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Characterization of the cytotoxic effect of extracellular ATP in J774 mouse macrophages

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Extracellular ATP (ATP_o) is known to be cytotoxic to many cell types through a mechanism which is largely unknown. Very recently this nucleotide has been shown to cause cell death by apoptosis, probably by interacting with specific cell-surface receptors. In the present study we have investigated the mechanism of ATP_o-dependent cytotoxicity in the macrophage-like mouse cell line J774. It has been previously reported that in this cell type ATP_o activates trans-membrane Ca^{2+} and Na^+ fluxes and a drastic increase in the plasma-membrane permeability to hydrophilic solutes smaller than 900 Da. These changes are followed by cell swelling and lysis. We show in the present study that, although this nucleotide triggers a rise in the cytoplasmic Ca^{2+} concentration, neither cell swelling nor lysis is Ca^{2+}-dependent. Furthermore, cell lysis is not dependent on Na^+ influx, as it is not prevented by iso-osmotic replacement of extracellular Na^+ with choline or *N*-methylglucamine. On the contrary, ATP_o-dependent cytotoxicity, but not the ATP_o-dependent increase in plasma-membrane permeability, is completely abrogated in sucrose medium. Under our experimental conditions ATP_o does not cause DNA fragmentation in J774 cells. We conclude from these findings that ATP_o does not cause apoptosis of J774 macrophages and promotes a Ca^{2+}- and Na^+-independent colloido-osmotic lysis.

INTRODUCTION

Several cell types are susceptible to the cytocidal activity of extracellular ATP (ATP_o), but the mechanism whereby this nucleotide acts is poorly characterized (Steinberg & Di Virgilio, 1991). In J774 and other cell types the effects of ATP_o are mediated by specific cell-surface receptors (P2 purinergic receptors) coupled to different early responses: generation of inositol 1,4,5-trisphosphate, release of Ca^{2+} from intracellular stores, Ca^{2+} influx from the extracellular milieu, Na^+ influx accompanied by plasma-membrane depolarization, and permeabilization of the plasma membrane to low-molecular-mass aqueous solutes (Gomperts, 1983; Steinberg & Silverstein, 1989; Dubyak, 1991). These changes could in principle be causally linked to cell death, as on the one hand it is thought that a perturbation of the plasma-membrane permeability leading to changes in intracellular ion homoeostasis may cause colloido-osmotic lysis, whereas on the other an increase in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and/or an inappropriate activation of protein kinase C have been invoked as triggers of apoptosis (McConkey *et al.*, 1990). We have previously shown that ATP_o promotes Ca^{2+}-independent apoptosis of a number of tumoral cell lines (Zanovello *et al.*, 1990; Murgia *et al.*, 1992), and similar results have been reported in mouse lymphocytes and endotoxin-primed macrophages (Zheng *et al.*, 1991; Hogquist *et al.*, 1991). However, the present results show that ATP_o does not cause apoptosis of J774 mouse macrophages, as under no conditions were we able to detect the typical ladder-pattern on agarose-gel electrophoresis of DNA from cell extracts. Furthermore, ATP_o caused an early swelling, probably owing to opening of the ATP_o-gated pore known to be expressed by these cells (Steinberg & Silverstein, 1987; Beyer & Steinberg, 1991), followed by disruption of intracellular organelles and the plasma membrane, changes indicative of colloido-osmotic lysis. ATP_o-triggered lysis was independent of the presence of extracellular Ca^{2+} and did not require a Na^+ influx; furthermore, although ATP_o also caused a transient $[Ca^{2+}]_i$ increase owing to Ca^{2+} release from intracellular stores, this fast $[Ca^{2+}]_i$ transient was unlikely to be a trigger for cell lysis, since it also occurred in the ATP-resistant J774 variant ATPRB2 that was refractory to the cytocidal effect of ATP_o. Rather surprisingly, lysis of ATP_o-pulsed cells was completely prevented in sucrose medium. Under these incubation conditions, the ATP_o-gated pore was fully activated and the plasma membrane was permeabilized, as shown by uptake of extracellular markers and cell swelling, yet no release of lactate dehydrogenase occurred and cells remained viable for several hours. Our results suggest that the mechanism responsible for ATP_o-dependent cell death is a feature of the target, and, very probably, depends on the P2 purinergic receptor subtype expressed on the plasma membrane.

