N', N'-tetra acetic acid (EGTA; 10 mmol/L final concentration) and cells were centrifuged through layers of silicone oil and 12% sucrose at room temperature for 1 minute in the microcentrifuge. The tips of the vials containing all pellets were cut and vigorously resuspended in scintillation fluid and radioactivity was measured. In all Ca$^{++}$ influx or efflux experiments triplicate samples were obtained for each datapoint and mean results calculated. These mean values were then treated as single determinations in subsequent statistical analysis.

Table 1. O$_2$- Response in FMLP Primed Neutrophils

<table>
<thead>
<tr>
<th>Preincubation Conditions</th>
<th>Release of O$_2$- mmol/L</th>
<th>FMLP</th>
<th>PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS with Ca$^{++}$</td>
<td>$5 \times 10^{-7}$ M</td>
<td>20 ± 3</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>Buffer</td>
<td>(a)</td>
<td>(c)</td>
<td></td>
</tr>
<tr>
<td>$5 \times 10^{-9}$ mol/L FMLP</td>
<td>$51 \pm 2$</td>
<td>$28 \pm 7$</td>
<td></td>
</tr>
<tr>
<td>Ca$^{++}$-free PBS and 3 mmol/L EGTA Buffer</td>
<td>$4 \pm 2$</td>
<td>$12 \pm 3$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(e)</td>
<td>(g)</td>
<td></td>
</tr>
<tr>
<td>$5 \times 10^{-9}$ mol/L FMLP</td>
<td>$3.6 \pm 2$</td>
<td>$2.8 \pm 4$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(f)</td>
<td>(h)</td>
<td></td>
</tr>
</tbody>
</table>

Cells were preincubated for 10 minutes with buffer or FMLP at a concentration of $5 \times 10^{-9}$ mol/L. In separate experiments, neutrophils were washed once and resuspended in Ca$^{++}$-free PBS containing 3 mmol/L EGTA before the addition of the priming dose of FMLP. Cells were then stimulated with $5 \times 10^{-7}$ mol/L FMLP or PMA for 5 minutes (n = 4, mean ± SEM) to assess O$_2$- production as detailed in Methods. In a Ca$^{++}$-free buffer, FMLP primed and stimulated O$_2$- response is abolished, but FMLP-primed cells respond to PMA as in Ca$^{++}$ containing buffer. P values: (a) vs (b) = .0001, (c) vs (d) = .0385, (e) vs (f) = .865, (g) vs (h) = .0012.
protocol (as described in the O2 assay) cells were resuspended in a chloroform-methanol (1:1) and H2O mixture (10 parts chloroform-methanol to one part H2O by volume), the upper layer aspirated, dried, and resuspended in 50 mm ammonium formate (pH 6.25), and then sonicated and frozen for later assay.

A Waters HPLC system (Millipore Corp, Millford, MA) with Whatman partisil 10 Sax column (Clifton, NJ) was used as described for separation of inositol phosphates. Standard preparations of radiolabeled IP3 were chromatographed during each run, with retention times observed of 49 to 51 minutes. Experimental samples were thawed at 4°C and quantitated by measuring peak radioactivity in fractions corresponding to the observed IP3 fractions.

Statistics. All P values were obtained using the paired t test.

RESULTS

Primed and Ca++ mobilization. The priming dose of FMLP (5 x 10^-9 mol/L) alone did not stimulate O2- production (data not shown). Neutrophils primed with 5 x 10^-9 mol/L FMLP and then stimulated with 5 x 10^-7 mol/L FMLP or PMA (400 nmol/L) demonstrated significant enhancement of O2- production compared with the control

cells (Table 1). As discussed below, a Ca++-free buffer abolished FMLP responses, but had no effect on PMA stimulation in either control or FMLP-primed cells.

