Transmembrane Signaling: An Ion-flux-independent Model for Signal Transduction by Complexed Fc Receptors

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ABSTRACT Fluxes of Na^+/K^+ that precede effector functions in stimulated phagocytes are thought to play a role in signal transduction. To examine this hypothesis, phagocytosis, phagosomal acidification, and superoxide anion generation (O_2^-) were stimulated in media in which the Na^+ was replaced with K^+ or choline$^+$. Counts of particles internalized and assessment of acidification of the phagosomes by acridine orange staining indicated that Na^+/K^+ fluxes were not necessary for phagocytosis or phagosomal acidification in J774.2 macrophages. Phagocytosis mediated by the ionophoretic Fc receptor$_{\gamma 2b/\gamma 1}$ of J774.2 macrophages was equally independent of a Na^+ gradient. Na^+/K^+ fluxes did not dictate the rate of O_2^- generation in human monocytes. Therefore, in at least these three effector functions, Na^+/K^+ fluxes stimulated by Fc- and non-specific receptor binding play neither a signaling nor an enhancing role. An ion-flux-independent model for transmembrane signaling by the Fc receptor is proposed.

Others have shown that there is an apparent dependence on the external Na^+ concentration for O_2^- generation and lysosomal secretion by neutrophils. These neutrophils had been pre-treated with NH_4^+ during a routine purification step. O_2^- generation stimulated by opsonized zymosan or phorbol myristate acetate, by monocytes or monocyte-derived macrophages, and phagocytosis of opsonized zymosan by J774.2 macrophages, showed dependence on external Na^+ only if these cells had been pre-treated with NH_4^+. Brief NH_4^+ pre-treatment would be expected to acidify the cytoplasm of the cells. The reversal of this acidification is known to require Na^+ for H^+ extrusion through the Na^+/H^+ antiport, thus explaining the apparent Na^+ dependence.

Mononuclear (monocytes, macrophages) and polymorphonuclear phagocytes (neutrophils) share certain functions. The binding of particulate or soluble ligands to surface receptors induces the production of microbicidal oxygen radicals, phagocytosis of particulate ligands, and lysosomal secretion, first to the outside of the cell and then into the phagosomal vacuole. Some of the surface receptors that are involved in initiating these pleiotypic responses, collectively called phagocyte effector functions, have been studied to determine how receptors signal these functions. It is known that internalization of ligand is not necessary for signaling. Immobilization of ligands on a non-phagocytosable surface (1) or treatment of phagocytes with cytochalasin B, an inhibitor of endocytosis, does not reduce inflammatory responses after receptor stimulation (2). Thus, stimulus-response coupling between a ligand-bound receptor and cytoplasmic effector functions is apparently a surface phenomenon.

Evidence, much of it indirect, suggests that the signal for phagocyte effector function is Na^+ influx. Stimulatory ligand-receptor interaction of phagocytic cells increases the permeability of their plasma membranes to Na^+ (3). This increased ion permeability results in a change in membrane potential (4) that is dependent on Na^+/K^+ fluxes (4–6). The membrane potential change always precedes ligand-stimulated inflammatory responses (2, 7–12). Membrane permeability to other ions, such as Ca^{2+} (3, 13), also increases, but Ca^{2+} influx does not account for the membrane potential changes (2), nor is it necessary for the stimulation of inflammatory responses (14). Membrane potential changes that are dependent on Na^+/K^+ fluxes have been recorded by microelectrode (5, 6, 12) and estimated by the distribution of the lipophilic cations (2, 4, 7, 9, 11) across the membrane in several cell types after the addition of stimulatory ligand. In a manner resembling the response of the postsynaptic membrane of muscle to Na^+

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influx, action potentials in macrophages can be elicited by microelectrode stimulation to an apparent threshold potential (15). Furthermore, a murine macrophage receptor that binds IgG2b and IgG1 immune complexes (16) and that triggers phagocytosis and release of various mediators of inflammation (17) forms a ligand-dependent ion channel (18). When incorporated into a planar lipid bilayer, this receptor shows ion channel activity selective for Na^+ and K^+ (19). The increase in ion conductance is dependent on addition of specific ligand. Antigen-antibody complexes that bind to this receptor stimulate Na^+/K^+ fluxes both in the intact cell and in the lipid bilayer or liposomes containing the isolated and reconstituted Fc receptor (FcR)[1] (17, 19, 20). A monoclonal antibody specific for this particular receptor induces cellular hyperpolarization that is dependent on Na^+/K^+ flux and also induces K^+ efflux from liposomes with the reconstituted receptor (19). Thus, there is little doubt that the increased conductance involves a ligand-binding dependent change in the FcR. The ionophoretic activity resulting in an increased Na^+ influx into the cell could be the transducing mechanism that signals phagocyte effector functions.

In an effort to directly link Na^+ influx to phagocyte effector functions, the dependence of these functions on external Na^+ has been studied in human neutrophils (7, 8). Measurement of the release of superoxide anion (O_2^-) and of lysosomal secretion shows that the rates of these effector functions are apparently dependent on the concentration of Na^+ in the medium. On the basis of this and other observations, Korchak and Weissmann (7) proposed a stimulus-response coupling for O_2^- generation and lysosomal secretion with Na^+ influx as a necessary part of the initiating event. Holian and Daniele (21), on the other hand, found that stimulated guinea pig alveolar macrophages generate 70% of normal levels of O_2^- in the absence of external Na^+. The present report is a re-examination of the role of Na^+ influx as the signal transducer or enhancer for phagocyte effector functions.

MATERIALS AND METHODS

Cell Preparation: The J774.2 murine macrophage cell line was grown in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 20% fetal calf serum, penicillin, streptomycin, and fungizone. Macrophages were washed in Earle's minimal essential medium and allowed to attach in the same medium to 1.2-cm (diameter) round glass coverslips for 3–4 h for a final cell confluency of 20–50%.

Human peripheral blood monocytes were isolated by Ficoll-Hypaque gradient (Pharmacia Fine Chemicals, Piscataway, NJ) as described by Boyum (22). The gradient-isolated cells were washed eight times with RPMI 1640 medium. Monocyte numbers were determined by counting adherent and non-adherent populations on a hemacytometer incubated for 5 min at 37°C. The final cell suspension contained ~25% contaminating lymphocytes. Human monocyte-derived macrophages were prepared by incubating monocytes in RPMI containing 10% homologous human serum at 37°C for 6 d.

NH_4^+-pre-treated monocytes and J774.2 macrophages were prepared by incubation in 155 mM NH_4Cl, 10 mM HEPES (pH 7.2) (titrated with KOH) for 10 min at 37°C or 22°C as indicated in text.

Earle's minimal essential medium, Dulbecco's modified Eagle's medium, penicillin, streptomycin, and fungizone were obtained from Gibco Laboratories, Grand Island, NY. HEPES buffered Hanks' medium and RPMI 1640 (tested for low endotoxin content) were obtained from M. A. Bioproducts, Walkersville, MD.

