Negative regulation of ligand-initiated Ca\textsuperscript{2+} uptake by PKC-βII in differentiated HL60 cells

HELEN M. KORCHAK,1 BARBARA E. CORKEY,2 GORDON C. YANEY,2 AND LAURIE E. KILPATRICK1

1Departments of Pediatrics and Biochemistry/Biophysics, University of Pennsylvania School of Medicine, The Joseph Stokes Jr. Research Institute of the Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104; and 2Obesity Research Unit, Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02118

Received 17 October 2000; accepted in final form 27 March 2001

Korchak, Helen M., Barbara E. Corkey, Gordon C. Yaney, and Laurie E. Kilpatrick. Negative regulation of ligand-initiated Ca\textsuperscript{2+} uptake by PKC-βII in differentiated HL60 cells. Am J Physiol Cell Physiol 281: C514–C523, 2001.—In phagocytic cells, fMet-Leu-Phe triggers phosphoinositide remodeling, activation of protein kinase C (PKC), release of intracellular Ca\textsuperscript{2+} and uptake of extracellular Ca\textsuperscript{2+}. Uptake of extracellular Ca\textsuperscript{2+} can be triggered by store-operated Ca\textsuperscript{2+} channels (SOCC) and via a receptor-operated nonselective cation channel(s). In neutrophilic HL60 cells, the PKC activator phorbol myristate acetate (PMA) activates multiple PKC isotypes, PKC-α, PKC-β, and PKC-δ, and inhibits ligand-initiated mobilization of intracellular Ca\textsuperscript{2+} and uptake of extracellular Ca\textsuperscript{2+}. Therefore PKC is a negative regulator at several points in Ca\textsuperscript{2+} mobilization. In contrast, selective depletion of PKC-β in HL60 cells by an antisense strategy enhanced fMet-Leu-Phe-initiated Ca\textsuperscript{2+} uptake but not mobilization of intracellular Ca\textsuperscript{2+}. Thapsigargin-induced Ca\textsuperscript{2+} uptake through SOCC was not affected by PKC-βII depletion. Thus PKC-βII is a selective negative regulator of Ca\textsuperscript{2+} uptake but not release of intracellular Ca\textsuperscript{2+} stores. PKC-βII inhibits a receptor-operated cation or Ca\textsuperscript{2+} channel, thus inhibiting ligand-initiated Ca\textsuperscript{2+} uptake.

Ca\textsuperscript{2+} mobilization; protein kinase C isotypes; inositol 1,4,5-trisphosphate; signal transduction

MOBILIZATION of intracellular Ca\textsuperscript{2+} and uptake of extracellular Ca\textsuperscript{2+} plays an important role in signal transduction for multiple cell responses (1, 33, 35). In neutrophils and HL60 cells differentiated to a neutrophil-like phenotype (dHL60 cells), ligands such as fMet-Leu-Phe elicit the twin signals of elevated cytosolic Ca\textsuperscript{2+} concentration and activation of protein kinase C (PKC). fMet-Leu-Phe triggers activation of a phospholipase Cβ, which cleaves phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) to generate inositol 1,4,5-trisphosphate (IP\textsubscript{3}), a trigger for release of intracellular Ca\textsuperscript{2+} stores, and diglyceride (DG), an activator of DG-dependent PKC (3, 15, 16, 18). Elevation of cytosolic Ca\textsuperscript{2+} is essential for optimal fMet-Leu-Phe-induced O\textsubscript{2} generation and degranulation (16, 30).

Non-excitable cells such as HL60 cells and neutrophils lack voltage-gated Ca\textsuperscript{2+} channels but possess receptor-operated Ca\textsuperscript{2+} channels. Ligands such as fMet-Leu-Phe trigger Ca\textsuperscript{2+} entry through receptor-operated nonselective cation channels that conduct Ca\textsuperscript{2+}, Na\textsuperscript{+}, and K\textsuperscript{+} (26). Ca\textsuperscript{2+}-activated cation nonselective channels have been demonstrated in dHL60 cells and in neutrophils (6, 19, 32, 42). In addition, IP\textsubscript{3}-initiated depletion of the endoplasmic reticulum (ER) Ca\textsuperscript{2+} stores can trigger uptake of extracellular Ca\textsuperscript{2+} via store-operated calcium channels (SOCC) (30). The SOCC can also be directly activated by the sarco(endo)plasmic Ca\textsuperscript{2+}-ATPase (SERCA) inhibitor thapsigargin (40). The Ca\textsuperscript{2+} channels in HL60 cells and neutrophils have not been fully characterized.

A role for PKC as a negative regulator of Ca\textsuperscript{2+} signaling has been demonstrated in multiple cell types, including dHL60 cells and neutrophils (12). Activation of PKC inhibits ligand-induced increases in cytosolic Ca\textsuperscript{2+} and ligand-induced IP\textsubscript{3} generation via inhibition of phospholipase C and may inhibit Ca\textsuperscript{2+} uptake (5, 9, 20, 22, 27, 39). These effects of PKC on Ca\textsuperscript{2+} mobilization were demonstrated in response to the phorbol ester phorbol myristate acetate (PMA), which activates multiple DG-dependent PKC isotypes including α, β, δ, and ε. dHL60 cells and neutrophils contain multiple isotypes of PKC, including Ca\textsuperscript{2+}/DG-dependent isotypes PKC-α and PKC-β, Ca\textsuperscript{2+}-independent DG-dependent isotype PKC-δ, and atypical phosphatidylerine-dependent, Ca\textsuperscript{2+}/DG-independent PKC-ζ (17). Activation of PKC can act as a positive signal in triggering cell responses such as adherence and O\textsubscript{2} generation. PKC-β is specifically required in positive signaling for activation of the NADPH oxidase for generation of O\textsubscript{2} but not for adherence by HL60 cells (17). However, isotype specificity has not been determined for the negative effects of PKC on Ca\textsuperscript{2+} signaling. It is not known whether a single PKC isotype is responsible for the inhibition of both intracellular Ca\textsuperscript{2+} mobil-
zation and uptake of extracellular Ca\textsuperscript{2+} triggered by PMA or whether different PKC isotypes target different points in Ca\textsuperscript{2+} signaling.

