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Inhibition of Extracellular ATP-Mediated Lysis of Human Macrophages by Calmodulin Antagonists

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Abstract Lysis of human culture-derived macrophages by extracellular ATP has recently been described, and treatment of macrophages with interferon-γ rendered those cells significantly more sensitive to lysis. In addition, cell death occurred more rapidly in interferon (IFN)-treated cells than in untreated macrophages. In an attempt to identify the mechanism by which extracellular ATP affects macrophages, as well as to explore the differences between interferon-γ-treated and untreated macrophages, selected metabolic inhibitors were included in the lytic assays. Of the compounds tested, three antagonists of calmodulin-linked pathways (trifluoperazine, KN-62, and calmidazolium) blocked the ATP-mediated lysis of both interferon-γ-treated and colony-stimulating factor-treated macrophages in a dose-dependent manner. Early signals of the ATP ligation of the P_{2Z} purinoceptors of human macrophages included increases in cytosolic [Ca^{2+}] and depolarization of the plasma membrane. However, the inclusion of calmodulin antagonists in these assays did not abrogate either effect. These results suggest that the mechanism which mediates the efflux of ^{51}Cr-labeled proteins from ATP-lysed macrophages is distinct from calcium mobilization and membrane depolarization, and may involve the generation of secondary pores/channels in the plasma membrane via a calmodulin-linked pathway.

Key words: cytotoxicity, purine nucleotides, interferon-gamma, colony-stimulating factor, purinoceptor, necrosis

The intracellular role of ATP as an energy source has been recognized for many years. However, its role as an extracellular mediator is not as well described, but is becoming increasingly complex as more functions are being discovered. This nucleotide has been shown to influence many biological processes, including membrane permeability, platelet aggregation, neurotransmission, cardiac function, smooth muscle contraction, and has recently been described as an effector molecule in cytotoxic T lymphocyte (CTL)-mediated lysis of tumor target cells [Gordon, 1986; Di Virgilio et al., 1990]. The lysis of antigen-presenting cells, specifically macrophages, by CTL has been proposed to be an important factor in maintaining homeostasis of the immune response [Braakman et al., 1987; Lu et al., 1986], although analysis of mechanisms leading to macrophage death is not complete but likely involves ATP.

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Burnstock proposed that the biological effects of extracellular adenine nucleotides and adenosine are mediated by membrane surface receptors, since these compounds do not enter cells and must therefore evoke biological responses via ligand-receptor binding which trigger specific signal transduction mechanisms. The two major receptors were termed purinoceptors, whereby the P_1-purinoceptors are preferentially activated by adenosine/AMP and the P_2-purinoceptors bind ADP/ATP [Burnstock, 1981]. More recently, a further division of the P_2-purinoceptors was suggested since various cells differed in their responses to ATP analogues [Burnstock and Kennedy, 1985].

Whatever the biological effects of ATP are, it is unclear what signal transduction pathways are involved, and how they are triggered. In many cell types, the functional effects induced by ATP are associated with increased intracellular Ca^{2+} concentrations [Dubyak et al., 1988; Dubyak, 1991; Greenberg et al., 1988; Pillai and Bickle, 1992]. In turn, increased cytosolic Ca^{2+} can activate a variety of secondary signal transduction processes, including activation of pro-

tein kinase C (PKC), stimulation of calmodulin-dependent kinases, and increasing phospholipase A_2 activity [Dubyak, 1988]. Recently, the P_{2Y} subclass of purinoceptors has been linked to G-protein activation, since many of the subsequent ATP-induced events are inhibitable by pertussis toxin treatment [Dubyak et al., 1988; Cowen et al., 1989; Murphy and Tiffany, 1990]. In addition to expressing P_{2Y} receptors, macrophages appear to also express the P_{2Z} purinoceptor, which is purportedly responsible for the permeabilization of those cells upon exposure to micromolar and millimolar amounts of ATP [Blanchard et al., 1991; Means et al., 1991]. In those studies, the binding of ATP with the P_{2Z} receptor apparently induces the formation of nonselective pores or channels in cell membranes which are permeable to ions and low molecular weight (<950 Da) organic molecules, leading to cell death [Steinberg and Silverstein, 1989]. However, the signal processes involved in ATP-driven cytolysis are largely unknown, and are the focus of the present studies. In our previous studies, only ATP, and not adenosine or other nucleotides, was able to kill macrophages, indicating the specificity of the purinoceptor of human macrophages [Blanchard et al., 1991].

We have also reported that disparate responses to ATP were seen by interferon-γ-(IFNγ)-treated macrophages (MIFN) and granulocyte-macrophage colony-stimulating factor (GM-CSF)-treated macrophages (MCSF). The former cells were significantly more sensitive and more rapidly killed by this purine nucleotide than the latter type of macrophage [Blanchard et al., 1991]. In the present report, we used various metabolic inhibitors to establish that calmodulin was a common denominator in the signal transduction mechanisms triggered by ATP that led to lysis of both MIFN and MCSF. This Ca^{2+}-binding protein is known to activate at least 20 different enzymes, and plays an important regulatory role in cell proliferation and activation [Means et al., 1991]. The present studies indicate that calmodulin is also involved in cell death mechanisms of macrophages signalled by ATP stimulation.

MATERIALS AND METHODS

Materials

ATP (tissue culture grade), 2′-3′-O-(4-benzoylbenzoyl)ATP (Bz-ATP), cholera toxin, pertussis toxin, theophylline, papaverine (solubilized in 95% ethanol), dantrolene (in Me_2SO), and trifluoperazine (TFP) (in 70% ethanol) were obtained from Sigma Chemical Co. (St. Louis, MO). 1-[N,O-*bis*-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) (in Me_2SO) was obtained from Calbiochem (La Jolla, CA). 1-[*bis*-(4-chlorophenyl)methyl]-3-[2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl]-imidazolium chloride (calmidazolium) (solubilized in Me_2SO) was from Boehringer Mannheim (Indianapolis, IN). {1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-N,N,N′,N′-tetraacetic acid, pentaacetoxymethyl ester} (fura-2/AM) and bis(1,3-diethylthiobarbituric acid)trimethine oxonol (bis-oxonol) (solubilized in ethanol) were purchased from Molecular Probes, Inc. (Eugene, OR). Human recombinant IFNγ was kindly provided by Genentech Corp. (South San Francisco, CA) and human recombinant GM-CSF was a generous gift from Immunex (Seattle, WA).

