Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 8267–8271, November 1990
Immunology

Extracellular ATP in T-lymphocyte activation: Possible role in effector functions

(cytolytic T lymphocytes/ectoprotein kinase/purinergic receptors)

ANTONIO FILIPPINI*, ROLF E. TAFFS, AND MICHAIL V. SITKOVSKY

Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

Communicated by Herman N. Eisen, August 3, 1990

ABSTRACT **We hypothesized that cytolytic T lymphocytes (CTL) may utilize extracellular ATP (ATP_o) during the effector phase of the CTL–target cell interactions and that CTL could be the source of ATP_o. It is demonstrated here that incubation of CTL with activating ligands [Con A or monoclonal antibody (mAb) to the T-cell antigen receptor (TCR)] results in the extracellular Ca^{2+}-independent accumulation of the ATP_o. The addition of the ATP-degrading enzymes into the mixture of CTL and target cells results in a strong inhibition of the CTL-mediated, TCR-triggered lethal-hit delivery to the target cell. In a parallel control experiment, the employed enzymes did not affect target cell-induced, TCR-triggered exocytosis of granules from CTL. Thus, the removal of ATP_o with enzymes does not interfere with the activation of CTL by the target cell but does block lytic events. Cloned helper T lymphocytes also accumulate ATP_o after incubation with anti-TCR mAb or Con A, suggesting the possibility that ATP_o, which acts in concert with ectoprotein kinases and/or purinergic receptors, may be of general use as a messenger in cellular interactions of T lymphocytes.**

The molecular mechanisms of the cell-mediated cytotoxicity are still poorly understood. It is generally accepted (1) that the recognition of the target cell (TC) by the T-cell antigen receptor (TCR) is required for the triggering of the biochemical events leading to the "lethal-hit" delivery (2, 3), and a substantial amount of suggestive evidence supports the hypothesis that TC death is due to the exocytosis of the pore-forming proteins from cytolytic T lymphocytes (CTL) (4, 5).

Exocytosis of cytolytic granules from CTL, as studied by using the serine esterase-release assay (6–10), was shown to be absolutely dependent on extracellular Ca^{2+} (10), while the TCR-mediated TC lysis can proceed even in the absence of extracellular Ca^{2+} (1, 11, 12). This leads to the conclusion that exocytosis of cytolytic granules may not be the only mechanism of CTL-mediated cytotoxicity (11–13).

In search for the alternative mechanism of cytotoxicity that would be consistent with the observations of the Ca^{2+}-independent lethal-hit delivery, we suggested (14, 15) the possibility of extracellular ATP (ATP_o) acting in concert with the cell-surface ATP_o-receptors and/or ectoprotein kinases (14–20) as a part of the molecular interactions leading to the CTL-induced TC death. Additionally, we characterized the CTL ecto-ATPase (15) and found that ATP_o can kill different tumor TC in both the presence and the absence of Ca^{2+}, whereas CTL are much more resistant to the lysis of ATP_o.

To further implicate the ATP_o in lymphocyte effector functions, it was necessary to explain the source of the ATP_o and to demonstrate the effect of ATP_o removal on the effector functions of CTL. It is shown here that activated CTL could be the source of the ATP_o. Addition of the ATP-degrading enzymes (during the assay) blocks the cytolytic process but does not affect other processes that follow the TC-induced, TCR-mediated transmembrane signaling in CTL.

MATERIALS AND METHODS

Cell Culture and Maintenance. CTL clone OE4 (anti-H-2^d specificity) was maintained as described (3). CTL were purified from dead cells by Ficoll/Hypaque centrifugation shortly before being used in assays. Special care was taken to assure the highest possible viability of CTL during the experiment. P815 mastocytoma cell line and EL4 tumor cell line were used as TC and were maintained in RPMI 1640 medium (Biofluids, Rockville, MD) containing 10% (vol/vol) fetal calf serum.

Peritoneal exudate CTL (alloimmune peritoneal exudate lymphocytes; PEL, anti-H-2^b specificity) were obtained 5 days after the secondary intraperitoneal immunization of BALB/c mice with 25×10^6 EL4 tumor cells (prepared as ascites in syngeneic C57BL/6 mice) (1). PEL were purified from crude peritoneal exudate cells by the removal of macrophages and other adherent cells on nylon wool columns (60 min at 37°C) followed by the elution of the nonadherent cells. D10.G4.1 helper T-cell line was maintained as described (21).

