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RESPONSES OF MOUSE LYMPHOCYTES TO EXTRACELLULAR ATP

II. Extracellular ATP Causes Cell Type-Dependent Lysis and DNA Fragmentation[1]

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Extracellular ATP (ATP_o) caused dose-dependent lysis of YAC-1 and P-815 mouse tumor cells. This event, assessed by ^{51}Cr release, was accompanied by sustained depolarization of the plasma membrane potential and Ca^{2+} influx. Plasma membrane depolarization and Ca^{2+} influx occurred within a few seconds of ATP_o addition to both cell types, whereas ^{51}Cr was released without apparent lag in YAC-1 cells and after 2 h in P-815 cells. Furthermore, a rise in $[Ca^{2+}]_i$ was required for ATP_o-dependent lysis of YAC-1 but not P-815 cells. In P-815 cells, ATP_o caused an early and $[Ca^{2+}]_i$-independent DNA fragmentation that occurred at lower nucleotide concentrations than those required to trigger ^{51}Cr release. Instead in YAC-1 cells very low concentrations of ATP_o caused early lysis (ED_{50} for lysis about 200 μM) accompanied by only barely detectable DNA fragmentation. Previous studies disclosed that lymphokine-activated killer cells are fully resistant to the membrane-perturbing effects of ATP_o. We show that lymphokine-activated killer cells also do not undergo DNA fragmentation even in the presence of high ATP_o concentrations. This study complements previous observations on the lytic effects of ATP_o and shows that this nucleotide can also cause DNA fragmentation, one of the earliest target cell alterations observed during CTL-mediated lysis.

Recent work on the effects of ATP_o[3] on intact cells has rekindled interest in the role of this purine nucleotide in intercellular communications (1–5). It is long known that immunocompetent cells, such as lymphocytes and macrophages, are responsive to ATP_o (6–9), but the role of ATP_o as an immunomodulator has remained elusive. In a previous report we showed that ATP_o causes depolarization of the plasma membrane potential and cytolysis in mouse thymocytes and splenocytes, as well as a number of mouse lymphoma cell lines (5). The effective ATP_o concentration depended on the lymphocyte type and on the presence or absence of divalent cations in the extracellular milieu. It was also observed by us (5) and by Sitkovsky and coworkers (10, 11) that lymphocytes with cytotoxic activity, i.e., CTL and LAK cells, that are resistant to cell-mediated lysis, were highly resistant to the membrane perturbing effects of ATP_o. These observations suggest a role for ATP_o in cell-mediated cytotoxicity (11, 12).

The molecular mechanism of CTL-mediated cytolysis is not yet understood. A growing body of recent evidence, however, suggests that one of the earliest changes induced by CTL on susceptible targets is widespread condensation of nuclear chromatin and DNA breakdown into nucleosome size fragments (13–16). This process, also called apoptosis, is the hallmark of "programed" cell death, and occurs during embryogenesis, normal tissue turnover, hormone-induced atrophy, and several other conditions (see Ref. 16 for review). The mechanism whereby CTL trigger cleavage of target DNA is unknown; although it is well established that pore-forming protein(s) contained in the granules of cytotoxic cells, as well as several other channel-forming agents may mediate membrane damage, their role in the induction of DNA breakdown is controversial (17, 18).

Our previous observations on the lytic properties of ATP_o led us to better characterize its effects on target cell lysis and nuclear integrity. By using two tumor cell lines, that exhibit different degrees of lysis and DNA fragmentation when challenged with pore-forming protein or CTL (17), we observed that ATP_o also triggers fragmentation of cellular DNA, and that this effect is cell-type dependent. Furthermore, we found that ATP_o-induced DNA fragmentation in P-815 cells was surprisingly independent of rises in the $[Ca^{2+}]_i$. Finally, LAK cells, which are resistant to cell-mediated as well as ATP_o-induced lysis, did not undergo DNA fragmentation at all ATP_o concentrations used.

These observations further substantiate the hypothesis that ATP_o may represent an effector molecule involved in cell-mediated cytotoxicity.

MATERIALS AND METHODS

Mice. Inbred C57 BL/6 (B6), BALB/c, and DBA/2 8- to 10-wk-old mice were purchased from the Charles River Laboratories (Calco Como, Italy).

Tumor cell lines. P-815 cells, obtained from a chemically induced

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[3] Abbreviations used in this paper: ATP_o, extracellular ATP; LAK, lymphokine activated killers.

