Transmembrane-Mediated Changes in $[Ca^{2+}]$ are Involved in the Signaling Pathway Leading to Macrophage Cytocidal Differentiation: Implications of Localized Changes in Intracellular $[Ca^{2+}]$ and of Interferon Priming on Ca^{2+} Utilization

Gregory A. Underwood* and David W.H. Riches†‡

Division of Basic Sciences, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80262, and *†Division of Pulmonary Sciences, Department of Medicine and †Department of Biochemistry, Biophysics, and Genetics, University of Colorado Health Sciences Center, Denver, Colorado 80262

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Macrophage cytocidal activation requires the sequential impingement on the macrophage of a priming stimulus (interferon $[IFN]\alpha$, β , or γ) and a triggering stimulus (such as polyinosinic acid:polycytidylic acid [poly $[I:C]$] or bacterial lipopolysaccharide). The mechanism of progression from the IFN-primed state to the cytocidal state is poorly understood. By quantifying the level of expression of a gene product (complement component factor B [Bf]) associated with cytocidal activation and through the use of phenotypically distinct populations of macrophages (unprimed and IFN-primed), we have investigated the functional necessity of changes in intracellular concentration of free calcium ions ($[Ca^{2+}]_i$) in signaling the transition from the primed to the cytocidal state. Elevating the $[Ca^{2+}]_i$ by incubation of unprimed macrophages with the calcium ionophore, ionomycin, failed to induce the expression of Bf. By contrast, Bf was expressed at high levels when IFN-primed macrophages were exposed to ionomycin, suggesting that priming induced within the macrophages the capacity to respond to a nonspecific change in $[Ca^{2+}]_i$. Quantification of the $[Ca^{2+}]_i$ in response to exposure to ionomycin revealed an initial transient elevation, followed by a secondary sustained component. No differences in these changes were observed between unprimed and IFN-primed macrophages. We therefore questioned if changes in $[Ca^{2+}]_i$ were also implicated in the transition between the primed and the cytocidal state using the ligand, poly $[I:C]$. In contrast to ionomycin, incubation of IFN-primed macrophages with poly $[I:C]$ did not sustain measurable increases in $[Ca^{2+}]_i$, yet fully stimulated the transition from the IFN-primed to the cytocidal state. However, incubation of IFN-primed macrophages with poly $[I:C]$ in the presence of 1) a Ca^{2+} /ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid buffer calculated to clamp the extracellular concentration of free calcium ions to a value approximately equal to the resting $[Ca^{2+}]_i$; 2) the calcium channel blocker verapamil; or 3) the intracellular Ca^{2+} antagonists (W-7, W-13, and TMB-8) substantially inhibited the induction of Bf. Collectively, these data support the following conclusions. First, that changes in $[Ca^{2+}]_i$ comprise an important element in the induction of progression from the IFN-primed to the cytocidal state. Second, the failure to detect global changes in $[Ca^{2+}]_i$ in response to the ligand, poly $[I:C]$, suggests that changes in $[Ca^{2+}]_i$ or Ca^{2+} movement may occur in either a spatially restricted or in an asynchronous cyclical fashion and are not detected by population fluorescence measurements. Third,

‡ Corresponding author.

the source of the relevant Ca^{2+} is extracellular. Fourth, our findings suggest that priming influences macrophage functional responses at a locus that is distal to the changes in $[\text{Ca}^{2+}]_i$, thereby potentially allowing signaling processes to be utilized to initiate different cellular responses.

INTRODUCTION

Macrophage cytotoxic activation is a two-step stimulus b2761/22-driven pathway of differentiation that confers on macrophages the capacity to destroy virally and neoplastically transformed cells (Evans and Alexander, 1970; Alexander and Evans, 1971; Hibbs *et al.*, 1972; Russell *et al.*, 1977; Ruco and Meltzer, 1978; Adams and Hamilton, 1984). The first step of this process, termed priming, is induced by exposure of uncommitted macrophages to a priming stimulus, usually interferon (IFN) α , β , or γ (Ruco and Meltzer, 1978; Pace and Russell, 1981; Pace *et al.*, 1985). At this stage, macrophages show an enhanced capacity to bind transformed target cells but are incapable of destroying them (Somers *et al.*, 1983). Full competence to lyse target cells, i.e., the expression of the nonspecific cytotoxic state, is achieved after IFN-primed macrophages have been exposed to trace amounts of a second triggering stimulus, such as bacterial lipopolysaccharide (LPS) (Ruco and Meltzer, 1978; Pace *et al.*, 1981), whole heat-killed *Listeria monocytogenes* or gram positive organisms (Pace *et al.*, 1985), lipid-associated proteins (Hogan and Vogel, 1987), polyribonucleotides (Taramelli and Varesio, 1981; Torres and Johnson, 1985), or a variety of other agents (Leu and Herriott, 1987).

During the transition from the primed to the cytotoxic state, the macrophage initiates the expression of many genes, such as tumor necrosis factor α (Koerner *et al.*, 1987), and metabolites, such as reactive nitrogen intermediates (Stuehr and Marletta, 1985; Ding *et al.*, 1988), that are normally repressed in uncommitted macrophages. Simultaneously, the synthesis of other products that have been suggested to be involved in the scavenging activities of the uncommitted macrophage, but that may not be required for the activities of the cytotoxically activated macrophage, are down-regulated (Imber *et al.*, 1982; Riches and Henson, 1986a,b). In work

previously reported from this laboratory, we have shown that the exposure of IFN-primed macrophages to the triggering stimuli, polyinosinic acid:polycytidylic acid (poly [I:C]) and LPS, induces the expression of the complement component factor B (Bf) (Riches *et al.*, 1988a), and we have applied this observation to begin to explore the early cellular and biochemical events that are responsible for initiating the progression from the IFN-primed state to the cytotoxic state. It is this latter aspect that forms the foundation of the work reported herein.

Changes in the intracellular concentration of free calcium ions ($[\text{Ca}^{2+}]_i$) have been implicated in the signal transduction mechanisms of many cellular responses in numerous cell types. The possible role of changes in $[\text{Ca}^{2+}]_i$ in promoting macrophage cytotoxic activation has been the subject of several studies, although evidence supporting its involvement has been elusive and inconsistent. For example, Drysdale *et al.* (1987) were unable to find any evidence of either changes in $[\text{Ca}^{2+}]_i$ or increases in $^{45}\text{Ca}^{2+}$ influx in mouse peritoneal macrophages stimulated with a high concentration of LPS. In another investigation, however, Prpic *et al.* (1987) were able to detect a small elevation in $[\text{Ca}^{2+}]_i$ in mouse macrophages stimulated with LPS. Similarly, a slow rise in $[\text{Ca}^{2+}]_i$ has been observed when a mouse macrophage cell line (RAW 264.7) was coincubated with both priming and triggering stimuli (Gorecka-Tisera *et al.*, 1986). There are at least two potential explanations for these discrepant conclusions. First, different studies have employed markedly different stimulation conditions that can result in the elicitation of multiple responses. For example, the use of high (μg) concentrations of triggering stimuli, which act by initially inducing IFN β expression leading to the autocrine priming of macrophages, and subsequent triggering (Torres and Johnson, 1985; Riches *et al.*, 1988a; Riches and Underwood, 1991) do not allow one to discriminate between those signaling mechanisms that are responsible for the induction of IFN β expression, the induction of the IFN-primed state, or the triggering of progression from the IFN-primed to the cytotoxic state. Moreover, under these conditions, the signaling event associated with the transition between the primed and the cytotoxically activated state would not be expected to be initiated until several hours after the application of the stimulus. Second, the possibility exists that more localized changes in $[\text{Ca}^{2+}]_i$, which hence are not detected by population

¹ Abbreviations used: Bf, complement component factor B; $[\text{Ca}^{2+}]_i$, intracellular concentration of free calcium ions; $[\text{Ca}^{2+}]_o$, extracellular concentration of free calcium ions; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; FCS, fetal calf serum; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HBSS, Hank's balanced salt solution; IFN, interferon; LPS, bacterial lipopolysaccharide; PBS, phosphate-buffered saline; poly [I:C], polyinosinic acid:polycytidylic acid; SDS, sodium dodecyl sulfate; SSS, standard saline solution; TCA, trichloroacetic acid; TMB-8, 8-(diethylamino) octyl 3,4,5-trimethoxybenzoate; W-13, *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide.

fluorescence measurements, do not appear to have been considered.