In this study, an HL60 cell clone, which is protein null for PKC-\(\beta\) but positive for PKC-\(\beta\)II, was used to probe a role for PKC-\(\beta\)II in regulation of ligand-initiated Ca\textsuperscript{2+} mobilization (11, 13). An antisense approach was used to selectively deplete PKC-\(\beta\)II but not PKC-\(\alpha\), PKC-\(\delta\), or PKC-\(\zeta\). Ca\textsuperscript{2+} uptake triggered by fMet-Leu-Phe, but not mobilization of intracellular Ca\textsuperscript{2+}, was enhanced in PKC-\(\beta\)-depleted cells. Thus PKC-\(\beta\)II is a selective, negative regulator of ligand-initiated Ca\textsuperscript{2+} uptake but, unlike the PKC activator PMA, was not an inhibitor of intracellular Ca\textsuperscript{2+} release. Ligand-initiated Ca\textsuperscript{2+} uptake by neutrophils and HL60 cells is mediated by a receptor-operated Ca\textsuperscript{2+} channel(s) and by an SOCC, which can also be activated by thapsigargin (40). Ca\textsuperscript{2+} uptake triggered by thapsigargin was not modulated by depletion of PKC-\(\beta\). These results indicate that PKC-\(\beta\)II selectively inhibits Ca\textsuperscript{2+} uptake via a thapsigargin-insensitive, receptor-operated Ca\textsuperscript{2+} channel.

MATERIALS AND METHODS

HL60 cell culture. A subclone of human promyelocytic HL60 leukemic cells, originally obtained from the American Type Culture Collection (Rockville, MD) expressed PKC-\(\alpha\), PKC-\(\beta\)II, PKC-\(\delta\), and PKC-\(\zeta\) but was protein null for PKC-\(\beta\)I (11, 13). These HL60 cells were grown in suspension culture in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% MEM vitamin solution, 0.1% gentamicin, and 10% heat-inactivated fetal bovine serum (FBS). The cell cultures were maintained at 37°C in a 5% CO\textsubscript{2} humidified atmosphere. HL60 cells were cultured in the presence of 1.3% DMSO for 6 days to initiate differentiation to a neutrophil-like phenotype (dHL60 cells).

Oligonucleotide synthesis and sequences. A 19-mer antisense oligonucleotide against the translation start site of human PKC-\(\beta\) was used for depletion of PKC-\(\beta\) in dHL60 cells as previously described (17). The 19-mer oligonucleotides had the following sequences: PKC-\(\beta\) antisense (\(\beta\)AS), 5’ AGC CGG GTC AGC CAT CTT G-3’; PKC-\(\beta\) sense (\(\beta\)SS), 5’ C AAG ATG GCT GAC CCG GCT 3’. Antisense and scrambled control oligonucleotides were synthesized as the phosphothioate derivatives and purified by HPLC.

Treatment of cells with oligonucleotides. HL60 cells were cultured in the presence of 1.3% DMSO for 4 days to initiate differentiation before treatment with the oligonucleotide. On day 4, the cells were washed and resuspended in Opti-MEM I reduced serum medium (GIBCO/BRL) at a cell concentration of 25 x 10\textsuperscript{6} cells/well. Oligonucleotides \(\beta\)AS or \(\beta\)MS (a scrambled \(\beta\) oligonucleotide) were suspended in Opti-MEM, at a final concentration of 400 nM. Delivery of the oligonucleotides was enhanced with the cationic lipid 1,2-dimyristoyl-trehalose-3-dimethylhydroxethyl ammonium bromide/cholesterol (DMRIE-C, 1:1 (M/M)) at 4 \muM/g. The cationic lipid/oligonucleotide mixture was added to the cells and incubated at 37°C for 4 h. An equal volume of RPMI 1640 medium containing 20% heat-inactivated FBS plus DMSO (1.3% final concentration) was then added, and the cells were cultured for an additional 24 h. The cells were harvested and suspended in HEPES buffer (pH 7.5) having the composition 150 mM Na\textsuperscript{+}, 5 mM K\textsuperscript{+}, 1.29 mM Ca\textsuperscript{2+}, 1.2 mM Mg\textsuperscript{2+}, 155 Cl\textsuperscript{−}, and 10 mM HEPES (17).

Western blots. Differentiated HL60 cell lysates (1 x 10\textsuperscript{6} cells/sample) were prepared by heating the cells at 95°C for 5 min in 2x SDS-PAGE sample buffer. The samples were briefly sonicated (12 s) to reduce viscosity. The dHL60 cell lysates were run on a 4–12% gradient SDS-PAGE, transferred to polyvinylidene difluoride membrane, and blocked for 1 h at room temperature with Tris-buffered saline, pH 7.5, containing 0.1% Tween 20 and 1% BSA/3% casein. To identify the different PKC isotypes, we incubated the membrane with a panel of PKC antibodies, followed by incubation with peroxidase-conjugated goat anti-rabbit IgG. Immunoreactive bands were visualized by Pierce SuperSignal ULTRA chemiluminescence substrate.

