

Trifluoperazine Inhibits Phagocytosis in a Macrophagelike Cultured Cell Line

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ABSTRACT Trifluoperazine, a drug that binds to Ca^{2+} -calmodulin and inhibits its interaction with other proteins, was found to inhibit growth and phagocytosis in a macrophagelike cell line, J774.16. Both effects were reversible and occurred at the same concentrations of drug (25–50 μM) that inhibited the activation of cyclic nucleotide phosphodiesterase by calmodulin *in vitro*. Fc-mediated phagocytosis was also depressed by W-7, a sulfonamide derivative that inhibits the activity of Ca^{2+} -calmodulin. In contrast, taxol, a drug that stabilizes cellular microtubules, had no effect on Fc-mediated phagocytosis although it inhibited cell growth at nanomolar concentrations. The inhibitory effects of trifluoperazine and W-7 on phagocytosis suggest that calmodulin may be involved in this complex cellular function.

The cloned cell lines J774.2 and J774.16 derive from a macrophagelike cell line established in culture from a murine reticulum cell sarcoma, J774 (1). These cultured lines maintain certain differentiated macrophagelike functions (2); they secrete lysozyme and plasminogen activator (3), and migrate and phagocytize antibody-coated erythrocytes via specific Fc receptors (1, 4). Since it is feasible to obtain stable variants in some of these functions, one can analyze macrophage-specific functions using biochemical and genetic tools. For example, variants defective in Fc-mediated phagocytosis (4), cAMP-dependent protein kinase (5) and adenylate cyclase (5) have facilitated analysis of the modulation of Fc-receptor function by cyclic AMP (6). We have previously reported (7) that trifluoperazine, a phenothiazine that binds to the Ca^{2+} -activated form of calmodulin (8–10) and inhibits its ability to interact with enzymes such as cyclic nucleotide phosphodiesterase (8), adenylate cyclase (11), membrane-bound Ca^{2+} -ATPase (12–14), myosin light chain kinase and myosin ATPase (15), and phospholipase A_2 (16), inhibits the growth of J774.2. This property was used as a strategy for selecting variants in J774.2 that are defective in calmodulin or in proteins that interact with calmodulin. To obtain information about the possible role of calmodulin in macrophage function, we studied the effect of two calmodulin-inhibitory drugs, trifluoperazine, and the sulfonamide derivative W-7 (13, 15, 17), and taxol, a drug that promotes microtubule assembly (18), on intact cells of the J774.16 line.

MATERIALS AND METHODS

Drugs

Trifluoperazine dihydrochloride (Tfp) was a gift from Dr. H. Green of Smith, Kline and French and *N*-(6-aminoethyl)-5-chloro-1-naphthalene sulfonamide (W-7) was a generous gift from Dr. H. Hidaka (Department of Pharmacology, School of Medicine, Mie University, Eobashi Tsu 514, Japan). Taxol was obtained from the National Cancer Institute, Bethesda, Md. Taxol and W-7 were dissolved in dimethyl sulfoxide at a concentration of 10 mM and stored in the dark at -20°C . The concentration of dimethyl sulfoxide in each experiment had no effect on control reactions. Tfp was freshly prepared in phosphate buffered saline (PBS) and protected from light.

Cells

The cloned cell line J774.16 was derived by Muschel et al. (4) from the murine reticulum cell sarcoma, J774 (1), and adapted to culture by Dr. M. Scharff, Albert Einstein College of Medicine. Cells were grown in monolayer culture in Dulbecco's modified Eagle's medium (DME; Gibco Laboratories, Inc., Grand Island, N. Y.) supplemented with penicillin, streptomycin, 1% glutamine, 1% nonessential amino acids and 20% heat-inactivated horse serum (Gibco Laboratories, Inc.). This supplemented DME is referred to as complete medium. The cells were maintained at 37°C in a humid incubator with an air mixture containing 8% CO_2 . Confluent cultures were harvested by vigorously pipetting the medium and cells. Before initiating a growth curve, cells were centrifuged at 1,000 rpm for 5 min and resuspended in complete medium.