In the present study, we have employed defined priming and triggering stimuli (IFN β and poly [I:C], respectively) and well-characterized macrophage phenotypic states to explore the functional necessity of changes in [Ca²⁺]_i in the progression of IFN-primed macrophages to the cytotoxic state. Our results suggest that transient global elevations in [Ca²⁺]_i are not involved in the induction of the cytotoxic state. However, localized or cyclical changes in [Ca²⁺]_i that are critically dependent on extracellular Ca²⁺ appear to comprise an essential element in the mechanism of signal transduction in these cells. Our results also localize the level of priming to a locus that is distal to the initial Ca²⁺-dependent signaling event.

MATERIALS AND METHODS

Animals

Eight week old C3H/HeJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained in a virus-free barrier room in the animal care facility at National Jewish for 2 wk before use. Euthanasia was by asphyxiation with carbon dioxide.

Reagents

Dulbecco's modified Eagle's medium (DMEM) was obtained from Whittaker Bioproducts Inc. (Walkersville, MD). Fetal calf serum (FCS) was purchased from Irvine Scientific (Santa Ana, CA). Twelve-well (22 mm diameter) culture plates were obtained from Costar (Cambridge, MA). Bacteriologic grade petri dishes (100 mm diameter) were obtained from VWR Inc. (Denver, CO). Poly [I:C], ATP, ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 8-(diethylamino) octyl 3,4,5-trimethoxybenzoate (TMB-8), N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (W-13), nifedipine, verapamil, and Fura 2-AM were purchased from Sigma Chemical (St. Louis, MO). Ionomycin, A23187, and bis-(*o*-aminophenoxy)-N,N,N',N'-tetraacetic acid-AM were purchased from Calbiochem (San Diego, CA). Mouse IFN β of specific activity 1.3 $\times$ 10⁸ interferon reference unit/mg was purchased from Lee Biomolecular Research, Inc. (San Diego, CA). Goat anti-human factor B IgG was obtained from Atlantic Antibodies (Scarborough, ME). L-[³⁵S]methionine (Trans³⁵S-label, >1000 Ci/mmol) was purchased from ICN Biomedicals (Irvine, CA). Scintiverse II was obtained from Fisher Scientific, Fair Lawn, NJ. All other reagents were of the highest possible purity.

Isolation and Culture of Mouse Macrophages

Mouse bone marrow-derived macrophages were obtained using a modification of the technique described by Stewart and previously described in detail (Stewart, 1981; Riches *et al.*, 1988a). The LPS-hyporesponsive strain of mouse, C3H/HeJ, was used throughout this study to minimize the possibility of stimulation of the cells by trace contamination of reagents with endotoxin (Pace *et al.*, 1985). The medium used for the isolation, culture, and stimulation of bone marrow macrophages was DMEM containing 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.37% (wt/vol) NaHCO₃, 10% (vol/vol) heat-inactivated FCS, and 10% (vol/vol) L929 cell-conditioned medium as a source of colony-stimulating factor-1. Bone marrow cells were flushed aseptically from the dissected femurs and tibias of C3H/HeJ mice with a jet of complete medium directed through a 25-gauge needle. The cells were adjusted to a density of $\sim 3 \times 10^5$ cells/ml of complete medium and were distributed in 2-

ml aliquots into 22-mm-diameter wells of 12-well tissue culture plates. Bone marrow-derived macrophages were also grown on 100-mm-diameter bacteriological grade petri dishes under loosely adherent conditions. The cells were maintained under a 10% (vol/vol) CO₂ atmosphere for 5 d. Thereafter, the complete medium was changed every other day until the cells were used. The cells generally were used on day 6 or 7. These culture conditions resulted in the formation of confluent monolayers of $\sim 1 \times 10^6$ macrophages per 22-mm-diameter well. Macrophages were primed by exposure to 100 U/ml of IFN β for 2–6 h and were then exposed to stimuli in complete medium, unless otherwise stated.

Analysis of Bf Expression

Macrophage monolayers were rinsed with Hank's balanced salt solution (HBSS) and biosynthetically pulse-labeled by incubation for 1 h at 37°C with 100 μ Ci/well of L-[³⁵S]methionine dissolved in 1 ml of Eagle's minimum essential medium lacking methionine. Under these conditions, the incorporation of isotope into Bf and total protein was found to be linear with respect to time for up to ≥ 3 h. At the end of the labeling period, the culture supernatants were removed and the cells were lysed in 1 ml of a detergent/inhibitor solution composed of 0.5% (vol/vol) Triton X-100, 0.25% (wt/vol) sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride, 100 μ g/ml leupeptin, and 10 mM EDTA dissolved in 10 mM phosphate-buffered saline (PBS) (150 mM), pH 7.6. The incorporation of [³⁵S]methionine into total protein was determined by precipitation with trichloroacetic acid (TCA) using the method described by Roberts and Paterson (1973) but in which ether was substituted by acetone.

The incorporation of [³⁵S]methionine into Bf was quantified by immunoprecipitation of cell lysates with 5 μ l of anti-human Bf (properdin factor B) IgG followed by adsorption to formalin-fixed *Staphylococcus aureus*. The immunoprecipitates were washed five times in PBS containing 1% (wt/vol) sodium dodecyl sulfate (SDS), 1% (vol/vol) Triton X-100, and 0.5% (wt/vol) sodium deoxycholate and were dissociated by boiling in Laemmli (Laemmli, 1970) sample buffer for 2 min before separating the labeled proteins by SDS-polyacrylamide gel electrophoresis through 7.5% polyacrylamide gels under reducing conditions. The radioactive bands were localized by fluorography, and the amount of [³⁵S]methionine incorporated into Bf was quantified by liquid scintillation counting of hydrolysates (15% [vol/vol] H₂O₂ for 18 h) of excised gel segments. The method of analysis has been previously described in detail (Cole *et al.*, 1985; Riches *et al.*, 1988a). The disintegrations per minute incorporated into Bf was normalized to the disintegrations per minute incorporated into total TCA precipitable protein and is expressed as the disintegrations per minute incorporated into Bf per 10⁷ dpm of total TCA precipitable protein. Each experiment was conducted in triplicate a minimum of three times.

Determination of [Ca²⁺]_i

Macrophage monolayers in 100-mm bacteriological grade petri dishes were loaded with Fura 2-AM using techniques similar to those described by Bonventre *et al.* (1986). The culture medium was removed, and the cells were placed in a standard saline solution (SSS) composed of 125 mM NaCl, 5 mM KCl, 1 mM KH₂PO₄, 5 mM dextrose, 1 mM NaHCO₃, 1 mM CaCl₂, 1 mM MgCl₂, and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.45). Fura 2-AM was added to a concentration of 4 μ M, and the cells were incubated for 10 min at 37°C in the dark. Complete medium was added to dilute the SSS/Fura 2-AM fourfold, and the cells were incubated an additional 30 min in the dark at 37°C. The macrophage monolayer was washed twice with HBSS, and the cells were suspended in complete medium via jetting through a 19-gauge needle. The cells were centrifuged at 1000 $\times$ g for 10 min and resuspended in SSS containing 10% heat-inactivated FCS and were kept on ice and in the dark until ready for use. All cells were used within 60 min of dye loading. Aliquots ($\sim 2 \times 10^6$ cells) were centrifuged as described above and re-

suspended in 2 ml prewarmed (37°C) SSS. Fluorescence measurements were made on a Hitachi F-4010 spectrofluorimeter operating at an excitation wavelength of 340 nm and an emission wavelength of 500 nm using a quartz cuvette held in a heated cell holder at 37°C with continuous stirring. Calibration of the fluorescence signal (F_1) was achieved by first adding 5 mM EGTA and 20 mM Trizma base (pH > 8.5) to quench any extracellular dye (F_2) and to allow its contribution to the fluorescence signal to be assessed by the immediate drop in fluorescence ($F_1 - F_2$). The minimum fluorescence ($F_{\min}$) was obtained by permeabilizing the cells with 50 μ M digitonin to quench the intracellular dye signal. Maximum fluorescence ($F_{\max}$) was obtained by the addition of 10 mM CaCl_2 . Values for intracellular calcium concentration ($[\text{Ca}^{2+}]_i$), corrected for leaked dye, were calculated from the following equation as described (Tsien and Pozzan, 1989)

$$[\text{Ca}^{2+}]_i = K_D(F_2 - F_{\min}) / (F_{\max} - F_1)$$

K_D is assumed to equal 224 nM (Grynkiewicz *et al.*, 1985).

