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EXTRACELLULAR CALCIUM RESULTS IN A CONFORMATIONAL CHANGE IN Mac-1 (CD11b/CD18) ON NEUTROPHILS

Differentiation of Adhesion and Phagocytosis Functions of Mac-1[1]

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Mac-1 is a leukocyte integrin involved in multiple adhesion phenomena and also in the phagocytosis of particles that are bound via CR1 and the IgG FcR. We examined the divalent cation requirements for the Mac-1-dependent processes of adhesion and receptor-mediated phagocytosis. With phorbol ester stimulation, both processes occurred in the absence of extracellular Ca^{+2}. In Ca^{+2}-containing buffer, IB4, an anti-β_2 mAb, inhibited both adhesion and CR1-mediated ingestion. In the absence of Ca^{+2}, IB4 no longer had any effect on ingestion, although it continued to inhibit adhesion to protein-coated plastic completely. This demonstrates that the role of Mac-1 in adhesion is distinct from its role in phagocytosis. In addition, 1) IB4 did not change intracellular Ca^{+2} homeostasis; 2) as little as 300 nM free extracellular Ca^{+2} could restore the ability of IB4 to inhibit ingestion; and 3) in the absence of extracellular Ca^{+2}, Mac-1 was more susceptible to enzymatic cleavage. Together these data suggest a Ca^{+2}-dependent conformational change in Mac-1, which allows distinction of the roles of Mac-1 in phagocytosis and adhesion.

Mac-1 (CD11b/CD18) is a leukocyte adhesion protein belonging to the integrin superfamily. It is a heterodimeric glycoprotein that in the integrin nomenclature is called $\alpha_M\beta_2$. α_M (CD11b) is unique to Mac-1; β_2 is found in LFA-1 and p150,95 as well Mac-1. The best defined ligand for Mac-1 is the complement fragment C3bi (1, 2), but evidence supporting an interaction between Mac-1 and Factor X, fibrinogen, endothelium, pathogens, and several extracellular matrix proteins has been presented (3–7). Phagocyte activation by agents such as FMLP, phorbol esters, ADP, and ATP can markedly affect the ligand binding functions of Mac-1, presumably by causing conformational changes in the molecule or in its molecular environment (3, 4, 8, 9). However, little direct data exist on this point.

The amino acid sequence of α_M derived from cDNA cloning predicts three divalent cation binding sites (10, 11). Mac-1 binding to many, if not all of its ligands is dependent on the presence of divalent cations, a property shared with many integrins. It is likely that this requirement for Ca^{+2} or Mg^{+2} reflects stabilization of a conformation of the molecule necessary for ligand binding.

In contrast to these ligand binding functions of Mac-1 stands its role in phagocytosis. Mac-1 is involved in phagocytosis mediated by several different receptors even when the opsonin involved does not bind to Mac-1 (12, 13). Because of the ability of Mac-1 to bind rather diverse ligands and because of its involvement in many in vitro adherence phenomena, it is possible to suppose that Mac-1 plays a rather nonspecific, "glue-like" role in these ingestion events. In contrast, Mac-1 may be involved in phagocytosis at a step beyond particle adherence. We recently have presented evidence that a subset of Mac-1 that interacts with the phagocyte cytoskeleton is required for normal receptor-mediated phagocytosis (13). Moreover, binding of IgG or C3b opsonized targets to phagocytes is normal on cells treated with anti-Mac-1 antibodies and on cells genetically deficient in Mac-1 even when ingestion is markedly abnormal (14). These data are most consistent with the hypothesis that Mac-1 plays a very specific role in ingestion, coordinating plasma membrane and cytoskeletal events.

To attempt to distinguish further between the role for Mac-1 in phagocytosis and its more conventional ligand binding function, we examined the divalent cation requirements of various Mac-1 functions. Removal of extracellular Ca^{+2} alone did not differentiate between the various Mac-1 functions tested. However, in the absence of extracellular Ca^{+2}, adhesion to protein-coated plastic could be differentiated from Mac-1 dependent phagocytosis using the mAb IB4 (anti-β_2). IB4 inhibited both phagocytosis and adhesion to protein-coated surfaces when extracellular Ca^{+2} is present. When these functions were tested in the absence of extracellular Ca^{+2}, IB4 continued to inhibit adhesion, but no longer had any effect on phagocytosis. This suggested the possibility of a Ca^{+2}-dependent conformational change in Mac-1, which affected the interaction of IB4 with the epitope of Mac-1 involved in phagocytosis. This hypothesis was supported by the demonstration of increased protease sensitivity of Mac-1 in the absence of extracellular Ca^{+2}. IB4 had no effect on intracellular Ca^{+2} homeostasis, and as little as 300 nM extracellular Ca^{+2} restored the inhibitory affect of IB4 on phagocytosis, suggesting a high

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affinity Ca^{+2} binding site in the Mac-1 molecule. Together these data demonstrate a Ca^{+2} dependent conformational change in Mac-1 which allows differentiation of the adhesion and phagocytic functions of Mac-1.

