Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 3534–3538, April 1994
Cell Biology

Bromophenacyl bromide binding to the actin-bundling protein l-plastin inhibits inositol trisphosphate-independent increase in Ca^{2+} in human neutrophils

(cytoskeleton/signal transduction/inositol phosphates/inflammation/monoclonal antibody)

CARLOS ROSALES*†, SAMUEL L. JONES*, DAVID MCCOURT‡, AND ERIC J. BROWN*§

*Division of Infectious Diseases, Department of Medicine, and ‡Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, MO 63110

Communicated by David M. Kipnis, January 6, 1994

ABSTRACT **Ligation of IgG Fc receptors on polymorphonuclear leukocytes causes an increase in the concentration of free intracytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) which arises from release of intracellular stores but is independent of inositol 1,4,5-trisphosphate. We found that bromophenacyl bromide (BPB), an alkylating agent which inhibits leukocyte degranulation, adherence, and phagocytosis, inhibited IgG-stimulated increases in $[Ca^{2+}]_i$ with an IC_{50} of 0.2 μM. In contrast, BPB had no effect on inositol 1,4,5-trisphosphate-dependent $[Ca^{2+}]_i$ increases induced by fMet-Leu-Phe, complement fragment C5a, ATP, or platelet-activating factor. Using a monoclonal antibody specific for BPB, we identified in polymorphonuclear leukocytes a single cytosolic protein of 66 kDa and isoelectric point pH 5.6 which bound BPB when intact cells were treated with the alkylating agent. This BPB-binding protein was identified as l-plastin, a Ca^{2+}-regulated actin-bundling protein. l-Plastin was found associated with the Triton X-100-insoluble cytoskeleton in polymorphonuclear leukocytes adherent to immune complexes, suggesting that BPB affects Fc receptor-mediated signal transduction by altering the actin cytoskeleton. Consistent with this hypothesis, both cytochalasin B and cytochalasin D inhibited the IgG-dependent increase in $[Ca^{2+}]_i$, without any effect on fMet-Leu-Phe-induced Ca^{2+} release. These data suggest that the actin cytoskeleton is essential for signal transduction from plasma membrane Fc receptors and that l-plastin has a critical role in activation of this pathway.**

The rise in intracytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by Fc receptor (FcR) ligation is necessary for fusion of secretory granules with the plasma membrane, increased expression of other receptors, phagosome–lysosome fusion, and perhaps chemotaxis (1–6). Increases in $[Ca^{2+}]_i$ can occur after ligation of either FcRII or FcRIII (7–9) and are produced entirely through release from intracellular stores, without a contribution from extracellular Ca^{2+} (7, 9, 10). This $[Ca^{2+}]_i$ rise, in contrast to fMet-Leu-Phe receptor stimulation, proceeds via a pertussis toxin-insensitive pathway which is independent of inositol 1,4,5-trisphosphate (IP_3) generation (10).

The identity of the intracellular mediator of Ca^{2+} release induced by FcR ligation remains an important unsolved problem. Although tyrosine phosphorylation is enhanced by immune complex activation of neutrophils (11, 12), this has not been directly associated with effector functions. In this work, we have found that bromophenacyl bromide (BPB) is a specific inhibitor of the FcR-mediated $[Ca^{2+}]_i$ rise. BPB is known to have important effects on several inflammatory functions of leukocytes, such as degranulation (13), adherence and spreading (14, 15), and macrophage phagocytosis (16), but the relevant target for its inhibition of leukocyte activation is unknown. Using a monoclonal antibody (mAb) to BPB as a probe, we found that l-plastin, a Ca^{2+}-regulated actin-bundling protein homologous to the brush-border cytoskeletal protein fimbrin (17), was the single detectable intracellular target modified by BPB at concentrations which completely inhibited $[Ca^{2+}]_i$ rise in response to FcR ligation. l-Plastin was found associated with the actin cytoskeleton in polymorphonuclear leukocytes (PMNs) after FcR ligation. Finally, cytochalasins, which disrupt actin filament assembly, were also specific inhibitors of the FcR-mediated $[Ca^{2+}]_i$ rise. These data demonstrate that rearrangements in the actin cytoskeleton triggered by FcR ligation are critical in release of Ca^{2+} from intracellular stores and suggest that l-plastin has a role in this process.