Detection of ATP_o Accumulation in Activated Cells and Measurements of the ATPase and Ecto-ATPase Activity. The luciferin/luciferase assay was used here to detect ATP_o in lymphocytes because this highly sensitive method has been successfully utilized before to demonstrate ATP_o release by other cells (e.g., ref. 18). An LKB 1250 luminometer was used to detect and to quantitate the luminescence. Monoclonal antibodies (mAbs) to the surface antigens were preadsorbed on the surface of the luminometer cuvette by incubating the solution of each mAb at indicated concentrations overnight. To detect the ATP_o accumulation, cells were dispersed in RPMI 1640 medium containing 1% bovine serum albumin, 10 mM Hepes, 2 mM glutamine, 1 mM sodium pyruvate, and 10 mM nonessential amino acid mixture, and 150 μl (total volume) of the dispersion was placed into cuvettes and incubated for different times in the 5% CO_2/95% air incubator as indicated in the figure legends. After incubation, cuvettes were centrifuged for 2 min at 1000 rpm (Sorvall RT6000B, H1000B rotor), and 50 μl of supernatant was taken for determination of lactate dehydrogenase activity as described (10) to assure that no cell damage had occurred during the incubation. The remaining 100 μl of supernatant and loosely pelleted cells were gently swirled (resuspended), and 100 μl of the luciferin/luciferase mixture

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ATP_o, extracellular ATP; CTL, cytolytic T lymphocyte(s); TC, target cell(s); mAb, monoclonal antibody; LFA-1, lymphocyte function-associated antigen 1; PEL, peritoneal exudate CTL; TCR, T-cell antigen receptor.

*On leave of absence from Institute of Histology and Embryology, School of Medicine, "La Sapienza" University, Rome, Italy.

was added while the cell suspension was gently mixed. The cuvette was immediately placed in the detection chamber of the luminometer. The optimal conditions of luminescence measurements were selected in preliminary experiments. By using the luminometer, reproducible measurements were obtained only in the presence of cells but not in the cell supernatants, indicating the detection of cell surface-associated ATP_o. However, with the use of a custom-made luminometer system, we were able to detect anti-TCR mAb-induced ATP_o accumulation during the first 1–3 min after addition of soluble mAb. The intensity of the luminescence was proportional to the concentration of ATP at the luminometer settings used in the experiments described here. The calibration curve was obtained in each experiment to convert the relative intensity of luminescence values into the concentration of ATP_o accumulated by activated cells. Total cellular content of ATP is defined as the amount of ATP detected by the luciferin/luciferase assay after the solubilization of cells with the nonionic detergent (0.5% Nonidet P-40).

Assays of ecto-ATPase activity of CTL and of ATPase activity of enzymes were performed as described in ref. 15 by measuring the ATP_o degradation.

Cytotoxicity Assays. Labeling of the TC and cytotoxicity assays were performed essentially as described (3).

Granule Exocytosis Assay. Antigen-bearing TC P815 and immobilized anti-TCR mAb F23.1 (22) or anti-CD3 mAb (2C11) (23) were used to induce secretion of granule-located enzyme N^{α}-benzyloxycarbonyl-L-lysine thiobenzyl ester esterase. The activity of secreted enzyme was measured as described (6, 10, 24).

Assay for the Effect of the ATP-Degrading Enzymes on CTL Cytotoxicity and on Granule Exocytosis. Hexokinase, apyrase, glycerokinase, and Na^+/K^+-ATPase from dog kidney were tested in preliminary experiments to confirm their ATPase activity by using essentially the same assay that was used for the detection of ecto-ATPase activity. Enzymes were dissolved in the medium and added into the functional assay at concentrations described in the legends to the figures. To test the effect of enzymes in pretreatment experiments, CTL or TC were preincubated for 2 hr at 37°C with the highest concentration of enzyme, washed, and then used in exocytosis or cytotoxicity assays. Standard errors and statistical significance were determined by using analysis of variance programs in the statistical analysis software package STATVIEW II (Macintosh).

Reagents and mAb. Human recombinant interleukin 2 was obtained from Bio-Gen. Phorbol 12-myristate 13-acetate was purchased from Sigma. The variable (V) region β chain (V_β)-specific anti-TCR antibody F23.1 (22) (mouse IgG2a) and anti-CD3 mAb 2C11 (23) were purified by affinity chromatography with the use of a protein A column. Orthovanadate was purchased from Fisher Scientific. Hexokinase (lot 11542026-05), glycerokinase (lot 11542026-05), luciferin, and luciferase were obtained from Boehringer Mannheim. Na^+/K^+-ATPase from dog kidney and ATPase from potatoes (apyrase) were purchased from Sigma. ATP (lot 76F-7040), leupeptin, antipain, and phenylmethylsulfonyl fluoride were from Sigma. [γ-^{32}P]ATP was obtained from Amersham.