MATERIALS AND METHODS

Cells

The J774 mouse macrophage cell line and the ATPRB2 J774 variant were grown in spinner cultures in Dulbecco's modified Eagle medium supplemented with 10% (v/v) heat-inactivated horse serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Unless otherwise indicated, experiments were performed in a saline solution containing (in mmol/l): 125 NaCl, 5 KCl, 1 $MgSO_4$, 1 Na_2HPO_4, 5.5 glucose, 5 $NaHCO_3$, 1 $CaCl_2$ and 20 Hepes (pH 7.4, 37 °C). This saline medium is subsequently also referred to as standard saline. In some experiments NaCl was replaced by an iso-osmotic concentration of choline chloride, methylglucamine or sucrose. In these Na^+-free buffers KCl was omitted, Na_2HPO_4 was replaced with K_2HPO_4 and $NaHCO_3$ with $KHCO_3$, and the pH was adjusted to 7.4 with Tris/HCl.

Enzyme release

Lactate dehydrogenase activity was measured by standard methods (Bergmeyer, 1963).

Abbreviations used: ATP_o, extracellular ATP; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; $Ca^{2+}{}_o$, extracellular free Ca^{2+}; $Na^+{}_i$, intracellular Na^+; $Na^+{}_o$, extracellular Na^+.

Measurement of $[Ca^{2+}]_i$

Loading with fura-2 acetoxymethyl ester and measurement of $[Ca^{2+}]_i$ were performed essentially as previously described (Di Virgilio *et al.*, 1988). To prevent fura-2 leakage and sequestration, 250 μM-sulphinpyrazone was present throughout the loading procedure and $[Ca^{2+}]_i$ measurement (Di Virgilio *et al.*, 1988). To measure $[Ca^{2+}]_i$ macrophages were suspended in a thermostatically controlled and magnetically stirred fluorimeter (Perkin–Elmer LS5) cuvette at a concentration of 0.5×10^6 cells/ml.

Measurement of cell swelling

Cell volume changes were monitored by measuring right-angle light scattering, at a wavelength pair of 540/550 nm, in a thermostatically controlled and magnetically stirred fluorimeter (Perkin–Elmer LS5) cuvette at a concentration of 0.5×10^6 cells/ml.

Measurement of Lucifer Yellow uptake

For Lucifer Yellow uptake, macrophage monolayers were incubated for 5 min at 37 °C in sucrose/saline in the presence of 5 mM-ATP_o, 1 mg of Lucifer Yellow/ml and 250 μM-sulphinpyrazone. After this incubation time, the monolayers were rinsed and kept in sulphinpyrazone-supplemented sucrose/saline.

Microscopy

Microscopic observations were performed with an Olympus (IMT-2 or BH-2) microscope equipped with a 40× objective.

Calculation of ATP^{4-}

The ATP^{4-} concentration, as a function of total ATP_o, Ca^{2+} and Mg^{2+} concentrations and pH, was determined as described by Fabiato (1988).

Data presentation

Results are expressed as means ± S.D. of triplicate determinations from a single experiment representative of at least three similar.

RESULTS AND DISCUSSION

Fig. 1 shows a dose–response curve for ATP_o-dependent release of lactate dehydrogenase from J774 cells and the ATP-resistant variant ATPRB2 cells. After a 6 h incubation in the presence of this nucleotide (5 mM), about 50% of total cellular-lactate dehydrogenase content was released in the supernatant of J774 cells, but not ATPRB2 macrophages. Total ATP_o threshold and EC_{50} for cytotoxicity were about 750 μM and 1.5 mM respectively, corresponding to a concentration of ATP^{4-}, the active ATP_o species, of 45 and 158 μM respectively, under our experimental conditions. As previously reported (Steinberg & Silverstein, 1989; Di Virgilio *et al.*, 1989), other nucleotides, such as UTP, ITP and CTP, even at very high concentration, could not mimic the cytocidal effect of ATP_o (results not shown). The cytotoxic effect of ATP_o was dependent on the presence of the membrane-permeabilizing P2z purinergic receptor, as the ATPRB2 mutant cell line, which was not killed by ATP_o, expresses a fully functional P2y receptor, but lacks the P2z (Greenberg *et al.*, 1988).

We and others have shown that activation by ATP_o of the P2z receptor and the subsequent increase in plasma-membrane permeability occur rapidly and without lag (Buisman *et al.*, 1988; Tatham & Lindau, 1990; Pizzo *et al.*, 1991). Release of lactate dehydrogenase, on the contrary, started about 1 h after addition of a maximally stimulatory ATP_o concentration and increased steadily throughout the incubation (Fig. 2). Release of lactate dehydrogenase from ATP_o-treated ATPRB2 cells and from control J774 cells incubated in the absence of the nucleotide is shown for comparison.