Growth Curves

Cells (2.8×10^5) were plated onto 60-mm tissue culture dishes in a total volume of 4 ml and allowed to attach to the dish for 2 h before addition of drug. At 24-h intervals, the medium was removed and 2 ml of DME containing 5.9×10^{-4} M EDTA was added to each plate. The cells were then scraped off the plate

with a rubber policeman and counted in a Coulter counter Model Zr. To determine whether inhibition of growth was reversible, the medium containing drug was removed, and cells were then washed twice with DME and incubated in complete medium without drug.

Preparation of Sheep Erythrocytes (SRBC) and Sheep Erythrocytes Coated with Anti-SRBC IgG (E[IgG])

SRBC in Alsever's solution (Scott Lab, Fiskeville, R. I.) were washed three times in DME, resuspended as a 5% (vol/vol) suspension in DME and incubated with an equal volume of Hanks' balanced salt solution containing rabbit antiserum to SRBC IgG (Cordis Laboratories, Inc., Miami, Fla.). The final dilution of antiserum was between 1:2,000 and 1:400. The mixture was incubated for 30 min at 37°C, washed three times in DME, and then resuspended in DME as a 0.5% (vol/vol) suspension.

Phagocytosis

Approximately 5×10^5 cells in 5 ml of complete medium were plated for 24 h at 37°C on sterile glass cover slips (13 mm) in 60-mm petri dishes. For the assay, the cover slips were rinsed once in DME and covered with a suspension of E[IgG]. The cells were incubated for 60 min at 37°C, rinsed for 15–20 s in a 1:5 dilution of Hanks' solution to lyse the adsorbed erythrocytes and then rinsed for 5 s in undiluted Hanks' solution. A minimum of 100 cells were evaluated for the number of SRBC ingested. They were scored as 0, 1, 2, 3, or ≥ 4 erythrocytes per macrophage.

Ingestion of Latex Beads

Polyvinyltoluene latex beads (2.97 μm diameter) (Dow Diagnostics, Dow Chemical Co., Indianapolis, Ind.) were washed 10 times with PBS and resuspended in complete DME. Cells were grown in 60-mm tissue culture dishes and incubated with either 0, 10, 25, 30, 45, or 60 μM Tfp for 1 or 3 h at 37°C. The medium was removed and a suspension of latex beads in complete DME containing the appropriate concentration of drug was added. After incubation for 1 or 3 h at 37°C, the cells were washed four times in DME and fixed as a monolayer for 45 min in 3% glutaraldehyde in DME and postfixed in 1% osmium tetroxide. A graded series of alcohol was used for dehydrating and embedding the monolayer in Epon. To quantitate phagocytic activity, the plastic-embedded cells were sectioned at 1 μm and stained with toluidine blue O and basic fuchsin. This made it possible to count the beads present in cell sections. Only cells with a delineated nucleus and plasma membrane were included. To ensure random sampling, cells were counted in order from corner to corner and 50 cells were sampled for each experimental point. For experiments designed to test the reversibility of the effects of Tfp, cells were incubated in either 0, 10, 25, 30, 45, or 60 μM of drug for 1 h, washed, and allowed to grow in the absence of drug for a 2nd h. A 1-h latex bead assay in the absence of drug followed.

Lysozyme Assay

Secretion of lysozyme was measured in macrophage-conditioned medium prepared by plating 3×10^6 cells on 100-mm tissue culture dishes in complete medium. 2 h after plating, 25 μM Tfp or 50 nM taxol was added to the medium. 10 h after addition of drug, medium was collected and 200- μl aliquots were assayed for lysozyme activity. Lysozyme was assayed turbidometrically (19) at room temperature in a spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with an automatic recorder. Conditioned medium (200 μl) was added to 0.25 mg/ml *Micrococcus lysodeikticus* (Worthington Biochemical Corp., Freehold, N. J.) in 0.067 M sodium phosphate buffer, pH 6.3, in a final volume of 1 ml. The decline in optical density of this solution was followed at 540 nm and the results presented as the change in optical density during the 10 min after addition of conditioned medium.