Preparation of Parasites for Use as Particles: *Toxoplasma gondii* was grown and harvested as previously described (23). Approximately 10^7 parasites were rendered non-invasive by one of two methods depending on the targeted receptor(s) on the macrophage. For the first method, parasites in 0.5 ml HEPES-buffered Hanks' medium were denatured by slow addition of 3.7% formaldehyde in phosphate-buffered saline (pH 7.2) to a final concentration of 3%. After 60 min at 22°C, they were washed three times with HEPES-buffered Hanks' medium and opsonized in a 1:300 dilution of rabbit antitoxoplasma serum that had been heated to inactivate complement. The suspension was incubated at 22°C for 60 min. Denatured, opsonized parasites were washed three times with Na^+ or Na^+-substituted medium for immediate use in a phagocytosis assay. Parasites, prepared in this way, are phagocytosed through Fc- and non-specific receptor binding.

For the second method, freshly harvested, highly invasive parasites were washed and incubated at 37°C for 20–60 min in phosphate-buffered saline (pH 7.2). This step reduced their invasiveness by ~95%. The parasites were then centrifuged at 700 *g* for 10 min and resuspended in 800 μl HEPES-buffered Hanks' medium containing 0.7% heat-inactivated fetal calf serum. A volume of 200 μl of the suspension was added to each of four 22-mm coverslips on which human fibroblasts had been grown to confluency. The few remaining invasive parasites penetrated the fibroblasts during a 20-min incubation at 37°C. The non-invasive parasites were washed off of the monolayers, pelleted, and added to Na^+ or Na^+-substituted medium. The success of this method for rendering parasites non-invasive was monitored by plaque titration on monolayers of human fibroblasts (23). Parasites prepared in this way do not bind to, and are not phagocytosed by, macrophages unless they are opsonized. Rabbit antiserum was added at a final dilution of 1:100 to parasite suspensions in Na^+ or Na^+-substituted medium immediately before adding them to the phagocytes. This method for opsonization was chosen to avoid antiserum-promoted aggregation of the non-denatured parasites. For opsonization that would link the antibody-coated parasites specifically to the FcRs of J774.2 macrophages that bind IgG2b and IgG1, antibody-antigen complexes, a mouse IgG2b monoclonal antibody (24) that is specific for an antigen on the surface of the parasite, was substituted for the rabbit antiserum, and the parasites were allowed a 20-min pre-incubation in the antibody before use in the phagocytosis assay.

Assay of Phagocytosis and Vacuolar Acidification: J774.2 monolayers on 1.2-mm coverslips in 1.6-mm wells of Linbro trays were washed with Na^+, K^+, or choline$^+$ medium, transferred to dry wells, and overlaid with 50 μl of parasites in Na^+, K^+, or choline$^+$ medium, prepared as described above. After a settling time of 2 min, during which most of the parasites came into contact with the cells, the cultures were incubated at 37°C for 20 min for relatively synchronized phagocytosis. A volume of 0.5 ml of the appropriate medium warmed to 37°C was added gently for a further incubation of 20 min. The cultures were then stained by transferring the coverslips to wells containing the same media used for the phagocytosis incubation but that also contained 10 μg/ml of acridine orange (AO) (25). The cells were allowed to concentrate the dye for 7–10 min at 22°C and the uptake of particles was immediately assessed by fluorescence microscopy. Phagosomes containing internalized *T. gondii* rapidly acidified, concentrated the AO dye, and emitted a red fluorescence. The distinctive crescent shape of the internalized parasites set them apart from secondary lysosomes, and the red fluorescence distinguished them from the extracellular parasites, which appeared light green.

Cells in the K^+ medium died ~10 min after AO staining and during the microscopic observations. Cell death in K^+ set a time limit within which the extent of phagocytosis and vacuolar acidification had to be determined. These effector functions were rapidly and reproducibly assessed by counting red crescent-shaped phagosomes at a 400-fold magnification. A field containing 30 to 100 phagocytosed parasites and 15 to 35 macrophages could be counted in <1 min.

The media used were the same as those previously described (17) with slight modifications. Na^+ medium contained 118 mM NaCl, 8.5 mM Na_2CO_3, 20 mM HEPES, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM glucose (pH 7.4). In two other media, Na^+ was substituted by replacing Na_2CO_3 with K_2CO_3, and NaCl with K or choline chloride (3X recrystallized [Sigma Chemical Co., St. Louis, MO]) for all but 5 mM of the NaCl (K^+ medium) or for both the NaCl and KCl (choline$^+$ medium). The osmolalities of the stock Na, K, and choline chlorides were determined or confirmed at the concentrations used by measurement with a Wescor 5100 osmometer. De-ionized, glass-distilled water was used for all media, and flame photometer determinations of contaminating Na^+ were made on all media and rinse water of all plasticware. Alternative media that are referred to as 0 and 140 mM Na^+ media were used for phagocytosis of opsonized zymosan by NH_4^+-pre-treated J774.2 cells. These media contained 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose, 10 mM HEPES titrated with 5 mM KOH, 0 mM Na^+ (140 mM choline chloride) or 140 mM NaCl (pH 7.45).

Nitroblue Tetrazolium (NBT) Reduction in Phagosomes: Qualitative NBT staining for O_2^- generation into the phagosomes was performed by a modification of the Baehner method (26, 27) on duplicate cultures grown on 1.2-cm coverslips in 1.6-cm Linbro plate wells. J774.2 macrophages

[1] *Abbreviations used in this paper:* AO, acridine orange; FcR, Fc receptor; LPS, lipopolysaccharide; NBT, nitroblue tetrazolium; O_2^-, superoxide anion; PMA, phorbol myristate acetate.

were overlaid with formaldehyde-denatured, opsonized parasites in 50 μl of Na^+, K^+, or choline$^+$ phagocytosis medium that also contained 0.05% NBT. Phagocytosis proceeded during a 20-min, 37°C incubation followed by another 45 min at 37°C after addition of 0.5 ml pre-warmed appropriate media. The cells were washed with the medium used for phagocytosis, fixed with 4% formaldehyde in phosphate-buffered saline for 30 min, and stained 5 min with 0.1% safranin 0 in 1% acetic acid. Dried stained cultures were examined microscopically for the presence of formazan, the black particulate product of NBT reduction by O_2^-, inside of the phagosomes that contained parasites.

Cytochrome c Reduction Assay for O_2^-: The rate of O_2^- generation by monocytes was measured by a modification of the spectrophotometric method of Babior et al. (28) using cytochrome *c* (Type II or VI [Sigma Chemical Co.]) that had been dialysed 12 h against medium that lacked Na^+. Human monocytes were added to assay medium for a final concentration of 0.2 to 1.0 × 10^6 cells/ml. The assay medium contained 1 mg/ml opsonized zymosan and 0.5 mg/ml cytochrome *c* in media containing 1 mM KCN, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose, 137 mM Na, K, or choline chloride, and 20 mM HEPES titrated with 10 mM NaOH (final pH 7.45). Alternative media that are referred to as 0- to 140-mM Na^+ media were used for some assays, as indicated in the text. These contained 1 mM KCN, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose, 10 mM HEPES titrated with 5 mM KOH, and 0–140 mM NaCl with 140–0 mM choline chloride for osmotic balance. When monocyte-derived macrophages were assayed for O_2^- generation, the assay medium also contained 0.1 μg/ml lipopolysaccharide (LPS) (from *Escherichia coli*, 055:B5 [Difco Laboratories, Inc., Detroit, MI]). When 50 mg/ml superoxide dismutase was added to the assay medium, KCN was omitted.