[^{32}P] End-Labeling of Poly [I:C]

Poly [I:C] was end-labeled with polynucleotide kinase and [^{32}P]- γ -ATP. Fifty micrograms of poly [I:C] were digested with 5 U of calf intestinal alkaline phosphatase at 37°C for 60 min. The reaction mixture was then twice extracted with phenol:chloroform (1:1), precipitated with 70% (vol/vol) EtOH, and resuspended in a volume of 50 μ l of 0.9% (wt/vol) RNase free NaCl. The efficiency of removal of the alkaline phosphatase was monitored by colorimetric assay with *p*-nitrophenyl phosphate (5 mM) at pH 10.0. One microgram of dephosphorylated poly [I:C] was end-labeled with 50 μ Ci of [^{32}P]- γ -ATP in the presence of 10 U of polynucleotide kinase for 30 min at 37°C. The reaction mixture was extracted with phenol:chloroform (1:1) and subjected to three rounds of precipitation with EtOH in the presence of 1 M ammonium acetate to remove unincorporated ATP. The purity of the labeled poly [I:C] was verified by electrophoresis under denaturing conditions through a 1% (wt/vol) agarose-formaldehyde gel. The specific activity of the labeled poly [I:C] ranged between 0.60 and 1.92×10^7 dpm/ μ g.

Binding of [^{32}P]Poly [I:C] to Macrophage Monolayers

The interaction of [^{32}P]poly [I:C] with monolayers of mouse bone marrow-derived macrophages was quantified at 0°C to prevent internalization of the ligand using a variation of a previously reported method (De Whalley and Riches, 1991). Monolayers of mouse macrophages in 24-well tissue culture plates were placed on ice in a cold room (4°C) and were rinsed with 1 ml of ice-cold DMEM buffered with 20 mM HEPES (pH 7.3) and containing 0.1% (wt/vol) bovine serum albumin (referred to as "binding medium"). [^{32}P]poly [I:C] was dissolved in ice-cold binding medium to a final concentration of 20 ng/ml. Binding was found to be saturable and was displaced by unlabeled poly [I:C] (Riches, unpublished observations). The cation dependency of binding was determined by incubating macrophage monolayers with 1 ml of binding medium containing 20 ng of [^{32}P]poly [I:C] in the absence or presence of 5 mM EGTA or 5 mM EDTA to chelate Ca^{2+} or Ca^{2+} and Mg^{2+} , respectively. After incubation on ice for 2 h, the binding medium was removed and the wells were rinsed with four consecutive aliquots of ice-cold binding medium alone. Finally, the monolayers were lysed with 0.5 ml of 0.1% (wt/vol) Triton X-100 in 0.9% (wt/vol) NaCl and transferred to scintillation vials. The wells were rinsed with a further 0.5-ml portion of 0.1% Triton X-100, and the washings were combined with the original lysates. The amount of radioactivity was determined by liquid scintillation spectrometry after the addition of 10 ml of Scintiverse II. Binding of [^{32}P]poly [I:C] to wells in the absence of cells was found to be negligible. The results are expressed as nanograms of poly [I:C] bound per milligram of cell protein in 2 h.

Figure 1. Effect of priming macrophages with IFN β on their capacity to express Bf in response to ionomycin. (A) Unprimed (○) or IFN β -primed (●) macrophages were exposed to increasing concentrations of ionomycin (1–10 μM) for 24 h and analyzed for Bf expression. (B) Monolayers of IFN β -primed macrophages were exposed to ionomycin (1–10 μM) for 24 h in the absence (●) or presence (○) of EGTA (5 mM). (C) Influence of ionomycin on the incorporation of [^{35}S]methionine into TCA-precipitable protein in unprimed (○) and IFN β -primed macrophages (●).

Statistics

The data are presented as means and standard errors of three experiments for each condition or as representative data derived from at least three experiments. Significance was calculated using paired t-

tests. For intracellular calcium concentrations, the means and standard errors for each group were calculated. Tukey's multiple comparison procedure was used to test pairwise differences in the means (Sokal and Rohlf, 1969).

RESULTS

Effect of Calcium Ionophores on Bf Expression

To determine whether increases in $[Ca^{2+}]_i$ could induce Bf production, unprimed and IFN β -primed (IFN β , 100 U/ml, 6 h) macrophages were exposed to the calcium ionophore, ionomycin (0.1–10 μ M), for 24 h before determining the level of Bf production. As illustrated in Figure 1A, incubation of IFN β -primed macrophages with ionomycin triggered a marked increase in the expression of Bf that peaked after challenge with 5 μ M ionomycin and declined at 10 μ M. By contrast, exposure of unprimed macrophages to ionomycin failed to induce the expression of Bf. Similar results were obtained using an alternative calcium ionophore, A23187, indicating that the response was not unique to ionomycin (Underwood and Riches, unpublished observations). The calcium dependence of the response to ionomycin is indicated by the finding that exposure of IFN β -primed macrophages to ionomycin in the presence of EGTA (5 mM) resulted in a complete inhibition of the induction of Bf by the ionophore (Figure 1B). Furthermore, the ability of IFN-primed macrophages to express Bf in response to ionomycin was not limited to IFN β as the priming agent but also occurred when the cells were primed with IFN γ and IFN α (Underwood and Riches, unpublished observations).

We determined whether this change in the synthesis of Bf was due to a more generalized effect of ionomycin on total cellular protein synthesis by quantifying the incorporation of [³⁵S]methionine into total TCA-precipitable protein in the presence and absence of the ionophore. Monolayers of unprimed and IFN β -primed (100 U/ml, 6 h) macrophages were stimulated with ionomycin (0.10–10.0 μ M) for 24 h before determining the level of cellular protein synthesis. As shown in Figure 1C, protein synthesis was consistently inhibited by ~25% in cells primed with 100 U/ml IFN β in confirmation of previous studies (Riches *et al.*, 1988a). However, this level of inhibition remained unchanged in the presence of increasing concentrations of ionomycin. Thus, these data indicate that priming with IFN β allows macrophages to express Bf in response to ionomycin by a mechanism that is independent of the overall level of cellular protein synthesis. Importantly, the measurement of the incorporation of [³⁵S]methionine into TCA-precipitable protein indicates that ionomycin is not exerting any toxic effect on the cells over the 24-h experimental period. Moreover, chelation of extracellular Ca²⁺ with EGTA neither inhibited incorporation of [³⁵S]methionine into TCA-precipitable protein nor influenced cell num-

Figure 2. Time course of ionomycin and poly [I:C]-induced factor B synthesis. Macrophage monolayers were primed with IFN β for 4 h before stimulation with ionomycin 5 μ M (●), or unprimed cells were stimulated with 1 μ g/ml poly [I:C] (○). Cells were stimulated for the time intervals indicated and were labeled with [³⁵S]methionine as described in MATERIALS AND METHODS during the final hour of the exposure period. This figure is representative of three experiments.

ber or cell morphology during the same time period, thus indicating that the macrophage is not sensitive to these experimental approaches. The requirement for micromolar concentrations of ionomycin is most likely explained on the basis of partitioning of the ionophore between serum proteins present in the culture medium and the macrophages.

The time course of induction of Bf synthesis by ionomycin was investigated by incubating monolayers of IFN β -primed (100 U/ml, 4 h) macrophages with ionomycin (5 μ M) for various time intervals up to 72 h. The level of Bf synthesis was determined during the last hour of the exposure period. As can be seen in Figure 2, synthesis of Bf was detected as early as 12 h after the addition of ionomycin and continued to rise in a linear fashion to the maximal level of expression at 18 h poststimulation. Thereafter, synthesis of Bf declined before reaching prestimulation levels ~48 h after application of the ionomycin. It will also be seen from Figure 2 that the expression of Bf in response to ionomycin is of more rapid onset and of shorter duration but of similar amplitude to that seen when macrophages are stimulated with the polyribonucleotide, poly [I:C] (1 μ g/ml).