MATERIALS AND METHODS

Buffers. HBSS (M. A. Bioproducts, Walkersville, MD) with 1% human serum albumin was used with either 1 mM Ca^{+2} and 1 mM Mg^{+2} (Ca^{+2} buffer), or 1 mM EGTA and 2 mM Mg^{+2} (EGTA-buffer).

mAb. IB4 (anti-CD18), 60.3 (anti-CD18), and Mo-1 (anti-CD11b) were the generous gifts of Dr. Sam Wright, Rockefeller University, New York, NY; Dr. Patrick Beatty, Fred Hutchinson Cancer Research Center, Seattle, WA; and Dr. R. F. Todd III, University of Michigan Medical School, Ann Arbor, MI; respectively (2, 15, 16). 10B5 (anti-CD11b) was prepared by immunization of mice with IB4 immunoprecipitates from PMN, and fusion of spleen cells with the nonsecreting meloma cell line P3X63AG8.6.5.3. Hybridoma culture supernatants were then screened for their inhibitory effect on Mac-1-dependent, CR1-mediated phagocytosis, and cells producing supernatants that consistently inhibited ingestion were cloned in soft agar. 10B5 specificity was determined by 1) binding to normal PMN and monocytes but not lymphocytes; 2) lack of binding to PMN from leukocyte adherence deficiency patients, and 3) immunoprecipitation of $\alpha_M\beta_2$.

Cells. Heparinized peripheral blood was obtained from normal volunteers and PMN isolated as described (17).

Phagocytic targets. Sheep E (M. A. Bioproducts) were opsonized with C4b (EC4b)[3] or C3b as described (18, 19). EC3bi were prepared by cleavage of C3b on sheep E opsonized with complement component 3b to the iC3b form by incubation in low ionic strength EDTA buffer with a 1/500 dilution of normal serum, as described (20).

Phagocytosis and attachment assays. Phagocytosis was assessed by a fluid phase assay in which 1×10^5 PMN were suspended in 300 μl of Ca^{+2} or EGTA-buffer. Cells were preincubated for 15 min at 37°C ± a Mac-1 mAb (30 μg/ml). Subsequently 1×10^7 EC4b and 15 ng/ml PDBu (Sigma Chemical Co., St. Louis, MO) were added, and centrifuged at 50 × *g* for 2 min. After incubation at 37°C for 30 min, uningested SRBC were lysed by the addition of 2 ml of 0.83% NH_4Cl and centrifugation at 500 × *g* for 5 min. Phagocytic index was quanitated as the number of opsonized E ingested per 100 PMN.

C3bi rosetting was determined in a similar assay ± 15 ng/ml PDBu. E were not lysed and attachment was quanitated as the number of EC3bi attached per 100 white blood cells.

For determination of the minimal amount of Ca^{+2} required to restore IB4 inhibition of phagocytosis, PMN were suspended in EGTA buffer and Ca^{+2} (100 to 900 μM) was added. Free Ca^{+2} was then calculated as described (21).

Adhesion assay. Adhesion of PMN to human serum albumin-coated Immulon plates (Dynatech Laboratories, Alexandria, VA) was determined as described (22). Briefly PMN were preincubated for 15 min at 37°C at 5×10^6/ml in Ca^{+2}- or EGTA-buffer ± a Mac-1 mAb at 30 μg/ml. PMN were then plated at 150 μl/well, and PDBu added to a final concentration of 20 ng/ml. Plates were incubated at 37°C for 10 min, washed with PBS, fixed with 1% glutaraldehyde and stained with Giemsa. Adhesion was quantitated by the addition of methanol and the measurement of absorbance at 570 nm.

IB4 binding. PMN were incubated with I^{125}-IB4 and increasing amounts of unlabeled IB4 in Ca^{+2} and EGTA buffers, on ice for 2 h. This was determined to be sufficient to establish equilibrium binding. The I^{125}-IB4 bound was quantitated by centrifuging the cells through phthalate oils at 12,000 × *g* for 2.5 min, and counting the cpm associated with the pellet. Nonspecific binding was considered to be the I^{125}-IB4 bound in the presence of a 100-fold excess of unlabeled antibody. The ability of mAb 60.3 to inhibit IB4 binding was determined similarly, by adding increasing amounts of unlabeled 60.3 simultaneously with ^{125}I-IB4.

Intracellular Ca^{+2} concentration. PMN were loaded with 2 μM fura-2 in Ca^{+2}-buffer as described (23). Intracellular Ca^{+2} concentration was determined in samples of 2×10^6 PMN/ml with an Hitachi F-2000 spectrofluorimeter (Hitachi, Danbury, CT) with a 37°C stirred cell. Fluorescence was measured using excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Cytoplasmic Ca^{+2} concentrations were calculated as described by Grynkiewicz et al. (24).