Measurement of cytosolic Ca\textsuperscript{2+} in fluo-3-loaded dHL60 cells. HL60 cells were incubated with 10 \muM of the acetoxymethyl ester of fluo-3 (fluor-3/AM) in HEPES buffer at 37°C for 5 min and then diluted 10 times to 1 ml with HEPES buffer at 37°C and incubated for a further 20 min. Suspensions were centrifuged (800 rpm, 10 min), and cells were resuspended in buffer at a concentration of 5 x 10\textsuperscript{6} cells/ml. Immediately prior to use, aliquots of 1 ml were microcentrifuged, and the cells resuspended in fresh 30°C buffer at 1 cell concentration of 2 x 10\textsuperscript{6} cells/200 \muL. The kinetics of fluorescence changes were monitored at 30°C in an unstirred suspension of preloaded cells, excitation 485 nm and emission 530 nm. Triton X-100 was added to measure F\textsubscript{max} (to calculate maximal Ca\textsuperscript{2+} concentration), and excess EGTA was added to measure F\textsubscript{0} (to calculate minimal Ca\textsuperscript{2+}). Cytosolic Ca\textsuperscript{2+} ([Ca\textsubscript{i}]\text{in}, in nM) was calculated as [Ca\textsubscript{i}]\text{in} = 390(F\textsubscript{max} – F0)/(F\textsubscript{max} – F) (23, 24).

Measurement of Ca\textsuperscript{2+} uptake as Mn\textsuperscript{2+}-induced quenching of fura-2. dHL60 cells were loaded with fura-2 using the same protocol as fluo-3 loading. Immediately prior to use, aliquots of cells were microcentrifuged, and the cells were resuspended in fresh 30°C buffer, in the presence or absence of 100 \muM Mn\textsubscript{Cl\textsubscript{2}}, at a cell concentration of 10 x 10\textsuperscript{6} cells/ml. Leak of dye from the cells was not observed over the time course of the experiment. The kinetics of fluorescence quenching triggered by fMet-Leu-Phe or thapsigargin, a SERCA inhibitor that triggers opening of SOCC (40), were monitored using a 360-nm excitation filter and a 530-nm emission filter. Fluorescence was corrected for nonspecific changes such as cell shape change, by subtraction of the values obtained in the absence of Mn\textsubscript{Cl\textsubscript{2}} from values obtained in the presence of Mn\textsubscript{Cl\textsubscript{2}}. Excitation at 380 nm is the calcium-insensitive isobestic point for fura-2; uptake of Mn\textsuperscript{2+} through a Ca\textsuperscript{2+} channel quenches fura-2 fluorescence (27).

Measurement of IP\textsubscript{3}. Generation of IP\textsubscript{3} was measured by a radioreceptor assay kit (model TRK1000, Amersham) (31). dHL60 cells (2 x 10\textsuperscript{6}) were suspended in 200 \muL HEPES buffer at 37°C and activated by 1 \muM fMet-Leu-Phe for 0, 5, 15, 30, and 60 s. The reaction was stopped by the addition of 40 \muL ice-cold 20% perchloric acid and kept on ice for 20 min. After centrifugation at 2,000 g for 15 min at 4°C, the supernatant was adjusted to pH 7.5 with ice-cold KOH. After centrifugation to remove KCl\textsubscript{4}, IP\textsubscript{3} was measured in the supernatant using the Amersham IP\textsubscript{3} assay system (model TRK1000). This assay is based on competition between unlabeled IP\textsubscript{3} in the sample and a fixed amount of [\textsuperscript{3}H]IP\textsubscript{3} for binding sites on an IP\textsubscript{3} binding protein (31). Determinations
were made in duplicate, and the results expressed as picomoles per 10⁶ cells.

Statistical analysis. Results are means ± SE (n = number of observations). Data were analyzed by Student's t-test.

Reagents. Cytochalasin B, BSA, PMA, and fMet-Leu-Phe were purchased from Sigma. Fluo-3/AM and fura-2/AM were obtained from Molecular Probes, and thapsigargin was from Biomol. PMA was stored as a concentrated stock solution in DMSO and diluted with HEPES buffer before use. fMet-Leu-Phe was stored as a stock solution in ethanol and diluted in buffer prior to use. Anti-peptide polyclonal antibodies, to PKC-α, PKC-β, PKC-βIII, and PKC-δ, and peroxidase-conjugated goat anti-rabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse monoclonal antibody to PKC-ζ was purchased from Transduction Laboratories.

RESULTS

Ligand-initiated increases in cytosolic Ca²⁺ in dHL60 cells: regulation by PKC. The kinetics of fMet-Leu-Phe-triggered increases in cytosolic Ca²⁺ were monitored in fluo-3-loaded dHL60 cells. Resting concentration of cytosolic Ca²⁺ was 88.9 ± 5.6 nM (Table 1). The addition of 1 μM fMet-Leu-Phe triggered a rapid increase in cytosolic Ca²⁺ that peaked by 18 s at a level of 264.4 ± 15.3 nM (Table 1, Fig. 1A). The stimulated increase in cytosolic Ca²⁺ subsequently declined toward resting levels by 2 min after addition of the stimulus. In the presence of EGTA, the peak concentration of cytosolic Ca²⁺ elicited by fMet-Leu-Phe was reduced to 123.5 ± 8.1 nM, a level that was significantly less than the peak attained in the presence of extracellular Ca²⁺ (46.7% control, P < 0.00002) (Table 1). Therefore, the ligand-induced increase in cytosolic Ca²⁺ in dHL60 cells is mobilized from both intracellular stores and by uptake from extracellular sources. PKC stimulated by the phorbol ester, PMA, has been shown to modulate ligand-induced Ca²⁺ movements in neutrophils and in differentiated HL60 cells (5, 9, 20, 22, 27, 39). Resting cytosolic Ca²⁺ was 88.9 nM (see above) in control cells and 85.1 ± 7.5 nM Ca²⁺ after preincubation with 1 μg/ml PMA for 5 min, a difference that was not statistically significant.