It has been previously reported that ATP_o-mediated cytotoxicity in rat hepatocytes is dependent on the presence of extracellular Ca^{2+} (Ca^{2+}_o) (Nagelkerke *et al.*, 1989). In contrast with these data, Fig. 3 shows that macrophages were fully susceptible to lysis in the absence of Ca^{2+}_o, and, if anything, release of lactate dehydrogenase was increased, probably because the ATP^{4-} concentration was higher in the absence of Ca^{2+}_o. Occurrence of lysis in the absence of Ca^{2+}_o does not rule out a trigger role for Ca^{2+} in ATP_o-dependent lysis, as J774 cells possess ATP_o-releasable intracellular Ca^{2+} stores; however, in agreement with previous results of Greenberg *et al.* (1988), we also observed a transient ATP_o-stimulated $[Ca^{2+}]_i$ rise in the ATP_o-resistant ATPRB2 variant, thus suggesting that mobilization of intracellular Ca^{2+} is unlikely to be the trigger for lysis (results not shown). Furthermore, addition of the Ca^{2+} ionophore

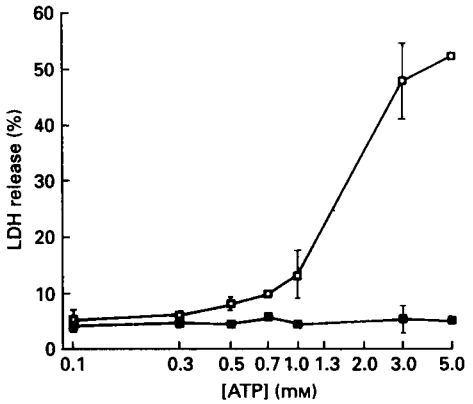

Fig. 1. Dependency on the nucleotide concentration of lactate dehydrogenase release from J774 and ATPRB2 macrophages

Monolayers of 2×10^5 cells/well were incubated in 24-well plates at 37 °C for 6 h in standard saline containing the indicated nucleotide concentration. At the end of the incubation, supernatants were collected, centrifuged to eliminate floating cells, and the lactate dehydrogenase (LDH) content was measured. LDH release is expressed as percentage of total content, determined by lysing an equal amount of cells with 0.1% Triton X-100. □, J774 cells; ■, ATPRB2 cells.

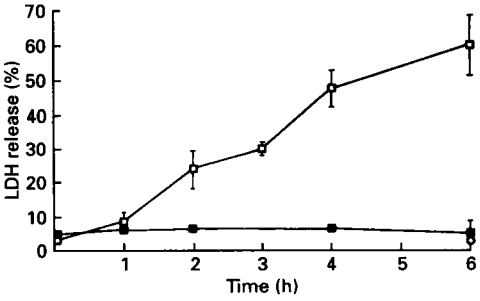

Fig. 2. Time course of lactate dehydrogenase (LDH) release from ATP_o-pulsed J774 and ATPRB2 macrophages

Macrophage monolayers (2×10^5/well) were incubated as described in Fig. 1 in Ca^{2+}-free 100 μM-EGTA-containing saline in the absence or presence of 3 mM-ATP_o. □, J774 cells; ■, ATPRB2 cells; ○, J774 cells incubated in the absence of ATP_o.

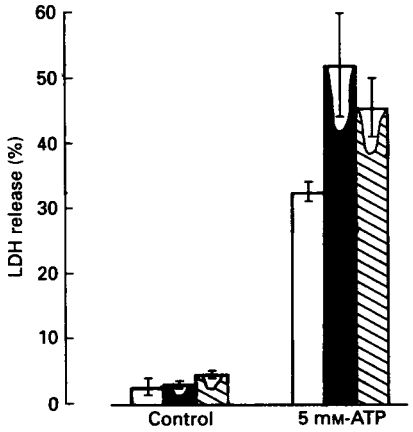

Fig. 3. ATP_o-triggered lactate dehydrogenase (LDH) release in Ca^{2+}-free medium

Macrophages (2×10^5/well) were incubated for 6 h in the presence of 5 mM-ATP_o under the following conditions: □, standard saline; ■, Ca^{2+}-free standard saline; ▨, standard saline containing 100 μM-EGTA.