Chymotrypsin cleavage of Mac-1. PMN were preincubated with 1 μM FMLP for 15 min at 37°C in Ca^{+2}-buffer, washed, and resuspended in Ca^{+2}- or EGTA-buffer. PMN were incubated for 1 h with chymotrypsin (100 μg/ml or 1 mg/ml) at 37°C. Control cells were left on ice. PMN were washed, and incubated for 1 h at 0°C with ^{125}I-10B5 (anti-CD11b mAb), centrifuged through oil, and the specific cpm associated with each pellet determined. Nonspecific binding was considered to be the ^{125}I-10B5 bound in the presence of a 100-fold excess of unlabeled antibody.

Total cell Mac-1 was quantitated by solubilization of PMN in 1% Triton-X100, immunoprecipitation with Mo-1 (anti-CD11b), followed by SDS-PAGE, silver staining (25), and densitometry of the CD11b band (EC densitometer, EC Apparatus Co., St. Petersburg, FL).

RESULTS

Ca^{+2} dependence of Mac-1 functions. We initially compared the Ca^{+2} requirements for Mac-1-dependent phagocytosis with the Ca^{+2} requirements for C3bi binding and PMN adhesion to protein-coated plastic. The Mac-1-dependent phagocytosis assay used was ingestion of EC4b by PMN stimulated with PDBu (13). C4b opsonization was used because this ligand binds to CR1 without cross-reactive binding to Mac-1. The ingestion of EC4b is dependent on Mac-1, as shown by the findings that patients with complete leukocyte adherence deficiency are totally unable to ingest these particles, and that some Mac-1 mAb inhibit this function (13, 14). Ingestion of EC4b occurred normally in the absence of extracellular Ca^{+2} (Fig. 1). C3bi binding and adhesion functions also were tested in the presence of phorbol esters. C3bi binding by unstimulated cells was dependent on extracellular Ca^{+2}; however, in the presence of phorbol esters the requirement for extracellular Ca^{+2} was at least partially eliminated (Fig. 1). The adhesion of phorbol ester-stimulated PMN to protein-coated plastic also was independent of extracellular Ca^{+2} under these assay conditions (Fig. 1). Although it is of interest that some "Ca^{+2} requiring" Mac-1 functions occurred in Ca^{+2}-deficient media when cells

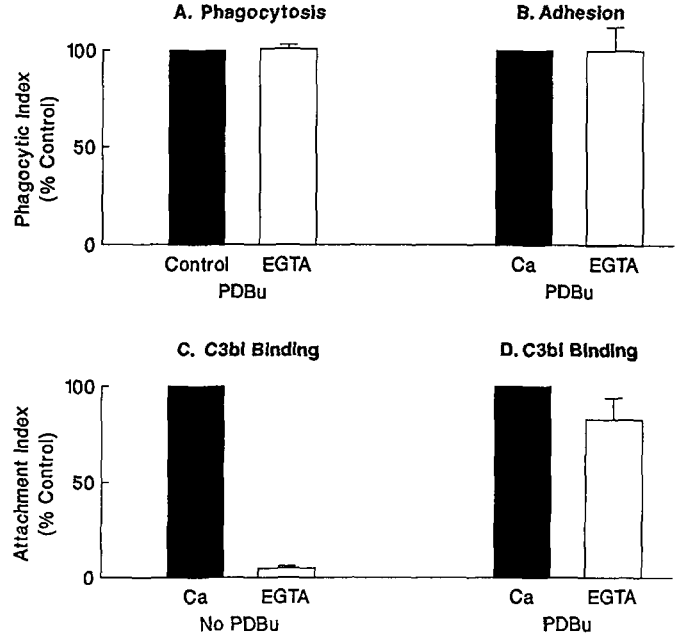

Figure 1. Ca^{+2} requirements of Mac-1-dependent functions. *A*, The phagocytosis of EC4b in response to phorbol esters was unchanged in the presence or absence of extracellular Ca^{+2}. *B*, Adhesion of PMN to HSA-coated plastic in the presence of phorbol esters was equivalent in Ca^{+2}- and EGTA-buffers. *C*, EC3bi binding in the absence of phorbol esters was markedly decreased in EGTA-buffer (AI = 1292 in Ca^{+2}, 64 in EGTA). *D*, EC3bi binding in the presence of phorbol esters was nearly equivalent in EGTA-buffer compared with Ca^{+2} buffer (AI = 1886 in Ca^{+2}, 1510 in EGTA). The data shown are the mean ± the SEM of three experiments.

[3] Abbreviations used in this paper: EC4b, sheep E opsonized with complement component 4b; EC3bi, sheep E opsonized with complement component 3bi; PDBu, phorbol dibutyrate; PMN, polymorphonuclear leukocytes.

were stimulated by phorbol esters, this observation did not differentiate among CD11b/CD18 conformations required for the various Mac-1 functions.