MATERIALS AND METHODS

General Methods. PMNs were purified and loaded with fura-2, fluorescence was measured, and $[Ca^{2+}]_i$ was calculated exactly as described (7, 10, 18). All buffers contained 1.5 mM Ca^{2+}. Agonists including aggregated IgG, immune complexes, and stock solutions of platelet-activating factor, fMet-Leu-Phe, and phorbol 12,13-dibutyrate were prepared, and phagocytosis of IgG-opsonized sheep erythrocytes was measured as described (7, 10, 18). The concentrations of fMet-Leu-Phe and phorbol dibutyrate used to stimulate IgG-mediated phagocytosis were 10 and 60 nM, respectively. These activators were added to PMNs at the same time as IgG targets. Complement fragment C5a (Abbott) and ATP (Calbiochem) were dissolved in water at 1000× and buffered to pH 7.4 with 1 M Tris. BPB (Sigma) was prepared as a 2 mM stock in ethanol. For $[Ca^{2+}]_i$ measurements, fura-2-loaded PMNs were incubated for 10 min on ice with BPB, or ethanol as a control, prior to activation by FcR ligands or other agonists. For experiments involving cytochalasins, fura-2-loaded PMNs were incubated with various concentrations of cytochalasins or with solvent (dimethyl sulfoxide) for 15 min at 37°C and then transferred immediately to the fluorimeter cuvette for measurement of $[Ca^{2+}]_i$. SDS/PAGE and Western blot analysis were performed exactly as described (19).

mAb Preparation. For immunization of mice with BPB as a hapten, BPB was covalently bound to keyhole limpet hemocyanin. Twenty milligrams of hemocyanin was incu-

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Abbreviations: BPB, bromophenacyl bromide; $[Ca^{2+}]_i$, intracytoplasmic concentration of free Ca^{2+}; PMN, polymorphonuclear leukocyte (neutrophil); FcR, IgG Fc receptor; mAb, monoclonal antibody; IP_3, inositol 1,4,5-trisphosphate.

†Present address: Department of Pharmacology, Campus Box 7365, University of North Carolina, Chapel Hill, NC 27599.
§To whom reprint requests should be addressed at: Campus Box 8051, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110.

bated with 1 mg of the dipeptide His-Lys (Sigma) in 1 ml of phosphate buffer at pH 7. One milliliter of 20 mM glutaraldehyde was added dropwise with stirring, and the mixture was incubated for 24 hr at room temperature. After dialysis, the His-Lys-derivatized hemocyanin was incubated with 0.4 mM BPB for 3 hr at room temperature to prepare BPB-hemocyanin. Anti-BPB mAb CAR-5 was selected for binding to BPB linked to ovalbumin via the His-Lys dipeptide linker, prepared as described above. CAR-5 recognized by immunoprecipitation and on Western blots *Naja naja* phospholipase A2 that had been treated with BPB. This phospholipase binds a single BPB per molecule of enzyme (20), demonstrating that CAR-5 was able to detect this low level of BPB binding to protein. Anti-plastin mAb was prepared from the fusion of spleens of BALB/c mice immunized with recombinant l-plastin (17). The anti-plastin mAb is an IgG1 which recognized native monocyte and PMN l-plastin by immunoprecipitation and on Western blots.