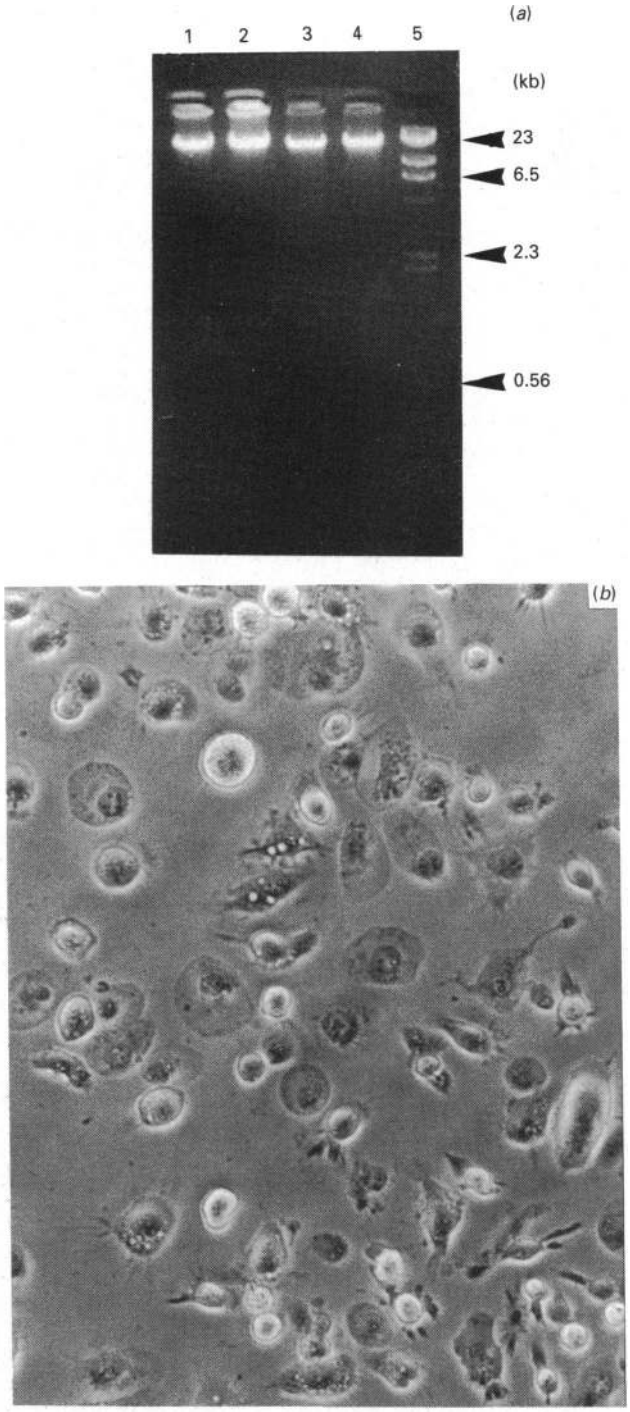

Fig. 4. ATP_o does not cause apoptosis of J774 and ATPRB2 macrophages

(*a*) Agarose-gel electrophoresis of total cellular DNA from J774 (lanes 1 and 2) and ATPRB2 (lanes 3 and 4) macrophages. First, 2×10^6 cells/sample were incubated in Dulbecco's modified Eagle medium supplemented with 10% horse serum and pulsed with 3 mM-ATP_o for 6 h. All samples were then lysed and DNA was extracted. Lanes 1 and 3, control cells; lanes 2 and 4, ATP_o-pulsed cells; lane 5, *Eco*RI digest of λ-phage DNA. (*b*) Phase-contrast micrograph of ATP-challenged J774 macrophages. The cell monolayer was incubated in standard saline solution supplemented with 5 mM-ATP for 6 h at 37 °C, then rinsed and photographed. Magnification ×400.

ionomycin after ATP_o showed that both cell types possessed intracellular Ca^{2+} stores of comparable size.

ATP_o has been shown to cause cell death by apoptosis of a number of cell types, such as lymphocytes, P-815 mastocytoma cells and L929 fibroblasts (Zanovello *et al.*, 1990; Zheng *et al.*, 1991; Murgia *et al.*, 1992). However, this mechanism of cell death does not seem to operate in J774 macrophages, since we were unable to detect ATP_o-dependent DNA fragmentation under our experimental conditions (Fig. 4*a*). Furthermore, ATP_o did not induce any of the morphological features of apoptosis, such as cell shrinkage and chromatin clumping; rather, it caused cell rounding, swelling and disruption of intracellular organelles (Fig. 4*b*), changes suggestive of a cytotoxic mechanism based on colloido-osmotic lysis. ATP_o-promoted volume changes started shortly after addition of the nucleotide, reached a plateau within 5 min and were partially reversed by addition of Mg^{2+} in excess of ATP_o (Fig. 5, trace *a*). Cell swelling depended on the activation of the ATP_o-gated pore (the P2z receptor), since ATPRB2 cells did not show any ATP_o-triggered volume changes (Fig. 5, trace *b*).