Ca^{+2} dependence of IB4 inhibition of phagocytosis. To confirm that the PMN adhesion and phagocytosis occurring in EGTA-buffer was still Mac-1 dependent, the inhibitory effects of Mac-1 mAb were tested. 60.3 (anti-β_2) and Mo-1 (anti-α_M) inhibited both adhesion and phagocytosis in either Ca^{+2}- or EGTA-buffers (Fig. 2). However, IB4, an anti-β_2 mAb, which was a potent inhibitor of adhesion and phagocytosis in Ca^{+2}-buffer, had no effect on phagocytosis in EGTA-buffer (Fig. 2). The possibility that the binding affinity of IB4 was altered by the absence of extracellular Ca^{+2}, was tested by determining the ID_{50} for cold IB4 inhibition of I^{125}-IB4 binding (Fig. 3). In Ca^{+2} buffer the ID_{50} for IB4 was 0.59 ± 0.05 μg/ml vs 0.62 ± 0.06 μg/ml (mean ± SEM, $n = 3$) in EGTA buffer. The K_d for IB4 binding calculated from these data was 1.19 nM in Ca^{+2} buffer and 1.79 nM in EGTA buffer. Therefore there was no evidence of a change in IB4 binding affinity in EGTA buffer. In addition IB4 continued to completely inhibit the adhesion to protein-coated plastic. This result suggested the possibility of a conformational change in Mac-1 that was dependent on extracellular Ca^{+2}. As a result of this conformational change, the IB4 epitope would no longer be in proximity to the domain of Mac-1 involved in phagocytic function, but would still be associated with the domain(s) of Mac-1 involved in adhesion. IB4 and 60.3 have been reported to have similar specificities based on competitive inhibition (Fig. 3), but their epitopes cannot be identical because 60.3 continued to inhibit phagocytosis in EGTA buffer whereas IB4 did not. The ID_{50} for IB4 inhibition of its own binding was about three-fold lower (0.6 μg/ml) than the ID_{50} for 60.3 inhibition of IB4 binding (1.5 μg/ml), which suggests close but not identical epitopes for IB4 and 60.3. These data are all consistent with the possibility of a Ca^{+2}-dependent conformational change in Mac-1 that affects the ability of IB4 to interfere with the ligand-independent function of Mac-1 in CR1 mediated phagocytosis.

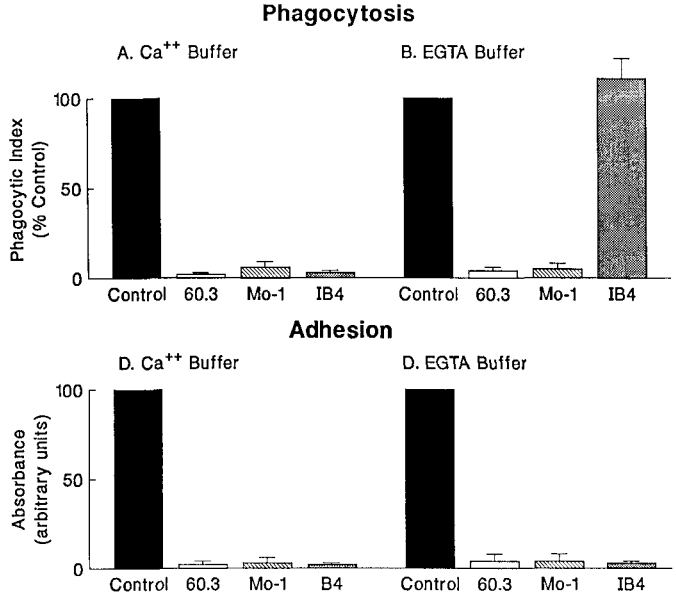

Figure 2. Effects of Mac-1 mAb on phagocytosis and adhesion. *A*, Phagocytosis of EC4b is completely inhibited by 60.3, Mo-1, and IB4 in Ca^{+2}-buffer. *B*, In EGTA-buffer, phagocytosis of EC4b continues to be inhibited by 60.3 and Mo-1. However, IB4 has no effect on phagocytosis in EGTA-buffer. *C* and *D*, Adhesion of PMN to protein coated plastic is inhibited by 60.3, Mo-1, and IB4 in both Ca^{+2}- and EGTA-buffers. Data represent the mean ± the SEM from three experiments.