Preparation of Human Culture-Derived Macrophages

Peripheral blood mononuclear cells were isolated from leukocyte buffy coats, obtained from normal volunteers at the Southwest Florida Blood Bank, as previously described [Blanchard et al., 1991]. Leukocytes were resuspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) containing 5% heat-inactivated human AB serum (Flow Laboratories, McLean, VA), 2 mM L-glutamine, 10 U/ml penicillin, 100 μg/ml streptomycin, 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, and 5×10^{-5} M β-mercaptoethanol, and will subsequently be referred to as complete medium, and contains 0.4 mM each of Ca^{2+} and Mg^{2+} in divalent ion concentration (GIBCO). Cells were then incubated on gelatin-coated tissue culture flasks for 1 h at 37°C to allow adherence of monocytes. Nonadherent cells were removed by washing the flasks with warm medium and the adherent cells were cultured with fresh medium in the presence of 1,000 U/ml of IFNγ for the generation of MIFN or with 1,000 U/ml of GM-CSF to produce MCSF for a final incubation time of 3 to 4 days. Culture-derived macrophages were recovered by vigorous pipetting of the monolayers after the addition of cold phosphate-buffered saline (PBS). All media and reagents contained less than 0.1 ng/ml of endotoxin as determined by the Limulus Amoebocyte Lysate assay (M.A. Biologics, Walkersville, MD).

Measurement of ATP-Mediated Cytotoxicity

Unless noted otherwise, a 2 h or a 6 h ^{51}Cr-release assay was used to measure the effect of ATP on cytokine-treated macrophages, and was performed essentially as previously described [Blanchard et al., 1991]. Macrophages were labeled for 1 h in 0.5 ml of medium with sodium [^{51}Cr] chromate, washed, and then added to serial dilutions of ATP in microtiter wells at 1×10^4 cells/well in a final volume of 0.2 ml in each well. All determinations were done in triplicate, and the SEM of all assays was calculated and was typically 5% of the mean or less. Student's *t*-tests were performed to identify significant differences between treatments.

To measure the effects on cell lysis of macrophages during short-term exposure to ATP, an oil separation method was used [Robb et al., 1981]. Briefly, 0.25 ml of radiolabeled macrophages (at 1×10^5 cells/ml) was layered over 0.1 ml of a binding oil mixture (comprised of 84% silicon oil [Aldrich] and 16% paraffin oil [Fisher Scientific]) in microcentrifuge tubes. In a 37°C water bath, 0.25 ml of medium or medium containing 2.5 mM ATP was quickly added. At 1 min intervals, tubes were removed and centrifuged at 12,000*g* for 30 s to sediment cells through the oil, and the supernatants were counted to determine rapid release of ^{51}Cr. At each time point, a medium control was included to determine spontaneous release, which was $<5\%$ lysis over 20 min for both MIFN and MCSF.

Fluorometric Measurement of Cytosolic Ca^{2+}

$[Ca^{2+}]_i$ was determined essentially as previously described [Liu et al., 1992]. Briefly, MIFN and MCSF were incubated in RPMI 1640 medium containing 2.5% fetal calf serum (Hyclone Labs, Logan, UT) and 10 μM fura2/AM at 37°C for 30 min, then diluted fivefold with medium and incubated an additional 30 min. Cells were then washed and resuspended at 2×10^6 cells/ml in PBS with or without 1 mM Ca^{2+}. In those experiments without Ca^{2+}, 1 mM EDTA was added to PBS to ensure that no residual cations were present. Fluorescence was measured with a Shimadzu RF-5000 fluorospectrophotometer (Columbia, MD) with a magnetic stirrer and thermostatically controlled cuvette holder maintained at 37°C.

Measurement of Membrane Potential

Changes in plasma membrane potential of macrophages stimulated with ATP was performed essentially as described [Soltoff et al., 1992]. Briefly, 8×10^5 washed macrophages were suspended in 2 ml PBS containing 1 mM Ca^{2+} and 100 nM bis-oxonol. Fluorescence was measured as described above at the wavelength pair 540–580 nm.

RESULTS

Kinetics of Lysis of MIFN and MCSF by ATP

To determine whether different mechanisms are involved in the lysis of MIFN and MCSF by ATP, it was important to fully characterize their kinetics and ultimate sensitivity to this purine nucleotide. While our earlier report indicated that MCSF were relatively insensitive to lysis by ATP within the 6 h assay period [Blanchard et al., 1991], it was apparent that longer incubations might result in more complete killing, thus approaching the effect of ATP on MIFN. For these experiments, ^{51}Cr-labeled MIFN and MCSF were incubated with serial dilutions of ATP, and assays were harvested at 2, 6, 12, 20, and 30 h (Fig. 1). At a concentration of 0.62 mM ATP, MIFN were rapidly killed, while their lysis at lower concentrations of ATP occurred more slowly. In contrast, the ATP-mediated lysis of MCSF differed from MIFN in that the former cells were less sensitive and required a longer incubation period to effect lysis. While 0.62 mM ATP was sufficient to kill more than 70% of MIFN at any time point assessed, this concentration evoked only 7 ± 0.5% lysis of MCSF at 2 h, which increased only to 55 ± 3.6% at 30 h of incubation. Even at the highest concentration of ATP used, the lysis of MCSF at 2 h was 19 ± 1.0% and was maximally 77 ± 4.1% at 30 h. These data indicate that increasing the amount of ATP in the assay could not elicit MCSF death at the same time course of lysis seen with MIFN.

Previous studies on the ATP-mediated lysis of murine macrophages and mast cells indicated that ATP opens a channel or pore in their membranes, resulting in very rapid release of intracellular contents and are generally small molecules less than 1,000 daltons in molecular weight [Murphy and Tiffany, 1990; Steinberg and Silverstein, 1989]. On the other hand, ^{51}Cr nonspecifically binds to macromolecules in the cytoplasm and is an indication of membrane disruption [Henney, 1974]. To determine the kinetics of

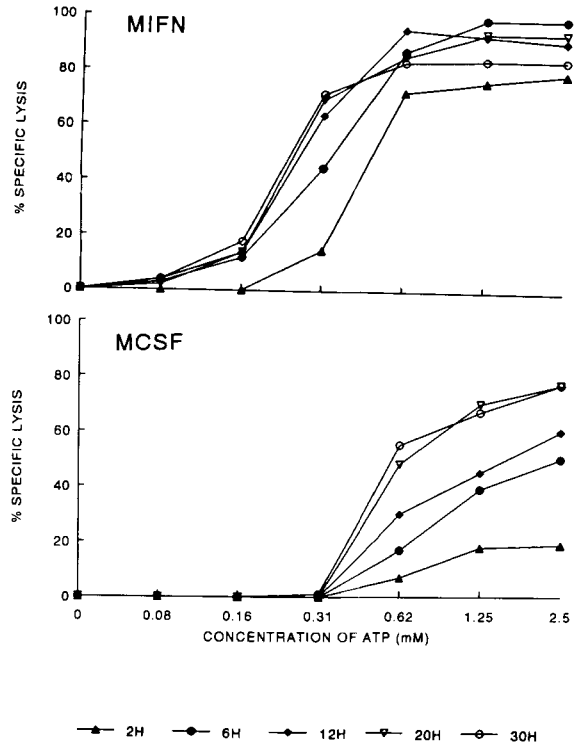

Fig. 1. Time course of ^{51}Cr-release from MIFN (**top**) and MCSF (**bottom**) stimulated with ATP. Macrophages were cultured for 3–4 days with the indicated cytokines, and used as target cells in a ^{51}Cr-release assay in the presence of increasing concentrations of ATP, as described under Materials and Methods. Supernatants were harvested at the indicated time during incubation, counted on a gamma counter, and the percent specific lysis was calculated. Numbers are means ± S.E. of triplicate determinations and data are representative of 3 experiments that were performed with similar results.