Based upon the Ca++-dependence of FMLP responses we then examined the correlation of O2- response to changes in Ca++ metabolism. There was a rapid increase in Ca++, on addition of the priming (5 x 10^-9 mol/L) and stimulating (5 x 10^-7 mol/L) doses of FMLP (Fig 1A, Table 2). Peak values were reached in approximately 15 seconds after the addition of FMLP at either concentration. Ca++ levels then gradually decreased and 2 minutes after the addition of 5 x 10^-9 mol/L FMLP, Ca++ levels plateaued at just above resting levels and remained at this level until the addition of 5 x 10^-7 mol/L FMLP. As compared with control neutrophils (treated with an equal volume of buffer), a rise in cytosolic Ca++ over baseline values in various experiments ranged from 100 to 200 nmol/L when cells were exposed to a priming dose (5 x 10^-9 mol/L) of FMLP, which is similar to values previously reported. The subsequent peak levels of cytosolic Ca++ achieved on stimulation with 5 x 10^-7 mol/L FMLP were significantly higher in primed cells compared

![Image](https://via.placeholder.com/150)

**Fig 1.** (A) Effect of priming with FMLP on Ca++, in fura-2-loaded neutrophils. (B) Ca++, response to the priming dose of FMLP in PT-treated neutrophils. Ca++ was calculated from the ratio of fura-2 fluorescence at two excitation wavelengths. The arrows indicate the times of addition of FMLP. The tracings represent four experiments.
Table 2. Effects of Priming With FMLP (5 x 10^{-8} mol/L) on Ca^{2+} i

<table>
<thead>
<tr>
<th>Preincubation Conditions</th>
<th>None</th>
<th>FMLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>72 ± 4 (a) (94 ± 4t)</td>
<td>281 ± 46 (d)</td>
</tr>
<tr>
<td>5 x 10^{-8} mol/L FMLP</td>
<td>179 ± 24 (b) (97 ± 5t)</td>
<td>394 ± 34 (c)</td>
</tr>
</tbody>
</table>

Fura-2 loaded neutrophils were preincubated for 10 minutes with buffer or primed with 5 x 10^{-8} mol/L FMLP. Baseline Ca^{2+} i values were determined initially and peak cytosolic Ca^{2+} levels following stimulation with 5 x 10^{-7} mol/L FMLP were determined by fluorescence measurement as detailed in Methods.

*Mean ± SEM, n = 6. FMLP primed cells showed significantly increased Ca^{2+} i levels on stimulation, as compared with buffer-treated cells (c v d = .0086; priming alone initiated a significant rise in Ca^{2+} i; P = .0053).

†Mean prestimulus Ca^{2+} i levels.

Table 3. FMLP-Stimulated [%Ca^{2+} i] Influx From Control and Primed Neutrophils

<table>
<thead>
<tr>
<th>Preincubation Conditions</th>
<th>[Ca^{2+} i] Influx (cpm) Stimulus*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>402 ± 76</td>
</tr>
<tr>
<td>5 x 10^{-8} mol/L FMLP</td>
<td>1,302 ± 352</td>
</tr>
</tbody>
</table>

Influx of Ca^{2+} into neutrophils was measured as described by Korchak et al. by adding [%Ca^{2+} i] to the cell medium just after the addition of the priming (5 x 10^{-8} mol/L) or stimulating (5 x 10^{-7} mol/L) dose of FMLP as detailed in Methods.

*Mean ± SEM, n = 5. FMLP primed cells showed a significant increased influx on stimulation with 5 x 10^{-7} mol/L FMLP compared with buffer-treated cells (P < .005).

Table 4. FMLP-Stimulated [%Ca^{2+} i] Efflux From Control and FMLP-Primed Neutrophils

<table>
<thead>
<tr>
<th>Preincubation Conditions</th>
<th>[%Ca^{2+} i] Efflux (cpm) Stimulus*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>5,856 ± 353</td>
</tr>
<tr>
<td>5 x 10^{-8} mol/L FMLP</td>
<td>7,919 ± 197</td>
</tr>
</tbody>
</table>

Neutrophils were loaded with [%Ca^{2+} i] by incubation for 1 hour at 37°C in PBS to which was added 100 uCi [%CaCl_2] and efflux measured as described by Korchak et al. and as detailed in Methods.

*Mean ± SEM of four experiments performed in triplicate. FMLP-primed cells showed no significant decrease in [%Ca^{2+} i] efflux on stimulation with 5 x 10^{-7} mol/L FMLP, as compared with buffer-treated cells (P > .18).

H^{+} and Na^{+}/Ca^{2+} exchange in priming with FMLP. There is a rapid and marked rise in pH, that is seen on addition of 5 x 10^{-8} mol/L FMLP, which is sustained and unchanged by the addition of the stimulating dose of FMLP (Fig 2). The addition of 50 mmol/L DA totally abolishes the pH response, but DA (50 mmol/L) has no effect on the rise in Ca^{2+} i levels during priming and primed stimulation (data not shown). These findings suggest that although there is a dramatic increase in pH with priming this is not crucial to activation, and the elevation in Ca^{2+} i upon priming does not occur via an amiloride-sensitive Na^{+}/Ca^{2+} exchange.