NK-resistant mastocytoma, were maintained in ascitic form by weekly i.p. passages in syngeneic DBA/2 recipient mice. The NK-sensitive YAC-1 line was subcloned in our laboratory and several cell clones with varying sensitivity to ATP_o were obtained. One of them, YAC-1 E 625-1, was chosen for these studies. This sub clone, hereafter referred to as YAC-1, was maintained by continuous in vitro culture in complete medium that consisted of DMEM (GIBCO, Glasgow, Scotland) supplemented with L-glutamine (2×10^{-3} M final concentration), HEPES (1×10^{-2} M final concentration), 2-ME (3×10^{-5} M final concentration), 100 IU of penicillin/ml, 100 μg of streptomycin/ml, and 10% heat-inactivated FCS (Flow Lab. Inc., Irvine, U.K.).

Cell cultures. LAK cells were generated by culturing B6 spleen cells at a final concentration of 3×10^6 cells/ml in complete medium containing 100 U/ml rIL-2 (Glaxo Institute for Molecular Biology S.A., Geneva, Switzerland) in 250-ml tissue culture flasks (Falcon 3024; Falcon, Los Angeles, CA). LAK cells were used after 5 days of culture in rIL-2-containing medium. Flow cytometry analysis of these cell populations showed that approximately 60% of LAK cells expressed the Thy-1.2 and Lyt-2.2 Ag.

Antibodies. Anti-Thy-1.2 mAb were purchased from NEN, Dreiech, Germany. Goat anti-mouse polyclonal Ab were obtained from Miles, Yeda, Israel.

^{51}Cr release and DNA fragmentation assay. Target cells (2×10^6) were labeled for 1 h at 37°C in 0.1 ml of DMEM plus 5% FCS containing 10 μCi of ^{125}IUdR (Amersham International plc, Amersham, Buckinghamshire, U. K.); 100 μCi of ^{51}Cr (NEN) were than added to the cell suspension and the incubation was continued for 1 additional h in the presence of both radionuclides.

Labeled cells (10^4/well) were incubated for various lengths of time, with different concentrations of ATP_o in duplicate wells of round bottom microtiter plates (Sterilin, Teddington, Middlesex, U.K.) in a final volume of 0.2 ml. At the end of the incubation, the plates were centrifuged ($200 \times g$ for 10 min), and 0.1 ml supernatant, containing fragmented, but not intact, DNA and ^{51}Cr released from lysed cells, was withdrawn for counting. The percentage of specific ^{51}Cr release was calculated according to the expression:

$$100 \times \frac{\text{(experimental release} - \text{spontaneous release)}}{\text{(maximum release} - \text{spontaneous release)}}$$

Pelleted cells were lysed by adding cold hypotonic buffer (20 mM EDTA, 5 mM Tris-Cl, 0.5% Triton X-100, pH 8) and then centrifuged at $13{,}000 \times g$ for 10 min to separate intact from fragmented DNA. The percentage of fragmented DNA was calculated according to the following expression:

% fragmented DNA

$$= \frac{(^{125}\text{IUdR } 200 \times g) + (^{125}\text{IUdR } 13{,}000 \times g)}{\text{total } ^{125}\text{IUdR}} \times 100$$

where (^{125}IUdR $200 \times g$) and (^{125}IUdR $13{,}000 \times g$) represent the ^{125}I radioactivity present in the 200 and $13{,}000 \times g$ supernatants, respectively, and total ^{125}IUdR is the sum of ^{125}I radioactivity present in the 200 and $13{,}000 \times g$ supernatants and the pellet (16). The percentage of specific fragmentation was determined by the same formula used for specific ^{51}Cr release and is abbreviated in the Figures as "^{125}I release."

Agarose gel electrophoresis and autoradiography of fragmented DNA. Analysis of DNA fragments was performed according to Schmidt et al. (19) with minor modifications. P-815 cells, labeled with ^{125}IUdR as described, were incubated (2.5×10^4/well) in duplicate samples with the various agonists or LAK cells, in round bottom microplates (Sterilin) in a final volume of 0.2 ml. At the end of the incubation, cells were collected from two wells, transferred to polypropylene tubes (Eppendorf) and lysed with 100 μl of PBS containing 10 mM EDTA and 1% Triton X-100, pH 7.4. After 10 min on ice, the lysates were centrifuged at $13{,}000 \times g$ for 30 s to separate intact from fragmented DNA. Supernatants (0.4 ml) were extracted with an equal volume of phenol/chloroform, pH 9. Aqueous phases were precipitated by treatment with two volumes of absolute ethanol, 0.3 mM sodium acetate, and 0.01 M $MgCl_2$, kept at −20°C overnight, resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 8, and then separated by electrophoresis on 1.2% agarose gel. DNA was subsequently transferred to a nylon membrane (Hybond-N, Amersham). The gel marker was the HAE digest of ΦX 174 RF and the calculated m.w. of the lower fragment in the "ladder" was about 200 bp. Autoradiography was performed using X-Omat-S x-ray film (Eastman Kodak, Rochester, NY) with X-Omatic intensifying screen (Kodak).