When 3 μg/ml phorbol myristate acetate (PMA) (Sigma Chemical Co.) was used as the stimulant, addition of PMA to the assay mixture that contained cells initiated the O_2^- generation. Monocytes were quickly washed three times in choline$^+$ medium, added to a pre-warmed O_2^--generation assay mixture, and incubated in 37°C shaker bath in 50 ml disposable polypropylene tubes (Sarstedt, Inc., Princeton, NJ) to which the cells did not adhere. Volumes of 0.9 ml were chilled in 1 ml Eppendorf tubes and centrifuged at 13,000 *g* for 1 min. Absorbance of the supernatants at 500 nm was measured. The absorbance of stimulated minus control values was converted to nmol reduced cytochrome *c* with $A_{550} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$. Na^+, K^+, and choline$^+$ media did not affect the spectral scans of oxidized and reduced cytochrome *c*. The PMA was stored at −20°C as 1 mg/ml in dimethylsulfoxide. Neither the dimethylsulfoxide added to the assay with the PMA nor five times that concentration had any effect when added alone.

It was important to assess the activity of the cells in as many Na^+ concentrations as possible in some of the experiments of this paper. Because these experiments required a cell-free or stimulant-free control for each Na^+ concentration, doubling the number of assays, and because early as well as late time points were desirable, duplicate determinations could not be done within an experiment. Instead, such experiments were done repeatedly, and several representative results are displayed in the figures. Continuous recording spectrophotometry was avoided because it can give data for only the first 5 min of the reaction, and because sequential assays require storage of phagocytes over a period of hours at 4°C or 22°C, either of which would have created problems in the interpretation of the data.

RESULTS

Phagocytosis and Phagosomal Acidification in the Absence of a Na^+ Gradient

To examine the importance of Na^+ influx as a signal transducer for phagocytosis and phagosomal acidification, I measured these functions in media in which the Na^+ gradient across the plasma membrane was abolished. Media were prepared by replacing most of the Na^+ in a simple HEPES buffered salt solution (Na^+ medium) with K^+ (K^+ medium) or with choline$^+$ (choline$^+$ medium). The K^+ medium equilibrates (approximately) the K^+ concentration across the membrane making it unlikely that there will be a significant K^+ conductance at the time of stimulation.

T. gondii, a 3 × 7-μm protozoan parasite, was chosen as the phagocytosable particle because its crescent shape allowed it to be readily distinguished even after internalization by the phagocyte. This obligate intracellular parasite actively enters cells even in the absence of any phagocytic activity. Therefore, the parasites used in the phagocytosis experiments were rendered non-invasive by a denaturing and non-denaturing means (described in Materials and Methods) so that they could serve exclusively as a source of readily recognized, phagocytosable particles. Phagocytosis and phagosomal acidification by cells of the murine macrophage cell line, J774.2, were assessed by AO uptake into phagosomes formed in Na^+, K^+, and choline$^+$ media.

In the first method for rendering parasites non-invasive, denaturation with formaldhyde produces particles that were phagocytosed through non-specific receptors on the cell surface. However, the overall rate of binding and uptake was greatly enhanced when denatured parasites were first opsonized with parasite-specific antibody. Since a rapid assay for phagocytosis was required, denatured parasites were always antibody-coated before use. Such treatment also targeted the parasites for the macrophage FcR. Phagocytosis, therefore, took place primarily by means of Fc-, but also by means of non-specific, receptors. Fig. 1, *A–C*, illustrates the appearance of the crescent-shaped parasites in dye-concentrating acidified

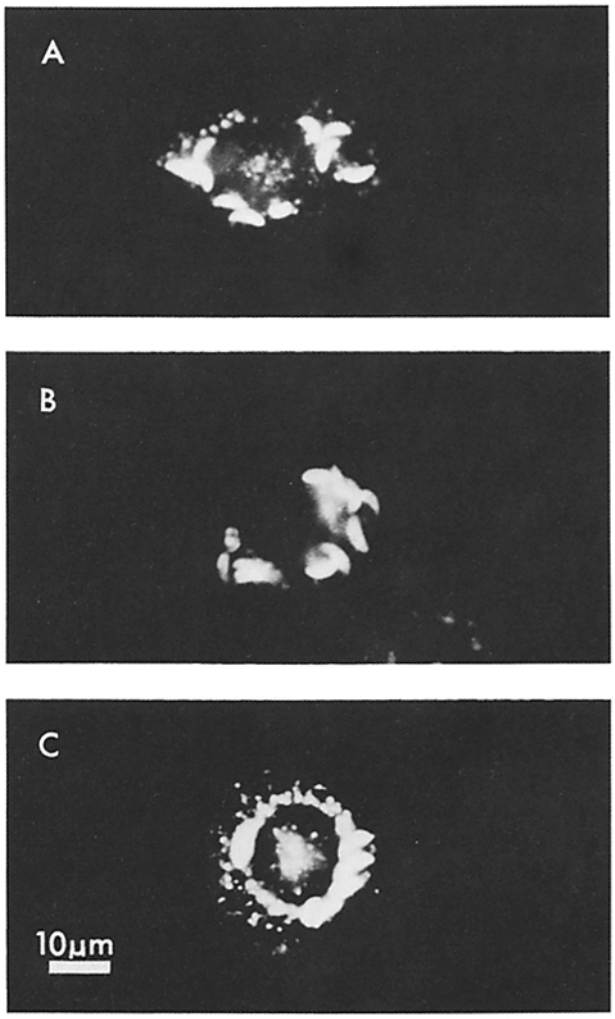

FIGURE 1 AO-stained phagosomes formed in Na^+ (*A*), K^+ (*B*), and choline$^+$ (*C*) media. J774.2 macrophage monolayer cultures were exposed to formaldehyde-denatured, opsonized *T. gondii* in Na^+, K^+, or choline$^+$ media as described in Materials and Methods. After 40 min at 37°C, acidic organelles were stained by AO in the media used for phagocytosis.

phagosomes. This qualitative assessment of the capacity for phagocytosis and vacuolar acidification in the presence and absence of a Na^+ gradient across its membrane indicated no apparent differences in uptake or acidification in the three media.

Parasites were also made non-invasive in a second, non-denaturing procedure. The non-denatured, non-invasive parasites differed from the formaldehyde treated *T. gondii* in that they did not bind to and were not appreciably phagocytosed by macrophages (antiserum-free control in Table I). Yet, when these treated parasites were added to macrophage monolayers in a medium that contained parasite-specific antibody, phagocytosis and phagosomal acidification progressed rapidly. Therefore, opsonization of these treated *T. gondii* produced particles that were avidly phagocytosed solely through IgG binding to FcRs. Quantitation of FcR-mediated uptake of non-denatured, non-invasive, antibody-coated parasites by J774.2 macrophages (Table I) supported the previous qualitative assessment. No significant differences in uptake followed by phagosomal acidification were observed in comparing cells incubated in Na^+ medium with those incubated in K^+ or choline$^+$ medium. Similar results (data not shown) were obtained when the opsonization was carried out with a parasite-specific IgG2b monoclonal antibody (24). The receptor for the Fc portion of this antibody is known to have ionophoretic activity when the appropriate ligand is bound (17). These results show that the Na^+ influx that follows binding is not the signal for phagocytosis or for phagosomal acidification since both occurred equally well in the absence of a Na^+ gradient in the K^+ and choline$^+$ media.