Immunoglobulin Binding Assay

The binding assay was adapted from that of Unkeless and Eisen (20) and is described in detail by Schneck et al. (6). Cells (5×10^5) were plated overnight in 16-mm Linbro wells (Flow Laboratories, Hamden, Ct.). 2–4 h before assay, the serum-containing medium was replaced by 0.02% lactalbumin hydrolysate (vol/vol) in DME. At the time of the assay, the medium was aspirated and replaced with 0.2 ml of a 1:1 mixture of 2% bovine serum albumin (BSA) in PBS and DME containing 1.5 μg of purified ^{125}I -IgG_{2a} myeloma protein and varying amounts of unlabeled IgG_{2a} (6). After 90 min of incubation at 4°C, the medium was withdrawn and the wells were washed three times with ice-cold PBS

containing 50 mM KI. Bound IgG was determined by removing the PBS and incubating the cells with 1 ml of 0.25% trypsin for 45 min at 37°C. The trypsin solution containing the released IgG was removed and assayed for ^{125}I . Trypsin treatment released ~90% of the SDS-soluble radioactivity. In experiments designed to test the effect of Tfp on IgG_{2a} binding, cells were preincubated for 3.5 h with 25 μM drug and the drug was present throughout the ensuing binding assay. The recovery of cells after the assay was 50% for the drug-treated cells and 90% for control cells. Binding was normalized to the final cell count. For the binding studies reported here, clone J774.2 rather than J774.16 was used since the Fc receptors have been characterized in this line (6) and the binding assays were technically easier with these cells. Fc-mediated phagocytosis in J774.2 is as inhibited by Tfp as it is in J774.16.

Cyclic Nucleotide Phosphodiesterase Activity

The assay mixture (40 μl) consisted of 50 mM Tris-HCl buffer, pH 7.4, 5 mM MgCl_2 , 200 μM cAMP, 2 μCi [^3H]cAMP, 2 mM dithiothreitol, 1 mg/ml BSA, and 20 μl of purified bovine brain cyclic nucleotide phosphodiesterase. Assays were performed for 10 min at 37°C in the presence of 1 μg of calmodulin and either 5 mM CaCl_2 or 5 mM EGTA. Reactions were terminated by addition of a solution (20 μl) containing 12.5 mM adenosine, 12.5 mM cAMP, 12.5 mM 5'-AMP, and 0.2 mM EDTA. The conversion of cAMP to 5'-AMP and adenosine was determined by spotting 3 μl of the assay mixture on PEI-cellulose thin layer plates as previously described (21). The preparation of purified bovine brain calmodulin and calmodulin-dependent cyclic nucleotide phosphodiesterase have been reported (7).

RESULTS

Effects of Tfp and Taxol on Cell Growth

Both Tfp and taxol inhibited the growth of J774.16. Addition of 25 μM Tfp to cells 2 h after plating resulted in cell death within 24 h (Fig. 1A). Inhibition was observed within a very narrow range of drug concentration; cells grew normally in 20 μM Tfp. Taxol was a more potent inhibitor of cell growth; 50% inhibition occurred at ~40 nM when observed at 72 hr (Fig. 1B).

Fc-mediated Phagocytosis in the Presence of Tfp, W-7, and Taxol

To assess the effect of drugs on Fc-mediated phagocytosis, cells that had been plated 16 h previously were incubated with either Tfp, W-7, or taxol for 1, 2, or 4 h at 37°C in complete medium. Upon completion of the incubation period, the medium was removed and replaced with 0.1 ml of a 0.5% suspension of E[IgG], and phagocytosis was assayed as described in Materials and Methods. The presence of drug during the 1-h incubation with E[IgG] did not alter the results. As shown in Figure 2A, Tfp inhibited Fc-mediated phagocytosis in a dose-