Effect of Ionomycin on $[Ca^{2+}]_i$

To begin to understand the mechanism of induction of Bf expression by ionomycin and the role of the IFN β -dependent priming step, we determined the effect of priming on changes in $[Ca^{2+}]_i$. Priming could

Figure 3. Effect of ionomycin, poly [I:C], and ATP on $[Ca^{2+}]_i$ in unprimed and IFN β -primed macrophages. Cells were loaded with Fura 2-AM, and the fluorescence signal was calibrated as described in MATERIALS AND METHODS. Stimuli were added as indicated in A-F. A and B illustrate the effect of ionomycin (1 μ M) on the $[Ca^{2+}]_i$ in unprimed and IFN β -primed macrophages, respectively. C and D show the effect of poly [I:C] (1 ng/ml and 1 μ g/ml, respectively) on $[Ca^{2+}]_i$ in IFN β -primed macrophages. E and F, respectively, illustrate the effect of ATP (10 μ M) on $[Ca^{2+}]_i$ in unprimed and IFN β -primed macrophages. Each tracing is representative of at least five experiments.

potentially influence the magnitude or the time course of changes in $[Ca^{2+}]_i$ in response to exposure to ionomycin. Macrophages were either incubated in medium alone for 6 h (unprimed) or were primed with 100 U/ml of IFN β . The cells were then loaded with Fura 2 as described in MATERIALS AND METHODS, and the $[Ca^{2+}]_i$ was determined spectrofluorimetrically. The average $[Ca^{2+}]_i$ in unprimed macrophages was 172 nM. This value was not significantly different ($p > 0.05$) in IFN β -primed macrophages (126 nM). Figure 3, A and B, respectively, shows that exposure of unprimed and IFN β -primed macrophages to ionomycin (1 μ M) led a rapid increase in $[Ca^{2+}]_i$ that peaked at similar levels ($p > 0.05$) in both cell populations (unprimed cells 312 nM; IFN β -primed cells 316 nM). Thereafter, the $[Ca^{2+}]_i$ declined to a sustained plateau ~3–4 min after the addition of the ionophore. There was no difference in the time course of changes in $[Ca^{2+}]_i$ between unprimed and IFN β -primed macrophages. Incubation of macrophages with concentrations of ionomycin $>1 \mu$ M stimulated the maximum fluorescence signal (F_{max}), thereby rendering the calculation of $[Ca^{2+}]_i$ impossible.

Effect of Poly [I:C] on $[Ca^{2+}]_i$

In view of the similar capacities of ionomycin and poly [I:C] to stimulate the expression of Bf in IFN β -primed macrophages, we questioned whether the mechanism of induction of Bf by the polyribonucleotide also involved an elevation in $[Ca^{2+}]_i$. IFN β -primed cells were loaded with Fura 2 and exposed to a concentration of poly [I:C] (1 ng/ml) sufficient to trigger Bf expression. It will be seen from Figure 3C that these conditions of stimulation did not induce any measurable increase in $[Ca^{2+}]_i$. The mechanism of interaction of poly [I:C] with macrophages has not been explored thoroughly, although binding of the polyribonucleotide does appear saturable and is competitively inhibited by pretreatment of the cells with a 30-fold excess of the ligand (De Whalley and Riches, unpublished observations), thus suggesting the involvement of a cell surface receptor. Therefore, by analogy to other stimulus-response coupling mechanisms (Michell *et al.*, 1976), it is possible that the induction of Bf expression is induced by ligation of only a small proportion of the ligand receptors, and hence, changes in $[Ca^{2+}]_i$ would not be detectable under these conditions. We therefore investigated the effect

of a 1000-fold higher concentration of poly [I:C] (1 µg/ml) on the [Ca²⁺]_i in IFNβ-primed macrophages with the aim of ligating "spare" receptors. As will be seen from Figure 3D, these conditions of stimulation also failed to elevate the [Ca²⁺]_i in IFNβ-primed macrophages. Additionally, macrophages stimulated with this higher concentration of poly [I:C] for 10 h demonstrated no change in [Ca²⁺]_i ([Ca²⁺]_i 175 nm; *p* > 0.05) compared with unstimulated cells. However, transient changes in [Ca²⁺]_i were detected in both unprimed (Figure 3E) and IFN-primed macrophages (Figure 3F) in response to ATP (10 µM), an agonist known to promote release of Ca²⁺ from internal stores (Greenberg *et al.*, 1988), indicating that our system was capable of detecting ligand-receptor-induced changes in [Ca²⁺]_i. The failure to detect a change in [Ca²⁺]_i in response to poly [I:C] was not related to compartmentalization of the Fura 2, as has been suggested in previous studies (Di Virgilio *et al.*, 1988). As will be seen in Figure 4, the conditions of loading mouse bone marrow-derived macrophages led to a uniform cytoplasmic fluorescence image when the cells were examined by fluorescence microscopy. Interestingly, and in contrast to previous studies using mouse peritoneal macrophages, Fura 2 appeared to be excluded from the vacuolar system of bone marrow-derived macrophages. This finding is consistent with our observation that the organic anion exchange inhibitor, probenecid, which blocks Fura 2 sequestration in peritoneal macrophages, showed no effect on the Ca²⁺ signal detected by spectrofluorimetry. A summary of the effects of the above

Figure 4. Fluorescence micrograph of Fura 2-loaded mouse bone marrow-derived macrophages showing diffuse cytoplasmic staining and the striking exclusion of the dye from the vacuolar compartment. Cells were loaded with Fura 2 as described in MATERIALS AND METHODS, were examined on a Nikon Diaphot inverted fluorescence microscope at an excitation wavelength of 365 nm, and were viewed through a 400 nm long pass dichroic filter at a magnification of 400×.

Table 1. Quantification of peak changes in [Ca²⁺]_i in marrow-derived mouse bone in response to various stimuli

Stimulus	[Ca ²⁺] _i (nM)	
	Unprimed cells	IFN-primed cells
No stimulus	172 ± 19 (8)	126 ± 17 (11)
Ionomycin (1 µM)	312 ± 21 (7) ^a	316 ± 21 (5) ^a
ATP (10 µM)	322 ± 19 (8) ^a	336 ± 25 (5) ^a
Poly [I:C] (1 ng/ml)	ND	113 ± 25 (5)
Poly [I:C] (1 µg/ml)	ND	152 ± 22 (2)

Values are the mean + SEM of the number of experiments indicated in parentheses.

^a Values that were significantly different (*p* < 0.01) from baseline (no stimulus). There were no significant differences between unprimed and IFN-primed cells for any stimulus (*p* > 0.05). In addition, there were no significant differences between the values obtained with ionomycin and those obtained with ATP (*p* > 0.05).

described ligands on [Ca²⁺]_i together with their statistical evaluation is presented in Table 1.

Attenuation of Trans-Plasma Membrane Ca²⁺ Movement

Although we were unable to detect global changes in [Ca²⁺]_i in response to poly [I:C], these findings do not exclude the possibility that localized changes in Ca²⁺, which are not detected by Ca²⁺-sensitive population fluorescence measurements, are involved in the signaling process in response to poly [I:C]. Recently reported data obtained both in fibroblasts (Kao *et al.*, 1990) and in cardiac myocytes (LeBlanc and Hume, 1990) have implicated such localized changes in [Ca²⁺]_i in the responses of these cells. Of particular relevance to our findings is the proposed localized changes in [Ca²⁺]_i in the induction of aldosterone secretion by adrenal glomerulosa cells (Kojima *et al.*, 1985a,b; Alkon and Rasmussen, 1988). In contrast to signaling involving release from internal stores or direct gating of Ca²⁺ through plasma membrane channels, both of which lead to changes in [Ca²⁺]_i, Ca²⁺ is hypothesized to be gated through a plasma membrane channel and then extruded to the extracellular compartment by a Ca²⁺ pump (Alkon and Rasmussen, 1988; Smallwood *et al.*, 1988). This sequence of events is thought to establish localized changes in [Ca²⁺]_i, or Ca²⁺ movement, in the region of the cell immediately adjacent to the plasma membrane. Consequently, such a mechanism does not provoke changes in [Ca²⁺]_i that are detectable by spectrofluorimetric or imaging techniques. The implication of this concept is that attenuation or Ca²⁺ gating across the plasma membrane will inhibit the execution of a given response. We have applied two approaches to address this issue, namely to eliminate the trans-plasma mem-

Figure 5. Attenuation of trans-plasma membrane movement of Ca²⁺. (A) Effect of deprivation of extracellular Ca²⁺. Monolayers of IFN β -primed macrophages challenged with poly [I:C] (1 ng/ml) in DMEM alone, containing a [Ca²⁺]_o of 1.88 mM, or in DMEM containing 2.21 mM EGTA and yielding a [Ca²⁺]_o of 122 nM. The cells were incubated for 24 h and analyzed for Bf expression as described in MATERIALS AND METHODS. (B) Divalent cation dependency of poly [I:C] binding to macrophages. Macrophage monolayers were exposed to [³²P]poly [I:C] (20 ng) in the presence of both Ca²⁺ (1.8 mM) and Mg²⁺ (0.8 mM), in the absence of Ca²⁺ or the absence of both Ca²⁺ and Mg²⁺. Binding was determined at 0°C for 2 h to prevent internalization of the labeled ligand.

brane Ca²⁺ gradient with a Ca²⁺/EGTA buffer and to inhibit Ca²⁺ channel activity with Ca²⁺ channel blockers.