Identification and Purification of the BPB-Binding Protein. PMN membranes and cytosol were separated by sonication of PMNs on ice in Hanks' balanced salts solution containing a cocktail of protease inhibitors (10), after incubation of intact PMNs with 1 mM diisopropyl fluorophosphate. Sonicated cells were centrifuged at 800 × *g* for 15 min to remove nuclei and unbroken cells and then at 100,000 × *g* for 45 min to separate membranes and cytosol. For purification of the BPB-binding protein, cytosol from BPB-treated PMNs was separated on a Mono Q HR5/5 anion-exchange column (Pharmacia). The sample was applied in 50 mM Tris/50 mM NaCl at pH 8.0 and a gradient to 500 mM NaCl was used to elute the retained proteins. The BPB-binding protein was eluted at ≈300 mM NaCl. The fractions containing anti-BPB-reactive material were concentrated and the proteins were separated by two-dimensional electrophoresis. The spot which contained the BPB-binding protein was detected by Coomassie blue staining of proteins after transfer to poly(vinylidene difluoride) membranes and confirmed by Western blotting with CAR-5. CNBr digestion of the protein spot on the poly(vinylidene difluoride) membrane was performed by standard techniques. Amino acid sequence was analyzed with an Applied Biosystems model 470A gas-phase sequencer and a model 120A phenylthiohydantoin (PTH) amino acid analysis system.

RESULTS

Effect of BPB on $[Ca^{2+}]_i$. BPB at 1 μM caused almost total inhibition of the IP_3-independent $[Ca^{2+}]_i$ rise induced by aggregated IgG (Fig. 1*A*) and by insoluble immune complexes (Table 1). In contrast to its inhibitory effect on $[Ca^{2+}]_i$ arising from FcR ligation, BPB had no effect on the fMet-Leu-Phe-induced increase in $[Ca^{2+}]_i$, which is IP_3-dependent (10) (Fig. 1*B*). The $[Ca^{2+}]_i$ rises in response to stimulation of PMNs with the complement fragment C5a, with platelet-activating factor, and with ATP also were not inhibited by BPB (Table 1). BPB inhibition of the IgG-induced $[Ca^{2+}]_i$ rise was dose dependent; half-maximal inhibition occurred at ≈0.2 μM BPB, without any effect on the fMet-Leu-Phe-induced $[Ca^{2+}]_i$ rise (Fig. 1*C*). Thus, the IP_3-independent increase in $[Ca^{2+}]_i$ induced by FcR ligation was specifically inhibited by BPB. At 3 μM, BPB did not affect PMN expression of FcRII or FcRIII (data not shown).

Previously, we found that PMNs increase their phagocytic activity in response to proinflammatory stimuli via two mechanisms. Phorbol esters and platelet-activating factor increase phagocytosis independent of a $[Ca^{2+}]_i$ rise, whereas fMet-Leu-Phe-enhanced ingestion is dependent on the $[Ca^{2+}]_i$ increase generated by FcR ligation (7). These experiments demonstrated that two distinct signaling pathways could result in IgG-mediated phagocytosis by activated PMNs. Since the Ca^{2+} response to aggregated IgG was inhibited by BPB, we examined the effect of BPB on IgG-