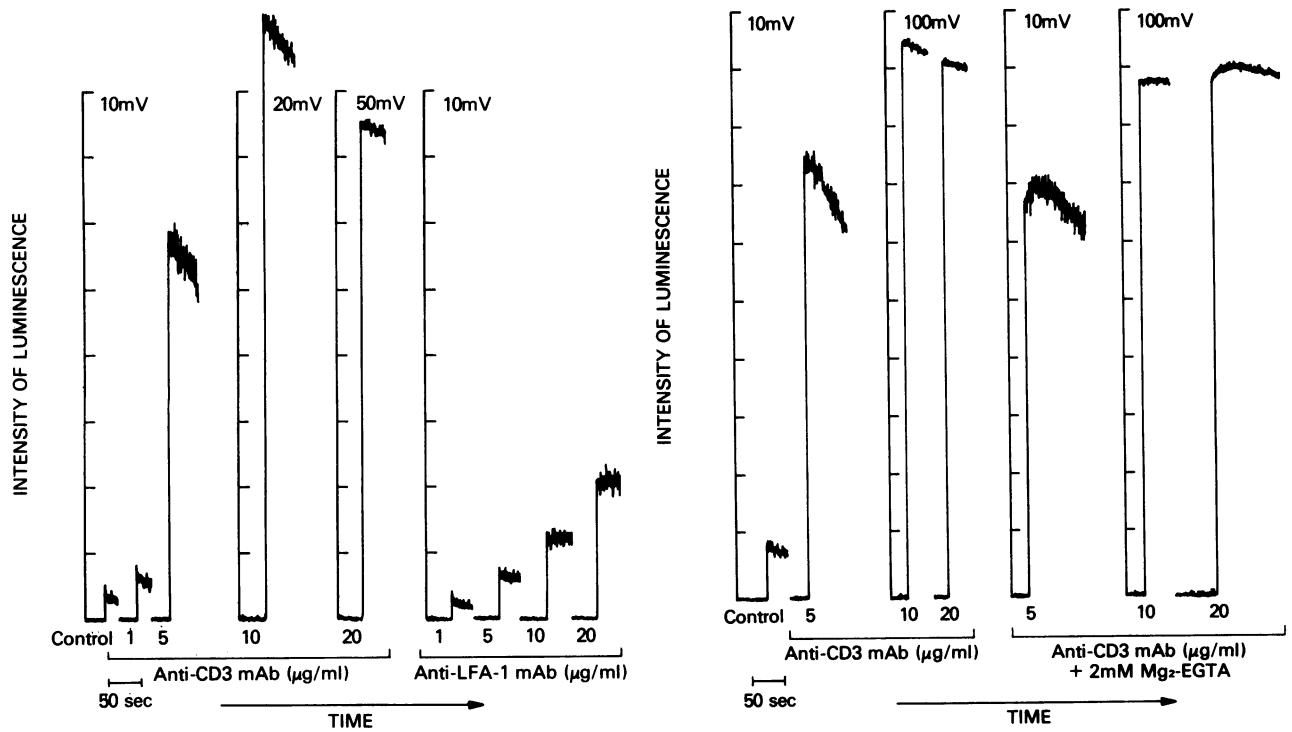

FIG. 1. Accumulation of ATP_o during incubation of CTL with immobilized mAb against the CTL surface proteins. The ordinate shows the intensity of luminescence (relative units in mV), which is proportional to the concentration of ATP available to the luciferin–luciferase reaction. The abscissa shows the concentration of mAb used during immobilization in the cuvettes of the luminometer. Time scale of the experiment is indicated by the 50-sec mark. The efficiency of incubation with mAb in triggering the CTL response was controlled by testing the parallel control samples for the granule exocytosis [Nα-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT)-esterase release] as the proof of the ability of immobilized mAb to activate CTL. Only those cells were tested for ATP_o accumulation that were undamaged (lactate dehydrogenase test; trypan blue exclusion test) during incubation. (*Left*) Accumulation of the ATP_o by PEL CTL is triggered by anti-TCR–T3 mAb. Cells were incubated for 30 min with immobilized mAb to the TCR–T3 complex or to lymphocyte function-associated antigen 1 (LFA-1). Note the different scales of ATP_o detection (e.g., the 50-mV scale with 20 μg of anti-CD3 mAb per ml and the 10-mV scale with 20 μg of LFA-1 mAb per ml correspond to the $\approx$5:1 differences in the concentration of ATP_o). (*Right*) Accumulation of ATP_o by PEL CTL is independent of extracellular Ca^{2+}. Results are shown of an experiment different from that described on the *Left*. PEL CTL were incubated for 45 min with immobilized anti-CD3 mAb in the absence or presence of Mg_2EGTA as indicated and were tested for ATP_o accumulation as described.