TABLE I

*Effect of the External Na^+ Concentration on FcR-mediated Phagocytosis of Non-invasive T. gondii**

Assay medium	Parasite-specific antibody	Total numbers counted: Parasites in phagosomes	Total numbers counted: Macrophages	Parasites/Macrophage
Experiment 1				
Na^+	+	524	206	2.54
K^+	+	317	116	2.73
Choline$^+$	+	694	258	2.69
Na^+	–‡	4	318	0.013§
Experiment 2				
Na^+	+	232	380	0.61
K^+	+	260	443	0.59
Choline$^+$	+	268	479	0.56
Na^+	–	17	700	0.024

J774.2 macrophage monolayers were overlaid with non-invasive parasites suspended in Na^+, K^+, or choline$^+$ medium. The Na^+-substituted media contained 5 mM Na^+ and amounts of K^+ or choline$^+$ to equalize the osmolarity. After a 45-min incubation at 37°C, and AO staining of the cultures, total counts of parasite phagolysosomes in 10 to 20 fields at × 400 were summed. Values from duplicate cultures were combined.

* A plaque titration in human fibroblast monolayers showed that the frequency of invasive parasites remaining in the suspension of non-invasive parasites was 2×10^{-6}.

‡ Normal mouse serum was added to the cultures lacking parasite-specific antiserum.

§ Similar control cultures that were incubated for 115 min did not show a significant increase in uptake over the indicated 45-min value.

Localization of Oxidative Intermediates in the Phagosomal Vacuole

A third phagocyte effector function, release of oxygen radicals, was assessed qualitatively by observation of the deposition of formazan in vacuoles that contain phagocytosed particles. Yellow NBT dye is ingested together with phagocytosed particles and is reduced by O_2^- to a black, insoluble dye, formazan (29, 30). Fig. 2, *A–C*, shows qualitatively similar deposition of formazan when formaldehyde-denatured, opsonized parasites were taken up by J774.2 macrophages in Na^+, K^+, and choline$^+$ media. These results suggest that oxygen radicals were released into the phagocytic vacuole even in the absence of Na^+ influx (9–12, 17) and in the absence of sustained hyperpolarizations (5, 11, 17) attributed to an increased K^+ permeability (5) as reported by other investigators.

O_2^- Generated in the Absence of a Na^+ Gradient

The production of a specific oxygen radical, O_2^-, was assayed quantitatively in stimulated human monocytes for two reasons. First, the initial rates of O_2^- generation were measured in order to answer the question: is Na^+ influx, if not a signal transducer, at least a mechanism by which the response of a stimulated cell is enhanced? Secondly, the control of O_2^- generation by freshly isolated human monocytes should more closely reflect phagocyte activity unselected by long term cell culture typical of the macrophage tumor cell lines. The J774.2 cell line could be constitutively enhanced or unenhanceable.

Monocytes were stimulated by opsonized zymosan in Na^+, K^+, and choline$^+$ media. Their production of O_2^-, as measured by the reduction of cytochrome *c*, did not require Na^+ influx for signaling or for enhancement (Fig. 3*A*). Chloride, another major ion in the assay medium, has an intracellular concentration that is about one third that of the assay medium (31). Replacement of NaCl by an osmotic equivalent of sucrose reversed the Cl^- gradient across the membrane, but only reduced the production of O_2^- by ~36% in this experiment (Fig. 3*B*), indicating that permeability changes in the membrane with respect to Cl^- are not essential to the signaling mechanism. Further substitutions: bicarbonate or phosphate buffers in place of HEPES or HEPES-Na_2CO_3 gave essentially the same results. The specificity of the assay for O_2^- production was indicated by the cell-free control levels of cytochrome *c* reduction when stimulated cells were assayed in the presence of the specific O_2^- scavenger, superoxide dismutase.

NH_4^+ Pre-treatment Introduces a Na^+ Requirement for O_2^- Generation

The above conclusion that stimulation of O_2^- generation in monocytes does not require a Na^+ gradient is inconsistent with published reports that O_2^- generation by neutrophils is dependent on the external concentration of Na^+. It seemed unlikely that these two cell types would have totally different mechanisms for signal transduction. Therefore, the methods for isolating these cells were compared to determine if there were any steps that could have altered the sensitivity of neutrophils to external Na^+. Using the Boyum isolation method (22), neutrophils, but not monocytes, co-isolate with large numbers of contaminating erythrocytes. Two purification steps are required to remove them. Monocytes, used as the test cell because their O_2^- generation response was independent of the external Na^+ concentration, were mock-purified by exposing them to each step of the neutrophil purifi-

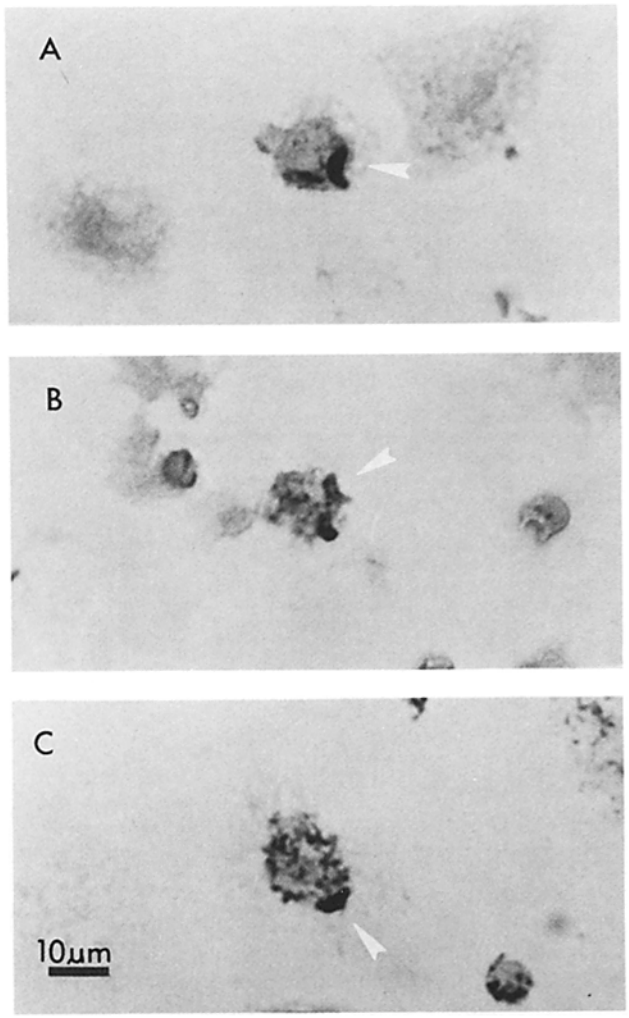

FIGURE 2 Formazan deposition in phagosomes formed in Na^+ (*A*), K^+ (*B*), and choline$^+$ (C) media. Phagocytosis of formaldehyde-denatured, opsonized *T. gondii* by J774.2 macrophages in Na^+ and Na^+-substituted media but containing NBT was carried out as described in the Materials and Methods. After 60 min at 37°C, the cultures were fixed, counterstained with safranin-O, and photographed. The black granular deposit in the crescent-shaped phagosome containing a parasite is formazan, the product of reduction of NBT by O_2^- released into the phagosomal vacuole.