IB4 does not generate Ca^{+2} influx. An alternative explanation for the lack of IB4 effect on phagocytosis in EGTA-buffer could be that the effect of IB4 on ingestion was indirect. Perhaps in Ca^{+2}-sufficient media IB4 generated a Ca^{+2} influx resulting in inhibition of phagocytosis, as is the case for the Ca^{+2} ionophore A23187 (27). This Ca^{+2} influx would be abolished by removal of Ca^{+2} from the extracellular media. To test this hypothesis, PMN were loaded with Fura-2 in Ca^{+2}-buffer and incubated with IB4. The intracellular Ca^{+2} concentration was monitored continuously using a spectrofluorimeter (Fig. 4). IB4 had no effect on the PMN intracellular Ca^{+2} concentration. Moreover, as previously shown, PDBu itself did not affect intracytoplasmic Ca^{+2} concentration (28), and the combination of IB4 and PDBu also had no effect on intracellular Ca^{+2} (data not shown). Therefore, removal of extracellular Ca^{+2} did not simply prevent the generation of an intracellular Ca^{+2}-dependent inhibition of phagocytosis by IB4.

Enzyme sensitivity of Mac-1. To further explore the possibility that removal of extracellular Ca^{+2} results in a conformational change in Mac-1, we tested the sensitivity of Mac-1 to proteolytic cleavage in Ca^{+2}- and EGTA-buffers. All PMN were initially treated with FMLP in Ca^{+2}-buffer to up-regulate the intracellular pool of Mac-1. PMN were subsequently incubated in either Ca^{+2}- or EGTA-buffer with various proteolytic enzymes. We chose to examine proteolytic sensitivity in whole cells because of the likelihood that detergent extraction itself could result in a significant and uncontrolled conformational change in Mac-1. Mac-1 present at the end of the incubation was quantitated by ^{125}I-10B5 (anti-CD11b) binding. After chymotrypsin treatment in Ca^{+2}-buffer, 10B5 binding decreased only 30%, suggesting only a small loss of its epitope from the cell surface. This is consistent with the relative insensitivity of Mac-1 to proteases, as reported by others (26). However, PMN in EGTA buffer consistently showed a 60% decrease in ^{125}I-10B5 binding after incubation with chymotrypsin (Fig. 5). This suggests that in the absence of extracellular Ca^{+2}, Mac-1 shows an increased sensitivity to chymotrypsin cleavage. Chymotrypsin cleavage of HLA-A was determined as a control using the mAb W6/32. Cleavage of this molecule was equivalent in Ca^{+2} and EGTA buffers (31 and 34%, respectively). Similar assays using trypsin, pronase or leukocyte elastase showed no difference in 10B5 (anti-CD11b) binding between Ca^{+2}- and EGTA-buffers (data not shown).

To confirm that the decrease in surface Mac-1 in EGTA-buffer in response to chymotrypsin was truly due to enzymatic cleavage, rather than some alteration in Mac-1 expression on the intact cells, a second type of experiment was performed. In this assay, after incubation with chymotrypsin, Mac-1 was quantitated by immunoprecipitation, followed by SDS-PAGE, silver staining, and densitometry. This allowed detection of total rather than just surface expressed Mac-1, because it has been previously demonstrated that a portion of the Mac-1 molecules remain intracellular even after FMLP treatment (29). In these experiments, loss of the Mac-1 epitope was less than in the preceding experiment, because the