Measurement of plasma membrane potential. Changes in plasma membrane potential were measured with the fluorescent dye bis(1,3-diethylthiobarbiturate) trimethineoxonal (bis-oxonol) at the wave length pair 540 to 580 nm, as previously described (20). Unless otherwise indicated, all experiments were performed at 37°C in saline medium containing 125 mM, NaCl; 5 mM, KCl; 1 mM, $MgSO_4$; 1 mM, KH_2PO_4; 5.5 mM, glucose; 1 mM, $CaCl_2$; and 20 mM, HEPES/NaOH buffer, pH 7.4, at 37°C; this saline medium is hereafter referred to as standard saline.

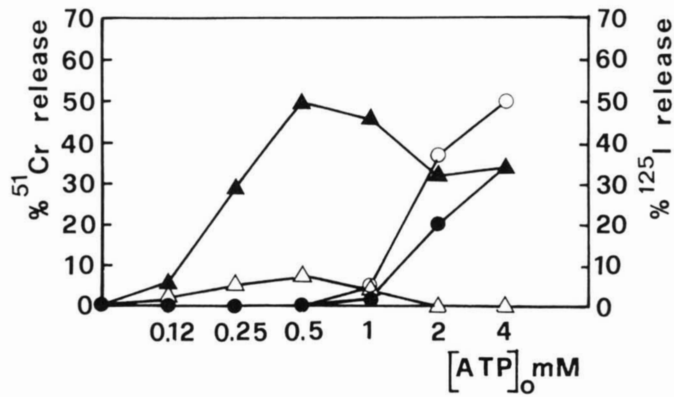
Measurement of $[Ca^{2+}]_i$. Changes in $[Ca^{2+}]_i$ were measured with the fluorescent indicator fura-2 as described previously (21). In some batches of P-815 and YAC-1 cells, considerable quenching of intracellular fura-2 fluorescence by endogenous heavy metals was observed. Therefore, where indicated, the membrane permeant chelator of heavy metals (N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine) was added at a concentration of 20 μM shortly after cell resuspension in the fluorimeter cuvette (22).

Fluorescence measurements. All fluorescence measurements were performed in a Perkin-Elmer LS5 spectrofluorimeter (Perkin-Elmer Corp., Norwalk, CT) equipped with a thermostatically controlled cuvette holder and magnetic stirrer.

RESULTS

Target specificity of ATP_o-dependent lysis and DNA fragmentation. The effects of ATP_o on cell membrane and DNA integrity were evaluated in two cell lines that differ significantly in sensitivity to CTL as well as perforin-induced lysis and DNA degradation: P-815 mastocytoma cells, that are fairly resistant to lysis but very susceptible to DNA degradation, and YAC-1 lymphoma cells, that, instead, are highly susceptible to lysis but relatively resistant to DNA degradation (17). Targets were labeled with ^{51}Cr and ^{125}IUdR, as cytoplasmic and DNA markers, and incubated for 4 h in the presence of increasing ATP_o concentrations. As shown in Figure 1, *upper panel*, P-815 cells underwent ATP_o-triggered, dose-dependent lysis and DNA degradation (35 and 50% specific ^{51}Cr and ^{125}IUdR release at 4 mM ATP_o, respectively); although the ATP_o threshold for both ^{51}Cr and ^{125}IUdR release was the same, 500 μM, DNA degradation was consistently higher than lysis at all ATP_o concentrations. In YAC-1 cells, ^{51}Cr release had a threshold at 50 μM and a maximum (50% specific release) at 500 μM ATP_o, and was accompanied by only 5 to 10% DNA fragmentation. At ATP_o concentrations of more than 500 μM there was a decline in lysis that was prevented by supplementing the incubation medium with extra Ca^{2+}, to keep the $[Ca^{2+}]_o$ constant also in the presence of the increasing Ca^{2+}-chelating power due to ATP_o (Fig. 1, *lower panel*). It is therefore likely that $[Ca^{2+}]_o$ availability was a limiting factor for ATP_o-induced lysis in YAC-1 cells. In contrast, DNA fragmentation in these cells was not increased by adding extra Ca^{2+} during the assay. As positive control in these experiments, we also included the K^+ ionophore valinomycin, a known and potent DNA fragmenting agent (23). Valinomycin caused high level DNA fragmentation in both P-815 and YAC-1 cells (100 and 50%, respectively), with an ED_{50} that was lower in the former (5 μM) than in the latter cells (10 μM) (data not shown).