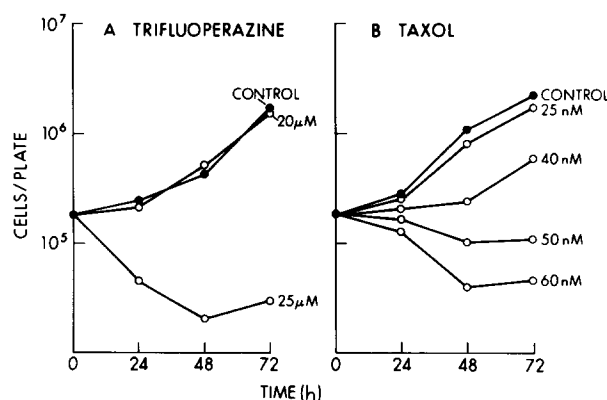


FIGURE 1 Effect of Tfp (A) and taxol (B) on the growth of J774.16. Cell growth was monitored as described in Materials and Methods. Control, ●, drug, ○.

FIGURE 2 Fc-mediated phagocytosis in J774.16 in the presence of Tfp (A), W-7 (B), and taxol (C). Cells (5×10^5) were plated on glass cover slips in complete medium. After 16 h, the indicated concentrations of drug were added for 1 (○), 2 (△), and 4 (□) h. At the end of the incubation, the cover slips were rinsed and phagocytosis was assayed as described in Materials and Methods using a 1:400 dilution of antiserum. The results cited are based on the number of cells ingesting four or more erythrocytes. 100 macrophages were counted for each experimental point. Phagocytosis in the control cells varied between 85 and 95%.

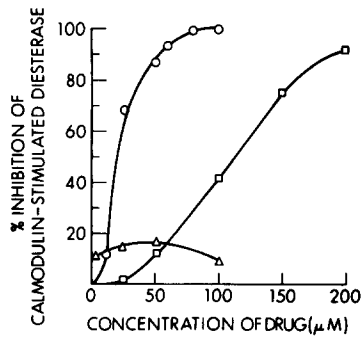
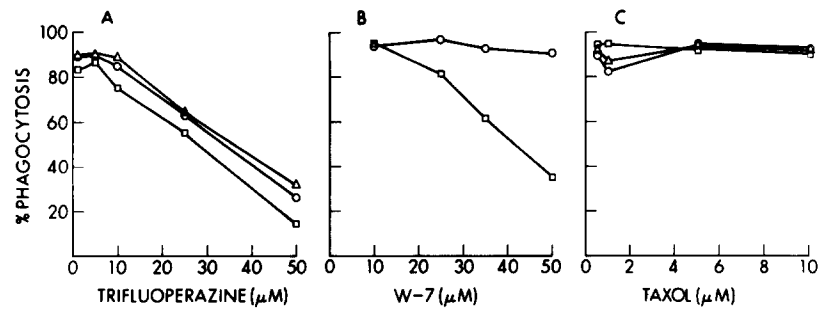


FIGURE 3 Inhibition of calmodulin-stimulated cyclic nucleotide phosphodiesterase activity by Tfp (○), W-7 (□), and taxol (△). Assays were performed as described in Materials and Methods. In the experiment depicted, the conversion of cyclic AMP to 5'-AMP was 2.4% in the absence of calmodulin and 42% in the presence of calmodulin.

dependent manner. At 25 μM Tfp, inhibition was seen after a 1-h exposure of the cells to drug. Fc-mediated phagocytosis was inhibited 28% after 1 or 2 h with 25 μM Tfp and 67% with 50 μM Tfp. Incubation for 4 h increased inhibition to 33% at 25 μM Tfp and to 82% at 50 μM Tfp. Very little inhibition of Fc-mediated phagocytosis was seen with W-7 after a 2-h incubation, but 62% inhibition was noted after a 4-h incubation with 50 μM drug (Fig. 2B). As shown in Fig. 3, W-7 was also less effective than Tfp in inhibiting the activation of cyclic nucleotide phosphodiesterase by Ca²⁺-calmodulin. On the other hand taxol, a drug that has a profound growth-inhibitory effect on these cells, did not affect phagocytosis (Fig. 2C) even at concentrations 100-fold in excess of those required to completely inhibit growth and had little effect on the activation of cyclic nucleotide phosphodiesterase by Ca²⁺-calmodulin (Fig. 3). A light microscope view of a field of Tfp-treated and control cells after incubation with E[IgG] is presented in Fig. 4. Only a few E[IgG] have been internalized by the drug-treated cells, whereas most control cells are filled with erythrocytes.