cation procedure. They were then assayed for O_2^- generation in the presence and absence of Na^+. Both steps in the "purification" procedure were found to introduce a Na^+ requirement for this effector function in monocytes (Table II). Cells exposed to 2% dextran in RPMI and chilled for a prolonged period, exaggerated here for the purpose of enhancement, were depressed in their ability to generate O_2^- in the absence of Na^+. The 23% reduction of the activity in the absence of Na^+ in the medium may have been a result of prolonged chilling that is known to alter intracellular Na^+ and K^+ concentrations due to slow ion flux across the membrane at 4°C with no countering Na^+/K^+ ATPase activity. This relatively small reduction of O_2^- generation activity in the absence of extracellular Na^+ is also seen in preparations of alveolar macrophages (21). Cells exposed to the erythrocyte lysis medium (155 mM NH_4Cl, 10 mM HEPES [pH 7.2]) were markedly depressed in O_2^- generation in medium that lacked Na^+. The 83% inhibition imposed by NH_4^+ pre-incubation and the 94% inhibition after exposure of cells to a combination of NH_4^+ pre-incubation and dextran at 4°C indicated that pre-treatment with NH_4^+ produced the sought-after Na^+-reversible inhibition.

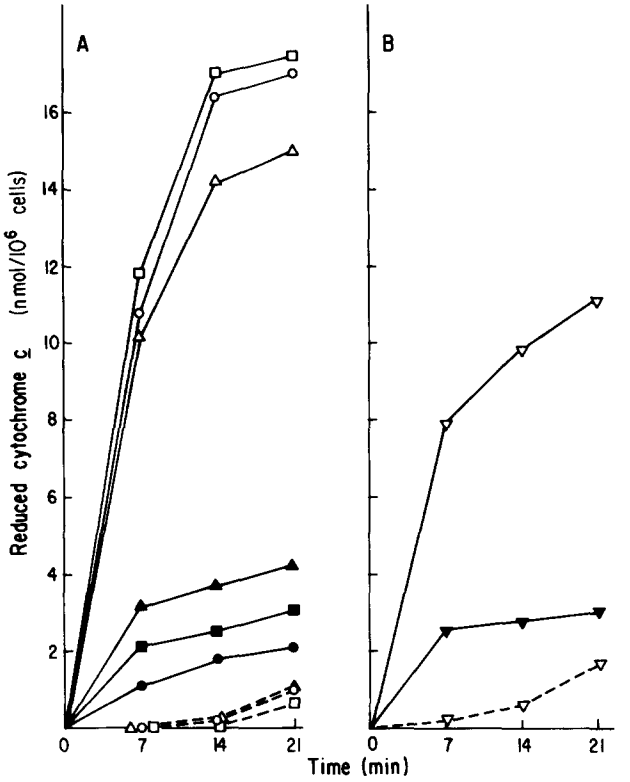

FIGURE 3 O_2^- generation by monocytes in the absence of a Na^+, K^+, or Cl^- transmembrane gradient. Freshly isolated human monocytes were incubated in RPMI medium for 4 h after which they were washed in choline$^+$ medium containing 10 mM Na^+. They were assayed for O_2^- generation in an assay mixture containing Na^+ (○), K^+ (△), choline$^+$ (□), or sucrose (▽) medium and opsonized zymosan. O_2^- generation in the absence of opsonized zymosan (closed symbols) and in the presence of opsonized zymosan and superoxide dismutase (open symbols, dashed line) are also indicated. O_2^- was determined by a spectrophotometric assay of cytochrome c reduction. Control values of cytochrome c reduced in the absence of cells were 0.4 nmol for Na^+ medium and 0.7 nmol for sucrose medium at 14 min. Control values determined for all time points have been subtracted. Levels of O_2^- generation by unstimulated cells were particularly high in this experiment. More typical values were 0.8–1.5 nmol at 15 min.

TABLE II

Effect of 2% Dextran (4°C) and NH_4^+ Pre-treatment on O_2^- Generation by Monocytes

Pre-treatment*	O_2^- generation activities, % of Na^+ control‡
2% dextran (4°C)	77.0
NH_4^+	17.5
2% dextran (4°C), then NH_4^+	5.5

* Monocytes were pre-treated with 2% dextran in RPMI for 2 h at 4°C, or 0.83% NH_4Cl-10 mM HEPES (pH 7.2) for 1 h at 37°C, or both. Cells were washed in 0 mM Na^+ medium before the O_2^- generation assay.

‡ Pre-treated monocytes in 0 or 140 mM Na^+ medium were assayed for O_2^- generation stimulated by opsonized zymosan and incubated for 17 h at 37°C. Results were expressed as (nmol reduced cytochrome c in 0 mM Na^+ medium/nmol reduced cytochrome c in 140 mM Na^+ medium) × 100.

Further examination of the effect of NH_4^+ on monocytes was necessary to establish its role in inducing a requirement for external Na^+ in the production of O_2^-. Korchak and Weissmann (7) and Simchowitz and Spilberg (8) used neutrophils that had been prepared by treatment with NH_4^+ to show that the rate of O_2^- production was a function of the external Na^+ concentration over a broad range. Monocytes, pre-treated with NH_4^+ or left untreated, were stimulated with opsonized zymosan and the generation of O_2^- was measured in media that contained 0, 4, 8, 15, 30, and 140 mM Na^+. The monocytes pre-treated with NH_4^+ (Fig. 4*A*), but not the untreated cells (Fig. 4*B*), were dependent upon the concentration of external Na^+ for the rate of O_2^- generation. When recovery from the NH_4^+-imposed inhibition was allowed to take place at 37°C in RPMI medium, the NH_4^+ pre-treated monocytes lost the dependence upon external Na^+ (Fig. 4*D*) that was expressed by cells not permitted such a recovery (Fig. 4*C*).

The possibility should be considered that the dependence upon external Na^+ imposed by prior treatment of monocytes with NH_4^+ could be the result of altered interaction of the opsonized zymosan with receptors. Although radiolabeled concanavalin A (7) and f-met-leu-phe (8) bind to NH_4^+ pre-treated neutrophils regardless of the external Na^+ concentration, particulate ligands such as opsonized zymosan may require receptor capping for stabilization of the stimulus on cell surfaces in a suspension culture. It is not known whether receptor capping is inhibited in a Na^+-reversible manner in NH_4^+-pre-treated cells. To eliminate the possibility that the NH_4^+ treatment resulted in a mechanical difficulty in the binding of particulate, multivalent ligands, PMA, a potent stimulator of the inflammatory response (32), was used to induce O_2^- production. PMA has been shown to activate cytoplasmic protein kinase C (33). This stimulant probably bypasses any binding and capping requirement for particulate ligand stabilization. PMA was added to NH_4^+-pre-treated monocytes that had been pre-incubated in media of various Na^+ concentrations from 0 to 140 mM (Fig. 5, *A* and *B*). The