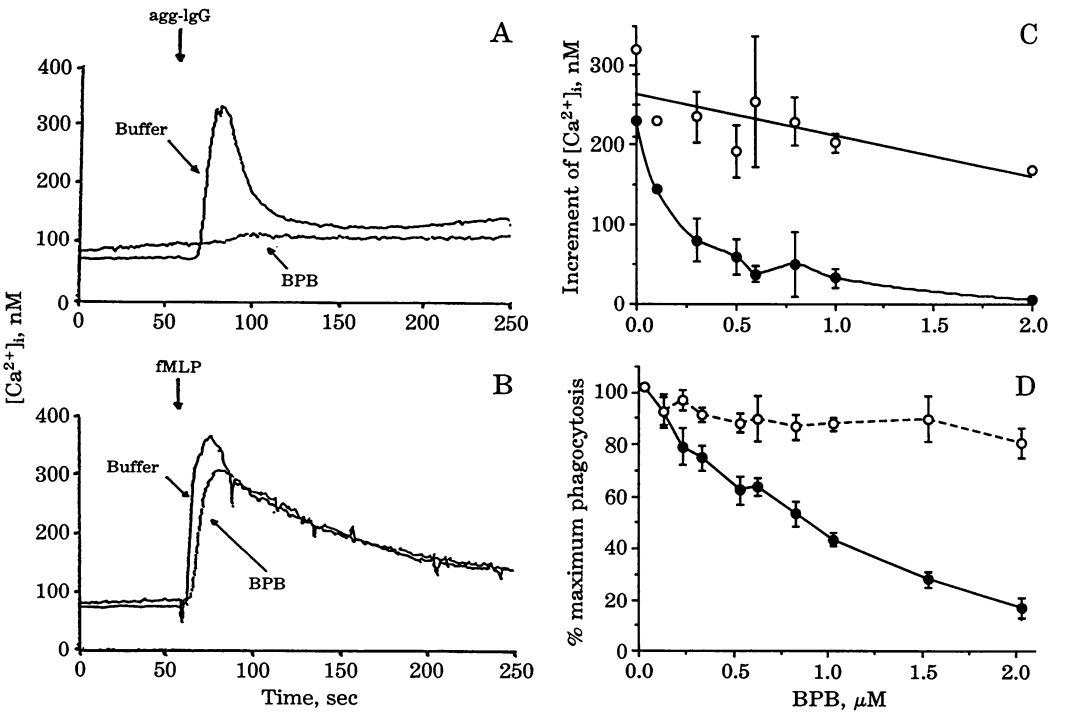

FIG. 1. Effect of BPB on IgG- and fMet-Leu-Phe-dependent increases in $[Ca^{2+}]_i$. (*A–C*) Fura-2-loaded PMNs were treated with vehicle (buffer) or 1 μM BPB (*A* and *B*) or various concentrations of BPB (*C*) on ice for 10 min. Both populations of cells were then incubated with aggregated IgG (agg-IgG, 300 μg/ml) (*A* and ● in *C*) or with 10 nM fMet-Leu-Phe (fMLP) (*B* and ○ in *C*). *A* and *B* show the average $[Ca^{2+}]_i$ vs. time; *C* shows the maximal increase in $[Ca^{2+}]_i$ vs. BPB concentration. (*D*) IgG-dependent phagocytosis by fMet-Leu-Phe-stimulated PMNs (●) and by phorbol dibutyrate-stimulated PMNs (○) vs. BPB dose. *A* and *B* are representative of four to six experiments. In *C* and *D*, error bars represent SEM for four and six experiments, respectively.

Table 1. Effect of BPB on $[Ca^{2+}]_i$ in PMNs

Agonist	Maximum $[Ca^{2+}]_i$, nM	
	Buffer	BPB
None	74 ± 6	80 ± 3
Agg-IgG	329 ± 21	98 ± 8
IC	336 ± 18	126 ± 7
fMLP	349 ± 1	346
C5a	455	467
ATP	365	355
PAF	382	395

Fura-2-loaded PMNs were treated with vehicle (Buffer) or 1 μM BPB and then stimulated with aggregated IgG (Agg-IgG, 300 μg/ml), a 1:25 dilution of immune complexes (IC), 10 nM fMet-Leu-Phe (fMLP), 10 nM C5a, 1 mM ATP, or 5 nM platelet-activating factor (PAF). When error is indicated, value is mean ± SEM of three or four independent experiments. When no error is indicated, value is average of two independent experiments.

mediated phagocytosis. Half-maximal inhibition of fMet-Leu-Phe-stimulated ingestion required ≈0.5 μM BPB (Fig. 1*D*). In contrast, BPB had no effect on the ingestion by phorbol ester-stimulated PMNs. Thus, BPB is not a general inhibitor of phagocytosis and is not generally toxic to cells, since Ca^{2+}-independent ingestion and the cytoskeletal rearrangements necessary for it were unaffected by concentrations of BPB which maximally inhibited Ca^{2+}-dependent ingestion and FcR-mediated $[Ca^{2+}]_i$ increase. This experiment reinforced our previous conclusion (7) that the $[Ca^{2+}]_i$ rise needed for Ca^{2+}-dependent ingestion must arise from FcR ligation, since the fMet-Leu-Phe-induced $[Ca^{2+}]_i$ increase was unaffected by BPB, but BPB inhibited phagocytosis by fMet-Leu-Phe-stimulated PMNs.