To evidence the pattern of DNA fragmentation induced by ATP_o, agarose gel electrophoresis analysis of ^{125}IUdR-labeled P-815 cells was performed (Fig. 2). Cells were incubated for 4 h with 4 or 2 mM ATP_o (Fig. 2, *lanes 4* and *5*), or with 15 μM valinomycin (*lane 3*) or LAK cells at an E:T ratio of 10:1 (*lane 2*). Incubation with ATP_o caused a massive and dose-dependent release of DNA fragments in the supernatants, similar to that obtained with valinomycin or with LAK cells as effectors. A very similar pattern of DNA fragmentation was observed by agarose gel electrophoresis and ethidium bromide stain-



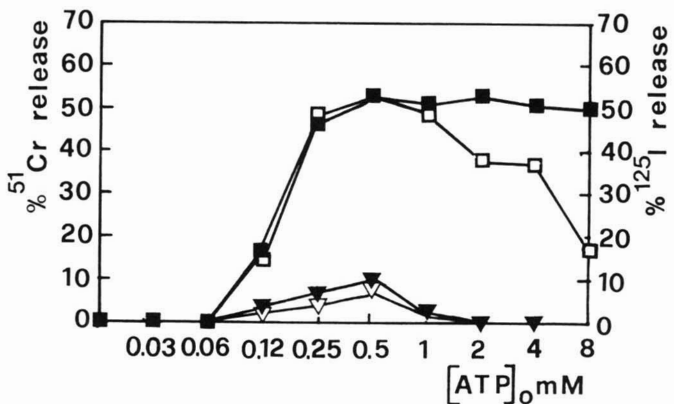


Figure 1. Effects of ATP$_o$ on ^{51}Cr and ^{125}IUdR release from P-815 and YAC-1 cells. *Upper panel*, specific release of ^{51}Cr (*filled symbols*) and ^{125}IUdR labeled DNA fragments (*open symbols*) was measured in duplicate samples of P-815 (*circles*) and YAC-1 (*triangles*) cells (10^4/well) incubated for 4 h at 37°C in the presence of increasing concentrations of ATP$_o$. Specific release of ^{51}Cr and of ^{125}IUdR-labeled DNA is abbreviated in all Figures as "^{51}Cr release" and "^{125}I release," respectively. ^{51}Cr and ^{125}IUdR spontaneous release never exceeded 20% of total incorporation. *Lower panel*, release of ^{51}Cr (*squares*) and ^{125}IUdR (*inverted triangles*) from YAC-1 cells incubated either under the same experimental conditions as in *upper panel* (*open symbols*), or in the presence of constant $[Ca^{2+}]_o$ (*filled symbols*). Data are averages from a single experiment representative of three other producing similar results.

ing of cells not labeled with ^{125}IUdR (not shown).

It has been suggested that a rapid damage of the target would prevent the initiation of the DNA degradation program (17). It is therefore possible that in YAC-1 cells, which are susceptible to much lower ATP$_o$ concentrations compared to P-815 cells, lysis occurs too early and thus prevents triggering of DNA fragmentation. To test this hypothesis, a time course experiment was performed with optimal ATP$_o$ concentrations of 4 and 0.5 mM, for P-815 and YAC-1 cells, respectively. As shown in Figure 3, ^{51}Cr release started without lag after the addition of ATP$_o$ to YAC-1 cells and reached near plateau levels (about 35% specific release) within 2 h. On the contrary, ^{51}Cr release from P-815 cells was delayed by about 2 h after ATP$_o$ addition and was still 10% after 4 h. Figure 3 also shows that DNA fragmentation preceded lysis in P-815 cells.

We recently reported that LAK cells, as with other cytotoxic cells (24, 25), are resistant to cell-mediated cytotoxicity as well as the depolarizing effects of ATP$_o$ (5, 26). As shown in Figure 4, ATP$_o$, at all concentrations tested, also failed to induce release of ^{51}Cr and ^{125}IUdR;

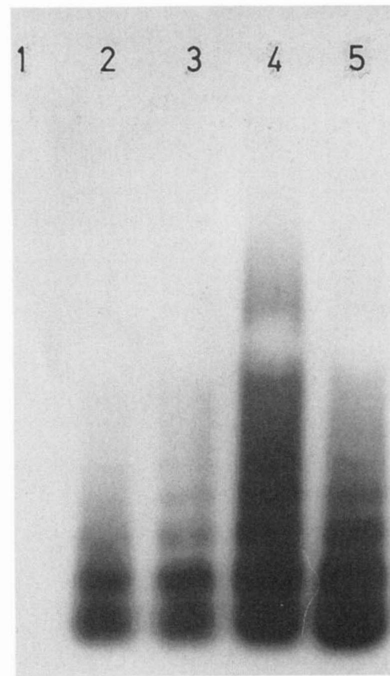


Figure 2. Analysis of DNA fragmentation induced by various treatments in P-815 cells. DNA was isolated as described in *Materials and Methods* from ^{125}IUdR-labeled P-815 cells (5×10^4) after 4 h of incubation under the following conditions: control (*lane 1*); incubation with LAK cells, E/T 10/1 (*lane 2*); 15 μM valinomycin (*lane 3*); 4 mM ATP$_o$ (*lane 4*); 2 mM ATP$_o$ (*lane 5*). Percent specific fragmented DNA, estimated from ^{125}IUdR release at the time of DNA isolation, was 48, 43, 51, and 34% in *lanes 2, 3, 4,* and *5*, respectively.