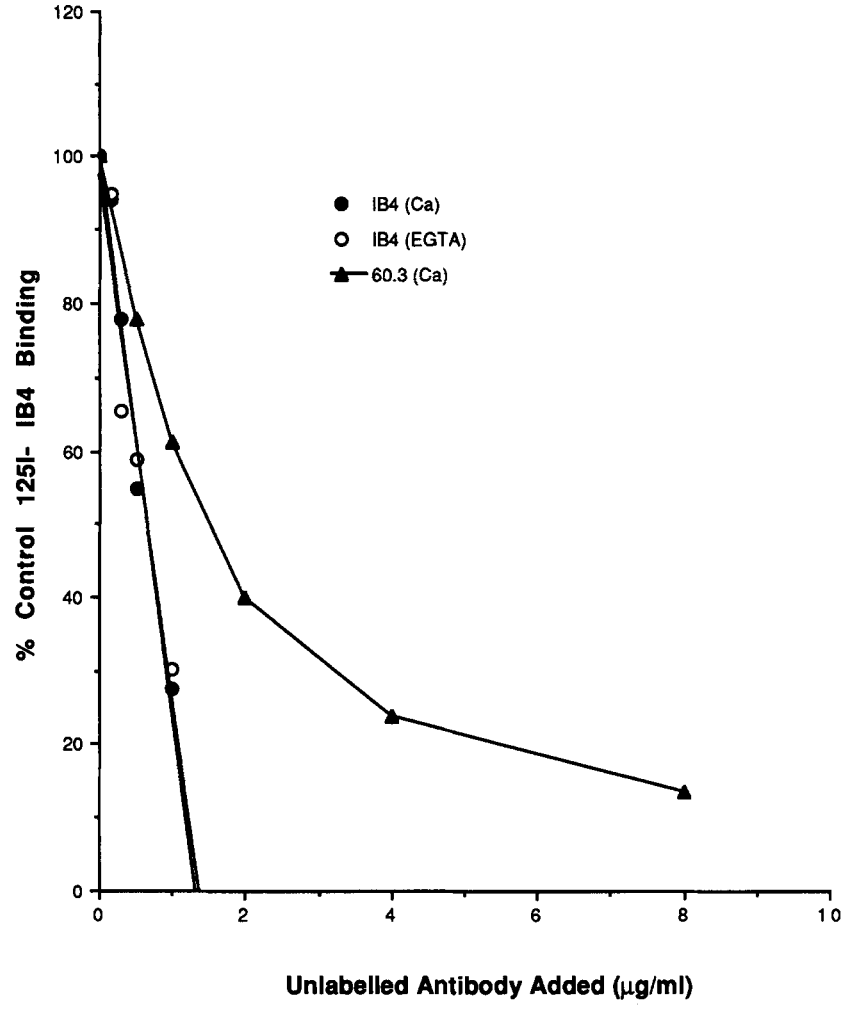

Figure 3. IB4 binding is equivalent in Ca^{+2} or EGTA-buffer. 60.3 competes partially for IB4 binding sites. PMN were incubated with I^{125}-IB4 with increasing amounts of unlabeled IB4 or 60.3. The ID_{50} for unlabeled IB4 was 0.59 μg/ml in Ca^{+2}-buffer, and 0.62 μg/ml in EGTA buffer. The ID_{50} for 60.3 to inhibit ^{125}I-IB4 binding was 1.5 μg/ml.

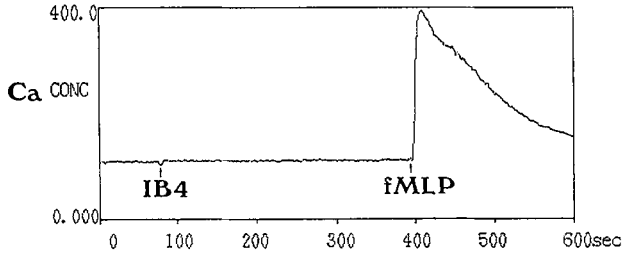

Figure 4. IB4 has no effect on intracellular Ca^{+2} concentration. PMN were loaded with 2 μM Fura-2 in Ca^{+2}-buffer. Intracellular Ca^{+2} concentration was continuously monitored using a spectrofluorimeter. IB4 (30 μg/ml) and FMLP (0.1 μM) were added where indicated. IB4 did not affect intracytoplasmic [Ca^{+2}] or the ability of FMLP to increase intracytoplasmic [Ca^{+2}].

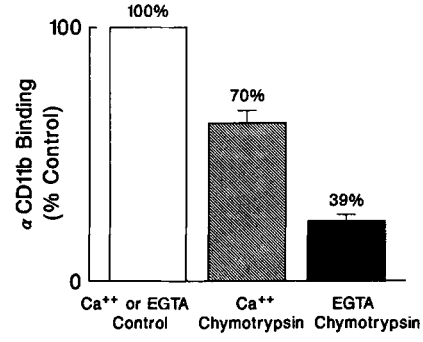

Figure 5. Mac-1 cleavage by chymotrypsin is increased in the absence of extracellular Ca^{+2}. PMN were treated with 1 μM FMLP in Ca^{+2}-buffer, and resuspended in Ca^{+2}- or EGTA-buffer. PMN were incubated with chymotrypsin (1 mg/ml) for 1 h at 37°C. Control cells were left on ice. PMN were then incubated with ^{125}I-10B5 (anti-CD11b), centrifuged through oil, and the specific cpm associated with each pellet determined. Data presented are the mean ± SEM from four experiments, each done in duplicate.

unexpressed intracellular pool was unaffected by the exogenous enzyme treatments. Nonetheless, chymotrypsin treatment in EGTA buffer again resulted in a significantly greater cleavage of Mac-1 than in Ca^{+2}-buffer (Fig. 6). This confirmed an increased sensitivity of Mac-1 to proteolytic cleavage in Ca^{+2}-deficient buffer. These results are consistent with a Ca^{+2}-dependent conformational change in Mac-1.

Restoration of IB4 activity. The minimum level of extracellular Ca^{+2} required to allow IB4 to inhibit phagocytosis was determined. As shown in Figure 7, only 300 nM extracellular Ca^{+2} was required to fully restore the inhibitory effect of IB4 on CR1-mediated ingestion. This extremely low Ca^{+2} concentration is in the range of the K_d of the high affinity Ca^{+2} binding sites described on gpIIb/IIIa, another integrin receptor (30). This result provides further evidence that IB4 does not inhibit phagocytosis via a plasma membrane Ca^{+2} channel, because these are inefficient at less than about 10 μM extracellular Ca^{+2} (23).