BPB Preferentially Binds l-Plastin. Because BPB exerts its inhibitory effect by covalent interaction with electron-donor side chains (20), we hypothesized that BPB would bind covalently to molecules in leukocytes which are required for FcR-dependent release of Ca^{2+} from intracellular stores. To identify the protein(s) modified by BPB, we developed a mAb against BPB. Remarkably, we found that when PMNs were treated with ≤1 μM BPB, only a single cytosolic protein of 65–70 kDa (66 ± 1 kDa, n = 10) was labeled with BPB (Fig. 2*A*). When PMNs were incubated with higher concentrations of BPB, other labeled proteins were observed. At 10 μM BPB, >25 bands were identified on a Western blot with the anti-BPB antibody (data not shown). When PMNs were treated with 1 μM BPB and then solubilized in 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), all reactivity with the anti-BPB antibody was retained by a 30-kDa-cutoff Amicon filter; thus, peptides or lipids unresolvable by SDS/PAGE did not detectably bind BPB. The 66-kDa protein had an isoelectric point of pH 5.6 and was found in human monocytes and lymphocytes as well as PMNs (data not shown). The 66-kDa protein was purified from cytosol of BPB-treated PMNs by a combination of Mono Q ion-exchange chromatography and two-dimensional electrophoresis. The purified BPB-labeled protein had a blocked amino terminus. CNBr cleavage followed by *o*-phthalaldehyde blocking (21) resulted in amino acid sequences of two fragments. These sequences corresponded exactly to l-plastin, a Ca^{2+}-regulated actin-bundling protein (22, 23) (Fig. 3). To corroborate that the purified BPB-binding protein was l-plastin, we performed Western blots with rabbit antisera raised against l-plastin and fimbrin (17), the chicken homologue of plastin (Fig. 2*B*). These antibodies detected a single protein in the cytosol from PMNs that comigrated with the 66-kDa BPB-binding protein. The Mono Q-purified BPB-binding protein also reacted with these anti-plastin antibodies, confirming that it was l-plastin (Fig. 2*B*).

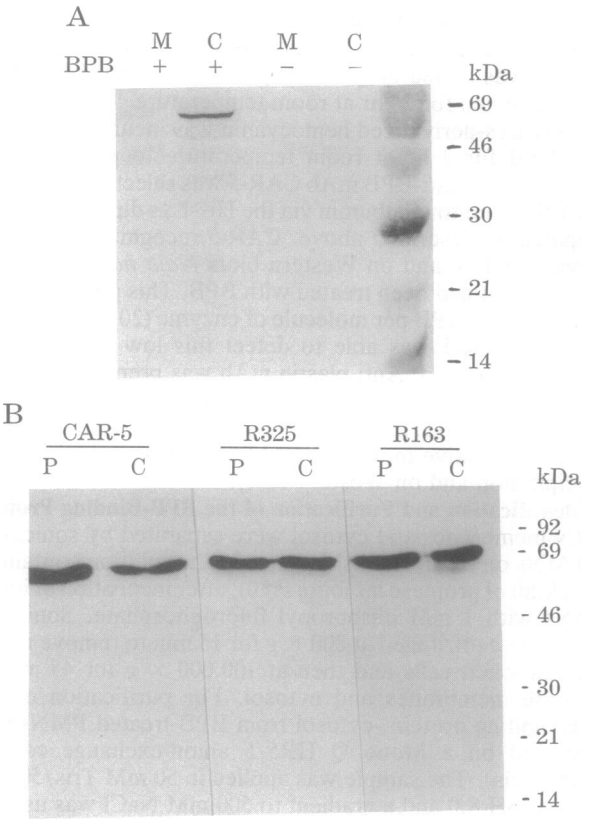

FIG. 2. Anti-BPB and anti-plastin detect the same protein in BPB-treated PMNs. (*A*) PMNs were treated with vehicle (−) or 1 μM BPB (+) as in Fig. 1, separated into membrane (M) and cytosolic (C) fractions, and subjected to SDS/PAGE. The separated proteins were transferred to a poly(vinylidene difluoride) membrane. Proteins with covalently attached BPB were detected with anti-BPB mAb CAR-5. (*B*) Cytosol from BPB-treated PMNs was separated on a Mono Q FPLC column and the fraction containing BPB reactivity (P) was electrophoresed in an SDS/polyacrylamide gel along with unfractionated cytosol (C). After transfer to poly(vinylidene difluoride), proteins were detected with CAR-5 (anti-BPB), R325 (rabbit anti-l-plastin), and R163 (rabbit anti-fimbrin).