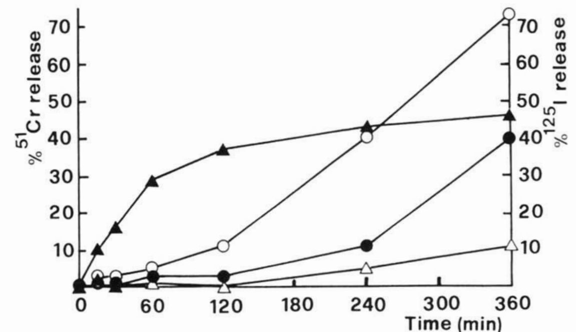


Figure 3. Kinetics of ATP$_o$-dependent ^{51}Cr and ^{125}IUdR release from P-815 and YAC-1 cells. P-815 (*circles*) and YAC-1 (*triangles*) cells (10^4/well) were incubated in duplicate samples in the presence of 4 and 0.5 mM ATP$_o$, respectively. ^{51}Cr (*filled symbols*) and ^{125}IUdR (*open symbols*) release were determined at indicated time of incubation. Spontaneous release never exceeded 20% of total incorporation. Data are from one experiment representative of three others that gave similar results.

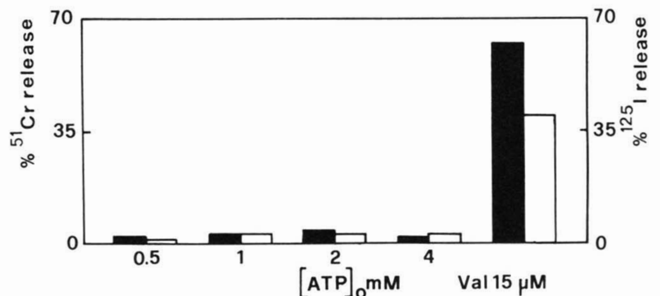


Figure 4. Absence of effect of ATP$_o$ in LAK cells. DNA fragmentation (■) and ^{51}Cr release (□) were measured in duplicate samples of LAK cells (10^4/well) after 4 h of incubation in the presence of increasing concentrations of ATP$_o$ or 15 μM valinomycin.

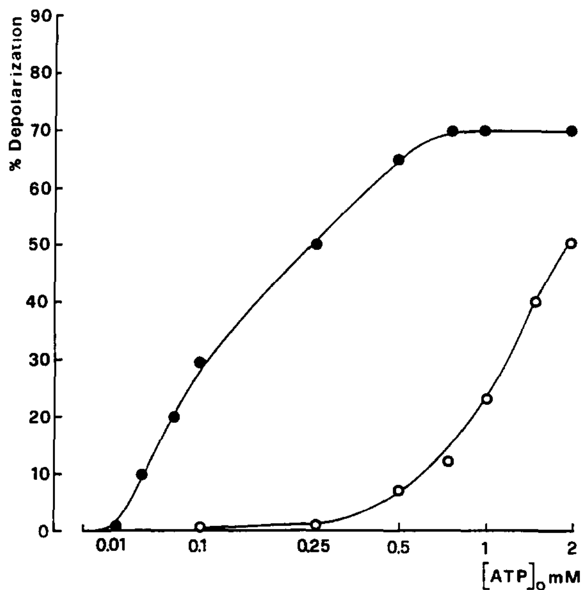


Figure 5. ATP_o dose-dependence of depolarization in P-815 and YAC-1 cells. P-815 (O) and YAC-1 (●) cells (5×10^5/ml) were incubated in the presence of 100 nM bis-oxonol, as described in *Materials and Methods*, and exposed to increasing ATP_o concentrations. Depolarization of plasma membrane potential is expressed as percent of maximal depolarization caused by 250 nM gramicidin D. Data are means of duplicate samples from a single experiment representative of several others giving similar results.