DISCUSSION

Mac-1 is involved in a diverse array of functions including 1) ligand binding (e.g., C3bi, factor X); 2) adhesion

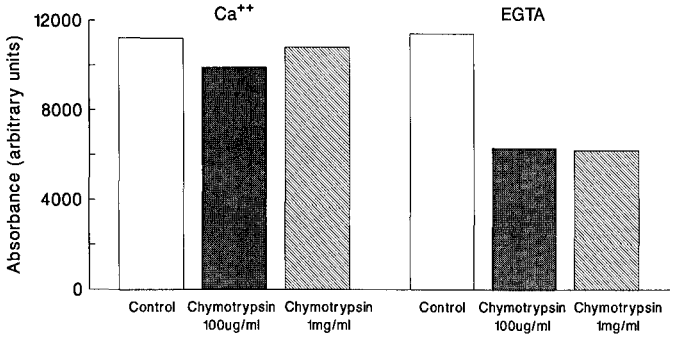

Figure 6. Total cell Mac-1 after chymotrypsin cleavage. Mac-1 shows increased sensitivity to proteolytic cleavage in the absence of extracellular Ca^{+2}. PMN were pretreated with 1 μM FMLP in Ca^{+2} buffer, resuspended in Ca^{+2}- or EGTA-buffer and incubated with chymotrypsin for 1 h at 37°C. Samples were then immunoprecipitated with Mo-1, followed by SDS-PAGE and silver staining. CD11b was quantitated by densitometry. Data are plotted as the density of the CD11b band present on the silver-stained gels.

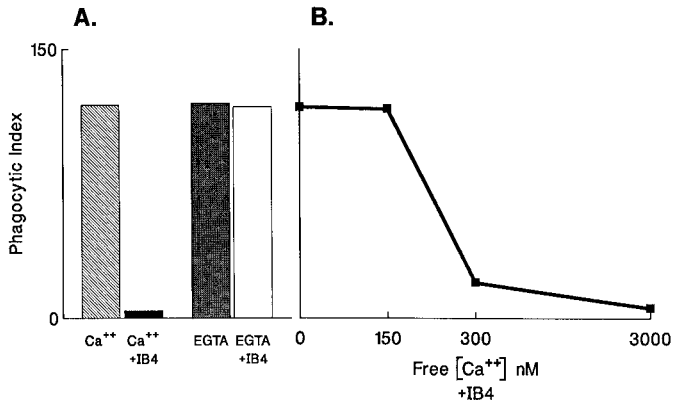

Figure 7. Restoration of IB4 inhibition of CR1-mediated ingestion by Ca^{+2}. *A*, IB4 inhibited EC4b phagocytosis in Ca^{+2}-buffer, but had no effect on phagocytosis in EGTA buffer. These data served as simultaneous controls for *B*, in which extracellular [Ca^{+2}] was varied. *B*, The inhibitory effect of IB4 on EC4b phagocytosis was quanitated in EGTA buffer with increasing amounts of free Ca^{+2} present. Only 300 nM free Ca^{+2} was required to restore the inhibitory effect of IB4 on EC4b phagocytosis. The data shown are representative of four similar experiments.

to cells and surfaces where the Mac-1 ligand remains unidentified (e.g., endothelial cell adherence, homotypic PMN aggregation, and adhesion to plastic and glass); and 3) phagocytosis of particles bound via the IgG FcR or CR1. Our data suggest that the role of Mac-1 in phagocytosis of particles bound by CR1 or IgG FcR occurs beyond the initial step of particle attachment, because PMN genetically deficient in Mac-1 rosette normally with IgG- and C3b-opsonized particles, but fail to amplify ingestion in response to inflammatory cytokines and extracellular matrix proteins (13, 14). However, the possibility remains that Mac-1 is merely providing a nonspecific, "glue-like" stabilization of particle binding that is not detected by rosette quantitation. If this were the case, then the role of Mac-1 in phagocytosis would be equivalent to Mac-1 mediated adhesion to glass or plastic.