FcR Ligation Induces l-Plastin Association with the Cytoskeleton. IgG FcR ligation in PMNs leads to a rapid increase in F-actin content and a redistribution of F-actin to the periphery of the cell. To determine whether FcR ligation could also induce a rearrangement of l-plastin, we examined l-plastin association with the Triton X-100-insoluble cytoskeleton of PMNs. When fixed prior to extraction, nonadherent PMNs showed diffuse cytoplasmic immunofluorescence of l-plastin. No plastin was found in the detergent-insoluble cytoskeletons of PMNs adherent to albumin- or poly(L-lysine)-coated surfaces. In contrast, indirect immunofluorescence for l-plastin was strongly positive in the Triton X-100-insoluble extracts of PMNs adherent to albu-

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BPB          MXPNTNXLFNAVGDGIVL
L-plast  146 MNPNTNDLFNAVGDGIVL 163
T-plast  149 MNPNTDDLFKAVGDGIVL 166

BPB          MXXXPEELLLRXANYHLENAG
L-plast  262 MKLSPEELLLRWANYHLENAG 282
T-plast  265 MKLSPEELLLRWANFHLENSG 285
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FIG. 3. Comparison of amino acid sequences of two peptides of the BPB-binding protein with plastin isoforms. Numbers designate position of aligning amino acids from the initiation methionine in the plastin sequences. Underlined residues differ between l-plastin and t-plastin. BPB, sequences of CNBr fragments of BPB-binding protein.

min–anti-albumin immune complexes (Fig. 4). Thus, FcR ligation induced an association of l-plastin with the detergent-insoluble cytoskeleton, presumably through a direct association with F-actin.

Cytochalasins Specifically Inhibit FcR-Mediated Increase in $[Ca^{2+}]_i$. Our data suggested the hypothesis that l-plastin provides a link between the cytoskeletal rearrangements induced by FcR ligation and release of Ca^{2+} from intracellular stores. Thus, it was possible that the actin cytoskeleton was involved in the IP_3-independent signal transduction pathway for Ca^{2+} release in PMNs. To test this, we examined $[Ca^{2+}]_i$ in PMNs treated with cytochalasins B and D. Both cytochalasin B and cytochalasin D inhibited the immune complex-induced $[Ca^{2+}]_i$ rise; neither had any effect on the fMet-Leu-Phe-induced increase in $[Ca^{2+}]_i$ (Fig. 5). Thus, disruption of the actin network with cytochalasins inhibited the IP_3-independent $[Ca^{2+}]_i$ rise induced by FcR ligation but did not affect the IP_3-dependent response to fMet-Leu-Phe.

DISCUSSION

These data show that low concentrations of BPB specifically inhibit FcR-mediated Ca^{2+} release without affecting the increase in $[Ca^{2+}]_i$ caused by PMN activation with fMet-Leu-Phe, complement fragment C5a, platelet-activating factor, or ATP. All these agents release Ca^{2+} from intracellular stores, but only FcR-mediated release is independent of IP_3. While BPB is known to inhibit some secreted phospholipases A2, the concentration needed to inhibit FcR-dependent Ca^{2+} release from intracellular stores is much lower that the amounts of BPB (100 μM) used to inhibit the secreted phospholipases *in vitro* (20). Intracellular phospholipases A2 differ markedly from the secreted forms of the enzyme; purified leukocyte phospholipase A2 is unaffected by BPB (24, 25); the partially purified Ca^{2+}-independent phospholipase A2 involved in IgG-mediated phagocytosis (26) is likewise unaffected by BPB (M. R. Lennartz and E.J.B., unpublished data). Thus, the inhibitory effect of BPB on Ca^{2+} release from intracellular stores is unrelated to any effect on phospholipase A2.