Using monoclonal antibodies, IgG_{2a}- and IgG_{2b}-mediated phagocytosis were separately analyzed in the presence of 25 μM Tfp and both were found to be depressed. The effect of Tfp on Fc-receptor expression also was assessed. Both control cells and cells treated with 25 μM Tfp bound ¹²⁵I-IgG_{2a} equally, indicating that Tfp at a concentration that inhibited Fc-mediated phagocytosis did not inhibit the expression of at least one of the major classes of Fc receptors present on the cell surface of J774.2 (6).

The effects of Tfp on Fc-mediated phagocytosis and on growth inhibition are reversible. Cells that were incubated with 25 μM Tfp for 1 h, washed, and grown in fresh complete medium in the absence of drug regained their capacity to carry

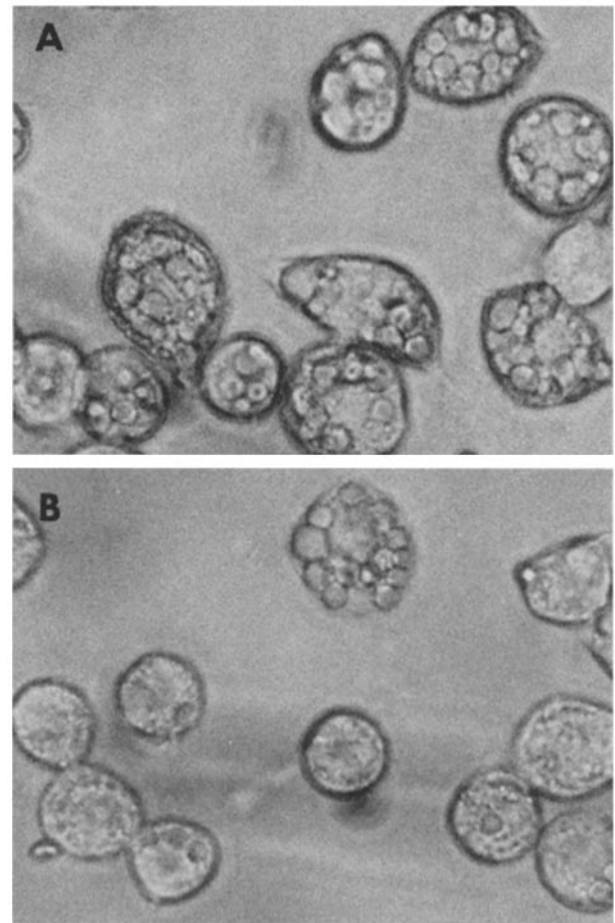


FIGURE 4 Fc-mediated phagocytosis in J774.16 in the absence (A) and presence (B) of Tfp. Cells were plated on glass cover slips and, in B, incubated with 25 μM Tfp for 2 h before assaying for phagocytosis in the presence of 25 μM Tfp. In the absence of drug (A), most macrophages have ingested several E[IgG]. A typical field was selected in (B) that clearly demonstrates the inhibition of phagocytosis in the presence of Tfp. $\times 2,700$.

out normal Fc-mediated phagocytosis within 1 h of drug removal (Fig. 5). Such cells continued to grow normally and gave no indication that they had been exposed to Tfp. The effect of treatment with 25 μM Tfp for 4 h was also reversible.