RESULTS AND DISCUSSION

Our hypothesis is based on the assumption that extracellular ATP is available and present during the interactions between CTL and TC. ATP_o has been detected previously in the blood and lymph *in vivo*, and its physiological role has been discussed (16, 17, 25). The possibility was tested that ATP_o is released by the CTL in response to the crosslinking of the TCR by the antigen-bearing TC. To avoid the presence of the large ATP-rich tumor TC in the assay, we used a model system for CTL–TC interactions in which cells were activated by immobilized anti-TCR mAbs (Fig. 1). Crosslinking of the CTL TCR by either anti-CD3 or anti-TCR mAb is sufficient to trigger delivery of the lethal hit by the CTL (even to non-antigen-bearing TC) (23, 26).

No significant extracellular ATP was detected when PEL CTL were incubated alone, whereas up to 15% of total cellular ATP content (in some experiments) was available for the luciferin/luciferase reaction after incubation of CTL with anti-CD3 mAb. Similar results were obtained with anti-TCR mAb and Con A (data not shown). Cloned CTL also accumulate ATP_o after the incubation with Con A or anti-TCR mAb (Fig. 2). Immobilized anti-LFA-1 mAb was much less efficient in the triggering of the ATP_o accumulation, as shown with both the CTL clone (Fig. 2) and with PEL CTL (Fig. 1). However, the difference between the anti-CD3 mAb and anti-LFA-1 mAb was more profound in PEL CTL than in the CTL clone (Figs. 1 and 2). The accumulation of cell surface-associated ATP_o in these experiments is most likely underestimated because of the presence of ATP-degrading enzymes on the CTL surface (15). It would be of interest to determine the spatial separation of ecto-ATPase(s) and of ATP_o.

The detection of the ATP_o accumulation by activated CTL (Figs. 1 and 2) provides direct proof, explains, and extends an early observation of Henney (27), who noticed that incubation of target cells with sensitized lymphocytes (polyclonal populations of CTL) resulted in higher amounts of liberated ATP than could be accounted for by the ATP content of the TC alone. We also tested other cells for the ability to accumulate ATP_o in response to Con A under the same conditions that induced ATP_o release from CTL (data not shown). Incubation of P815, EL4, or WH231 cells with Con A did not induce the accumulation of ATP_o. However, incubation of helper T-cell clone D10.G4.1 with Con A (data not shown) or with immobilized anti-TCR mAb (Fig. 2) did result in the accumulation of ATP_o, suggesting that ATP_o accumulation could be the general property of T lymphocytes in response to the TCR crosslinking.

It was recently concluded (13) that any alternative mechanism of CTL-mediated cytotoxicity should explain the ability of CTL to kill TC in the presence of Mg_2EGTA. Indeed, we found that the addition of Mg_2EGTA to the incubation medium did not prevent the accumulation of ATP_o (Fig. 1 *Right*) by anti-CD3 mAb-activated CTL.

If ATP_o were indeed an intermediate of cellular interactions in the effector phase of the CTL response, then the removal of ATP_o would be expected to inhibit the lymphocyte response. To test it, we used different ATP-degrading enzymes, like Na^+/K^+-ATPase, hexokinase, and glycerokinase. We found that these enzymes inhibited CTL-mediated cytotoxicity in a concentration-dependent manner, prompting the detailed investigation of their effects on CTL response.

The efficiency of these enzymes in the removal of ATP_o is demonstrated in Fig. 3 *Left*. The addition of the hexokinase in the cytotoxicity assay (Fig. 3 *Right*) strongly inhibited antigen-specific CTL-mediated cytotoxicity. Similar results were obtained with glycerokinase and other enzymes tested (data not shown). ATPase from potatoes (apyrase) was also inhibitory but not as strong as other tested enzymes, and not all batches of enzyme were equally potent.