The specific inhibitory effect of BPB is mimicked by cytochalasins, which also block FcR-mediated but not fMet-Leu-Phe-mediated release of Ca^{2+} from intracellular stores. This suggests that the inhibitory effect of BPB is exerted through an effect on the cytoskeleton. This hypothesis is strongly supported by the discovery that BPB at ≤ 1 μM binds detectably only to l-plastin, a Ca^{2+}-regulated actin-bundling

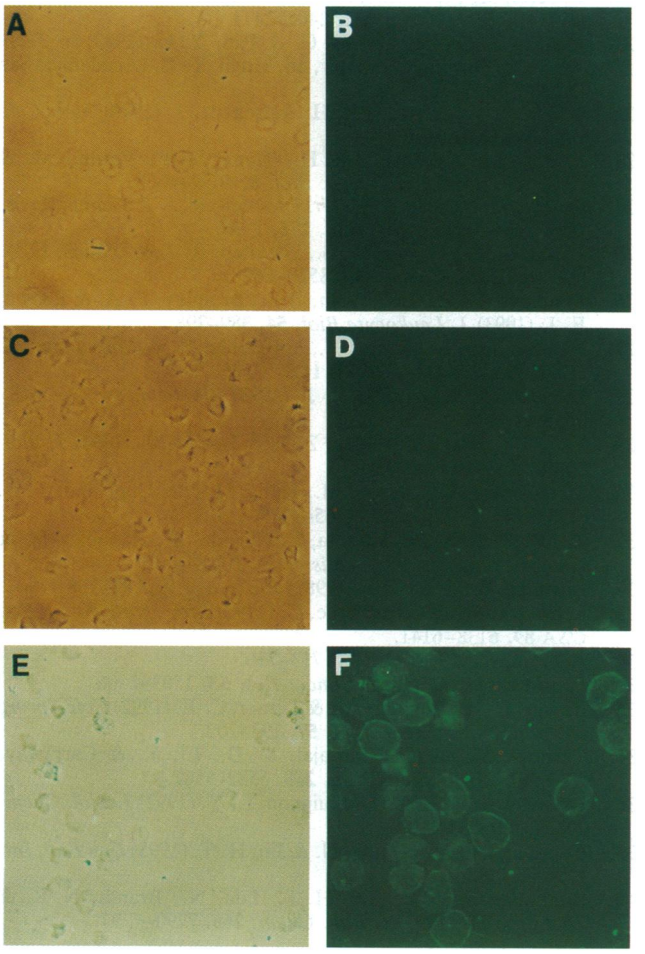

FIG. 4. Immunofluorescence localization of l-plastin in PMNs. PMNs were incubated on glass coverslips coated with bovine serum albumin (BSA)–anti-BSA immune complexes (*A* and *C*) or BSA alone (*B*) for 30 min at 37°C. Cells were extracted with Triton X-100 for 1 min and then fixed with methanol. Coverslips were stained with control mAb 6F2 (19) (*A*) or anti-l-plastin mAb (*B* and *C*) followed by fluoresceinated $F(ab')_2$ anti-mouse IgG. Like cells on BSA, PMNs adherent to poly(L-lysine) showed no staining with anti-l-plastin mAb (data not shown). (×240.)