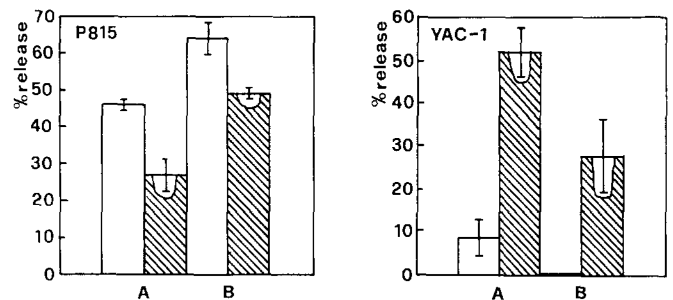


Figure 6. Effect of $[Ca^{2+}]_o$ chelation on ATP_o-triggered lysis and DNA fragmentation. P-815 (*left panel*) and YAC-1 (*right panel*) cells were incubated with 4 and 0.5 mM ATP_o, respectively, in the absence (*A*) or presence (*B*) of 2 mM EGTA. ^{51}Cr (*hatched bars*) and $^{125}IUdR$ (*open bars*) release were measured after 4 h of incubation. Values represent means ± SD of three separate experiments each performed in duplicate. Spontaneous release never exceeded 20% of total incorporation.

on the contrary, incubation with valinomycin caused about 60% fragmentation of cellular DNA and 40% release of ^{51}Cr from these cells.

A rapid and drastic change in membrane permeability to ions is one of the earliest cellular alterations caused by ATP_o and is probably responsible for lysis and DNA fragmentation (5). We therefore investigated the correlation between changes in plasma membrane potential and DNA fragmentation and lysis. YAC-1 cells were depolarized by very low ATP_o doses (threshold about 10 μM), whereas P-815 cells required high ATP_o concentrations (250 μM) (Fig. 5). Moreover, in YAC-1 ATP_o dose dependence for depolarization was almost overlapped by that for ^{51}Cr release, whereas in P-815 cells it was significantly shifted to lower ATP_o concentrations; in fact, 2 mM ATP_o caused 50% depolarization and only 20% lysis of P-815 cells. However, it should be noted that the fluorescence technique we used to measure plasma membrane potential changes did not enable us to discriminate whether a fraction of the entire cell population or all cells were depolarized by ATP_o, albeit incompletely.

Ca^{2+} dependency of ATP_o-triggered lysis and DNA breakdown. Several workers suggested that lysis and DNA fragmentation require a $[Ca^{2+}]_i$ rise in the target cell (17, 23, 27). However, the results shown in Figure 1 indicate that ATP_o lytic efficiency in P-815 and YAC-1 cells is differently affected by changes in $[Ca^{2+}]_o$. Hence we further investigated the Ca^{2+} requirement for cytolysis and DNA breakdown by challenging P-815 and YAC-1 cells with ATP_o in the absence of extracellular Ca^{2+} and in the presence of 2 mM EGTA. DNA breakdown was increased in P-815 cells by $[Ca^{2+}]_o$ chelation whereas the low DNA release observed in YAC-1 cells was completely suppressed (Fig. 6). Release of ^{51}Cr by P-815 cells was also increased by $[Ca^{2+}]_o$ chelation although it was decreased by about 50% in YAC-1 cells. These results suggest that the $[Ca^{2+}]_i$ dependency of lysis and DNA fragmentation is not absolute but is rather a property of the cell type investigated, thus further stressing the target cell contribution to its own killing.

It could be argued that $[Ca^{2+}]_o$ independence of ATP_o-induced lysis and DNA fragmentation in P-815 cells was due to ATP_o ability to release Ca^{2+} from intracellular stores; however, Figure 7 shows that the ATP_o-dependent rise in $[Ca^{2+}]_i$ in both YAC-1 and P-815 cells (traces *a* and *c*, respectively) was entirely explained by influx from the extracellular medium, because it was suppressed by chelation of $[Ca^{2+}]_o$ with excess EGTA (traces *b* and *d*). ATP_o inability to raise $[Ca^{2+}]_i$ in the absence of $[Ca^{2+}]_o$ was not due to a lack of $[Ca^{2+}]_i$ stores in these cell types, because the Ca^{2+} ionophore ionomycin caused a rise in $[Ca^{2+}]_i$ in the absence of $[Ca^{2+}]_o$ in both cell types (Fig. 7, traces *b* and *d*). Furthermore, when YAC-1 cells, sensitized by mouse anti-Thy-1.2 mAb, were challenged with goat-anti mouse Ab, an increase in $[Ca^{2+}]_i$ was elicited (Fig. 7, trace *e*). This latter experiment indicates that $[Ca^{2+}]_i$ can be mobilized in YAC-1 cells not only by a Ca^{2+} ionophore but also by a surface-acting agonist. It is therefore likely that ATP_o inability to raise $[Ca^{2+}]_i$ in YAC-1 and P-815 cells in the absence of $[Ca^{2+}]_o$ reflects a genuine dissociation of the ATP_o receptor from the signaling system responsible for Ca^{2+} release from intracellular stores.