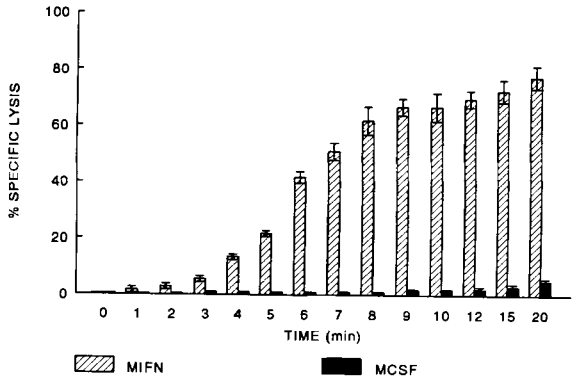

Fig. 2. Rapid time course of ^{51}Cr-release from MIFN and MCSF stimulated with ATP. Radiolabelled macrophages were prepared as described for Figure 1, except that 0.25 ml of cells was layered over 0.1 ml of an oil mixture in a microfuge tube. Medium or ATP-containing medium (0.25 ml) was quickly added and tubes were incubated at 37°C. At the indicated times, tubes were centrifuged at 12,000g for 30 s to remove radiolabelled cells, and the supernatants collected and counted, as described. Final concentration of ATP was 1.25 mM (MIFN) or 2.5 mM (MCSF). Data presented are means ± S.E. of triplicate determinations, and are representative of 2 experiments that were performed with similar results.

ATP lysis of MIFN, ^{51}Cr release was determined at 1 min intervals by centrifuging radiolabelled cells through an oil mixture in microcentrifuge tubes, and counting the supernatants (Fig. 2). However, unlike the rapidly increased permeability seen by murine macrophages after ATP stimulation, the release of ^{51}Cr-labeled macromolecules from human MIFN was barely detectable at 3 min of incubation with ATP, with half-maximal lysis apparent only after 6 min of incubation.

Effect of Metabolic Inhibitors on ATP-Mediated Lysis of Macrophages

From the kinetic studies, lysis of human macrophages was apparently not simply due to immediate leakage via ATP-induced pore or channel formation in the plasma membrane, but cell death could be mediated by intracellular signaling systems. To explore which ATP-mediated signal transduction might be involved, inhibitors of several different metabolic pathways were examined for their ability to modulate ATP cytolysis of MIFN and MCSF. For these experiments, cytotoxicity assays using MIFN were incubated for only 2 h to ensure that the responses to ATP were solely mediated by IFN-related mechanisms. MCSF were stimulated with ATP for 6 h since this period was sufficient to demonstrate significant lysis. (Untreated macrophages were not routinely included in these assays because no significant differences in their responses to ATP were noted between MCSF and control cells in preliminary studies, and cytokine-treated macrophages were typically more viable than cells incubated in medium alone; approximately 90% for the former cells, and 75% for the latter.)

As shown in Table I, the addition of 1 μM staurosporine, a PKC inhibitor, had no significant effect on the ATP-mediated lysis of either macrophage target. Similarly, the G-protein inhibitor, pertussis toxin, was not able to block the effect of ATP. The adenylate cyclase activator, cholera toxin, also had no effect. However, two calmodulin antagonists, calmidazolium and TFP, were found to decrease ATP-mediated lysis of MIFN and MCSF. At the ATP concentrations tested, calmidazolium suppressed the lysis of MIFN more effectively than that of MCSF, while

TFP was uniformly suppressive on both macrophage targets. The specific multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) antagonist, KN-62 [Tokumitsu et al., 1990], blocked the effect of ATP on both MIFN and MCSF. To further define the role of this ubiquitous Ca^{2+}-binding protein, two selective inhibitors of phosphodiesterases, papaverine and theophylline, were also evaluated for their ability to block ATP-mediated lysis of macrophages, since many of these enzymes are known to be activated by Ca^{2+}/calmodulin. As shown, neither papaverine nor theophylline had any significant effect on either MIFN or MCSF. It should be noted that all the reagents used had no deleterious effect on cell viability, and the vehicles of all of these inhibitors, either Me_2SO or ethanol, had no effect on the ATP-mediated lysis of either macrophage targets at the concentrations used in these assays (data not shown).

Dose Response Curves of Calmodulin Inhibitors

To further examine the inhibitory effect of the calmodulin-linked antagonists, TFP, KN-62, and calmidazolium, dose dependence curves were generated using various concentrations of each of these compounds (Fig. 3). As noted above, the lytic assays using MIFN were performed for 2 h in the presence of 0.62 mM ATP, and MCSF targets were lysed by 2.5 mM ATP in a 6 h assay. The indicated inhibitors were added to macrophages 15 min before the introduction of ATP. KN-62 appeared to be the most effective inhibitor with both MIFN and MCSF, with as little as 0.12 μM seen to block 52 ± 3% and 25 ± 2% of the ATP-mediated lysis of each target, respectively. The higher concentrations of KN-62 almost completely abrogated the effect of ATP on these target cells. It should also be noted that the addition of KN-62 at the same time as ATP, and as late as 5 min later, effectively blocked the lytic effect of this purine nucleotide (data not shown), indicating that preincubation with the inhibitor was not necessary. Similarly, TFP was uniformly effective in inhibiting ATP-mediated lysis of both MIFN and MCSF. The third compound, calmidazolium, appeared to be somewhat more effective in blocking the lysis of MIFN than MCSF at all concentrations, although 95% and 90% inhibition, respectively, was noted at 5 μg/ml of calmidazolium. Finally, there was no direct toxicity of any of these compounds at the concentrations used in these assays seen against either MIFN or MCSF as assessed by trypan blue exclusion and by the spontaneous release of ^{51}Cr-labeled cells.