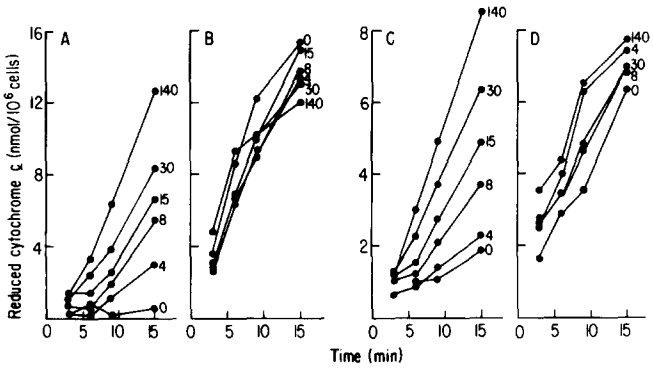

FIGURE 4 Na^+ concentration dependence for O_2^- generation by monocytes pre-treated with NH_4^+. (*A*) O_2^- generation by NH_4^+-pre-treated monocytes stimulated by opsonized zymosan. The numbers labeling each curve indicate the millimolar Na^+ concentration present in that assay medium. (*B*) O_2^- generation by untreated monocytes (from the same lot of cells as *A*). (*C*) O_2^- generation by another population of NH_4^+-pre-treated monocytes. (*D*) O_2^- generating capacity of pre-treated cells from *C* after a "recovery" incubation in RPMI medium at 37°C for 6 h. Background cytochrome c reduction in the absence of cells was subtracted from all the time points and averaged 0.4–0.15 nmol/ml at 15 min. No samples were taken at zero time and therefore no correction was made for the initial absorbance differences.

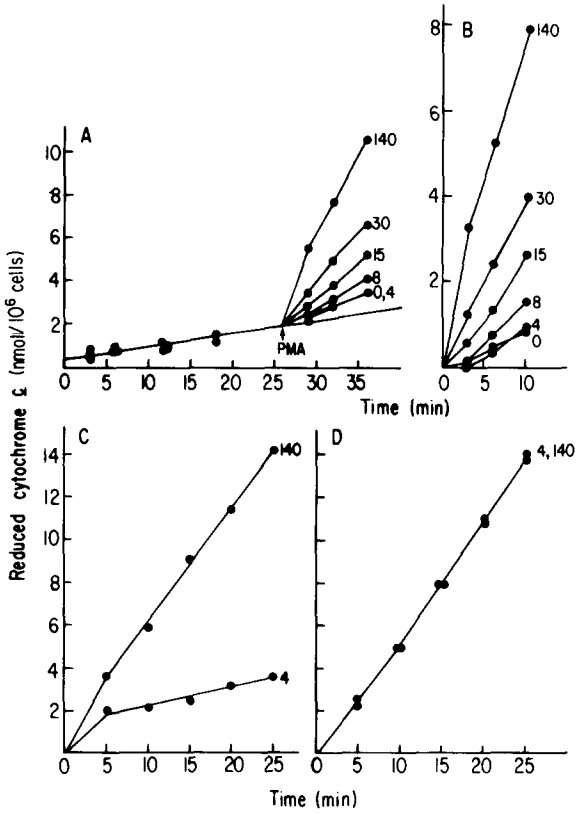

FIGURE 5 Dependence on external Na^+ for O_2^- generation by PMA-stimulated monocytes and macrophages pre-treated with NH_4^+. (*A*) Monocytes, exposed to NH_4^+, were added at zero time to O_2^- generation assay medium that contained 0, 4, 8, 15, 30, or 140 mM Na^+ but lacked stimulant. PMA was added to each assay at 26 min. (*B*) PMA-stimulated O_2^- generation of *A* was replotted after subtracting the background cytochrome c reduction that occurred in the presence of cells but in the absence of stimulant. (*C*) Monocyte-derived macrophages were incubated with 0.1 μg/ml LPS for 24 h at 37°C. They were then pre-treated with NH_4^+, after which one half were allowed to recover in RPMI medium while the other half were assayed for O_2^- generation in 4 and 140 mM Na^+ assay medium containing LPS. PMA was the stimulant. (*D*) The NH_4^+-pre-treated, LPS-activated macrophages assayed under the same conditions as in C after having been allowed to recover in RPMI medium for 60 min. Control curves of O_2^- generation in the presence of cells and LPS but in the absence of PMA were subtracted from *C* and *D*. Values for reduced cytochrome c in these controls at 15 min were 0.35 nmol in *C*, and 0.40 nmol in *D*. Control and experimental time points included zero time samples.

background rates of cytochrome *c* reduction in the absence of the stimulant were essentially the same for all Na^+ concentrations. Although PMA more closely induced linear rates of O_2^- generation than were seen with opsonized zymosan, the overall Na^+ concentration dependence for effector function was maintained. The half maximal rate of O_2^- generation was supported by 30 mM Na^+. The results indicated that the Na^+-reversible NH_4^+ inhibition was probably not simply due to inhibition of the capping required for interaction of particles and multiple receptors.

Another possible explanation for the Na^+-reversible effect of pre-treatment with NH_4^+ is that some activator contaminated the media used to reverse the effect of NH_4^+. This possibility was examined with cells that had been incubated in RPMI medium that had been tested and found to be essentially free (<0.071 ng/ml) of endotoxin. Subsequent

incubation in saturating concentrations of a known activator and maintainance of that concentration throughout the assay should eliminate the influence of contaminating activator and it should eliminate variations of cell activity that may occur if NH_4^+ pre-treatment decreases the activated state of the cells. Monocytes that were incubated for 6 d show the granulocytic morphology of macrophages. Monocyte-derived macrophages obtained in this way were activated for 24 h in 100 ng/ml LPS (34). All of the activated macrophage cultures were then pre-treated with NH_4^+. One half of the cultures were stimulated with PMA after a short incubation in media that contained LPS and 4 or 140 mM Na^+ (Fig. 5*C*). The other half of the cultures were allowed to recover from the NH_4^+ treatment by incubation in RPMI medium that contained LPS. These latter cultures were then stimulated with PMA in media of different Na^+ concentrations as described above (Fig. 5*D*). The activated cells did not generate O_2^- unless stimulated with PMA (see legend of Fig. 5). These cells were as susceptible to NH_4^+ inhibition as were freshly isolated monocytes, and showed an initial Na^+ dependence and recovery to the independent state similar to that seen in Fig. 4, *A–D*. Therefore, inadvertant addition of activator to the media used for Na^+-reversal or for monocyte recovery is unlikely to be the explanation for the difference in O_2^- generating capacity of cells in media with low and high Na^+.