Previous work using Mac-1 mAb has not differentiated the Mac-1 adhesion and phagocytic functions. Inasmuch as many Mac-1 functions are believed to be dependent on divalent cations, it was possible that this divalent cation requirement would be useful in the differentiation of these Mac-1 functions. Of interest is that although PMN aggregation, another Mac-1 function, is known to require extracellular divalent cations, receptor aggregation can occur to at least some extent in their absence (31). One form of Mac-1-dependent phagocytosis, the phorbol ester-stimulated ingestion of particles bound via CR1 is clearly independent of extracellular Ca^{+2} (32) (Fig. 1). Interestingly, both adhesion to protein-coated plastic and EC3bi rosetting, functions of Mac-1 thought to require Ca^{+2}, occurred at least to some extent in the absence of extracellular Ca^{+2} after phorbol ester stimulation. This could be related to an increase in Mac-1 activity in activated cells, either because phorbol esters stimulate ligand independent aggregation of Mac-1 (33), which results in more effective ligand binding, or because they induce phosphorylation (34, 35) or some other covalent change in Mac-1 that results in a ligand binding conformation even in the absence of Ca^{+2}. Although, the divalent cation requirement alone was not helpful in differentiating these Mac-1 functions, the change in IB4 activity in the absence of extracellular Ca^{+2} did serve to differentiate Mac-1-dependent adhesion from phagocytosis. Although IB4, an anti-β_2 antibody, can inhibit binding to protein-coated surfaces in the absence of extracellular Ca^{+2}, it cannot inhibit phagocytosis. This observation suggests that epitopes of the molecule are involved in adhesion that are not involved in Mac-1's role in phagocytosis. The fine specificity and sublety of this distinction is emphasized by the fact that another anti-β_2 mAb, 60.3, inhibits both functions in EGTA-buffer, and that 60.3 and IB4 can actually cross-inhibit each other's binding to some extent (36) (Fig. 3).

There are several potential explanations for the change in IB4 activity in the absence of extracellular Ca^{+2}. One possibility was that IB4 was generating a Ca^{+2} influx in Ca^{+2}-containing media, as has been reported for other Mac-1 mAb, resulting in a 2° inhibition of phagocytosis similar to the effect of a Ca^{+2} ionophore on phagocytosis. This possibility was excluded by direct measurements that showed that IB4 had no effect on intracellular Ca^{+2} levels. An alternate explanation for the loss of IB4 inhibition of phagocytosis in Ca^{+2}-free media, was that Ca^{+2} was required for optimal IB4 binding, either by causing a conformational change in Mac-1 or by being directly involved in the IB4 binding site. However, binding studies with I^{125}-IB4 showed no evidence of a change in binding affinity in Ca^{+2}-free media. This raised the possibility of a conformational change in Mac-1 that allowed IB4 to continue to bind, yet no longer interact with the epitope involved in phagocytosis. The finding that Mac-1 on intact PMN is more sensitive to chymotrypsin cleavage in the absence than in the presence of extracellular Ca^{+2}, supports the idea of a Ca^{+2}-dependent conformational change in Mac-1. Data to date support the concept that divalent cations can stabilize the ligand binding conformation of all integrins, suggesting that integrins in general undergo divalent cation-dependent conformational changes. However, an alternative hypothesis, that divalent cations form a tripartite complex with integrin receptor and ligand has been proposed (30). In comparisons of Ca^{+2} and Mg^{+2}, different receptors show different effects. Mg^{+2} has a greater effect than Ca^{+2} on fibronectin binding to gp IIb/IIIa and VLA-5 (37, 38). In contrast, VLA-4 and the leukocyte response integrin require Ca^{+2} but not Mg^{+2} for α- and β-chain association and Arg-Gly-Asp binding, respectively (39, 40). Some functions of Mac-1 such as C3bi binding and adhesion to endothelium

are optimal on resting or FMLP-stimulated PMN only in the presence of both Ca^{+2} and Mg^{+2} (8). This suggests that Ca^{+2} and Mg^{+2} may have independent effects on the conformation of various integrins. Our hypothesis of a conformation of Mac-1 particularly dependent on Ca^{+2}, and modulated by a binding site with an affinity for Ca^{+2} less than 1 μM, is similar to suggestions made about the role of Ca^{+2} in the conformation of gpIIb/IIIa, an homologous integrin of platelets (30). This may represent only one of several possible Ca^{+2} binding sites on Mac-1 because ATP and ADP induction of factor X and fibrinogen binding to CD11b/CD18 requires extracellular Ca^{+2} in excess of 2 mM (3, 4). Similarly, the conformational change in gpIIb/IIIa detected by mAb PMI-1 requires mM concentrations of either Ca^{+2} or Mg^{+2} (41).

In summary the change in activity of IB4 seen in the absence of extracellular Ca^{+2} has allowed the differentiation of Mac-1-mediated adhesion to protein-coated plastic from the phagocytic function of Mac-1. Thus, these data, along with all the data accumulated so far, strongly support the hypothesis that Mac-1 is acting in CR1-mediated phagocytosis by playing a fundamental role in the ingestion process, rather than as a way of stabilizing the rosetting of a phagocyte with its target. The data are most consistent with the hypothesis that Mac-1 plays a critical role in communication between the plasma membrane and the cytoskeleton during ingestion mediated by several or perhaps all phagocytically competent receptors. This signaling role for Mac-1 is distinct from its involvement in adhesion phenomena. We speculate that the specific signals generated by Mac-1 in ligand binding, adhesion, and phagocytosis may be conformation dependent. In this way the presence of extracellular Ca^{+2} may alter cell responses by affecting Mac-1 conformation, without any alteration in intracytoplasmic Ca^{+2}. This could represent a new role for extracellular Ca^{+2} in intracellular signaling processes.

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