Lysis of Macrophages in Ca^{2+}-Free Medium

The ligand that activates P_{2Z} receptors, responsible for mediating lysis of macrophages, is shown to be the tetrabasic anion ATP^{4-}, which is present as only a minor equilibrium component in solutions containing divalent cations [Steinberg and Silverstein, 1987]. To completely evaluate the role of Ca^{2+} in the ATP-mediated lysis of human macrophages, cytotoxicity assays were performed in Ca^{2+}-free/Mg^{2+}-free Hanks' balanced salts solution (HBSS) supplemented with 2 mg/ml bovine serum albumin (BSA) and 5 mM HEPES buffer (Fig. 4). As expected, the lysis of both MIFN and MCSF was greater in the

TABLE I. Effect of Metabolic Inhibitors on ATP-Mediated Lysis of Macrophages†

		% Specific lysis ± S.E. (% inhibition)	
Inhibitor	Concentration	MIFN + 0.62 mM ATP	MCSF + 2.5 mM ATP
None	—	74 ± 3.3	38 ± 1.0
Staurosporine	1 μM	67 ± 3.5 (9)	38 ± 1.9 (0)
Pertussis toxin	1 μg/ml	73 ± 2.4 (1)	39 ± 2.0 (0)
Cholera toxin	200 ng/ml	73 ± 6.1 (1)	37 ± 0.9 (3)
Calmidazolium	2 μg/ml	16 ± 1.0 (78)*	29 ± 0.7 (24)*
KN-62	1 μM	12 ± 0.9 (84)*	8 ± 0.4 (79)*
TFP	10 μM	11 ± 0.7 (85)*	6 ± 0.5 (84)*
Theophylline	2 μM	69 ± 5.3 (11)	38 ± 2.3 (0)
Papaverine	100 μM	72 ± 5.5 (3)	39 ± 1.8 (0)

†Radiolabelled macrophages were used as target cells in the presence of the indicated concentration of ATP in a 2 h (MIFN) or 6 h (MCSF) ^{51}Cr-release assay. The indicated inhibitors were added to macrophage target cells for 15 min at ambient temperature prior to the addition of ATP. Data are representative of 3–6 experiments performed with similar results.
*Significant inhibition of lysis as compared to control samples ($P < 0.05$).

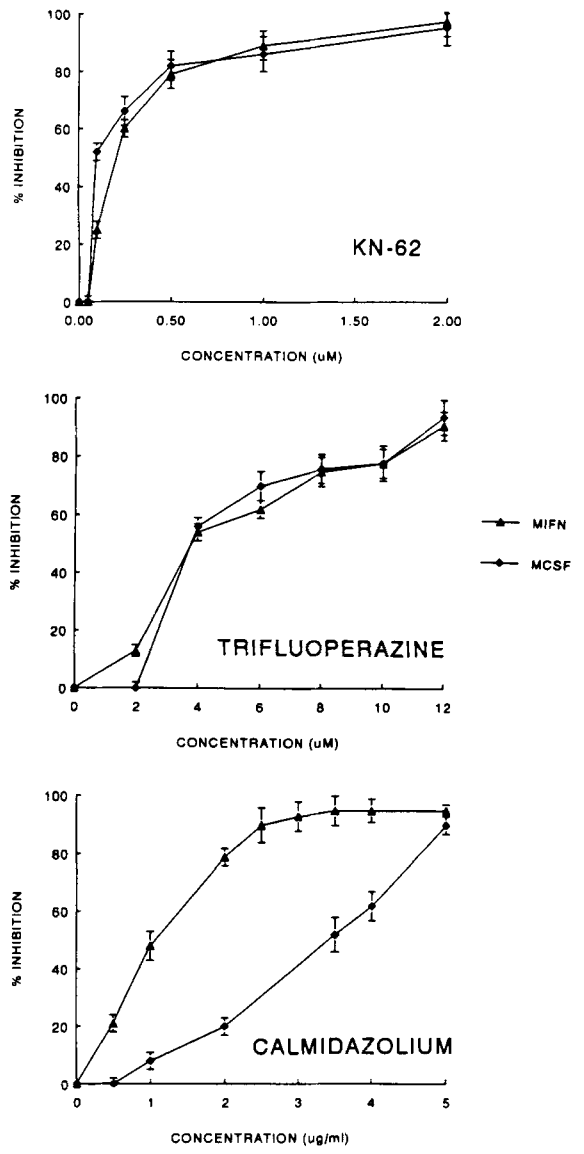

Fig. 3. Dose dependence on KN-62 **(top)**, trifluoperazine **(center)**, or calmidazolium **(bottom)** of decreased lysis of MIFN and MCSF by ATP. Cytokine-treated macrophages were used as target cells in a 2 h (MIFN) or a 6 h (MCSF) ^{51}Cr-release assay in the presence of the indicated concentrations of each inhibitor. Cells were pre-incubated with inhibitors for 15 min at ambient temperature, and then MIFN were challenged with 0.62 mM ATP, with control specific lysis of 78 ± 4%, and MCSF were lysed with 2.5 mM ATP, with lysis of 43 ± 2%. Numbers are means ± S.E. of the calculated percent inhibition compared to control lysis using triplicate determinations. Data are representative of 3 experiments that were performed with similar results.

absence of Ca^{2+} in the medium, a condition which effectively increased [ATP^{4-}], and suggested the involvement of the P_{2Z} purinoceptor. Additionally, the presence of 5 μM KN-62 abrogated ATP-mediated lysis of macrophages, whether extracellular Ca^{2+} was present in the medium or not.

Cytosolic [Ca^{2+}] Changes in MIFN and MCSF

Since activation of calmodulin requires Ca^{2+}, it was necessary to determine whether ATP stimulation of human macrophages resulted in increased cytosolic [Ca^{2+}], whether from extracellular influx or release from intracellular stores. Furthermore, ATP-induced Ca^{2+} mobilization has been reported in human monocytic cells at sub-lytic (10 μM) concentrations, presumably via a P_{2Y} purinoceptor [Cowen et al., 1989]. These experiments were therefore performed using two concentrations of ATP, sub-lytic (50 μM for MIFN and 250 μM for MCSF) and lytic (1 mM for both cells). As seen in Figure 5, in the absence of extracellular Ca^{2+}, both concentrations of ATP were able to increase [Ca^{2+}]$_i$ in MIFN and MCSF (the number in parentheses is Δ[Ca^{2+}]$_i$ induced by ATP). As expected, the addition of 1 mM Ca^{2+} to the medium resulted in greater levels of cytosolic Ca^{2+}. From 4 experiments, the increases in cytosolic [Ca^{2+}] were not statistically significant whether induced by the sub-lytic or the lytic concentrations of ATP. It should also be noted that at ATP concentrations of as little as 1 μM, detectable changes in [Ca^{2+}]$_i$ were seen for both MIFN and MCSF (data not shown).