To eliminate the trans-plasma membrane Ca²⁺ gradient, IFN β -primed macrophages (100 U/ml, 2 h) were exposed to poly [I:C] (1 ng/ml) in a Ca²⁺/EGTA buffer calculated to establish an extracellular concentration of free calcium ions ([Ca²⁺]_o) of 150 nM. The concentration of EGTA required to obtain this concentration was calculated using a computer program that was kindly provided by Dr. Willy Scheurs (Dept. of Biochemistry, Colorado State University, Fort Collins, CO). Spectrofluorimetric determination of the [Ca²⁺] of the protein-free culture medium with Indo-1 (Grynkiewicz *et al.*, 1985) verified that culture medium containing 2.21 mM EGTA had a free [Ca²⁺] of 122 $\pm$ 20 (mean $\pm$ SE) nM and approximated the previously determined [Ca²⁺]_i of macrophages (Table 1). As illustrated in Figure 5A, under these conditions the expression of Bf in response to poly [I:C] was reduced by $\sim$ 90% compared with that seen in culture medium containing a [Ca²⁺]_o of 1.8 mM. To verify that the attenuation of Bf expression was not simply due to an impairment of the binding of poly [I:C] to macrophages, we prepared [³²P]poly [I:C] by end-labeling with [³²P]- γ -ATP in the presence of polynucleotide kinase and examined the cation depen-

dency of poly [I:C] binding to macrophages. As will be seen in Figure 5B, the binding of [³²P]poly [I:C] to macrophage monolayers was not impaired by Ca²⁺ deprivation with EGTA, although interestingly, binding was reduced by $\sim$ 50% when both Ca²⁺ and Mg²⁺ were chelated with EDTA, suggesting that both divalent cation-dependent and independent mechanisms control poly [I:C] interaction with macrophages.

Second, we determined if calcium channel blockers inhibited the induction of Bf expression by poly [I:C]. Monolayers of IFN β -primed macrophages (100 U/ml, 4 h) were exposed to a triggering concentration of poly [I:C] (1 ng/ml) for 24 h at 37°C in the presence of increasing concentrations (0.1–100 μ M) of the calcium channel blocker verapamil. As shown in Figure 6, verapamil was found to inhibit poly [I:C]-induced Bf production in a concentration-dependent fashion, with a significant inhibition being observed initially at 1 μ M. To verify the specificity of this antagonist, IFN β -primed macrophages were exposed to ionomycin (5 μ M) in the presence of verapamil. As will also be seen in Figure 6, verapamil failed to inhibit the calcium ionophore-induced expression of Bf ($p = 0.383$) at a concentration of 10 μ M, although 100 μ M verapamil was found to be inhibitory. This latter finding suggests that at higher concentrations, verapamil exerts inhibitory effects distal to the Ca²⁺ signal. Experiments were also conducted using nifedipine, an alternative Ca²⁺ channel blocker. However, nifedipine was found to inhibit both the poly

Figure 6. Effect of the calcium channel blocker verapamil on the induction of Bf expression by IFN β -primed macrophages in response to poly [I:C] and ionomycin. IFN β -primed macrophages were pretreated with increasing concentrations of verapamil (0.1–100 μ M) for 30 min before adding poly [I:C] to a final concentration of 1 ng/ml or ionomycin to a final concentration of 5 μ M. The cells were incubated for 24 h before analyzing the level of expression of Bf as described in MATERIALS AND METHODS. This figure is representative of three experiments.

[I:C] and the ionomycin-induced synthesis of Bf to similar extents and hence, due to this lack of specificity, was not studied further.

Effect of Intracellular Ca²⁺ Antagonists on Bf Expression

These findings of a requirement for a trans-plasma membrane Ca²⁺ gradient in the induction of Bf expression by IFN β -primed macrophages and the absence of detectable changes in [Ca²⁺]_i imply that the changes in [Ca²⁺]_i may be subtle. To further verify the Ca²⁺-dependence of the signaling mechanism, we determined the influence of three intracellular Ca²⁺ antagonists on the induction of Bf in IFN β -primed macrophages by both poly [I:C] and ionomycin. Monolayers of IFN β -primed macrophages were pretreated with the calmodulin antagonists W-7 (25 μ M) and W-13 (50 μ M) (Tanaka and Hidaka, 1980) for 30 min or with TMB-8 (an inhibitor that stabilizes Ca²⁺ binding to intracellular sites [Chiou and Malagodi, 1975]) and then stimulated with either poly [I:C] (1 ng/ml) or ionomycin (5 μ M) for 24 h in the continued presence of each inhibitor. As shown in Figure 7A, W-7, W-13, and TMB-8 all inhibited Bf synthesis to varying extents in response to poly [I:C]. A similar inhibition of Bf expression was seen when IFN β -primed macrophages were exposed to ionomycin in the presence of these inhibitors (Figure 7B), although W-7 was less effective compared with W-13 and TMB-8. These concentrations of inhibitors did not diminish the viability of the cells. Indeed, TMB-8 was found to increase the incorporation of [³⁵S]methionine into total TCA-precipitable protein production by ~30% in poly [I:C]-stimulated cells and by 8% in ionomycin stimulated cells (Underwood and Riches, unpublished observations).

DISCUSSION

Changes in the [Ca²⁺]_i have been implicated in the signal transduction mechanisms of a profusion of cellular responses in eukaryotic cells (as reviewed by Berridge, 1986). In many situations, the second messenger function of Ca²⁺ is transmitted in a global fashion, thereby resulting in an elevation in [Ca²⁺]_i throughout the cytosolic compartment. The source of Ca²⁺ can be either the internal compartment of the endoplasmic reticulum or by the opening of Ca²⁺ channels in the plasma membrane, allowing an in-rushing of Ca²⁺ from the extracellular compartment. The functional necessity of such global increases in [Ca²⁺]_i in the immediate responses of cells, such as in the stimulation of secretion of luteinizing hormone from pituitary cells in response to gonadotropin-releasing hormone (Conn *et al.*, 1980) or in the activation of T cells (Gelfand *et al.*, 1987), is well established. However, more subtle changes in [Ca²⁺]_i have been implicated in the induction of some cellular responses. For example, Alkon and Rasmusson (1988)

Figure 7. Influence of intracellular Ca²⁺ antagonists on the induction of Bf expression in IFN β -primed macrophages. Monolayers of IFN β -primed macrophages were pretreated with W-7 (25 μ M), W-13 (50 μ M), or TMB-8 (75 μ M) for 30 min before the addition of either poly [I:C] (1 ng/ml) or ionomycin (5 μ M). The cells were stimulated for 24 h and analyzed for Bf expression as described in MATERIALS AND METHODS. A illustrates the effect of inhibitors on the induction of Bf by poly [I:C]; B shows the effects of the agents on the ionomycin-induced expression of Bf.

and Kao *et al.* (1990) have suggested that spatially restricted changes in [Ca²⁺]_i or in Ca²⁺ flow occur in adrenal glomerulosa cells in response to stimulation with angiotensin II. These localized changes in [Ca²⁺]_i have been proposed to be detected by proteins that sense changes in [Ca²⁺]_i, such as protein kinase C or calmodulin, which subsequently transmit the signal downstream to other components of the signaling pathway. Alternatively, asynchronous oscillations in [Ca²⁺]_i have been detected by single cell analyses of many different cell types (Tsien and Tsien, 1990), including inflammatory cells (Kruskal and Maxfield, 1987), and have also been suggested to play important roles in the activation of cellular functions (Richter *et al.*, 1990). Importantly, in both situations, changes in [Ca²⁺]_i are not always detected by population fluorescence measurements.