To observe the inhibition, ATP-degrading enzymes must be present during the assay because the pretreatment of CTL

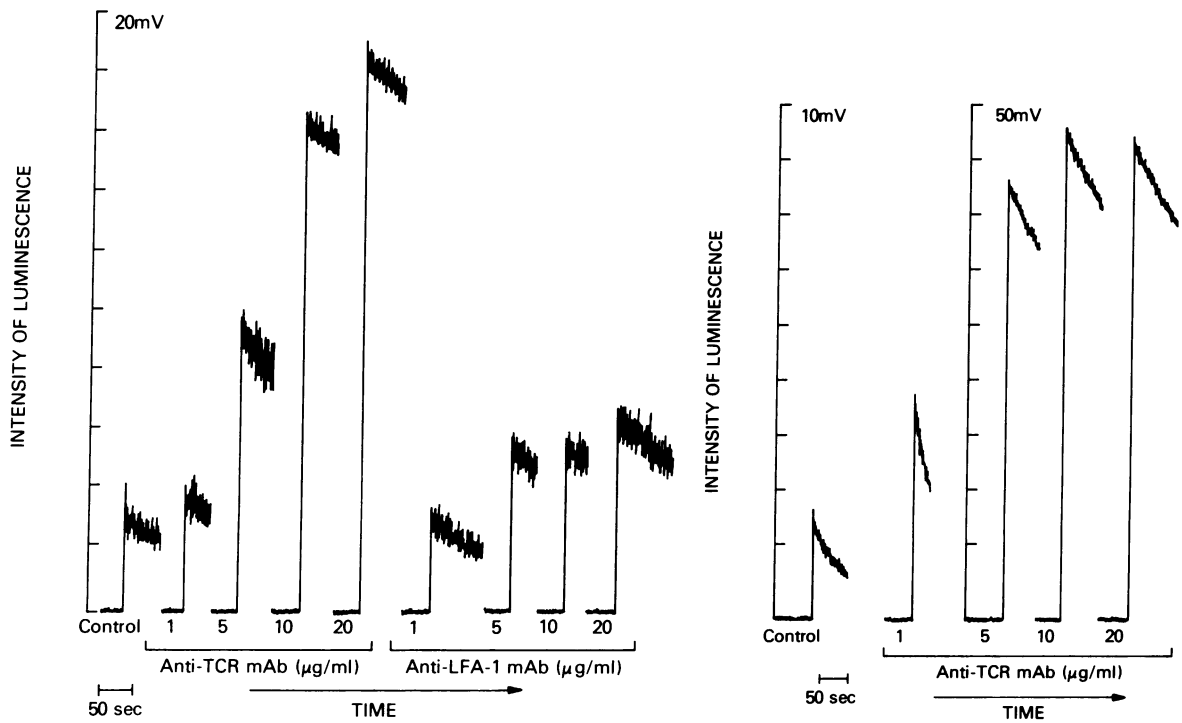

FIG. 2. Accumulation of the ATP_o by the CTL clone OE4 (*Left*) and by helper T-cell clone D10.G4.1 (*Right*) is triggered by anti-TCR mAb. Cells were incubated with immobilized mAb as described. In control cuvettes, cells (OE4) were tested for mAb-induced granule exocytosis. While a minor increase in ATP_o is seen with anti-LFA-1 mAb (*Left*), no effect of anti-LFA-1 mAb on granule exocytosis was detected. Anti-TCR mAb (F23.1) was able to induce granule exocytosis and ATP_o accumulation in CTL OE4 (*Left*) and ATP_o accumulation by D10.G4.1 helper T-cell clone.