As depletion of extracellular Ca^{2+} resulted in increased sensitivity of cells to lysis, presumably by the elevation of the more effective anion, ATP^{4-}, and since Ca^{2+} in required for the activation of calmodulin, it is necessary to block the release of cytosolic Ca^{2+} to confirm a role for Ca^{2+}/calmodulin in the ATP-induced lysis of macrophages. This is achieved by the inclusion of dantrolene, a drug which inhibits the release of Ca^{2+} from intracellular stores [Munshi et al., 1993; Ward et al., 1986], in the lytic assays. In preliminary studies, 80 μM of dantrolene was found to decrease the ATP-stimulated [Ca^{2+}]$_i$ increase in both MIFN and MCSF, from 253 to 38 nM and from 288 to 53 nM, respectively. As shown in Figure 6, there is a dose-dependent abrogation of the ATP-mediated lysis of both MIFN and MCSF by increasing concentrations of dantrolene. The assay was performed in Ca^{2+}-free/Mg^{2+}-free HBSS medium, which was supplemented with 2 mM EDTA to ensure complete elimination of extracellular Ca^{2+} and avoid potential complications by its influx.

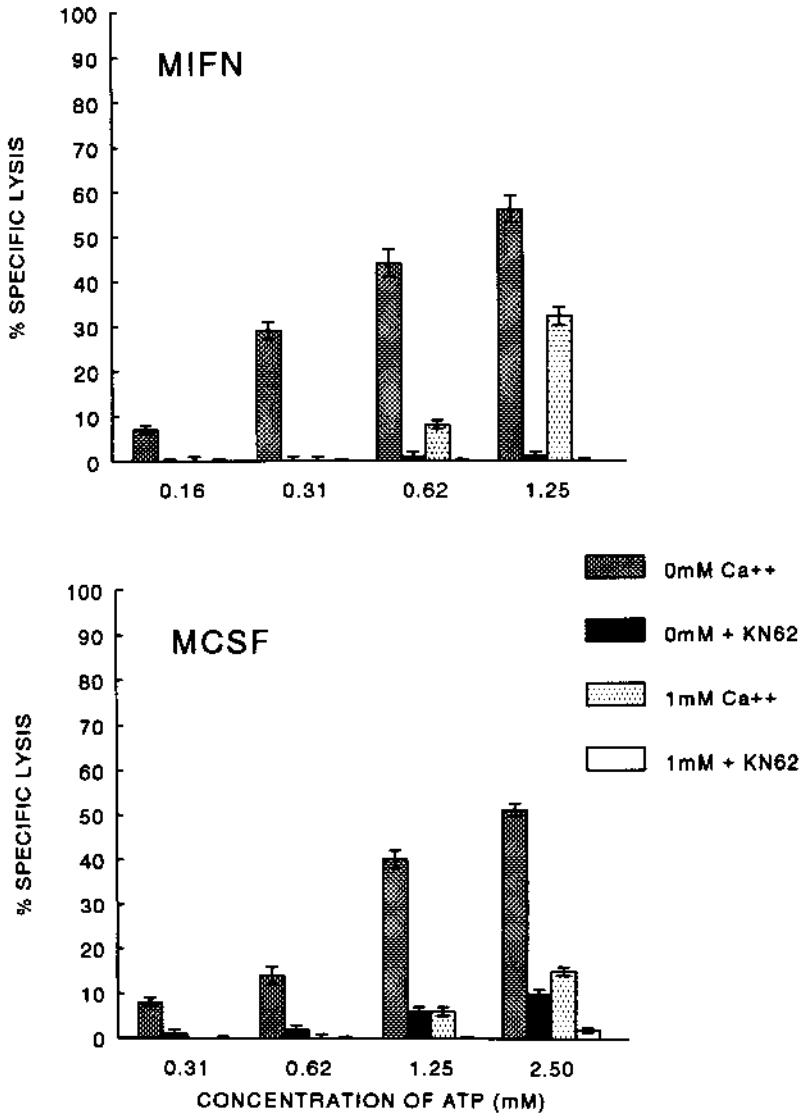

Fig. 4. Effect of extracellular Ca^{2+} on lysis of macrophages; inhibition by KN-62. Cytokine-treated macrophages were used as target cells in a 2 h (MIFN, **top**) or a 6 h (MCSF, **bottom**) ^{51}Cr-release assay in the presence of the indicated concentrations of ATP. The medium used in these assays was Ca^{2+}-free/Mg^{2+}-free HBSS, containing 2 mg/ml BSA ("0 mM Ca^{2+}"), or containing 1 mM $CaCl_2$ ("1 mM Ca^{2+}"). Assays were preincubated for 15 min at ambient temperature in the absence or presence of 5 μM KN-62, where indicated, prior to the addition of ATP. Data presented are means ± S.E. of triplicate determinations, and are representative of 2 experiments that were performed with similar results.

Effect of KN-62 on ATP-Induced Changes in Cytosolic [Ca^{2+}]

Previous studies have shown that KN-62 can directly inhibit Ca^{2+} influx in insulin-secreting cells by direct interaction with specific L-type Ca^{2+} channels [Li et al., 1992]. To determine whether KN-62 blockage of ATP-mediated lysis of macrophages was due to a similar inhibition of calcium mobilization, 2 μM KN-62 was added to fura-2 loaded MIFN and MCSF 3 min prior to the addition of 1 mM ATP (Table II). At this concentration of KN-62, ATP-mediated lysis of macrophages was almost completely abrogated, yet there was no significant inhibition of changes in cytosolic [Ca^{2+}], in both the presence or absence of extracellular Ca^{2+}.