Table 1. Effect of pretreatment with 1 μg/ml PMA on fMet-Leu-Phe-induced changes in cytosolic Ca²⁺ in the presence or absence of extracellular Ca²⁺.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>n</th>
<th>Resting Cytosolic [Ca²⁺], nM</th>
<th>Peak Cytosolic [Ca²⁺], nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>12</td>
<td>88.9 ± 5.6</td>
<td>264.4 ± 15.3</td>
</tr>
<tr>
<td>EGTA</td>
<td>7</td>
<td>69.9 ± 3.8</td>
<td>123.5 ± 8.1</td>
</tr>
<tr>
<td>PMA</td>
<td>11</td>
<td>85.1 ± 7.5</td>
<td>180.0 ± 15.3</td>
</tr>
<tr>
<td>PMA + EGTA</td>
<td>7</td>
<td>42.5 ± 6.6</td>
<td>69.4 ± 11.0</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of observations. Fluo-3-loaded dHL60 cells were pretreated for 5 min with 1 μg/ml phorbol 12-myristate 13-acetate (PMA) in the presence or absence of 1.25 mM EGTA, prior to the addition of 1 μM fMet-Leu-Phe. Peak cytosolic [Ca²⁺] in buffer vs. EGTA-pretreated cells, P < 0.006. Peak cytosolic [Ca²⁺] in buffer vs. PMA-pretreated cells, P < 0.006. Peak cytosolic [Ca²⁺] in buffer vs. PMA + EGTA-pretreated cells, P < 0.006 (Table 1). However, after activation by 1 μM fMet-Leu-Phe, the peak cytosolic Ca²⁺ was significantly reduced to 180.0 ± 15.3 nM (n = 11) in cells pretreated for 5 min in the presence of 1 μg/ml PMA compared with a level of 264.4 ± 15.3 nM in control cells (68.1% control, P < 0.01) (Fig. 1A, and Table 1). Furthermore, pretreatment with PMA decreased the peak cytosolic Ca²⁺ levels attained in the presence of EGTA from 123.5 ± 7.5 nM Ca²⁺ in control cells...
exposed to 1 μM fMet-Leu-Phe, to 69.4 ± 11.0 nM Ca\(^{2+}\) in PMA-pretreated cells (P = 0.05) (Table 1). The difference in ligand-induced increase in cytosolic Ca\(^{2+}\) in the presence and absence of EGTA represents uptake of Ca\(^{2+}\) from the medium. The difference in peak Ca\(^{2+}\) triggered by fMet-Leu-Phe in the presence and absence of extracellular Ca\(^{2+}\) was 140.9 nM in control cells but only 110.6 nM in dHL60 cells pretreated with PMA. These findings suggest that PMA pretreatment inhibits both mobilization of intracellular Ca\(^{2+}\) stores and uptake of extracellular Ca\(^{2+}\).

Ca\(^{2+}\) uptake pathways were more directly assessed as fMet-Leu-Phe-induced uptake of Mn\(^{2+}\) into fura-2-loaded cells and quenching of fura-2 fluorescence, measured at the isosbestic point, which is not Ca\(^{2+}\) sensitive. Mn\(^{2+}\) is a good surrogate for Ca\(^{2+}\) since it is not pumped out of the cells and thus serves as a relatively selective monitor for Ca\(^{2+}\) entry (20, 27). Addition of 1 μM fMet-Leu-Phe triggered a prompt increase in Mn\(^{2+}\) and uptake of Ca\(^{2+}\) in the presence and absence of extracellular Ca\(^{2+}\). Mn\(^{2+}\) flux was most rapid in the first minute but which continued over the 5 min monitored after addition of the stimulus (Fig. 1B). fMet-Leu-Phe triggered a loss of fluorescence of 215.6 ± 71.9 arbitrary fluorescence units (AFU) (n = 8) by 1 min in control cells and 602.3 ± 83.5 AFU (n = 8) by 5 min after addition of the stimulus. When the dHL60 cells were pretreated for 5 min with 1 μg/ml PMA, the extent of Mn\(^{2+}\) influx and thus of fluorescence quenching was reduced to only 64.2 ± 30.0 AFU (n = 7) by 1 min and 207.8 ± 70.8 AFU (n = 7) by 5 min after addition of the stimulus, which is significantly less than the quenching attained in control cells (P < 0.025 for 1 min and P < 0.01 for 5 min, paired Student’s t-test) (Fig. 1B). Thus PMA, an activator of PKC, inhibited ligand-induced Ca\(^{2+}\) (Mn\(^{2+}\)) uptake. Therefore, PMA, which is an activator of DG-dependent α-, β-, and δ-isotypes of PKC, but not DG-independent PKC-ζ, inhibited ligand-initiated increases in cytosolic Ca\(^{2+}\) in the presence and in the absence of extracellular Ca\(^{2+}\). These findings implicate a DG-dependent PKC isotype(s) as a negative regulator of ligand-initiated mobilization of intracellular Ca\(^{2+}\) and uptake of Ca\(^{2+}\) from the extracellular milieu. We questioned whether one PKC isotype was responsible for regulation of Ca\(^{2+}\) mobilization, or whether different PKC isotypes selectively regulated specific pathways of Ca\(^{2+}\) mobilization.

Depletion of PKC-β by an antisense strategy. To test a role for PKC-β in regulating ligand-initiated Ca\(^{2+}\) mobilization, dHL60 cells were treated with β-antisense (βAS) and control β-missense oligonucleotides for 48 h (see MATERIALS AND METHODS). The HL60 βI-null cells were shown to express PKC-βII but not PKC-βI (Fig. 2A). Previous work demonstrated that these cells also contained α-, δ-, and ζ-isotypes of PKC (13). Pretreatment with 400 nM βAS selectively depleted PKC-βII (Fig. 2A) but not PKC-α, PKC-δ, or PKC-ζ as previously shown (13), in these differentiated HL60 cells. Densitometry demonstrated that the level of PKC-βII declined from a level of 600 ± 64 density units (DU) (n = 6) in control βMS treated cells, to a level of 383 ± 87 DU (n = 6) (52.1 ± 11.6% of control, P < 0.001) (Fig. 2B).