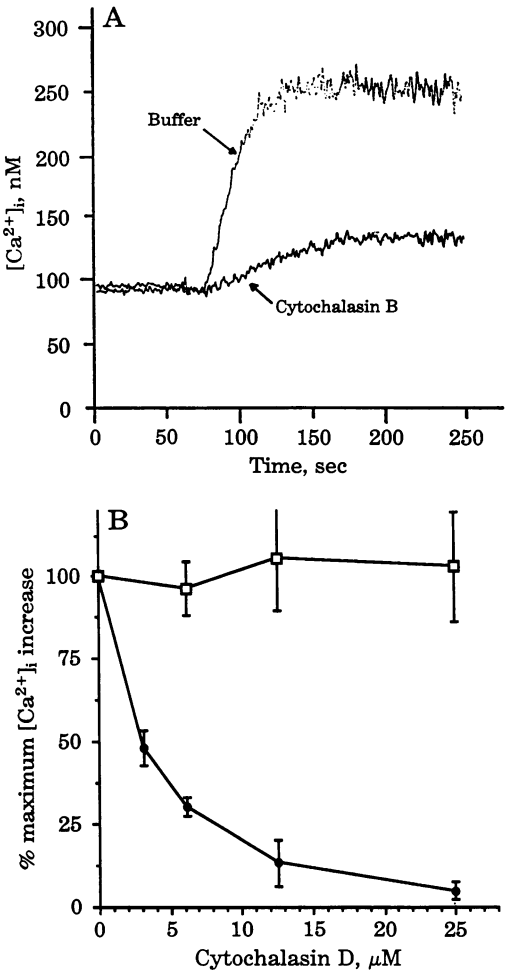

FIG. 5. Effect of cytochalasins on $[Ca^{2+}]_i$ during PMN activation. (*A*) Fura-2-loaded PMNs were incubated with vehicle (buffer) or 25 μM cytochalasin B for 15 min at 37°C and then incubated with a 1:25 dilution of albumin–anti-albumin immune complexes. Data are representative of three experiments. (*B*) PMNs were incubated as in *A* with various concentrations of cytochalasin D and then with immune complexes (●) or 10 nM fMet-Leu-Phe (□). Data, plotted as the maximal increase in $[Ca^{2+}]_i$ vs. cytochalasin D concentration, are the mean of three experiments.

protein. At this concentration, BPB does not cause a general disintegration of the actin cytoskeleton, since Ca^{2+}-independent phagocytosis is unaffected. Thus, BPB's specific inhibition of Ca^{2+} release from intracellular stores must reflect interference with a very specific step in FcR-mediated cytoskeletal assembly. Preliminary data indicate that BPB does not affect actin–plastin interaction *in vitro* or in response to FcR ligation, suggesting that l-plastin interaction with an as yet unidentified component of the membrane cytoskeleton is critical for signal transduction for Ca^{2+} release from intracellular stores.

l-Plastin is a 68-kDa cytosolic protein which is highly homologous to t-plastin and to fimbrin, a cytoskeletal protein of the intestinal brush border (17, 23). l-Plastin has no known function but has several properties consistent with a role in leukocyte activation-dependent cytoskeletal reorganization. Both plastins and fimbrin express actin binding sites which are homologous to those contained in spectrin and α-actinin and, in addition, have potential Ca^{2+} binding sites of the EF-hand type. l-Plastin has been shown to bind and bundle F-actin *in vitro* in a Ca^{2+}-regulated manner (27, 28). Its exclusive expression in leukocytes (29) and its phosphorylation in response to leukocyte activators such as interleukins 1 and 2 and phorbol esters (30, 31) support the hypothesis that l-plastin has a role in cytoskeletal rearrangements during leukocyte activation.

A connection between membrane cytoskeleton reorganization and biochemical pathways of signal transduction has received much attention (32–36). Cytoskeletal proteins regulate signal transduction by modulation of enzymatic activities through direct interaction with phosphatases and kinases (37, 38). Interestingly, the membrane cytoskeleton, and ankyrin in particular, has been found to inhibit IP_3-dependent release of Ca^{2+} from intracellular stores by direct competition with IP_3 for the IP_3 receptor (39, 40). Together with our data, this suggests that the cytoskeleton may regulate IP_3-dependent and IP_3-independent Ca^{2+} release oppositely. l-Plastin's reversible association with F-actin, its phosphorylation during leukocyte activation, and the regulation of its function by Ca^{2+} concentration make it a likely mediator of cross talk between the leukocyte cytoskeleton and signal transduction cascades.

We thank Drs. Wenyan Shen, Paul Matsudaira, and Ching-Shwun Lin for l-plastin cDNA and for rabbit anti-l-plastin and anti-fimbrin antisera. We thank the Washington University Hybridoma Facility for help in preparing anti-BPB and anti-l-plastin mAbs, Dr. John Cooper for helpful discussions, and Dr. Fred Lindberg for critical review of the manuscript. This work was supported by Grants AI24674 and GM38330 from the National Institutes of Health.

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