Effect of KN-62 on Bz-ATP Induced Changes in Cytosolic [Ca^{2+}]

Bz-ATP is reportedly a selective agonist of the P_{2Z} purinoceptor [Dubyak et al., 1988], and has been shown to be more potent than ATP in the

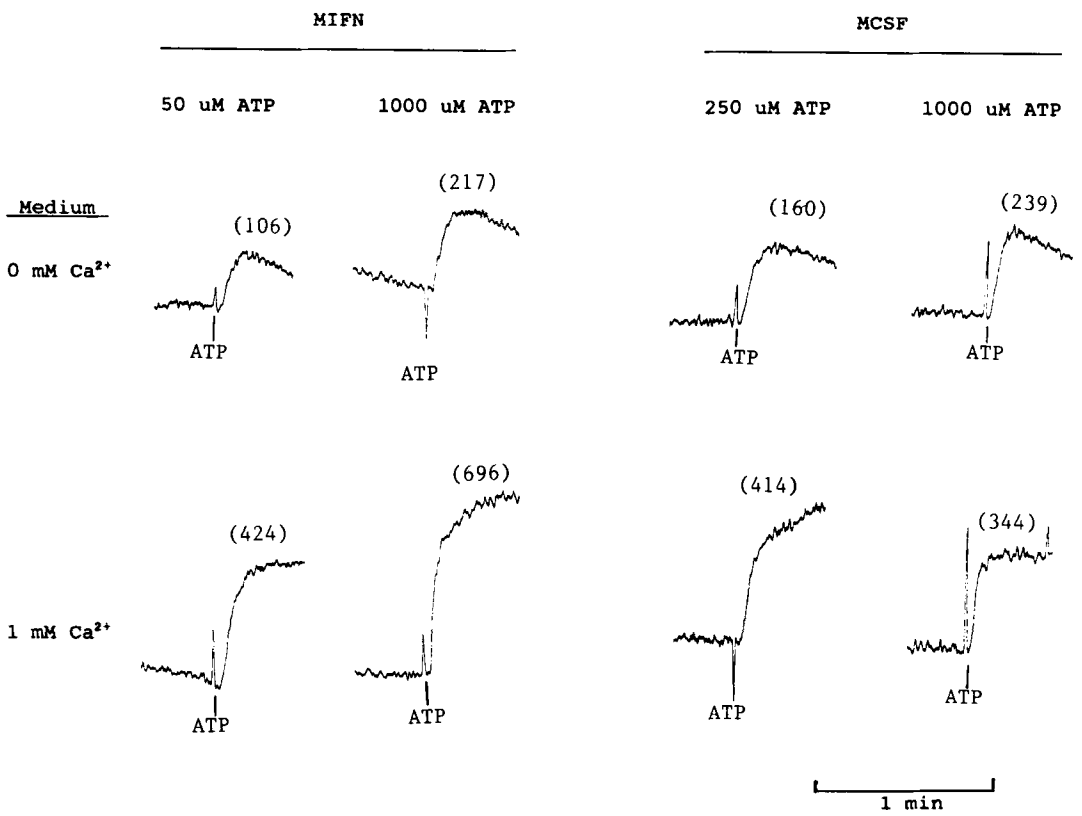

Fig. 5. Measurement of $[Ca^{2+}]$ influx and release from intracellular stores in ATP-stimulated macrophages. Cytosolic $[Ca^{2+}]$ in MIFN **(left)** and MCSF **(right)** loaded with fura-2/AM was measured as described under Materials and Methods. All measurements were made at 37°C using 2×10^6 cells/ml in PBS with or without 1 mM Ca^{2+}. At the indicated point, either a sub-lytic concentration of ATP (50 μM for MIFN and 250 μM for MCSF) or a lytic concentration (1 mM of ATP for both MIFN and MCSF) was added. Baseline $[Ca^{2+}]_i$ for MIFN prior stimulation with ATP was 322 ± 15 nM, and for MCSF was 223 ± 12 nM. Number in parentheses represents the change in cytosolic $[Ca^{2+}]$ in nM after ATP addition, and tracings represent 1 of 4 experiments that were performed with similar results.

lysis of murine [Greenberg et al., 1988; El-Moatassim and Dubyak, 1992] and human macrophages (lysis of MIFN by 0.63 mM ATP and Bz-ATP was 35 ± 2% and 78 ± 2% specific lysis, respectively). In previous studies on the effect of Bz-ATP on cytosolic Ca^{2+} mobilization, concentrations of this ATP analogue greater than 30 μM quenched the fura-2 signal [McMillian et al., 1993], and our preliminary experiments confirmed this observation. As shown in Figure 7A, 30 μM of Bz-ATP effectively stimulated release of Ca^{2+} from intracellular stores in both MIFN and MCSF. The maximal $[Ca^{2+}]_i$ was unable to be determined since the addition of Triton X-100 to the cells (second arrow) resulted in quenching of the signal. However, it was apparent that the presence of 2 μM KN-62 did not have a significant effect on the magnitude of Bz-ATP-stimulated Ca^{2+} release. Similarly, in the presence of 1 mM extracellular calcium (Fig. 7B), KN-62 had little effect. Although the kinetics of increase seemed slightly slower with KN-62, the overall changes in $[Ca^{2+}]$ was the same when the peaks are super-imposed.

Effect of KN-62 on ATP-Induced Depolarization of Macrophage Membrane Potential

Another consequence of binding of the P_{2Z} receptor with ATP is depolarization of the plasma membrane [Di Virgilio et al., 1988]. As shown in Figure 8, both MIFN and MCSF responded to ATP by loss of membrane potential, with the magnitude of reaction of MIFN being larger than MCSF. In addition, KN-62 did not suppress the depolarization of either type of macrophage.

DISCUSSION

Consequences of the interaction of ATP with cellular receptors leads to a variety of effects,

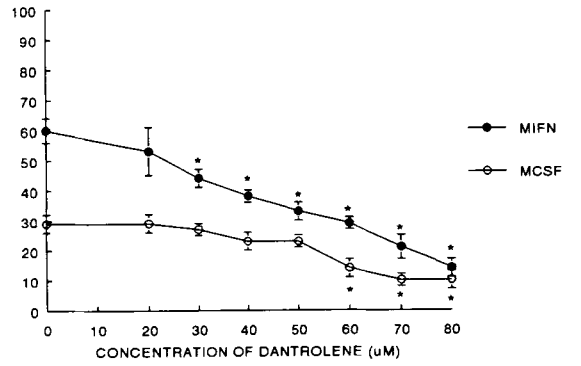

Fig. 6. Inhibition of ATP-mediated lysis of macrophages by dantrolene. Cytokine-treated macrophages were used as target cells in a 2 h (MIFN) or a 6 h (MCSF) ^{51}Cr-release assay in the presence of the indicated concentrations of dantrolene. The medium used in these assays was Ca^{2+}-free/Mg^{2+}-free HBSS, containing 2 mg/ml BSA and 2 mM EDTA. Assays were pre-incubated for 15 min at ambient temperature in the absence or presence of dantrolene, prior to the addition of 0.13 mM ATP (for MIFN) or 1.25 mM (for MCSF). Data presented are means ± S.E. of triplicate determinations, and are representative of 3 experiments that were performed with similar results. Asterisks indicate statistically significant inhibition of lysis ($P < 0.05$) as compared with medium controls.