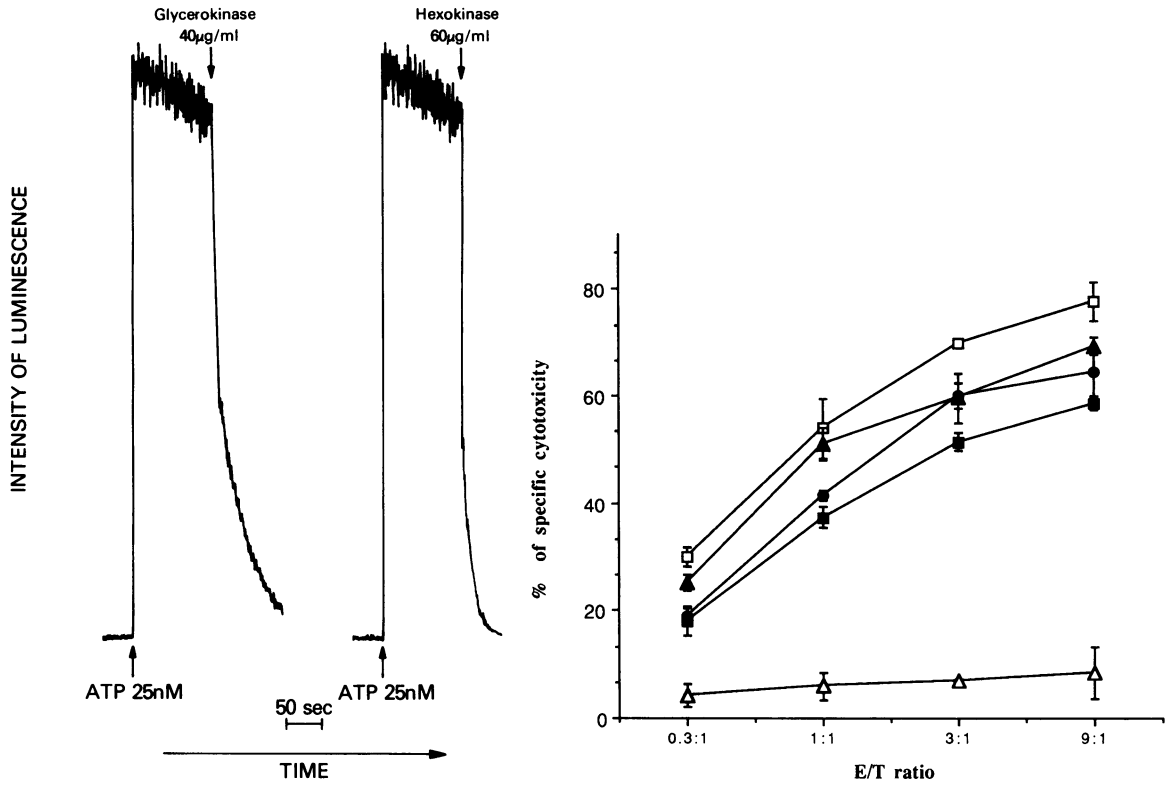

FIG. 3. Effect of the ATP-degrading enzymes on the CTL-mediated TC lysis. (*Left*) Demonstration of the ATPase activity of the glycerokinase and hexokinase. Addition of enzyme results in the fast degradation of exogeneously added ATP_o (25 nM), which is reflected in the rapid decline of the ATP-dependent luminescence. (*Right*) Effect of hexokinase (60 μg/ml) on CTL cytotoxicity. □, No enzyme in the cytotoxicity assay (control); ▲, preincubation of TC with enzyme, followed by washing of cells; ●, preincubation of CTL with enzyme, followed by washing of cells; ■, preincubation of both CTL and TC with enzyme, followed by washing of cells; △, enzyme is present during the assay. E/T ratio, effector (CTL OE4) to target (P815) cell ratio.

or TC with hexokinase or with glycerokinase was much less inhibitory (Fig. 3 *Right*).

The obvious caveat in the use of the enzymes described above to implicate the extracellular ATP in the lethal-hit delivery by CTL is the possible effects they may have on other important steps that precede the lethal hit. To control for such "nonspecific" effects of enzymes, we simultaneously tested in parallel experiments the effect of hexokinase (Fig. 4) and glycerokinase (data not shown) (*i*) on TC-induced, CTL-mediated lethal-hit delivery to the TC (Fig. 4 *Left*) and (*ii*) on the TC-induced granule exocytosis from CTL (Fig. 4 *Right*). Both of these responses are triggered by the TCR-crosslinking by TC surface antigens during engagement of CTL with the TC in CTL–TC conjugates. It was