DISCUSSION

It was recently shown that ATP_o is a powerful lytic agent in mouse cells (5, 11, 28). The mechanism whereby ATP_o causes cell death seems straightforward, because it causes dramatic alterations in plasma membrane permeability; it thus seems likely that cells die as a consequence of this damage, and the ensuing alteration in Na^+, K^+, and Ca^{2+} homeostasis (29). Plasma membrane damage, however, is not the only lethal event triggered by ATP_o, as it also induces DNA degradation into nucleosome size fragments, a phenomenon which is a striking feature of CTL-mediated lysis.

Susceptibility to undergo DNA fragmentation was inversely correlated with susceptibility to undergo depolarization and lysis. In fact, although P-815 cells are depolarized and lysed by fairly high ATP_o concentrations and after a lag of about 2 h, they exhibit massive DNA fragmentation; on the contrary, YAC-1 cells are rapidly depolarized and lysed by very low ATP_o doses, but undergo

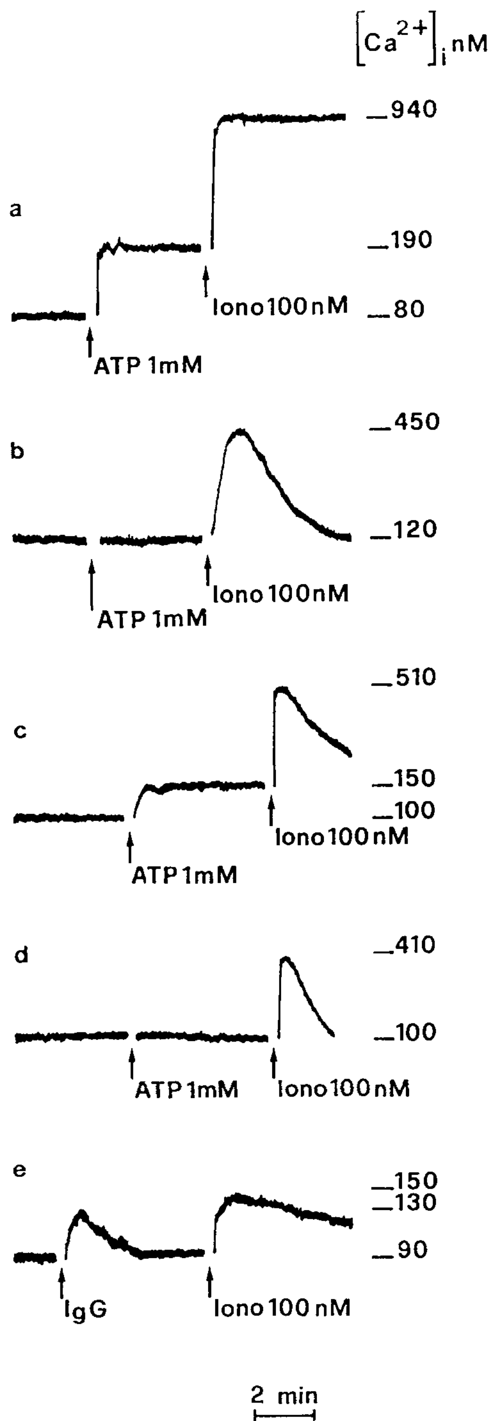


Figure 7. Effect of ATP_o on $[Ca^{2+}]_i$ homeostasis. YAC-1 (traces *a*, *b*, and *e*) and P-815 cells (traces *c* and *d*) were loaded with fura-2/AM and suspended in the fluorimeter cuvette at a concentration of 5×10^5/ml in the presence of 20 μM TPEN. In traces *a* and *c* the incubation medium contained 1 mM Ca^{2+}; in traces *b*, *d*, and *e* the incubation medium was Ca^{2+}-free and EGTA (2 mM) containing. Ionomycin was added at a concentration of 100 nM. In trace *e*, goat anti-mouse IgG (IgG) were added at a concentration of 32 μg/ml to YAC-1 cells previously sensitized by mouse anti-Thy 1.2 mAb. IgG addition caused a sudden fluorescence jump due to IgG intrinsic fluorescence. This jump was subtracted in trace *e* to calculate the true Ca^{2+}-sensitive increase in fura-2 fluorescence.

almost undetectable DNA fragmentation.