TABLE II. Effect of KN-62 on ATP-Induced Changes in Cytosolic $[Ca^{2+}]$*

Inhibitor	$[Ca^{2+}]o$ (mM)	$\Delta[Ca^{2+}]i$ MIFN	$\Delta[Ca^{2+}]i$ MCSF
Me_2SO	1	394	310
KN-62	1	380	272
Me_2SO	0	131	120
KN-62	0	150	151

*Cytosolic $[Ca^{2+}]$ in MIFN and MCSF loaded with fura-2/AM was measured as described under Materials and Methods. All measurements were made at 37°C using 2×10^6 cells/ml in PBS with or without 1 mM Ca^{2+}, as indicated. To determine whether KN-62 inhibited the influx or release from intracellular stores of Ca^{2+}, KN-62, or its vehicle Me_2SO, was added to the assay at a final concentration of 2 μM at 3 min prior to the addition of 1 mM ATP. Numbers represent the change in cytosolic $[Ca^{2+}]$ in nM, and are representative of 1 of 3 experiments that were performed with similar results.

depending on the type of receptor involved and in intracellular signalling pathways stimulated. On murine and human macrophages, evidence exists for the presence of at least two types of purinoceptors: P_{2Y} receptors, which mediates Ca^{2+} mobilization and activates inositol phospholipid hydrolysis via G-protein-dependent mechanisms [Dubyak, 1991; Fine et al., 1989; Xing et al., 1992], and P_{2Z} receptors, which induces rapid formation of pores/channels that allows permeabilization of membranes to both ions and small organic molecules [Steinberg et al., 1987; Steinberg and Silverstein, 1989]. Electrophysiological studies of murine mast cells and macrophages suggest the ATP-induced formation of variable-sized pores, whose diameter reportedly increase in size at higher concentrations of ATP^{4-} ionic species [Tathan and Lindau, 1990]. In those cells, pore formation was reversed by the removal of ATP from the medium, and the addition of Mg^{2+} served to assist in sealing the membrane pores. Additionally, we and others have shown that the continuous presence of ATP was not needed for lysis of susceptible target cells to occur. Human macrophages [Blanchard et al., 1991] and murine thymocytes [Di Virgilio et al., 1989], when incubated for 15 min with ATP, washed, and then cultured without extracellular ATP, are as readily killed in 6 h as if they were incubated the entire period with ATP. Taken together, these studies suggest that ATP-driven pore formation may be required but not sufficient to cause loss of cell viability in some cells, and that intracellular signaling events are required to effect lysis.

The effects of P_{2Z} receptor activation (i.e., collapse of membrane potential and elevation of cytosolic $[Ca^{2+}]$) were thought to be directly generated by formation of pores/channels [Steinberg and Silverstein, 1989]. Secondary responses, such as activation of G-proteins, kinases, and other enzymes, were therefore dependent on a signaling cascade induced by those primary actions. Recently, however, binding of the P_{2Z} receptor of murine macrophages by ATP was shown to directly activate phospholipase D activity, suggesting that pore formation may not be the only immediate consequence of ATP^{4-} stimulation of macrophages [El-Moatassim and Dubyak, 1992].

The mechanism by which ATP causes cell death remains unclear. In some studies, the loss of viability of ATP-stimulated hepatocytes correlated with dissipation of mitochondrial membrane potential, and required a sustained increase in cytoplasmic $[Ca^{2+}]$ [Zoeteweij et al., 1992]. Other investigators have suggested that ATP might stimulate apoptosis of some cells [Zychlinsky et al., 1991], although our preliminary studies using human macrophages indicate that their cell death is morphologically consistent with necrosis rather than apoptosis (manuscript submitted). The focus of the present study, therefore, was to dissect the molecular path-

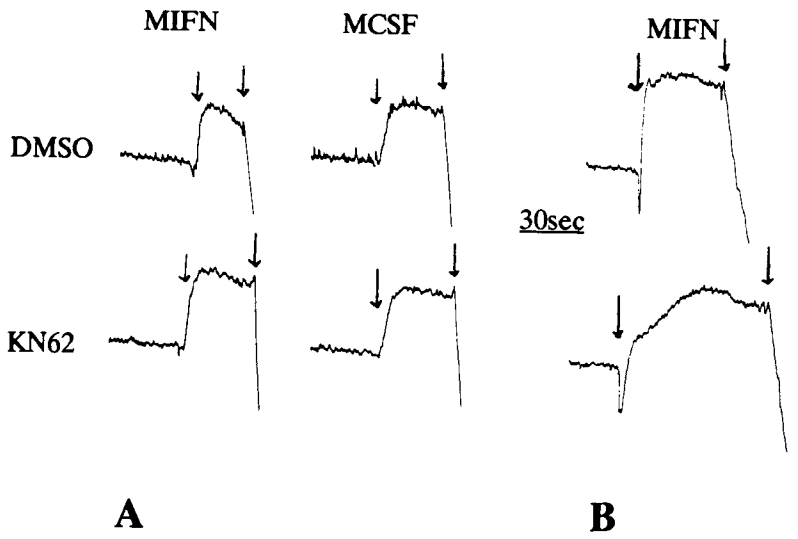

Fig. 7. Measurement of $[Ca^{2+}]$ influx and release from intracellular stores in Bz-ATP-stimulated macrophages. Cytosolic $[Ca^{2+}]$ in MIFN and MCSF loaded with fura-2/AM was measured as described under Materials and Methods. All measurements were made at 37°C using 2×10^6 cells/ml in PBS with 0 mM Ca^{2+} (**A**) or with 1 mM Ca^{2+} (**B**), and in the presence of 2 μM KN-62 or the equivalent amount of its vehicle Me_2SO ("DMSO"). At the first arrow, Bz-ATP was added to a final concentration of 30 μM, and the second arrow indicates when Triton X-100, to a final concentration of 0.5%, was added to effect permeabilization of the cells. Tracings represent 1 of 3 experiments that were performed with similar results.