Depletion of PKC-β and Ca\(^{2+}\) mobilization. Resting cytosolic Ca\(^{2+}\) levels were not affected by depletion of PKC-β by βAS pretreatment (Table 2). Cytosolic Ca\(^{2+}\) concentration was 119.8 ± 14.4 nM (n = 6) in cells treated with βAS, compared with a level of 99.9 ± 7.4 nM in control cells treated with βMS (Fig. 3A), and not significantly different from resting cytosolic Ca\(^{2+}\) levels in untreated cells (Fig. 1A). In contrast, antisense depletion of PKC-β enhanced the fMet-Leu-Phe-triggered increase in cytosolic Ca\(^{2+}\). In cells pretreated with BMS and activated with 1 μM fMet-Leu-Phe, cytosolic Ca\(^{2+}\) peaked at 270.2 ± 15.9 nM (Fig. 3A). In contrast, cells pretreated with βAS underwent an enhanced peak increase in cytosolic Ca\(^{2+}\) of 374.0 ± 16.5 nM (n = 6) (Fig. 3A, Table 2), a difference that was
significantly different from the response in control βMS-treated cells (P < 0.002).

In the presence of 1.25 mM EGTA, where cytosolic Ca$_{2+}$ changes were only from intracellular stores, resting cytosolic Ca$_{2+}$ was 69.0 ± 3.7 nM (n = 5) and 65.6 ± 7.3 nM (n = 5) in βAS- and βMS-pretreated cells, respectively (Table 2). When the cells were activated by addition of 1 μM fMet-Leu-Phe (Fig. 3B), cytosolic Ca$_{2+}$ rose to peak values of 121.0 ± 6.0 nM (n = 5) and 113.4 ± 5.6 nM (n = 5) in βAS- and βMS-pretreated cells, respectively, a difference that was not statistically significant. Thus no alteration in ligand-initiated mobilization of intracellular Ca$_{2+}$ stores was observed in PKC-β-depleted cells. The increase in peak cytosolic Ca$_{2+}$ observed in the presence of extracellular Ca$_{2+}$ in PKC-β-depleted cells may reflect an increase in Ca$_{2+}$ uptake from extracellular sources.

We next determined whether PMA still retained the ability to downregulate cytosolic Ca$_{2+}$ mobilization in PKC-β-depleted dHL60 cells. Pretreatment with PMA had no effect on resting cytosolic Ca$_{2+}$ concentration, but in control βMS-pretreated cells the peak cytosolic Ca$_{2+}$ level was 198.8 ± 25.3 nM (n = 5) when cells were pretreated for 5 min with PMA (Table 2), compared with a peak level of 270.2 ± 15.9 nM (n = 6) in βMS cells not exposed to PMA [72.2 ± 8.5% (n = 5) of control βMS-pretreated cells, P < 0.02]. When DHL60 cells were depleted of PKC-β by treatment with βAS, pretreatment with PMA for 5 min significantly reduced the fMet-Leu-Phe-induced peak cytosolic Ca$_{2+}$ to 202.0 ± 24.8 nM (n = 5), a level that was similar to the level of 198.8 ± 25.3 nM observed in PMA-pretreated βMS-pretreated cells (Table 2) but that was significantly reduced from the peak level of 374.0 ± 16.5 nM observed in βAS-pretreated cells in the absence of PMA [54.1 ± 7.4% (n = 5) of control βAS-pretreated cells, P < 0.0002] (Table 2). These findings suggest that alterations in cytosolic Ca$_{2+}$ in PKC-β-depleted cells were due to changes in ligand-initiated uptake of extracellular Ca$_{2+}$ and not due to alterations in mobilization of intracellular Ca$_{2+}$ stores. Furthermore, the ability of PMA to inhibit ligand-initiated release of intracellular Ca$_{2+}$ stores was retained in PKC-β-depleted cells. Peak cytosolic Ca$_{2+}$ in the presence of EGTA was reduced from 121.0 ± 6.0 to 89.3 ± 12.0 nM after pretreatment by PMA (Table 2). These findings indicate a possible role for PKC-α or PKC-δ in the regulation of fMet-Leu-Phe-induced release of Ca$_{2+}$ from intracellular Ca$_{2+}$ stores.

The role of PKC-β in regulation of ligand-induced Ca$_{2+}$ uptake pathways was more directly investigated by measuring Ca$_{2+}$ uptake as Mn$_{2+}$-induced fluorescence quenching in fura-2-loaded DHL60 cells. Greater quenching of fura-2 fluorescence in response to fMet-Leu-Phe was observed in PKC-β-depleted (βAS) cells compared with control (βMS) cells (Fig. 3C). fMet-Leu-Phe triggered a loss of fluorescence by 1 min after addition of the stimulus of 113.0 ± 49.9 AFU (n = 6) in control βMS-pretreated cells, while in PKC-β-depleted cells (βAS) the decrease in fluorescence was 219.7 ± 51.8 AFU (n = 6) (Fig. 3C). The loss of fluorescence by 5 min after addition of fMet-Leu-Phe was 507.7 ± 73.3 AFU (n = 6) in control βMS-pretreated cells, whereas in PKC-β-depleted cells (βAS) the decrease in fluorescence was increased to 850 ± 136.0 AFU (n = 6), an increase that was significantly increased compared with the response in control cells (P < 0.04 paired t-test) (Fig. 3C). Therefore, fMet-Leu-Phe-induced Ca$_{2+}$ uptake was enhanced in PKC-β-depleted DHL60 cells, further implicating PKC-β in negative regulation of ligand-induced Ca$_{2+}$ uptake.