Replacement of Na^+_o with other extracellular univalent cations did not abrogate ATP_o-mediated cytotoxicity, as release of lactate dehydrogenase was inhibited (by about 60%) in choline, but slightly enhanced (by about 25%) in *N*-methylglucamine medium (Fig. 6). On the contrary, iso-osmotic replacement with sucrose allowed a complete inhibition of cell death. A possible explanation for the protective action of sucrose could be that at low ionic strength ATP_o was unable to activate the P2z receptor and thus trigger the plasma-membrane permeability changes involved in colloido-osmotic lysis. However, this is not the case, as neither uptake of the extracellular fluorescent marker Lucifer Yellow nor cell swelling (Fig. 7) was inhibited in sucrose medium. As shown in Fig. 7, under these experimental conditions the increase in cell volume was not reverted by addition of Mg^{2+}, probably because after resealing of the plasma membrane cells were unable to remove quickly the excess of osmotically active sucrose accumulated in the cytoplasm.

The cytocidal activity of ATP_o is long known, but little information is available on the plasma-membrane receptors, intracellular messengers and mechanism involved. To this purpose, the J774 mouse macrophage-like cell line is a very useful model, since it is equipped with well-characterized plasma-membrane ATP_o receptors and fairly well-known trans-membrane signal-transduction pathways (Steinberg & Silverstein, 1989; Greenberg *et al.*, 1988). Furthermore, Steinberg and

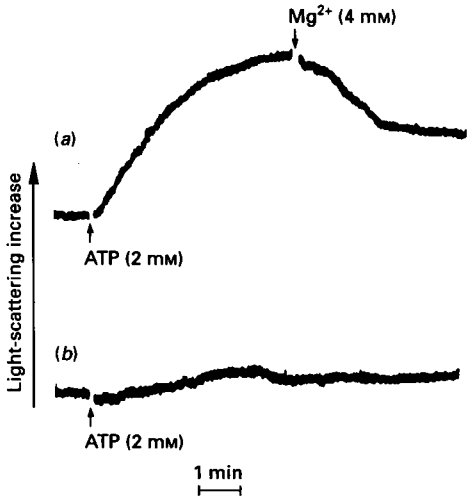

Fig. 5. ATP_o induces swelling of J774 but not ATPRB2 macrophages

For kinetic measurement of swelling, cells (2.5×10^5/ml) were suspended in Ca^{2+}-free standard saline and incubated at 37 °C in a fluorimetry cuvette as described in the Materials and methods section. ATP_o was 2 mM and $MgSO_4$ 4 mM. An upward deflection indicates an increase in light-scattering. Trace (*a*), J774 cells; trace (*b*), ATPRB2 cells.

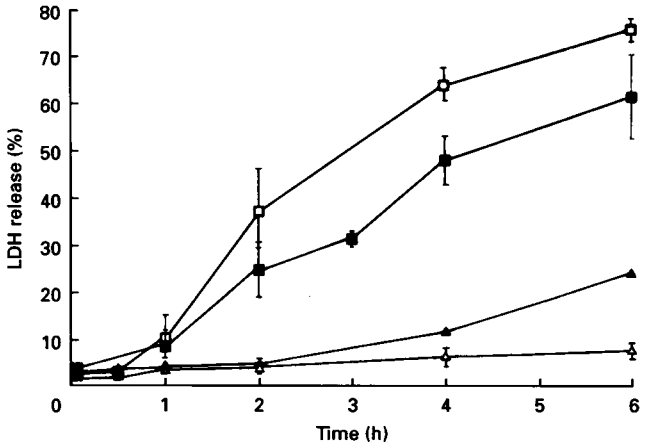

Fig. 6. Effect of Na^+ replacement on ATP_o-mediated lysis of J774 macrophages

Macrophage monolayers were incubated at 37 °C in the absence or presence of 3 mM-ATP_o for the indicated times under the following conditions: ■, standard saline; □, Na^+-free saline containing 125 mM-*N*-methylglucamine; ▲, Na^+-free saline containing 125 mM-choline; △, Na^+-free saline containing 250 mM-sucrose. Abbreviation: LDH, lactate dehydrogenase.