NH_4^+ Pre-treatment Introduces a Na^+ Requirement for Phagocytosis

It was important to determine whether the NH_4^+ pre-treatment of cells inhibited only the O_2^- generation pathway or if it produced a more general condition of non-responsiveness to the binding of a stimulatory ligand. To answer this question, I used in a phagocytosis assay, the same media and the same particulate stimulant, opsonized zymosan, used to assess O_2^- generation by inhibited cells. Instead of measuring the initial rates of O_2^- generation by NH_4^+-pre-treated monocytes, phagocytosis was measured in pre-treated J774.2 macrophages. Cell monolayers on glass coverslips were pre-treated with NH_4Cl-HEPES for 10 min at 22°C, rinsed with 0 mM Na^+ medium, and overlaid with opsonized zymosan in 0 and 140 mM Na^+ medium. Microscopic assessment of the phagocytic capacity of pre-treated cells, after a 20-min incubation at 37°C and a 10-min AO staining, revealed complete inhibition of phagocytosis only in the Na^+-deprived cultures (Fig. 6*A*) while normal phagocytosis was observed in cultures incubated in 140 mM Na^+ medium (Fig. 6*B*). Thus, NH_4^+ pre-treatment of macrophages renders them incompetent for phagocytosis in the absence of external Na^+.

NH_4^+ Pre-treatment Acidifies Macrophage Cytosol—Reversed by Na^+

When J774.2 macrophages were incubated in NH_4Cl-HEPES for 10 min at 37°C and then stained with AO in medium lacking Na^+, the AO was concentrated by the entire cell, rather than just by the lysosomes (Fig. 6*C*), indicating that the cytosolic pH had decreased as a result of NH_4^+ pre-treatment followed by Na^+ deprivation. If Na^+ medium was added, the concentrated AO was released from the cytosol, but not from the lysosomes, of most of the cells (not shown). AO was not concentrated by the cytosol but only by the lysosomes of NH_4^+-pre-treated cells stained in medium that contained Na^+ (Fig. 6*A*). This indicated that the NH_4^+-pre-treated macrophages had become acidified under the conditions used for the phagocytosis assay and that the proton concentration of the cytosol decreased with the addition of Na^+. Further consideration of the relevance of acidified cytosol to the inhibition of phagocytosis in J774.2 macrophages as well as to the inhibition of O_2^- generation in monocytes and monocyte-derived macrophages will be found in the Discussion.

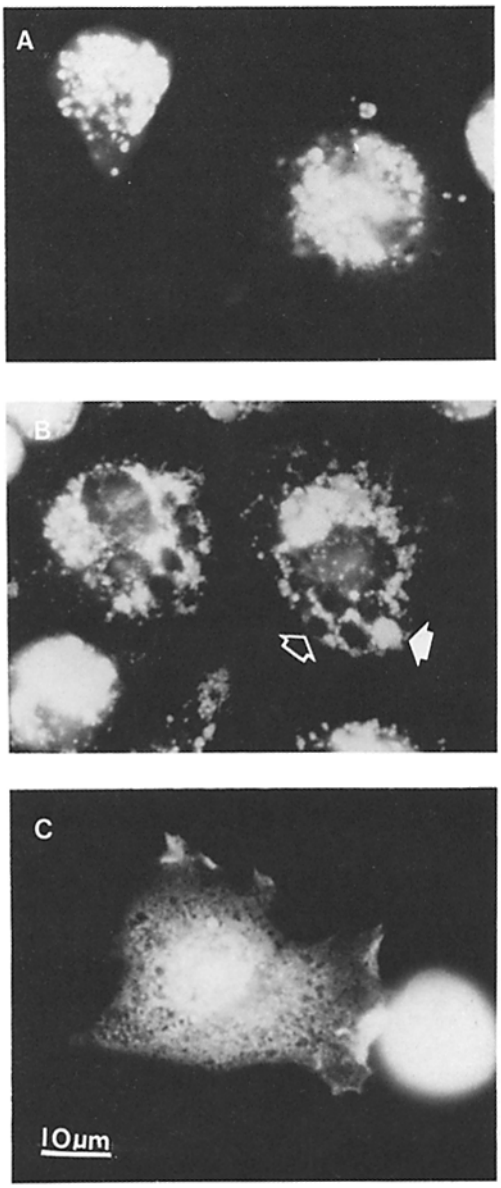

FIGURE 6 AO-stained lysosomes (*A*), lysosomes and acidified phagosomes (*B*), and acidified cytoplasm (C) of NH_4^+-pre-treated J774.2 macrophages. NH_4^+-pre-treated cells were incubated for 20 min with opsonized zymosan in 0 (*A*) or 140 mM (*B*) Na^+ medium. Washed cells were stained with AO in Na^+ medium. Some cells were incubated and stained in 0 mM Na^+ medium (C). The fluorescent dye is concentrated by the cytosol as well as the lysosomes of *C*. Open arrow points to zymosan in a phagosome; closed arrow indicates zymosan in a stained (acidified) phagosome.

DISCUSSION

The Nature of the Transmembrane Signal

There are two general models for information transfer, called signal transduction, across membranes. In one model,

some substance crosses the membrane as a result of ligand-receptor interaction in a manner similar to the Na^+ influx through the bound acetylcholine receptor or Ca^{2+} channel gating in the stimulated mast cell membrane. In the other model, a conformational change in a bound transmembranous receptor activates an effector on the cytoplasmic side of the membrane. An example of this model is the agonist activation of the beta-adrenergic receptor which then binds and activates the cytoplasmically disposed adenyl cyclase.

As noted in the Introduction, binding of stimulatory ligands such as antigen-antibody complexes, concanavalin A, f-met-leu-phe, or PMA (1–12, 17–21) to different membrane receptors of mammalian phagocytes brings about changes in permeability, ion fluxes, and transmembrane potential. One of these receptors, the FcR that binds antigen-antibody complexes formed with IgG of the $\gamma 2b/\gamma 1$ subclasses, acts as a channel for Na^+ and K^+ in a ligand-dependent manner (17–20). As a result of these observations, there is a frequently encountered belief that phagocyte effector function is a result of transmembranous signaling through ion fluxes (7–9, 11, 17–20, 35, 36).

The data in the present report show that the channel activity of the FcR cannot account for the signal transduction mechanism for three effector functions: phagocytosis, phagosomal acidification, and O_2^- generation. Phagocytosis through binding of Fc- and non-specific receptors or through binding of the ionophoretic $FcR_{\gamma 2b/\gamma 1}$ of J774.2 macrophages did not require Na^+/K^+ fluxes. Acidification of the phagosome was equally Na^+-insensitive, as was O_2^- generation. Whether acidification of the vacuoles took place through the action of a phagosomal H^+-ATPase or through lysosomal fusion was not determined, although fusion with vacuoles containing killed parasites has been seen to occur, within the interval allowed for phagocytosis here, in peritoneal macrophages (37). It may be that lysosomal fusion with the phagosomal vacuole and secretion to the outside of the cell are also not dependent on transmembrane ion fluxes.

The initial rate of O_2^- generation, stimulated by opsonized zymosan binding to Fc- and non-specific receptors, was unaffected by varying or substituting external Na^+. This showed that enhancement of response is also not achieved by permitting Na^+ influx at the time of agonist-dependent membrane permeability changes. Therefore, Na^+/K^+ fluxes are neither sufficient for transmembrane signaling nor necessary for the maximal rate of response.

Other ions in the assay media known to exist in a gradient across the membrane include Cl^-, Ca^{2+}, and H^+. The data of Fig. 3*B* shows that external Cl^- was not essential for O_2^- generation. Wright and Silverstein (38) and Smolen et al. (14) have demonstrated that extracellular Ca^{2+} is not necessary for phagocytosis and inflammatory responses, respectively, although the level of the responses may be reduced in its absence. Intracellular pH change (39) requires normal Na^+ concentrations in the medium for H^+ exchange through the homeostatic Na^+/H^+ exchanger (40–43), yet signal transduction proceeds in the absence of external Na^+. Thus, if a gradient-driven ion flux is the signal for phagocyte functions, the ion involved cannot be Na^+, K^+, Ca^{2+}, Cl^-, and probably is not H^+.