The role of PKC-β in regulation of ligand-induced Ca$_{2+}$ uptake pathways was more directly investigated by measuring Ca$_{2+}$ uptake as Mn$_{2+}$-induced fluorescence quenching in fura-2-loaded DHL60 cells. Greater quenching of fura-2 fluorescence in response to fMet-Leu-Phe was observed in PKC-β-depleted (βAS) cells compared with control (βMS) cells (Fig. 3C). fMet-Leu-Phe triggered a loss of fluorescence by 1 min after addition of the stimulus of 113.0 ± 49.9 AFU (n = 6) in control βMS-pretreated cells, while in PKC-β-depleted cells (βAS) the decrease in fluorescence was 219.7 ± 51.8 AFU (n = 6) (Fig. 3C). The loss of fluorescence by 5 min after addition of fMet-Leu-Phe was 507.7 ± 73.3 AFU (n = 6) in control βMS-pretreated cells, whereas in PKC-β-depleted cells (βAS) the decrease in fluorescence was increased to 850 ± 136.0 AFU (n = 6), an increase that was significantly increased compared with the response in control cells (P < 0.04 paired t-test) (Fig. 3C). Therefore, fMet-Leu-Phe-induced Ca$_{2+}$ uptake was enhanced in PKC-β-depleted DHL60 cells, further implicating PKC-β in negative regulation of ligand-induced Ca$_{2+}$ uptake.

Table 2. Changes in cytosolic Ca$_{2+}$ triggered by 1 μM fMet-Leu-Phe in control and in β-PKC-depleted dHL60 cells

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Resting Cytosolic [Ca$^{2+}$], nM</th>
<th>Peak Cytosolic [Ca$^{2+}$], nM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>βAS</td>
<td>βMS</td>
</tr>
<tr>
<td>Buffer</td>
<td>6</td>
<td>119.8 ± 14.4</td>
</tr>
<tr>
<td>EGTA</td>
<td>5</td>
<td>69.0 ± 3.7</td>
</tr>
<tr>
<td>PMA</td>
<td>5</td>
<td>90.7 ± 14.8</td>
</tr>
<tr>
<td>PMA + EGTA</td>
<td>5</td>
<td>53.6 ± 10.8</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = number of observations. dHL60 cells were pretreated with βMS (control) or βAS (β-PKC-depleted), and then loaded with Fluo-3. Control and β-PKC-depleted dHL60 cells were pretreated for 5 min with 1 μM PMA in the presence or absence of 1.25 mM EGTA, prior to the addition of 1 μM fMet-Leu-Phe. PKC, protein kinase C; βAS, PKC-β antisense; βMS, PKC-β missense. Peak cytosolic [Ca$^{2+}$] in βAS vs. βMS-pretreated cells, P < 0.002.
SOCC opening triggered by thapsigargin is regulated by PKC. A role for PKC has been suggested in the regulation of SOCC and receptor-operated Ca\(^{2+}\) or cation nonselective channels in HL 60 cells (26, 30). The SERCA inhibitor, thapsigargin, inhibits the Ca\(^{2+}\)-ATPase responsible for pumping Ca\(^{2+}\) into the ER and induces Ca\(^{2+}\) uptake through the SOCC without inducing hydrolysis of phosphoinositides. Thapsigargin triggered Ca\(^{2+}\) uptake in dHL60 cells, concordant with activation of SOCC (30). Addition of 100 nM thapsigargin to fluo-3-loaded dHL60 cells in the presence of extracellular Ca\(^{2+}\), triggered an increase in cytosolic Ca\(^{2+}\) from 90.9 ± 5.9 nM \((n = 5)\) to 235.9 ± 24.8 nM \(\text{Fig. 5A}\). This increase in cytosolic Ca\(^{2+}\) was due to uptake of extracellular Ca\(^{2+}\), since only a small increase in cytosolic Ca\(^{2+}\) was observed in the absence of extracellular Ca\(^{2+}\) \(\text{Fig. 5A}\). When cells were pretreated with 1 \(\mu\)g/ml PMA for 5 min, thapsigargin triggered an increase of cytosolic Ca\(^{2+}\) from 92.7 ± 8.1 to only 142.5 ± 17.0 nM \(\text{Fig. 5A}\), a difference that was significantly different from control \((P < 0.025)\). Therefore, PMA reduced Ca\(^{2+}\) uptake through SOCC upon addition of thapsigargin implicating PKC as a regulator of SOCC.

When Ca\(^{2+}\) uptake was measured as Mn\(^{2+}\) uptake in fura-2-loaded cells, a slow but persistent Ca\(^{2+}\) uptake of 2,255 ± 159 AFU/5 min \(n = 5\) was triggered in dHL60 cells by 100 nM thapsigargin \(\text{Fig. 5B}\). When the cells were preincubated for 5 min with 1 \(\mu\)g/ml PMA, the thapsigargin-initiated Ca\(^{2+}\) uptake was re-
duced to 1,328 ± 287 AFU/5 min (P < 0.02 paired Student's t-test), indicating that a PKC isotype(s) activated by PMA could act as a negative regulator of SOCC.