PT insensitivity of FMLP priming. We next examined the role of inositol phosphate metabolism in FMLP-primed cells because of the well-documented association of this activity to the release of Ca^{2+} i stores. We have previously shown that the Na^{+}/H^{+} antiporter plays a role of a significant metabolic regulator of pH, in activated neutrophils, which influences receptor-coupled reactions proximal to the reduced nicotinamide-adenine dinucleotide phosphate (NADPH)-oxidase. Simchowitz and Crago have recently shown that an amiloride-sensitive Na^{+}/Ca^{2+} exchange may be responsible for the increase in [%Ca^{2+} i] influx and rise in free Ca^{2+} i, seen following stimulation with FMLP. We therefore examined the role of Na^{+}/

[pdf document image of Fig 2]
PT-INSENSITIVE FMLP NEUTROPHIL PRIMING

Table 5. FMLP-Stimulated IP2 Generation in Primed Neutrophils

<table>
<thead>
<tr>
<th>Preincubation Conditions</th>
<th>Stimulus</th>
<th>IP2 (nM) ± SEM</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5 x 10^-9 mol/L FMLP</td>
<td>194 ± 6</td>
<td>3</td>
</tr>
<tr>
<td>Buffer</td>
<td>5 x 10^-8 mol/L FMLP</td>
<td>206 ± 26</td>
<td>6</td>
</tr>
</tbody>
</table>

Cells were either primed or buffer treated, and stimulated with 5 x 10^-8 mol/L FMLP for 1 minute, and as detailed in Methods. There is no significant increase in IP2 in cells primed and stimulated with FMLP compared with cells receiving the FMLP stimulating (5 x 10^-9 mol/L) dose alone.

*Mean ± SEM, n = 3.

lation dose of FMLP alone (Table 5). Thus, the rise in Ca2+, appeared to bypass inositol turnover. These data were supported by the insensitivity of FMLP priming to PT treatment.

The FMLP receptor has been shown to be coupled to a PT-sensitive G protein that initiates a transduction process of receptor-ligand coupled reactions to enzymatic effector function.27,28 Preincubation of neutrophils with PT (500 ng/mL) for 2 hours at 37°C completely blocked O2- generation in cells that were primed with 5 x 10^-9 mol/L FMLP and stimulated with 5 x 10^-7 mol/L FMLP, but there was no such inhibitory effect on cells primed with 5 x 10^-9 mol/L FMLP and stimulated with PMA (Fig 3). In PT-treated neutrophils, the incremental rise in Ca2+ upon a priming dose of FMLP was greater than twofold (Fig 1B), and this increment was not statistically different from that observed in untreated cells. These results substantiate the insensitivity of the primed response to PT.

Extracellular calcium independence of priming. Calcium independence of priming (Table 1) offered another difference between the priming process and the G-protein coupling system characterized in unprimed FMLP-stimulated neutrophils. The necessity of including extracellular Ca2+ in the buffer system has been established for various FMLP-elicited responses, eg, respiratory burst, degranulation, actin polymerization, and chemotaxis.28 When we repeated the priming studies in Ca2+-free PBS, containing EGTA (3 mmol/L), the primed neutrophil O2- response was not diminished as compared with Ca2+-containing buffer (Table 1). Although a full dose of FMLP could not elicit O2- generation above baseline in primed cells incubated in the Ca2+-free buffer, the PMA-primed response was unaffected. (The extracellular Ca2+ dependence of PMA elicited function is discussed below.) It is of note that the intracellular rise in Ca2+ during priming, although lower than in Ca2+-containing PBS buffer, showed more than a fourfold increase over the baseline (111 ± 10 x 27 ± 3 [mean ± SEM], n = 4, P < .006). These data suggest that mobilization of Ca2+ is crucial for FMLP priming, and it is the relative rise over baseline rather than a threshold Ca2+ level that determines the primed O2- response.

The role of Ca2+ in PMA-stimulated responses. In PMA-stimulated neutrophils, the lack of change in Ca2+ levels, and the absent requirement for extracellular Ca2+ for either O2- production or degranulation, has suggested that Ca2+ plays no role in the neutrophil response to this agonist.22,29 However, the data correlating with the Ca2+ dependence of PMA elicited function is not threshold level that determines the response. To help differentiate these alternative sites of control, we re-examined the Ca2+ requirements for PMA neutrophil stimulation.