The involvement of changes in [Ca²⁺]_i in the stimulation of different macrophage functional activities has been questioned in several laboratories, and data have been reported showing that elevations in [Ca²⁺]_i accompany the stimulation of eicosanoid release in response to soluble stimuli (Riches *et al.*, 1988b), as well as in the formation of reactive oxygen intermediates such as O₂⁻ (Riches *et al.*, 1988b; Hagenlocker *et al.*, 1990). Infor-

mation concerning the possible involvement of changes in $[Ca^{2+}]_i$ in the induction of macrophage cytotoxic activation, however, has been less lucid and, in general, difficult to detect (Gorecka-Tysera *et al.*, 1986; Drysdale *et al.*, 1987; Prpic *et al.*, 1987). Nevertheless, Ca^{2+} has been suggested to play a pivotal role in macrophage cytotoxic differentiation, although the locus of its involvement has not been determined.

Macrophage cytotoxic activation requires the sequential impingement of a priming stimulus (usually IFNs) and a triggering stimulus (such as LPS or poly [I:C]), and previously reported studies have employed either unprimed macrophages (Drysdale *et al.*, 1987; Prpic *et al.*, 1987) or macrophages in which the priming and triggering agents were added simultaneously (Gorecka-Tysera *et al.*, 1986). Using phenotypically distinct macrophage populations (i.e., unprimed and IFN-primed) and quantifying the level of synthesis of a gene product (Bf) whose expression is associated with cytotoxic activation (Riches *et al.*, 1988a), we have questioned the role of Ca^{2+} in mediating the transition from the IFN-primed to the cytotoxic state. Specifically, we have 1) determined the functional effects of artificially elevating $[Ca^{2+}]_i$ with Ca^{2+} ionophores, 2) quantified changes in $[Ca^{2+}]_i$ when IFN-primed macrophages are exposed to a triggering stimulus, and 3) determined the effects of attenuating Ca^{2+} movement across the plasma membrane in the induction of cytotoxic activation.

Exposure of unprimed macrophages to the Ca^{2+} ionophores, ionomycin and A23187, failed to induce the expression of Bf. By contrast, macrophages that previously had been primed with IFN acquired the capacity to express Bf in response to Ca^{2+} ionophores by a process that was absolute in its dependence on Ca^{2+} and that was of similar amplitude to that induced by the polyribonucleotide poly [I:C]. However, a kinetic analysis revealed that the induction of Bf expression by IFN-primed macrophages was of more rapid onset in response to ionomycin than that induced by poly [I:C]. It is possible that the increased rate of responsiveness to ionomycin may be a consequence of circumvention of the conventional signaling pathway utilized by poly [I:C]. The expression of Bf in response to ionomycin was also more transient in nature than that induced by poly [I:C]. Although the reasons for this are unclear, prolonged exposure to calcium ionophores or exposure to supraoptimal concentrations of these agents may negatively control Bf expression and would be consistent with the observed decline in Bf expression at concentrations of ionomycin in excess of 5 μ M observed in the absence of cellular toxicity. Spectrofluorimetric quantification of the changes in $[Ca^{2+}]_i$ in unprimed and IFN-primed macrophages on challenge with ionomycin indicated a two-phase elevation in $[Ca^{2+}]_i$. These data are in agreement with previously reported data (Gelfand *et al.*, 1987) and are consistent with an immediate and

transient release of Ca^{2+} from internal stores, followed by a sustained elevation in $[Ca^{2+}]_i$ as a result of an inward movement of Ca^{2+} from the extracellular phase. The changes in $[Ca^{2+}]_i$ were indistinguishable between unprimed and IFN-primed populations of macrophages, thus indicating that priming did not influence the extent or time course of the changes in $[Ca^{2+}]_i$.

In contrast to the elevation of $[Ca^{2+}]_i$ induced by Ca^{2+} -ionophores, the induction of progression from the IFN-primed to the cytotoxic state by a triggering concentration of poly [I:C] was not associated with a detectable rise in $[Ca^{2+}]_i$. Similarly, when IFN-primed macrophages were stimulated with a concentration of poly [I:C] one thousand times greater than that required to trigger Bf expression, with the aim of saturating ligand binding as has been applied in other systems (Michell *et al.*, 1976), the $[Ca^{2+}]_i$ remained at a constant baseline level. The failure to detect an increase in $[Ca^{2+}]_i$ did not appear to be due to inappropriate selection of time points because loading macrophages with Fura 2 at time points after exposure to poly [I:C], although less desirable, failed to show an elevation in $[Ca^{2+}]_i$. Moreover, in other work, we have shown that another macrophage gene, IFN β , which is a member of a cluster of genes activated during cytotoxic activation, is elevated after 1 h of exposure of IFN-primed macrophages to poly [I:C] (Riches and Underwood, 1991). Thus, the signaling events for this gene are activated within the time course of measurements made in the present study. The induction of Bf expression by stimulation of IFN-primed cells with poly [I:C] was, nevertheless, a Ca^{2+} -dependent response, as illustrated by the finding of a strong attenuation of the specific synthesis of Bf when the $[Ca^{2+}]_o$ was clamped at the $[Ca^{2+}]_i$. By quantifying ligand binding to macrophages, data were obtained to show that this attenuation of Bf expression was not due to a reduction in ligand binding in the nominal absence of Ca^{2+} . Bf expression was similarly blocked when IFN-primed macrophages were exposed to poly [I:C] in the presence of either the Ca^{2+} -channel blocker verapamil or the intracellular Ca^{2+} antagonists W-7, W-13, and TMB-8. These collective findings thus suggest that the changes in $[Ca^{2+}]_i$ implicated in the mechanism of signal transduction by poly [I:C] are subtle. By contrast, Ca^{2+} -ionophores are less selective and transfer Ca^{2+} through membranes across which there is a sizable Ca^{2+} gradient. Thus, many of the changes in $[Ca^{2+}]_i$ triggered by ionomycin may have occurred in a spatial or in a temporal fashion that are not essential to the induction of Bf expression.

On the basis of these data, we speculate that a localized or a cyclical change in $[Ca^{2+}]_i$ promoted by an inwardly directed movement of the divalent cation through the plasma membrane is required to initiate the progression of the IFN-primed macrophage to the cytotoxic state in response to poly [I:C]. The mechanism(s)

by which these proposed changes in the permeability of the macrophage plasma membrane to Ca²⁺ are induced are largely unknown, although the finding that verapamil antagonized the progression from the IFN-primed to the cytotoxic state are suggestive that a Ca²⁺ channel may be involved. Little is known about the occurrence of Ca²⁺ channels in macrophages, although the detection of action potentials has been interpreted as possible evidence for the existence of voltage-operated Ca²⁺ channels (McCann *et al.*, 1983; Woehlck and McCann, 1986). Ca²⁺ channel blockers have proven to be useful in determining the involvement of Ca²⁺ channels in other cells (Birn *et al.*, 1984). However, additional effects of calcium channel blockers (Levina, 1983; Chang *et al.*, 1987), including an inhibition of voltage-dependent K⁺ channels, have been observed (DeCoursey *et al.*, 1985), and hence it remains a possibility that other mechanisms of Ca²⁺ exchange may be involved. For example, Na⁺/Ca²⁺ exchange has been reported to be the major mechanism of entry of Ca²⁺ into the cell during stimulation of superoxide anion production in neutrophils in response to the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (Simchowitz and Cragoe, 1988; Simchowitz *et al.*, 1990). Similarly, recently reported data suggest that the induction of contraction of cardiac myocytes may be induced by Na⁺/Ca²⁺ exchange, which, in the absence of stimulation, serves to exchange intracellular Ca²⁺ for extracellular Na⁺, thereby maintaining a low [Ca²⁺]_i, but which in response to Na⁺ influx through a Na⁺-channel reverses and allows extracellular Ca²⁺ to enter the cell (LeBlanc and Hume, 1990) in exchange for intracellular Na⁺. Like second-messenger and voltage-activated Ca²⁺ channels, Ca²⁺ appears to be restricted in its movement to the area of the cytosol adjacent to the plasma membrane that has been referred to as the "fuzzy space" (Lederer *et al.*, 1990). Local changes in [Ca²⁺]_i are also regulated by the plasma membrane located Ca²⁺-ATPase that actively extrudes Ca²⁺ from most cell types (Waisman *et al.*, 1982; Smallwood *et al.*, 1988), including macrophages (Lew and Stossel, 1980) and neutrophils (Volpi *et al.*, 1983).