Signaling of cytoplasmic function after stimulation of one specific type of receptor can affect several pathways that are not interdependent. For example, the bound FcR is capable of signaling phagocytosis, O_2^- generation, and lysosomal secretion (17). Yet O_2^- generation and lysosomal secretion can proceed even when phagocytosis is inhibited with cytochalasin B (2), and O_2^- generation proceeds independently of lysosomal secretion in stimulated neutrophil cytoplasts (44, 45). Perhaps there are multiple signaling mechanisms associated with the individual receptors, one of which might be Na^+ influx-signaling of a function not tested in this report. The likelihood of multiple transmembrane signaling mechanisms diminishes with each Na^+-insensitive effector function studied. Nevertheless, ion influx, triggered or channeled by receptor binding, could promote a cytoplasmic function (possibly homeostatic) other than the pleiotypic functions described above. At least for the stimulation of phagocytosis, phagosomal acidification, and O_2^- generation, the FcR must be signaling by undergoing an agonist-dependent conformation change. This change could confer stimulatory activity on its cytoplasmic domain or it could govern a lateral interaction in the membrane with other membrane components involved in a stimulatory pathway.

Protein kinase C is thought to mediate inflammatory responses of neutrophils (46–50). Diacylglycerol, the natural endogenous ligand of the kinase and a hydrolysis product of the polyphosphoinositides, stimulates O_2^- generation (48) and, in conjunction with Ca^{2+}, lysosomal enzyme release (47) in neutrophils. Agonist-dependent breakdown of polyphosphoinositides, precursors of the kinase activator, has been confirmed in neutrophils by Volpi et al. (51). In fact, there is evidence to suggest that protein kinase C mediates cell response to stimulation by Ca^{2+}-mobilizing-agonists in many different cell types (46). PMA, a potent stimulant of the inflammatory response, is thought to bypass the initial steps of the agonist-dependent phosphoinositide pathway, by mimicking the effect of one of its products, diacylglycerol, through direct activation of the kinase (33, 46, 49, 50). However, PMA also induces permability changes in the plasma membrane of neutrophils (9, 11, 45, 52). An interesting finding in the present report (Fig. 5*D*) was that Na^+ influx was not required for and did not enhance PMA stimulation of O_2^- generation. This eliminates the possibility that Na^+ influx or Na^+-induced, voltage-dependent changes in the membrane, in themselves, could be inducers of cell response by acting concurrently with the presumed protein kinase C activation.

Na^+ Reversible Inhibition Imposed by an "NH_4^+-Prepulse"

The inconsistancy between the results of this report and those of previous reports in which neutrophils showed a dependence on Na^+ for lysosomal secretion and O_2^- generation (7, 8) prompted a search for the basis of the differences between neutrophil and monocyte responses. An important finding was that the neutrophil purification procedure, used in the earlier investigations, inadvertantly introduced an artifactual requirement for Na^+. The agent that introduced a Na^+ dependence in neutrophils was identified as NH_4^+. Brief exposure to NH_4^+, the main component of erythrocyte lysis medium in the Boyum isolation method (22), inhibited O_2^- generation in human monocytes and phagocytosis in J774.2 macrophages. In each case, the inhibition was dependent upon Na^+ for reversal. The kinetics of dependence on the Na^+ concentration for the rate of O_2^- generation by neutrophils (7, 8) were reproduced using monocytes, but only if the monocytes were first exposed to NH_4Cl before stimulation.

Beyond the artifactual Na^+ dependence by neutrophils isolated by the Boyum method, there are no other accounts of a Na^+ requirement for stimulus-response in phagocytes.

There is a plausible explanation for the inhibition imposed by NH_4^+ on phagocytes as well as for the observation that the inhibition was reversed by Na^+. NH_4^+ pre-treatment of cells is known to induce rapid acidification of cytoplasm (40, 43, 53, 54). Cytoplasmic acidification using an "NH_4^+-prepulse," the term adopted (53) to emphasize the brevity of the NH_4^+ pre-treatment and to distinguish it from the prolonged use of NH_4Cl as a lysosomotropic agent (55, 56), occurs in human fibroblasts (40), Chinese hamster lung cells (43), squid giant axon, mouse soleus muscle, and several other cell types (53, 54). Cytoplasmic acidification by transient exposure to NH_4^+ would be expected to occur in phagocytes since the mechanism of acidification seems to be diffusion of NH_3 across the plasma membrane. The intracellular NH_3 is protonated to form NH_4^+, and thus alkalinizes the cytoplasm. Influx of H^+ through the H^+/Na^+ antiport or in the form of small amounts of NH_4^+ brings the cytosolic pH back to neutrality. When the NH_4Cl in the medium is removed, NH_4^+ leaves mainly as NH_3, thereby loading the cells with an excess of protons. (For fuller details see references 55 and 56.) External Na^+ is required for reversal of the acidification in other cell types by promoting H^+ extrusion through the Na^+/H^+ antiport (40–43). In the present report, pre-treated J774.2 macrophages that were washed in a Na^+-free medium did indeed become acid, and medium containing normal levels of Na^+ was observed to reverse the acidification.

There is no direct evidence that cytoplasmic acidification is the mechanism by which an NH_4^+-prepulse inhibits phagocytosis and O_2^- generation. However, several correlations, summarized below, suggest that this is the case. (*a*) The NH_4^+-introduced inhibition of phagocyte effector functions was reversed by Na^+. (*b*) The Na^+ concentration (30–40 mM) that supports the half maximal rate of O_2^- generation in NH_4^+-prepulsed human neutrophils (7) and monocytes (present report) was the same as that for the half maximal rate of pH rectification in acidified human fibroblasts (40) and A431 cells (41). (*c*) AO, usually concentrated only by acidic organelles, concentrated in the cytosol of NH_4^+–pre-treated J774.2 macrophages. This indicated that macrophages can become acidified under the conditions used for the phagocytosis assay. The AO-indicated acidity disappeared with the addition of Na^+. (*d*) Two functionally independent pathways, phagocytosis and O_2^- generation, were both inhibited by NH_4^+ in a Na^+-reversible manner suggesting a general, pathway-independent, inhibitory condition. (*e*) The Na^+-reversible inhibition was independent of the type of stimulant used: opsonized zymosan binding to Fc- and non-specific surface receptors or PMA binding presumably to cytoplasmic protein kinase C (33, 46, 49, 50). Again, a general condition of non-responsiveness is suggested.

In summary, the present data indicate that monocytes, macrophages, and (it may be inferred) neutrophils require external Na^+ to reverse an NH_4^+-prepulse–introduced inhibition but not to signal or enhance phagocytosis, phagosomal acidification, or O_2^- generation. It is proposed that transmembrane signal transduction resulting from agonist interaction with an external domain of the FcR is due to an ion-flux–independent stimulus of cytoplasmic response presumably through a conformational change in the receptor.

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