We studied the function of Ca2+ in the cellular response by first acutely reducing Ca2+ to negligible levels with the addition of EGTA (2 mmol/L) to PBS2+: the O2- response under these conditions was not inhibited (data not shown). However, if the cells were depleted of intracellular Ca2+ by prolonged incubation in Ca2+-free buffer, the O2- response became dependent on extracellular Ca2+ as shown in parallel control experiments. A dose-response requirement for extracellular Ca2+ was established with an approximate one-half nmol/L (PBS Fig 4). The Ca2+ levels in fact were shown to be depleted under these conditions. Using the fluorescent indicator fura-2, we established that neutrophil Ca2+ levels decreased from 48.3 ± 6.2 nmol/L (PBS2+) to 13.0 ± 4.4 nmol/L (PBS) (n = 5). In cells incubated in Ca2+-free, 1 mmol/L EGTA PBS, the Ca2+ levels were further decreased below a level of accurate determination. Although these levels are lower than those measured by the Ca2+ indicator quin2,22,29,33 they are in agreement with those reported for fura-2.24 When Ca2+-depleted cells were tested for viability by trypan blue exclu-
C-independent activity to that which controls the primed O_2 response. Whether the rise in Ca^2+ is pivotal or a parallel differentiation observed with the Ca^2+ rise depleted neutrophils and correlates with the Ca^2+ rise dependence for PMA stimulation is evident in Ca^-free conditions. PK-C would predict such a regulatory role for Ca^2+.

Certainly the Ca^2+ dependence of PK-C would predict such a regulatory role for Ca^2+. In any case, the extracellular Ca^2+ dependence for PMA stimulation is evident in Ca^-depleted neutrophils and correlates with the Ca^2+ rise observed with FMLP priming. Thus, we are unable to differentiate whether the rise in Ca^2+ is pivotal or a parallel activity to that which controls the primed O_2 response. However, we can assert that the priming process is PK-C–independent in that 1-(5-isooquinoline sulfonyl)2-methyl-piperazine (H-7), an inhibitor of PK-C as well as other kinases, had no effect on primed stimulation with FMLP but abolished the primed O_2 response in cells stimulated with PMA (Fig 5).

DISCUSSION

The data presented here confirm previous reports that neutrophils are primed by exposure to low concentrations of FMLP to release increased amounts of O_2^- when stimulated with a higher concentration of this agonist (second stage of stimulation). At the priming dose, FMLP does not generate neutrophil O_2^- production but a rise in Ca^2+ and an increase in pH are noted. Although we have dissociated the pH change with priming (discussed below) our results are consistent with others who showed that the priming process, using Ca^2+ ionophore, exhibits a correlation in the rise of Ca^2+ and augmented O_2 production. Both primed responses to FMLP and PMA appear related to this Ca^2+ rise. Our studies of PMA Ca^2+ dependence are consistent with these results.

Although the identity of the Ca^2+ dependent site of control is subject for future study, our findings have further defined this role of endogenous Ca^2+, stores. We have demonstrated that FMLP priming occurs in a Ca^2+ free buffer and that no increase in Ca^2+ influx over resting values occurs during the primary process. The Ca^2+ rise is thus due to mobilization of Ca^2+. Earlier studies suggested that intracellular sources of Ca^2+ are functionally more important than extracellular influx for eliciting the respiratory burst and degranulation responses in (unprimed) FMLP-stimulated neutrophils when either Ca^2+ was chelated or release blocked, or the cells were Ca^2+ depleted.