The conclusions drawn from this study can also be considered in the context of other investigations of the potential role of changes in [Ca²⁺]_i in the expression of macrophage cytotoxic activity. It has been consistently difficult to find evidence of global changes in [Ca²⁺]_i in macrophages during differentiation to the cytotoxic state and this would be consistent with our proposal of localized or cyclical changes in [Ca²⁺]_i. Furthermore, in studies where measurements of ⁴⁵Ca²⁺ movement have been made, these have focused on the influx of ⁴⁵Ca²⁺ (Gorecka-Tisera and McCulloch, 1986; Drysdale *et al.*, 1987) and no changes have been observed in this parameter. Under the conditions in which these measurements were made, however, the model of activation

proposed herein would predict such a result because the compensatory activation of Ca²⁺ extrusion would make it difficult to detect a difference in the level of influx.

The data obtained from this study also suggest that the level of IFN-priming, at least in the case of induction of Bf expression by ionomycin, is expressed distal to the changes in [Ca²⁺]_i. Thus, priming appears to allow the ionophore-induced elevation in [Ca²⁺]_i to become productively coupled with elements that control the expression of the genes associated with the macrophage cytotoxic state. In unprimed cells, this coupling is speculated to be in an inactive state, thereby allowing changes in [Ca²⁺]_i to be coupled to the induction of different macrophage functions. Consistent with this conclusion is the finding that the capacity of macrophages to synthesize and secrete eicosanoids in response to Ca²⁺-ionophores and other stimuli (Boraschi *et al.*, 1984, 1985; Browning and Ribolini, 1987) is lost when macrophages are primed with IFNs. Thus, priming agents such as IFNs may allow common pathways of transmembrane signaling to be "switched" from their "default" condition to elicit different cellular responses in macrophages and thus emphasize the functional importance of the priming step. It would seem plausible that proteins capable of sensing [Ca²⁺]_i, such as protein kinase C or calmodulin, may contribute to this hypothetical switching mechanism. Future studies will be aimed at addressing this question.

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REFERENCES

- Adams, D.O., and Hamilton, T.A. (1984). The cell biology of macrophage activation. *Annu. Rev. Immunol.* 2, 283-318.
- Alexander, P., and Evans, R. (1971). Endotoxin and double stranded RNA render macrophages cytotoxic. *Nature New Biol.* 232, 76-78.
- Alkon, D.L., and Rasmussen, H. (1988). A spatial-temporal model of cell activation. *Science* 239, 998-1005.
- Berridge, M.J. (1986). Inositol phosphates as second messengers. In: *Phosphoinositides and Receptor Mechanisms*, ed. J.W. Putney, New York: Alan R. Liss, 25-45.
- Birn, D.L., Berger, M., and Fleisher, T.A. (1984). The interference of T cell activation by calcium channel blocking agents. *J. Immunol.* 133, 2904-2909.

- Bonventre, J.V., Skorecki, K.L., Kreisberg, J.I., and Cheung, J.Y. (1986). Vasopressin increases cytosolic calcium concentration in glomerular mesangial cells. *Am. J. Physiol.* 251, F94-F102.
- Boraschi, D., Censini, S., Bartalini, M., Scapigliati, G., Barbarulli, G., Vicenzi, E., Donati, M.B., and Tagliabue, A. (1984). Interferon inhibits prostaglandin biosynthesis in macrophages: effects on arachidonic acid metabolism. *J. Immunol.* 132, 1987-1992.
- Boraschi, D., Censini, S., Bartalini, M., and Tagliabue, A. (1985). Regulation of arachidonic acid metabolism in macrophages by immune and nonimmune interferons. *J. Immunol.* 135, 502-505.
- Browning, J.L., and Ribolini, A. (1987). Interferon blocks interleukin 1-induced prostaglandin release from human peripheral monocytes. *J. Immunol.* 138, 2857-2863.
- Chang, J., Blazek, E., and Carlson, R.P. (1987). Inhibition of phospholipase A2 activity by nifedipine and nisoldipine is independent of their calcium channel blocking activity. *Inflammation* 11, 353-364.
- Chiou, C.Y., and Malagodi, M.H. (1975). Studies on the mechanism of action of a new Ca²⁺ antagonist, 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride in smooth and skeletal muscles. *Br. J. Pharmacol.* 53, 279-285.
- Cole, F.S., Auerbach, H.S., Goldberger, G., and Colten, H.R. (1985). Tissue specific pretranslational regulation of complement production in human mononuclear phagocytes. *J. Immunol.* 134, 2610-2616.
- Conn, P.M., Marian, J., McMillian, M., and Rogers, D. (1980). Evidence for calcium mediation of gonadotropin releasing hormone action in the pituitary. *Cell Calcium* 1, 7-20.
- DeCoursey, T.E., Chandy, K.G., Gupta, S., and Cahalan, M.D. (1985). Voltage-dependent ion channels in T-lymphocytes. *J. Neuroimmunol.* 10, 71-95.
- De Whalley, C.V., and Riches, D.W.H. (1991). Influence of the cytotoxic macrophage phenotype on the degradation of acetylated low density lipoproteins: dual regulation of scavenger receptor activity and of intracellular degradation of endocytosed ligand. *Exp. Cell Res.* 192, 460-468.
- Ding, A.H., Nathan, C.F., and Stuehr, D.J. (1988). Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J. Immunol.* 141, 2407-2412.
- Di Virgilio, F., Steinberg, T.H., Swanson, J.A., and Silverstein, S.C. (1988). Fura-2 secretion and sequestration in macrophages. A blocker of organic anion transport reveals that these processes occur via a membrane transport system for organic anions. *J. Immunol.* 140, 915-920.
- Drysdale, B.E., Yapundich, R.A., Shin, M.L., and Shin, H.S. (1987). Lipopolysaccharide-mediated macrophage activation: the role of calcium in the generation of tumoricidal activity. *J. Immunol.* 138, 951-956.
- Evans, R., and Alexander, P. (1970). Cooperation of immune lymphoid cells with macrophages in tumour immunity. *Nature* 228, 620-622.
- Gelfand, E.W., Mills, G.B., Cheung, R.K., Lee, J.W.W., and Grinstein, S. (1987). Transmembrane ion fluxes during activation of human T lymphocytes: role of Ca²⁺, Na⁺/H⁺ exchange and phospholipid turnover. *Immunol. Rev.* 95, 59-87.
- Gorecka-Tisera, A.M., and McCulloch, M.A. (1986). Extracellular calcium is not an absolute requirement for tumoricidal activation of RAW-264 macrophage-like cell line. *J. Leukocyte Biol.* 40, 203-214.
- Gorecka-Tisera, A.M., Snowdowne, K.W., and Borle, A.B. (1986). Implications of a rise in cytosolic free calcium in the activation of RAW-264 macrophages for tumor cell killing. *Cell. Immunol.* 100, 411-421.
- Greenberg, S., DiVirgilio, F., Steinberg, T.H., and Silverstein, S.C. (1988). Extracellular nucleotides mediate Ca²⁺ fluxes in J774 macrophages by two distinct mechanisms. *J. Biol. Chem.* 263, 10337-10343.
- Gryniewicz, G., Poenie, M., and Tsien, R.Y. (1985). A new generation of calcium indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260, 3440-3450.
- Hagenlocker, B.E., Walker, B.A., and Ward, P.A. (1990). Superoxide responses of immune complex-stimulated rat alveolar macrophages. Intracellular calcium and priming. *J. Immunol.* 144, 3898-3906.
- Hibbs, J.B.J., Lambert, L.H.J., and Remington, J.S. (1972). Control of carcinogenesis: a possible role for the activated macrophage. *Science* 177, 998-1000.
- Hogan, M.M., and Vogel, S.N. (1987). Lipid A-associated proteins provide an alternate "second signal" in the activation of recombinant interferon-gamma-primed, C3H/HeJ macrophages to a fully tumoricidal state. *J. Immunol.* 139, 3697-3702.
- Imber, M.J., Pizzo, S.V., Johnson, W.J., and Adams, D.O. (1982). Selective diminution of the binding of mannose by murine macrophages in the late stages of activation. *J. Biol. Chem.* 257, 5129-5135.
- Kao, J.P.Y., Alderton, J.M., Tsien, R.Y., and Steinhardt, R.A. (1990). Active involvement of Ca²⁺ in mitotic progression of Swiss 3T3 fibroblasts. *J. Cell Biol.* 111, 183-196.
- Koerner, T.J., Adams, D.O., and Hamilton, T.A. (1987). Regulation of tumor necrosis factor (TNF) expression: interferon-gamma enhances the accumulation of mRNA for TNF induced by lipopolysaccharide in murine peritoneal macrophages. *Cell. Immunol.* 109, 437-443.
- Kojima, I., Kojima, K., and Rasmussen, H. (1985a). Characteristics of angiotensin II-, K⁺- and ACTH-induced calcium influx in adrenal glomerulosa cells. Evidence that angiotensin II, K⁺, and ACTH may open a common calcium channel. *J. Biol. Chem.* 260, 9171-9176.
- Kojima, I., Kojima, K., and Rasmussen, H. (1985b). Role of calcium fluxes in the sustained phase of angiotensin II-mediated aldosterone secretion from adrenal glomerulosa cells. *J. Biol. Chem.* 260, 9177-9184.
- Kruskal, B.A., and Maxfield, F.R. (1987). Cytosolic free calcium increases before and oscillates during frustrated phagocytosis in macrophages. *J. Cell Biol.* 105, 2685-2693.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
- LeBlanc, N., and Hume, J.R. (1990). Sodium current-induced release of calcium from cardiac sarcoplasmic reticulum. *Science* 248, 372-376.
- Lederer, W.J., Niggli, E., and Hadley, R.W. (1990). Sodium-calcium exchange in excitable cells: fuzzy space [comment]. *Science* 248, 283.
- Leu, R.W., and Herriott, M.J. (1987). Triggering of interferon gamma-primed macrophages by various known complement activators for non-specific cytotoxicity. *Cell. Immunol.* 106, 114-121.
- Levina, L. (1983). Inhibition of the A23187-stimulated leukotriene and prostaglandin biosynthesis of rat basophil leukemia (RBL-1) cells by nonsteroidal antiinflammatory drugs, antioxidants and calcium channel blockers. *Biochem. Pharmacol.* 32, 3023-3026.
- Lew, P.D., and Stossel, T.P. (1980). Calcium transport by macrophage plasma membranes. *J. Biol. Chem.* 255, 5841-5846.
- McCann, F.V., Cole, J.J., Guyre, P.M., and Russell, J.A.G. (1983). Action potentials in macrophages derived from human monocytes. *Science* 219, 991-993.
- Michell, R.H., Jafferji, S.S., and Jones, L.M. (1976). Receptor occupancy dose-response curve suggests that phosphatidylinositol breakdown