Silverstein have selected mutants which are completely resistant to ATP_o and thus very helpful for investigating intracellular processes involved in ATP_o-dependent cytotoxicity (Steinberg & Silverstein, 1987; Greenberg *et al.*, 1988). In J774 cells ATP_o did not cause any of the changes typical of apoptosis, such as cell shrinkage, chromatin condensation and DNA fragmentation. On the contrary, it promoted cell swelling, disruption of intracellular organelles and the plasma membrane, changes which are clear indication of colloido-osmotic lysis. Rather intriguingly, Hogquist *et al.* (1991) reported ATP_o-triggered DNA fragmentation in mouse macrophages primed by a 48 h incubation in the presence of bacterial lipopolysaccharide, suggesting that

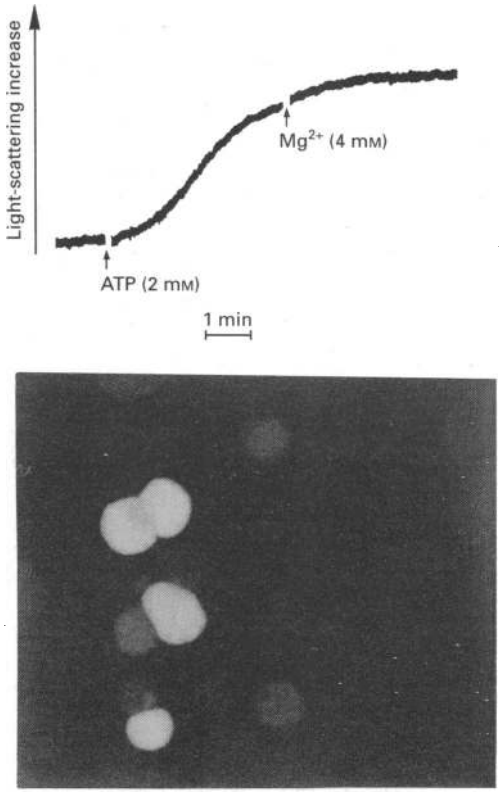

Fig. 7. ATP_o induces swelling of J774 macrophages in Na^+-free sucrose-containing medium

Upper panel, ATP_o-triggered light-scattering increase. Lower panel, Lucifer Yellow uptake. For light-scattering measurement, experiments were performed essentially as described in Fig. 5, except that NaCl was iso-osmotically replaced with sucrose. For Lucifer Yellow uptake, see the Materials and methods section. Magnification × 330.

activation by inflammatory agents can modulate macrophage responses to ATP_o. This conclusion is further supported by the observation that sensitivity to ATP_o is drastically enhanced in γ-interferon-primed human macrophages (Blanchard *et al.*, 1991).

The intracellular processes triggered by ATP_o and responsible for cell death are still unknown. A $[Ca^{2+}]_i$ rise is clearly neither necessary nor sufficient, since on the one hand ATP_o is fully active in the absence of $Ca^{2+}{}_o$, and on the other ATPRB2 mutants undergo ATP_o-stimulated $[Ca^{2+}]_i$ changes, yet they are not killed. Likewise, a Na^+ influx is not required, as replacement with either choline or *N*-methylglucamine does not abrogate cytotoxicity. To our surprise, incubation in sucrose medium completely prevented lysis. Under these conditions the plasma membrane was fully permeabilized, as shown by cell swelling and uptake of extracellular markers, yet release of lactate dehydrogenase was abrogated. Incubation in sucrose medium may prove helpful to minimize cell damage during experiments involving protracted plasma-membrane permeabilization.

An open issue is whether the high ATP_o concentrations required to induce cell death are ever reached *in vivo*. ATP is known to be released from several cells, e.g. platelets, adrenal chromaffin cells, cytotoxic T lymphocytes and various nerve terminals (Dubyak, 1991). Basal ATP_o plasma concentration is about 1–5 μM, whereas peaks up to 20 μM have been measured after extensive intravascular platelet aggregation (Born & Kratzer, 1984). However, it cannot be excluded that much higher ATP_o concentrations can be reached within protected environments, such as those formed during platelet–endothelial-cell or cytotoxic-target-cell interaction.

In conclusion, our results show that ATP_o promotes Ca^{2+}- and Na^+-independent cell lysis and strengthen the view that cytotoxic factors may use different cytocidal pathways in different cell targets.

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