These studies however do not address the physiologic relevance of these manipulations, since increased Ca^2+ influx is observed under normal conditions. The absence of augmented Ca^2+ influx during priming establishes a strong parallelism with the Ca^2+ free buffer experiments, which together show that the rise in Ca^2+ is due to mobilized Ca^2+ stores. Note that there is an increased Ca^2+ influx accompanying the increase in free Ca^2+ levels during second-stage stimulation with FMLP in primed cells. Whether this increased influx alone explains for the elevated cytosolic Ca^2+ seen during second-
stage FMLP stimulation cannot be concluded from these data. We and others have demonstrated that Na+/H+ antiporter-regulated pH_{i} significantly influences NADPH-oxidase activation. Sinchowitz and Cragoe recently showed that FMLP activated the Na+/Ca^{2+} coutertransport system and that the increase in Ca^{2+} influx and rise in free Ca^{2+} seen following FMLP stimulation may be accounted for by a carrier-mediated exchange of external Ca^{2+} for internal Na^{+}. This system, like the Na+/H+ antipporter, is inhibited by amiloride analogs. Our data indicate that priming with FMLP is associated with activation of the Na+/H+ antipporter, as indicated by the rise in pH_{i}; DA, which blocks Na+/H+ as well as Na+/Ca^{2+} exchange, abolishes the pH response but has no effect on the rise in Ca^{2+}. This clearly indicates that the Ca^{2+} flux occurring with priming is not regulated by the Na+/H+ antipporter or Na+/Ca^{2+} countertransport system.

Based on the well-established link between FMLP-initiated mobilization of Ca^{2+}, and inositol phosphate turnover, we sought to establish this pathway as the mechanism of elevated Ca^{2+}. However, both parameters of this pathway indicated that FMLP primed the neutrophil independent of the 41 kDal G-protein linked to inositol phosphate metabolism: (1) we were unable to show a significant increase in IP_{2} levels in primed cells stimulated with 5 x 10^{-7} FMLP compared with cells receiving the stimulating dose alone, and (2) whereas preincubation of neutrophils with PT abolished the O_{2} response to FMLP, primed O_{2} generation was seen in PT-treated cells that were primed with FMLP and then stimulated with PMA. While the FMLP O_{2} response is transduced through a PT-susceptible G-protein, PMA stimulation bypasses this step; we have now shown that FMLP priming is independent of this PT site. Furthermore, we have found that H-7, a kinase inhibitor, has no effect on primed stimulation with FMLP while blocking PMA responses (Fig 4), suggesting that FMLP priming and primed stimulation involve distinct transduction mechanisms other than PK-C.

These results are of interest in at least two respects. Unlike FMLP-stimulated effector functions (ie, respiratory burst, chemotaxis, degranulation), priming is neither dependent on extracellular Ca^{2+}, nor the PT-sensitive coupled G-protein system. What is the explanation of how priming and the concomitant Ca^{2+} rise are related to these other activities? One possibility is that we are observing effects subject to different sensitivities of either Ca^{2+} or PT ribosylation. In this scenario, effector functions are dependent on higher Ca^{2+} concentrations for Ca^{2+}-dependent transduction activities—the Ca^{2+} being recruited from the extracellular compartment—while priming has no such requirement. This hypothesis is consistent with the primed O_{2} response in our studies where correlatives change in Ca^{2+}, (and Ca^{2+} influx during second stage FMLP stimulation) corresponded to augmented O_{2} production. This explanation also could be applied to differing PT sensitivity, where the occupied portion of high-affinity nucleotide receptors independent of guanine nucleotides is sufficient for the priming response, while effector functions (ie, O_{2} production) require more occupied receptors. Previous studies, using pulse binding techniques, have established that different neutrophil responses require varying FMLP-receptor saturations: 50% maximal responses of O_{2} production required 30% receptor occupancy, whereas Ca^{2+} changes require only 5% occupancy. Consistent with this explanation, we noted that the priming process both increases FMLP receptor number and shifts more receptors to a higher affinity state.

A second hypothesis suggests that some FMLP receptors are coupled to a transduction system other than that defined by PT. The changes in Ca^{2+}, and pH in this scheme would then be induced by one uncharacterized transduction process and the effector functions would be coupled to the PT-sensitive G-protein system. In this second model, neutrophil responses are linked in parallel, where Ca^{2+}-independent, PT-independent linked functions control elements of Ca^{2+} mobilization (and pH), while other FMLP receptors with higher receptor occupancy requirements initiate the G-protein cascade (PT- and Ca^{2+}-dependent) leading to O_{2} production, degranulation, and chemotaxis, for example. By the law of parsimony the first model might be preferred, but we risk omitting a potentially important avenue of investigation if we do not further characterize the heterogeneous responses of FMLP-receptor binding. This is of particular interest considering the recent demonstration of isolated FMLP receptors linked to PT-insensitive low molecular weight G proteins. By our second model, priming offers a potentially useful system by which to pursue the functional consequences of FMLP-receptor heterogeneity.

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