- may be intrinsic to the mechanism of the muscarinic cholinergic receptor. *FEBS Lett.* 69, 1–5.
- Pace, J.L., and Russell, S.W. (1981). Activation of mouse macrophages for tumor cell killing. I. Quantitative analysis of interactions between lymphokine and lipopolysaccharide. *J. Immunol.* 126, 1863–1867.
- Pace, J.L., Russell, S.W., LeBlanc, P.A., and Murasko, D.M. (1985). Comparative effects of various classes of mouse interferons on macrophage activation for tumor cell killing. *J. Immunol.* 134, 977–981.
- Pace, J.L., Taffet, S.M., and Russell, S.W. (1981). The effect of endotoxin in eliciting agents on the activation of mouse macrophages for tumor cell killing. *J. Reticuloendothel. Soc.* 30, 15–21.
- Prpic, V., Weiel, J.E., Somers, S.D., DiGuiseppi, J., Gonias, S.L., Pizzo, S.V., Hamilton, T.A., Herman, B., and Adams, D.O. (1987). Effects of bacterial lipopolysaccharide on the hydrolysis of phosphatidylinositol-4,5-bisphosphate in murine peritoneal macrophages. *J. Immunol.* 139, 526–533.
- Riches, D.W.H., Channon, J.Y., Leslie, C.C., and Henson, P.M. (1988b). Receptor-mediated signal transduction in mononuclear phagocytes. *Prog. Allergy* 42, 65–122.
- Riches, D.W.H., and Henson, P.M. (1986a). Bacterial lipopolysaccharide suppresses the production of catalytically active lysosomal acid hydrolases in human macrophages. *J. Cell Biol.* 102, 1606–1614.
- Riches, D.W.H., and Henson, P.M. (1986b). Functional aspects of mononuclear phagocyte involvement in lung inflammation. *Ann. N.Y. Acad. Sci.* 465, 6–14.
- Riches, D.W.H., Henson, P.M., Remigio, L.K., Catterall, J.F., and Strunk, R.C. (1988a). Differential regulation of gene expression during macrophage activation with a polyribonucleotide. The role of endogenously derived IFN. *J. Immunol.* 141, 180–188.
- Riches, D.W.H., and Underwood, G.A. (1991). Expression of IFN β during the triggering phase of macrophage cytotoxic activation. Evidence for an autocrine/paracrine role in the regulation of this state. *J. Biol. Chem.* 266, 24785–24792.
- Richter, J., Olsson, I., and Andersson, T. (1990). Correlation between spontaneous oscillations of cytosolic free Ca²⁺ and tumor necrosis factor-induced degranulation in adherent human neutrophils. *J. Biol. Chem.* 265, 14358–14363.
- Roberts, B.E., and Paterson, B.M. (1973). Effect of translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. *Proc. Natl. Acad. Sci. USA* 70, 2330–2334.
- Ruco, L.P., and Meltzer, M.S. (1978). Macrophage activation for tumor cytotoxicity: development of macrophage cytotoxic activity requires completion of a sequence of short-lived intermediary reactions. *J. Immunol.* 121, 2035–2042.
- Russell, S.W., Doe, W.F., and McIntosh, A.T. (1977). Functional characterization of a stable, noncytolytic stage of macrophage activation in tumors. *J. Exp. Med.* 146, 1511–1520.
- Simchowitz, L., and Cragoe, E.J.J. (1988). Na⁺-Ca²⁺ exchange in human neutrophils. *Am. J. Physiol.* 254, C150–C164.
- Simchowitz, L., Foy, M.A., and Cragoe, E.J.J. (1990). A role for Na⁺/Ca²⁺ exchange in the generation of superoxide radicals by human neutrophils. *J. Biol. Chem.* 265, 13449–13456.
- Smallwood, J.I., Gugi, B., and Rasmussen, H. (1988). Regulation of erythrocyte Ca²⁺ pump activity by protein kinase C. *J. Biol. Chem.* 263, 2195–2202.
- Sokal, R.R., and Rohlf, F.J. (1969). *Biometry*, San Francisco: W.H. Freeman and Co.
- Somers, S.D., Mastin, J.P., and Adams, D.O. (1983). The binding of tumor cells by murine mononuclear phagocytes can be divided into two qualitatively distinct types. *J. Immunol.* 131, 2086–2093.
- Stewart, C.C. (1981). Murine mononuclear phagocytes from bone marrow. In: *Methods for Studying Mononuclear Phagocytes*, ed. D.O. Adams, P.J. Edelson, and H. Koren, New York: Academic Press, 5–20.
- Stuehr, D.J., and Marletta, M.A. (1985). Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. *Proc. Natl. Acad. Sci. USA* 82, 7738–7742.
- Tanaka, T., and Hidaka, H. (1980). Hydrophobic regions function in calmodulin-enzyme(s) interactions. *J. Biol. Chem.* 255, 11078–11080.
- Taramelli, D., and Varesio, L. (1981). Activation of murine macrophages. I. Different patterns of activation by polyI:C than by lymphokine or LPS. *J. Immunol.* 127, 58.
- Torres, B.A., and Johnson, H.M. (1985). Lipopolysaccharide and polyribonucleotide activation of macrophages: implications for a natural triggering signal in tumor cell killing. *Biochem. Biophys. Res. Commun.* 131, 395–401.
- Tsien, R., and Pozzan, T. (1989). Measurement of cytosolic free Ca²⁺ with quin 2. In: *Methods in Enzymology*, ed. S. Fleischer and B. Fleischer, San Diego: Academic Press, 230–262.
- Tsien, R.W., and Tsien, R.Y. (1990). Calcium channels, stores, and oscillations. *Annu. Rev. Cell. Biol.* 6, 715–760.
- Volpi, M., Naccache, P.H., and Sha'afi, R.I. (1983). Calcium transport in inside-out membrane vesicles prepared from rabbit neutrophils. *J. Biol. Chem.* 258, 4153–4158.
- Waisman, D.M., Smallwood, J., Lafreniere, D., and Rasmussen, H. (1982). The role of band III in calcium transport across the human erythrocyte membrane. *FEBS Lett.* 145, 337–340.
- Woehlck, H.J., and McCann, F.V. (1986). Action potentials in human macrophages are calcium spikes. *Cell Biol. Int. Rep.* 10, 517–525.