Depletion of PKC-β and Ca^{2+} uptake triggered by thapsigargin. The role of PKC-β in regulation of thapsigargin-induced Ca^{2+} uptake was investigated using dHL60 cells pretreated with βAS and βMS. Thapsigargin-induced Ca^{2+} uptake was measured in fluo-3-loaded cells (Fig. 6A). In control dHL60 cells treated with βMS, thapsigargin triggered an increase in cytosolic Ca^{2+} from 103.6 ± 8.1 nM (n = 4) in resting cells to 153.7 ± 9.5 nM in cells treated for 5 min with thapsigargin (Fig. 6A), representing uptake of Ca^{2+} through the SOCC. Similarly, in PKC-β-depleted dHL60 cells treated with βAS, thapsigargin triggered an initial increase in cytosolic Ca^{2+} levels from 90.6 ± 11.1 nM (n = 4) in resting cells to 147.8 ± 11.1 nM after addition of thapsigargin (Fig. 6A). Thus no significance difference in thapsigargin-induced uptake of extracellular Ca^{2+} was noted between cells depleted of PKC-β by βAS and the control cells treated with βMS.

Similarly, when Ca^{2+} uptake triggered by thapsigargin-induced opening of the SOCC was measured as Mn^{2+} uptake into fura-2-loaded cells, an equivalent decrease in fluorescence triggered by 100 nM thapsigargin was observed in PKC-β-depleted (βAS) cells compared with control (βMS) cells (Fig. 6B). Thapsigargin triggered a loss of fluorescence of 1,736 ± 371

Fig. 5. Effect of PMA on thapsigargin-induced changes in cytosolic Ca^{2+} and in Ca^{2+} uptake. A: dHL60 cells were loaded with fluo-3 and incubated for 5 min at 30°C in the presence or absence of 1 μg/ml PMA in Ca^{2+}-free buffer, before the addition of 100 nM thapsigargin (arrow), and after a further 4 min of 1.25 mM Ca^{2+}. Changes in cytosolic Ca^{2+} were monitored as fluorescence of fluo-3. This experiment is representative of 3.

B: dHL60 cells preloaded with fura-2 were incubated in the presence of 100 μM MnCl₂ for 5 min at 30°C before the addition of 100 nM thapsigargin (arrow). Changes in Ca^{2+} uptake were monitored as Mn^{2+}-induced quenching of fura-2 (see MATERIALS AND METHODS). This experiment is representative of 3.

Fig. 6. Effect of depletion of PKC-β on Ca^{2+} uptake triggered by thapsigargin. A: dHL60 cells were pretreated with βAS or βMS and then loaded with fluo-3 and monitored for 5 min at 30°C in buffer, followed by addition of 100 nM thapsigargin (arrow). Changes in cytosolic Ca^{2+} were monitored as fluorescence of fluo-3. This experiment is representative of 4. B: dHL60 cells were pretreated with βAS or βMS, were loaded with fura-2, and incubated in the presence of 100 μM MnCl₂ for 5 min at 30°C before the addition of 100 nM thapsigargin (arrow). Changes in Ca^{2+} uptake were monitored as Mn^{2+}-induced quenching of fura-2 (see MATERIALS AND METHODS). This experiment is representative of 4.
AFU/5 min in control βMS-treated cells, whereas in PKC-β-depleted cells (βAS) the decrease in fluorescence was 1,896 ± 371 AFU/5 min (113.6 ± 8.1% control, n = 5), a difference that was not statistically significant. Therefore, PKC-β is a negative regulator of ligand-initiated Ca\(^{2+}\) uptake. However, PKC-β has no direct effect on SOCC, since thapsigargin-induced Ca\(^{2+}\) uptake through SOCC was not affected in PKC-β-depleted dHL60 cells. Thus PKC-β was not responsible for the PMA-induced inhibition of SOCC. The increase in Ca\(^{2+}\) uptake observed in PKC-β-depleted dHL60 cells occurs through a thapsigargin-insensitive channel such as the receptor-operated nonselective cation channel.

**DISCUSSION**

Alterations in intracellular Ca\(^{2+}\) homeostasis has profound effects on many cell functions. A role for PKC as a negative regulator of Ca\(^{2+}\) homeostasis has been proposed in differentiated HL60 cells, neutrophils (5, 9, 12, 20, 22, 27, 39), and in other cell types (21, 28, 34, 36, 37). Three steps in Ca\(^{2+}\) signaling have been implicated as targets for negative regulation by PKC. 1) PKC-dependent phosphorylation inhibits activation of phospholipase Cβ and reduces the subsequent production of IP\(_3\) and release of intracellular Ca\(^{2+}\) stores. 2) Phosphorylation of the plasmaemmal Ca\(^{2+}\)-ATPase by PKC activates Ca\(^{2+}\) efflux, which is responsible for the return of cytosolic Ca\(^{2+}\) to resting levels (4, 14, 41). 3) Activation of PKC inhibits the rate of Ca\(^{2+}\) uptake from the extracellular milieu. The chemotactic peptide fMet-Leu-Phe triggers mobilization of intracellular Ca\(^{2+}\) stores and uptake of extracellular Ca\(^{2+}\) by dHL60 cells. Pretreatment of dHL60 cells with the PKC activator PMA elicits inhibition of both ligand-triggered release of intracellular Ca\(^{2+}\) stores and uptake of extracellular Ca\(^{2+}\). Since PMA activates multiple isoforms of PKC, including PKC-α, PKC-β, and PKC-δ, these studies do not demonstrate isotype specificity. We questioned whether the inhibitory effect of PMA on both release of intracellular Ca\(^{2+}\) stores and uptake of extracellular Ca\(^{2+}\) was due to the activation of a single PKC isotype or, alternatively, whether different PKC isoforms regulated discrete Ca\(^{2+}\) movements.