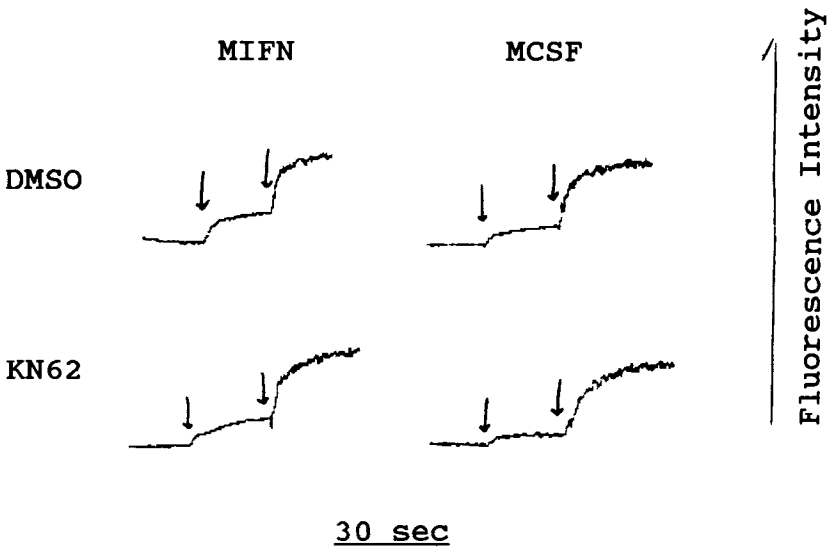

Fig. 8. Changes in membrane potential induced by ATP: effect of KN-62. ATP-induced depolarization of MIFN (**left**) and MCSF (**right**) was measured as described under Materials and Methods. All measurements were made at 37°C using 4×10^5 cells/ml in PBS with 1 mM Ca^{2+}, and with 100 nM bis-oxonol. KN-62, at a final concentration of 2 μM, or the equivalent amount of its vehicle Me_2SO ("DMSO") was added 3 min prior to ATP. At the first arrow, ATP was added to a final concentration of 1 mM, and the second arrow indicates when Gramicidin D, to a final concentration of 100 nM, was added to completely depolarize macrophage plasma membranes. Tracings represent 1 of 4 experiments that were performed with similar results.

way(s) responsible for the ATP-mediated lysis of human macrophages. To perform these experiments, a variety of metabolic inhibitors were chosen. While the specificity of many of these compounds remains questionable, their concerted use can allow the dissection of ATP-induced signal transduction pathways in both MIFN and MCSF. Also, since many of these compounds affect multiple systems, it was important to use several similar and overlapping inhibitors to pinpoint the most important pathway. In this regard, the inability of either

pertussis toxin or cholera toxin to block ATP-mediated lysis of MIFN and MCSF indicates that G-proteins are not involved in cell death. P_{2Y} purinoceptors appear to utilize GTP-binding proteins as a second messenger system [Cowen et al., 1989; Dubyak et al., 1988; Murphy and Tiffany, 1990], and macrophages are known to express this subclass of receptors [Cowen et al., 1989; Greenberg et al., 1988]. Nevertheless, the use of pertussis and cholera toxins in our system allowed us to eliminate the involvement of P_{2Y} purinoceptors in ATP lysis of MIFN and MCSF.

The efficacy of the accepted calmodulin-specific antagonists, TFP and calmidazolium, to block ATP-mediated lysis of macrophages suggested that this Ca^{2+}-binding protein is involved in triggering cell death. While some reports have indicated that these compounds can also suppress PKC activity [Aftab et al., 1991; Mazzei et al., 1984], the inability of staurosporine, a potent PKC inhibitor, to interfere with ATP-mediated lysis theoretically eliminates the involvement of this kinase in the lytic pathway. Interestingly, TFP was shown in other studies to augment the permeabilization of transformed murine 3T3 cells by ATP, and it was suggested that Ca^{2+}/calmodulin may play a role in the maintenance of membrane integrity of these cells [De and Weisman, 1984].

At this point, the specificity of KN-62 for CaM II kinase is the most convincing evidence on the involvement of calmodulin in ATP-mediated lysis of macrophages. This enzyme is a multimeric complex that has broad substrate specificity and requires activation by Ca^{2+}/calmodulin, and seems to be a common point in the lysis of both MIFN and MCSF. It is a "multifunctional" enzyme that is well characterized in the mammalian central nervous system, but its role in other cells is less defined [Colbran et al., 1989; Schulman, 1988]. The ability of KN-62 to completely block lysis of both MIFN and MCSF suggests that this enzyme complex is important in the signal transduction pathway triggered by ATP, and proposes a new role for CaM II kinase, i.e., mediating cell death. Alternatively, it is also possible that the specificity of KN-62, like other metabolic inhibitors, is not absolute and this compound may affect other enzyme systems not yet identified.

From the results of the present study, it is tempting to speculate that the following events occur during ATP-mediated lysis of human macrophages: a) ATP initially induces increased cytosolic $[Ca^{2+}]$, via pore formation or release from intracellular stores; b) elevated $[Ca^{2+}]_i$ activates calmodulin, which in turn stimulates Ca^{2+}/calmodulin-dependent enzymes; and c) cell lysis is subsequently induced by increased activity of CaM II kinase, or related enzyme, possibly by mediating secondary membrane pore formation. This hypothesis depends upon the contentions that a) ATP-induced Ca^{2+}-influx and ATP-induced ^{51}Cr-efflux occur via distinct pores/channels, and b) ^{51}Cr release is dependent on increased cytosolic $[Ca^{2+}]$. This latter point is demonstrated by lysis of both MIFN and MCSF in Ca^{2+}-free medium, which increases the relative concentration of the P_{2Z} ligand, ATP^{4-}, and is presumably due to release of Ca^{2+} from intracellular stores [Pillai and Bickle, 1992].

While direct evidence for the existence of two distinct pores/channels is not yet available, some data suggest their presence. First, kinetic studies demonstrate a temporal distance between increased cytosolic $[Ca^{2+}]$ (seconds) and ^{51}Cr-release (minutes). Second, both lytic and sublytic concentrations of ATP were found to stimulate equivalent increases in $[Ca^{2+}]_i$ in both MIFN and MCSF, probably via activation of both P_{2Y} and P_{2Z} receptors. It is unlikely that the P_{2Y} receptor is required as a co-signal for lysis of macrophages since ATP-mediated killing occurs under conditions that favor activation of the P_{2Z} receptor (absence of extracellular divalent cations). Finally, the calmodulin inhibitor KN-62 does not abrogate either membrane depolarization or calcium mobilization in ATP-stimulated macrophages, suggesting that these responses may be part of the initial signaling event, but not the formation of the secondary pores. Thus, while increased cytosolic $[Ca^{2+}]$ is necessary to activate calmodulin-linked processes, it is not sufficient to trigger lysis of macrophages, and a second signal, yet unknown, is required for complete expression of ATP-mediated lysis.

We propose that ATP, purportedly released from stimulated CTL, serves as a mediator in the lysis of antigen-presenting macrophages, which would effectively down-regulate an immune response and restore homeostasis. MIFN are more efficient antigen-presenting cells, due to their increased MHC class II expression [Virelizier et al., 1984], and their concomitant sensitivity to ATP-mediated lysis correlates with the need to eliminate these cells. In other studies, we have identified a putative ATP receptor, using a monoclonal antibody that blocks ATP-

mediated lysis of MIFN, whose expression on macrophages is upregulated by treatment with IFNγ, and not GM-CSF (manuscript in preparation). Further studies are required to fully characterize this receptor, as well as its biological significance.

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