The Ca^{2+}-dependency of the ATP_o-induced responses in YAC-1 but not in P-815 cells, further strengthens the thesis that the intracellular pathways responsible for cell lysis and DNA degradation are most likely target cell-dependent, as proposed earlier for CTL-mediated cytolysis (30). Much debate surrounds the role of Ca^{2+} as the intracellular signal responsible for activation of intracellular pathways leading to cell death. Several reports have very recently questioned the need for a rise in $[Ca^{2+}]_i$ during cell lysis mediated by a number of agents, such as several CTL clones and TNF (31–33); ATP_o represents an additional example of Ca^{2+}-independent cytotoxic molecule, albeit to a different extent in the two cell lines tested in this study. Lysis inhibition by $[Ca^{2+}]_i$ chelation also explains the decline in ^{51}Cr release at high ATP_o concentrations in YAC-1 cells, because under these conditions, most of the $[Ca^{2+}]_o$ is chelated, and therefore reproduces nearly "Ca^{2+}-free" incubation conditions.

Our observation that DNA fragmentation in P-815 cells is dissociated from a $[Ca^{2+}]_i$ rise is rather intriguing in the light of the numerous observations suggesting that a rise in $[Ca^{2+}]_i$ is needed to activate the endonucleases responsible for DNA breakdown (23, 27). However, Hameed et al. (17) reported that only the chelation of both $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$ inhibited DNA breakdown completely in P-815 cells challenged with α-staphylotoxin. As ATP is known to be associated with $Ins1,4,5P_3$ generation and $[Ca^{2+}]_i$ mobilization in several cell types (1, 2, 4), an obvious explanation would be that ATP_o also causes the release of Ca^{2+} from intracellular stores, and therefore an increase in $[Ca^{2+}]_i$ even in the absence of $[Ca^{2+}]_o$. However, this is not the case in mouse lymphocytes (29) and in the mouse tumor cell lines tested in this study. Neither P-815 nor YAC-1 cells showed any $[Ca^{2+}]_i$ increase in the absence of $[Ca^{2+}]_o$. In fact, ATP_o barely raised $[Ca^{2+}]_i$ even when Ca^{2+} was present in the extracellular medium. Hence it is clear that the inability of EGTA to block DNA fragmentation in P-815 cells is not due to a residual increase in $[Ca^{2+}]_i$ also occurring in the absence of $[Ca^{2+}]_o$. Accordingly, a Ca^{2+}-independent pathway in TNF-mediated DNA fragmentation has been previously reported (33). In addition, studies in isolated nuclei indicate that endonucleases are already active at physiologic resting $[Ca^{2+}]_i$ levels and are only slightly (about 30% of maximal) stimulated by further $[Ca^{2+}]_i$ increases to 0.5 to 1 μM (27).

Alternatively, it may also be advanced that, in the absence of $[Ca^{2+}]_o$, ATP_o triggers a localized (intranuclear) $[Ca^{2+}]_i$ increase that can be detected only by single cell microfluorimetry.

Our observations stress the target specificity of DNA fragmentation even when a soluble triggering agent, such as ATP_o, is used, and suggest a dependence of this process on the kinetics of lysis. In fact, YAC-1 cells are lysed very rapidly by ATP_o, whereas P-815 cells release ^{51}Cr after a 2 to 4 h lag. Therefore, in YAC-1 cells, as opposed to P-815 cells, it is possible that lysis occurs too abruptly to permit initiation of the internal DNA degradation program. Moreover, the rate of DNA release in P-815 targets precedes lysis, a kinetic that is usually typical of the "internal disintegration" process described in CTL-induced lysis (15). It is worth mentioning that P-815 cells also exhibit low sensitivity to lysis by pore-forming proteins and by CTL (17).

We previously showed that LAK cells, which are resistant to cell-mediated lysis, are also refractory to depolarization of the plasma membrane potential and ATP_o-induced lysis (5). Accordingly, ATP_o also failed to induced DNA breakdown in these cells. LAK cells, however, are susceptible to DNA degradation induced by an agonist,

such as valinomycin, that bypasses surface receptors.

It has been suggested that cytotoxic cells resistance to ATP_o is due to their very active surface ATPase that would efficiently hydrolyze ATP_o in the vicinity of the plasma membrane (11). However, this hypothesis faces a major inconsistency, because ecto-ATPases are strictly dependent on Mg^{2+} for their activity, but cytotoxic cells remain fully refractory to ATP_o even in the presence of millimolar EDTA concentrations (5). We rather prefer the alternative suggestion that cytotoxic cells are refractory to ATP_o because they lack the ATP_o receptor, or possess a receptor that is functionally uncoupled from the effector system that causes ion fluxes and DNA fragmentation.

In conclusion, we have described some interesting similarities between ATP_o lytic effects and CTL-mediated lysis, notably the early DNA fragmentation that precedes cell lysis and the selective resistance of cytotoxic cells to the lytic properties of ATP_o. The indication that stimulated effector cells release ATP (11, 34), although it is as yet unknown whether lytic ATP concentrations are reached in close proximity of the target cell plasma membrane during effector-target cell interaction, supports the hypothesis that ATP_o may be involved in cell-mediated lysis.

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