Effect of Tfp on Phagocytosis of Unopsonized Particles

Incubation of J774.16 with 25 μM Tfp for 1 or 3 h, plus an additional hour in the presence of latex beads, depressed ingestion of the beads (Fig. 6). As the concentration of Tfp was

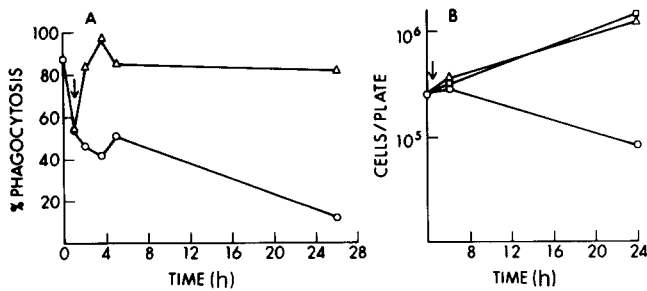


FIGURE 5 Reversibility of effects of Tfp on Fc-mediated phagocytosis (A) and cell growth (B) in J774.16. For phagocytosis, cells were prepared as described in the legend to Fig. 2. 25 μM Tfp were added to the complete medium (○). After 1 h (\downarrow), the medium containing drug was aspirated from half of the plates and fresh complete medium minus drug was added to these plates (Δ). At the indicated times, Fc-mediated phagocytosis was assayed as described in Materials and Methods using a 1:400 dilution of antiserum. For the reversal of cell growth, cells were grown in the presence of 25 μM Tfp for 1 h before the medium was removed (\downarrow). Cells were washed twice with DME, and fresh medium plus 25 μM Tfp (○) or minus drug (Δ) was added. A control growth curve (□) carried out completely in the absence of drug was done at the same time. Cell growth was monitored as described in Materials and Methods.

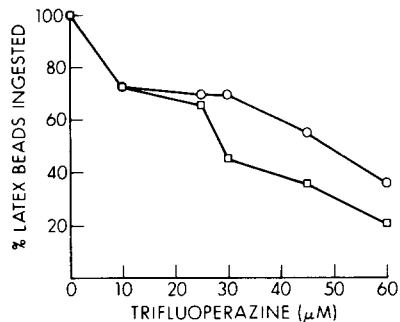


FIGURE 6 Ingestion of latex beads in J774.16 in the presence of trifluoperazine. Cells ($2.5\text{--}5 \times 10^6$) were plated in tissue culture dishes and after 2 h were incubated for either 1 h (○) or 3 h (□) with the indicated concentrations of Tfp. The cells were assayed after 1 h for latex bead ingestion in the presence of drug as described in Materials and Methods. 50 cell sections were counted for each

increased there was progressive inhibition of internalization (Fig. 6) in a pattern similar to that observed for Fc-mediated phagocytosis. As in the case of Fc-mediated phagocytosis, the effect of 25 μM trifluoperazine on latex bead ingestion was reversible.

Effect of Tfp on Lysozyme Secretion

Conditioned medium obtained from cells that had been incubated with either 25 μM Tfp or 50 nM taxol for 10 h was assayed for lysozyme activity. During a 10-min assay the change in absorbance at 540 nm was 0.13 for control, 0.14 for cells treated with 25 μM Tfp, and 0.16 for cells treated with 50 nM taxol.

Other Effects

Cells that had been incubated with either 25 μM Tfp or 50 nM taxol for 10 h excluded trypan blue to the same extent as control cells (>90%). Cells incubated with 25 μM Tfp for 2 or 5 h showed ~25% inhibition of both [³H]uridine and [³H]-

thymidine incorporation into TCA-precipitable material (data not shown), whereas taxol (1 μM) had no effect on macromolecular synthesis during a 2-h incubation. Macromolecular synthesis was measured as previously described (22). Thus, Tfp, unlike taxol, affects either uptake of these nucleic acid precursors and/or nucleic acid synthesis under the same conditions that it inhibits phagocytosis.