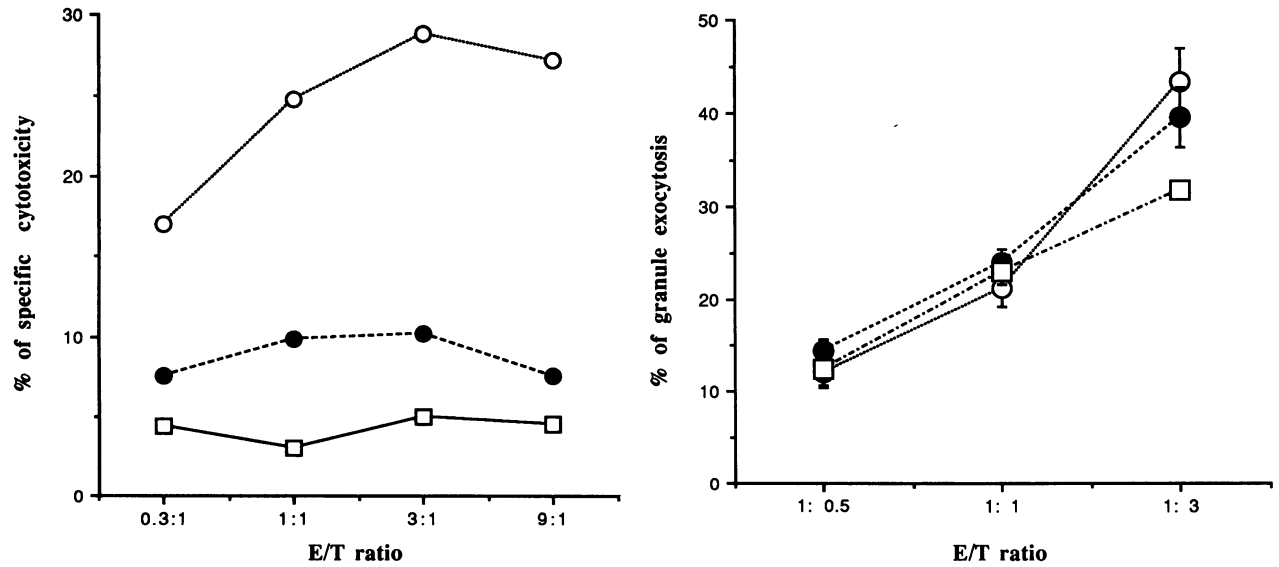

FIG. 4. Effect of hexokinase on the TC lysis by CTL and on the TC-induced exocytosis of granules from CTL. (*Left*) Inhibition of the cytotoxicity by hexokinase at 25 μg/ml (●) and 50 μg/ml (□) during the assay. ○, Control (no hexokinase is present in the assay). (*Right*) TC-induced granule exocytosis from CTL is not affected by the presence of hexokinase at 25 μg/ml (●) or 50 μg/ml (□). ○, Control (no enzyme is present during the assay). E/T ratio, effector (CTL OE4) to target (P815) cell ratio.

found that at the concentrations used, these enzymes strongly inhibited CTL-mediated killing of the TC but did not effect TC-triggered exocytosis from CTL. At higher concentrations of enzymes, both exocytosis and cytotoxicity could be inhibited (data not shown). These data show that the inhibition of cytotoxicity by ATP-degrading enzymes is not simply due to their interference into CTL–TC interactions that precede the lethal hit.

While these experiments suggest the role for ATP_o in the processes of cell-mediated cytotoxicity, no clue is yet available as to how extracellular ATP-dependent mechanism brings about the CTL-mediated TC death. Possible mechanisms by which ATP_o can act on the TC include: (*i*) its well-documented abilities to permeabilize cellular membranes (19, 20); (*ii*) interaction with purinergic receptors (16, 17) on the CTL and TC surface; and/or (*iii*) participation in the ectoprotein kinase (28) reactions or in the additive effects of ATP_o and yet unknown intermediates or in both. In this respect, interesting data were reported by Di Virgilio and colleagues (29), who demonstrated the resistance of lymphocytes with cytotoxic activity to the permeabilizing effects of ATP. Since the ATP^4-form of ATP is largely responsible for its cell permeabilizing properties, it remains to be established if the conditions of cytotoxicity assay allow for a significant proportion of ATP_o molecules to be in the ATP^4-form.

In this study we describe the ability of both CTL and helper T cells to accumulate ATP_o in response to TCR-crosslinking. This suggests a general role of ATP_o in the effector functions of T lymphocytes. When secreted by CTL toward the TC, ATP_o may contribute to TC death, while secretion of ATP_o by helper T cells toward the antigen-presenting cell (macrophage or B lymphocyte) may modulate the action of lymphokines by involving the purinergic receptors or ectoprotein kinases or both in synergistic interactions. Indeed, it has been shown that ATP_o can initiate the blastogenesis of the phorbol 12-myristate 13-acetate-treated medullary population of thymocytes (30) and can synergize with epidermal growth factor, platelet-derived growth factor, or insulin in triggering the growth of fibroblasts (31). This hypothesis may stimulate studies of CTL–TC and helper T cell–antigen-presenting cell interactions from a different point of view. It is also possible that there are still other, yet unidentified, low molecular weight intermediate(s) that upon release by stimulated lymphocytes participate in their effector functions.