Phagocytic cells such as HL60 cells and neutrophils possess a number of PKC isoforms, PKC-α, PKC-β1, PKC-βII, PKC-δ, and PKC-ζ. In this study, we have demonstrated a role for PKC-βII in negative regulation of ligand-initiated uptake of extracellular Ca\(^{2+}\), but not of intracellular Ca\(^{2+}\) release. An antisense strategy was used to selectively deplete PKC-βII from a clone of HL60 cells, which is protein null for PKC-βII but positive for α-, βII, δ-, and ζ-isotypes of PKC. Selective depletion of PKC-δ, i.e., PKC-βII, enhanced the fMet-Leu-Phe-triggered increase in cytosolic Ca\(^{2+}\) in the presence of extracellular Ca\(^{2+}\). In contrast, depletion of PKC-βII had no effect on fMet-Leu-Phe-elicted increase in cytosolic Ca\(^{2+}\) in the absence of extracellular Ca\(^{2+}\), indicating that PKC-βII was a negative regulator of fMet-Leu-Phe-induced Ca\(^{2+}\) uptake but not of ligand-induced release of intracellular Ca\(^{2+}\) stores.

Signaling for release of intracellular Ca\(^{2+}\) stores involves fMet-Leu-Phe-induced activation of phospholipase Cβ (2, 39) and cleavage of PIP\(_2\) to generate IP\(_3\). Phospholipase Cβ2 is a substrate for PKC (5). The finding that generation of IP\(_3\) was not affected by PKC-βII depletion is concordant with our findings that ligand-initiated release of intracellular Ca\(^{2+}\) stores was not affected by PKC-βII depletion. This finding indicates that any effect of PKC-βII must occur independently of phospholipase Cβ activation. Furthermore, the PMA-induced inhibition of release of intracellular Ca\(^{2+}\) stores still occurred in PKC-β-depleted cells, demonstrating that the negative effect of PMA on Ca\(^{2+}\) mobilization was not solely dependent on PKC-β and may be due to another PKC isoform such as PKC-α or PKC-δ or to a non-PKC-dependent mechanism. Indeed, a role for PKC-α has been demonstrated in negative regulation of phospholipase C in visual signal transduction in Drosophila (25).

A role for PKC-βII in negative regulation of Ca\(^{2+}\) uptake was confirmed by the finding that PKC-βII depletion also enhanced fMet-Leu-Phe-induced uptake of Mn\(^{2+}\), which acts as a surrogate for Ca\(^{2+}\) during opening of the ligand-operated Ca\(^{2+}\) channels in HL60 cells (27) and of SOCC in numerous cell types (1). A similar role for PKC-β in the regulation of Ca\(^{2+}\) uptake has been demonstrated in platelets and lymphocytes (10, 43).

Two distinct channels for Ca\(^{2+}\) uptake are potential targets for PKC. fMet-Leu-Phe triggers Ca\(^{2+}\) uptake via a nonselective cation channel(s) that is activated by elevation of cytosolic Ca\(^{2+}\) (6). Ca\(^{2+}\)-mobilizing ligands such as fMet-Leu-Phe also activate SOCC, initiated by IP\(_3\)-triggered emptying of ER Ca\(^{2+}\) stores. The SERCA inhibitor thapsigargin can bypass ligand-initiated depletion of Ca\(^{2+}\) stores and directly activate the store-operated Ca\(^{2+}\) entry pathway (40). In dHL60 cells, thapsigargin triggered enhanced Ca\(^{2+}\) uptake monitored as increased cytosolic Ca\(^{2+}\) or alternatively as increased Mn\(^{2+}\) uptake. Thapsigargin-induced Ca\(^{2+}\) uptake was inhibited by pretreatment of the cells with PMA and activation of PKC, in agreement with other workers (9, 20, 22, 27, 39). However, the Ca\(^{2+}\) uptake through SOCC activated by thapsigargin was not affected by depletion of PKC-βII. Therefore, the SOCC per se is not regulated by PKC-βII. Thus PKC-βII could regulate a non-SOCC Ca\(^{2+}\) channel such as the receptor-operated nonselective cation channel. Indeed, thapsigargin and fMet-Leu-Phe triggered an additive Ca\(^{2+}\) (Mn\(^{2+}\)) uptake (results not shown), indicating that fMet-Leu-Phe and thapsigargin activate different channels. Alternatively, PKC-βII might not act directly on SOCC but might play a role in the ligand-initiated signaling for the opening of SOCC.

These studies demonstrate a selective role for PKC-βII in the negative regulation of ligand-initiated Ca\(^{2+}\) uptake but not mobilization of intracellular Ca\(^{2+}\) stores. Since modulation of PKC-βII affected receptor-operated Ca\(^{2+}\) uptake but had no effect on thapsigarin-
gin-initiated Ca\textsuperscript{2+} uptake, these findings suggest a role for PKC-\(\beta\)II in the signaling for activation of Ca\textsuperscript{2+} uptake through a receptor-operated nonselective cation channel rather than the SOCC. PKC-\(\beta\)II is involved in positive signaling for \(O_2\) generation (17) but also plays a role in signaling crosstalk in which activation of PKC also downregulates Ca\textsuperscript{2+} uptake. Ca\textsuperscript{2+} uptake leads to localized changes in Ca\textsuperscript{2+} concentration, so that modulation of Ca\textsuperscript{2+} uptake could regulate cell function at the membrane. Downregulation of Ca\textsuperscript{2+} uptake could serve as a turn-off mechanism for Ca\textsuperscript{2+}-dependent responses such as \(O_2\) generation or serve as an essential element in signaling for functions such as cell shape changes and chemotaxis (8).

This work was supported by National Institutes of Health Grant AI-24840.

REFERENCES

23. Merritt JE, McCarthy SA, Davies MPA, and Moores KE. Use of fluo-3 to measure cytosolic Ca\textsuperscript{2+} in platelets and neutrophils. Loading cells with the dye, calibration of traces, measurements in the presence of plasma, and buffering of cytosolic Ca\textsuperscript{2+}. Biochem J 269: 513–519, 1990.