DISCUSSION

One approach to studying complex cellular functions such as phagocytosis is to select variants defective in different steps of the process and analyze both their function and pertinent biochemical pathways. The macrophagelike cell lines derived from J774 are particularly useful models for the study of phagocytosis since Fc- and non-Fc-mediated phagocytosis are easily assayed and stable variants defective in phagocytosis can be obtained. Although the first step in Fc-mediated phagocytosis appears to be specific binding of an opsonized particle to macrophage cell surface Fc receptors (23–28) little is known about how this interaction initiates the series of events that subsequently result in phagocytosis. In a recently completed analysis of Fc receptors in phagocytic variants of J774.2 (6), one variant was found to be defective in IgG_{2a} phagocytosis but exhibited normal binding of monomeric IgG_{2a}. It is likely that this variant was defective in some step distal to the binding function of the Fc receptor for IgG_{2a}. Isolation of a series of such variants could be extremely useful in establishing the biochemical steps involved in phagocytosis. A complementary approach is to analyze biochemical pathways that may be involved in phagocytosis. Calmodulin (see references 29–31), a ubiquitous Ca²⁺-binding protein, is a component of a number of Ca²⁺-dependent enzymes and may mediate the effects of Ca²⁺ on a wide variety of cellular functions such as microtubule assembly (32) and clathrin recruitment to the cell surface in receptor-mediated endocytosis of IgM in cultured human lymphoblastoid cells (33). The discovery that phenothiazines and W-7 inhibit the activity of calmodulin has provided a tool for appraising the role of calmodulin in phagocytosis by intact macrophages. In a study on ingestion of zymosan particles by rabbit polymorphonuclear leukocytes, Elferink (34) reported that chlorpromazine inhibited phagocytosis but did not discuss the possibility that the effect of the phenothiazine resulted from interference with the action of calmodulin. We have previously reported (7) that calmodulin constitutes ~1% of the soluble protein of J774.2 and that it activates bovine brain cyclic nucleotide phosphodiesterase and is immunologically similar or identical to calmodulin purified from bovine brain. Extracts of J774.2 exhibit Ca²⁺-calmodulin-dependent protein phosphorylation and myosin light chain kinase activity and complexes of ¹²⁵I-calmodulin and soluble proteins of J774.2 have been detected by polyacrylamide gel electrophoresis (7). To evaluate the potential role of calmodulin in the differentiated macrophagelike functions of J774 we studied the effect of the phenothiazine, Tfp, and the sulfonamide derivative, W-7, on phagocytosis in J774.16, a more highly phagocytic clone than J774.2. Both drugs were found to reversibly inhibit Fc-mediated as well as latex bead phagocytosis. The concentration of trifluoperazine (25 μM) required to inhibit Fc-mediated and non-Fc-mediated phagocytosis was the same as that needed to inhibit growth and similar to the concentration that inhibited activation of bovine brain cyclic nucleotide phosphodiesterase by calmodulin in vitro. Taxol, a drug that interacts specifically with microtubules, had no effect on either phagocytosis or the

activation of cyclic nucleotide phosphodiesterase at concentrations greatly exceeding those that inhibited the growth of J774.16. Although the studies reported herein do not constitute unambiguous evidence for the involvement of Ca^{2+} -calmodulin in phagocytosis, they do suggest the involvement of calmodulin in one or more steps distal to the interaction of particles with the macrophage cell membrane.

ADDENDUM

W-12 and W-13 are sulfonamide derivatives that act as calmodulin antagonists. W-12 lacks a single chloride present in W-13 and this alteration results in W-12 being a weaker calmodulin antagonist (Dr. H. Hidaka, personal communication). We found that 30 $\mu\text{g}/\text{ml}$ W-13 inhibited bovine brain calmodulin-stimulated cyclic nucleotide phosphodiesterase activity by 56% and Fc-mediated phagocytosis in J774.16 by ~65%. W-12 at 40 $\mu\text{g}/\text{ml}$ did not inhibit the phosphodiesterase and inhibited phagocytosis only 20%. This data provides a further indication that calmodulin may be involved in phagocytosis.

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