1. Berke, G. (1989) in *Fundamental Immunology*, ed. Paul, W. E. (Raven, New York), 2nd Ed., pp. 735–764.
2. Russell, J. H. (1986) *J. Immunol.* **136,** 23–27.
3. Takayama, H., Trenn, G. & Sitkovsky, M. V. (1988) *J. Biol. Chem.* **263,** 2330–2336.
4. Henkart, P. (1985) in *Mechanisms of Host Resistance to Infectious Agents, Tumours and Allographts*, eds. Steinman, R. M. & North, R. J. (Rockefeller Univ. Press, New York), pp. 205–216.
5. Podack, E. R. (1986) *J. Cell. Biochem.* **30,** 133–170.
6. Pasternack, M. S. & Eisen, H. N. (1985) *Nature (London)* **314,** 743–745.
7. Pasternack, M., Verret, C. R., Liu, M. A. & Eisen, H. N. (1986) *Nature (London)* **332,** 740–743.
8. Henkart, P., Berrebi, G., Takayama, H. & Sitkovsky, M. (1987) *J. Immunol.* **139,** 2398–2405.
9. Takayama, H., Trenn, G., Humphrey, W., Bluestone, J. A., Henkart, P. A. & Sitkovsky, M. V. (1987) *J. Immunol.* **138,** 566–569.
10. Takayama, H. & Sitkovsky, M. (1987) *J. Exp. Med.* **166,** 725–743.
11. Trenn, G., Takayama, H. & Sitkovsky, M. V. (1987) *Nature (London)* **330,** 72–74.
12. Ostergaard, H. L., Kane, K. P., Mescher, M. F. & Clark, W. R. (1987) *Nature (London)* **330,** 71–72.
13. Sitkovsky, M. V. (1988) *Immunol. Rev.* **103,** 127–160.
14. Filippini, A., Taffs, R. & Sitkovsky, M. (1989) *J. Immunol. Immunopharmacol.* **9,** 118–119.
15. Filippini, A., Taffs, R. E., Agui, T. & Sitkovsky, M. V. (1990) *J. Biol. Chem.* **265,** 334–340.
16. Burnstock, G. (1981) in *Receptors and Recognition*, ed. Burnstock, G. (Chapman and Hall, London), pp. 1–44.
17. Gordon, J. I. (1986) *Biochem. J.* **233,** 309–319.
18. Rojas, E., Pollard, H. B. & Heldman, E. (1985) *FEBS Lett.* **185,** 323–327.
19. Rozenqurt, E. & Heppel, L. A. (1979) *J. Biol. Chem.* **254,** 708–714.
20. Steinberg, T. H., Newman, A. S., Swanson, J. A. & Silverstein, S. C. (1987) *J. Biol. Chem.* **262,** 8884–8888.
21. Kaye, J., Porcelli, S., Tite, J., Jones, B. & Janeway, C. A., Jr. (1983) *J. Exp. Med.* **158,** 836–856.
22. Staerz, U. D., Rammensee, H. G., Benedetto, J. D. & Bevan, M. J. (1985) *J. Immunol.* **134,** 3994–4000.
23. Leo, O., Foo, M., Sachs, D. H., Samelson, L. E. & Bluestone, J. A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1374–1378.
24. Takayama, H., Trenn, G. & Sitkovsky, M. V. (1987) *J. Immunol. Methods* **104,** 183–190.
25. Rapaport, E. & Fontaine, J. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1662–1660.
26. Kranz, D. M., Tonegawa, S. & Elsen, H. N. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7922–7926.
27. Henney, C. S. (1973) *J. Immunol.* **110,** 73–84.
28. Kiibler, D., Pyerin, W., Bill, O., Hotz, A., Sonka, J. & Kinzel, V. (1989) *J. Biol. Chem.* **264,** 14549–14555.
29. Di Virgilio, F., Bronte, V., Callavo, D. & Zanovello, P. (1989) *J. Immunol.* **143,** 1995–1960.
30. El-Moatassim, C., Dornand, J. & Mani, J.-C. (1987) *Biochim. Biophys. Acta* **927,** 437–444.
31. Huang, N., Wang, D. & Heppel, L. A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 7904–7908.