ANTIGEN RECEPTOR–REGULATED EXOCYTOSIS IN CYTOTOXIC T LYMPHOCYTES

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Cytotoxic T lymphocyte (CTL)-mediated lysis of target cells is believed to involve cytolytic molecules located in intracellular granules (1, 2). The granules in cytotoxic cells also contain proteoglycans (3), trypsin-type serine esterases (*N*-α-benzyloxycarbonyl-L-lysin thiobenzyl esterase; BLT-E) (4, 5) and it was suggested that a low pH environment in the granules may control intragranular enzyme activity in mast cells (6) and possibly in CTL. It was assumed that CTL deliver the lethal hit to the target cell due to exocytosis of the cytolytic granules (1, 7, 8), and it was demonstrated recently that secretion of intragranular enzyme in CTL is regulated by the occupation/crosslinking of the CTL antigen receptor by the target cell surface antigens (4, 9) or by immobilized anti-TCR monoclonal antibodies (9). It is generally accepted (10–13) that exocytosis is triggered by a transmembrane signals, involves movement of secretory vesicles to the plasma membrane, fusion of secretory granule membranes with the cell plasma membrane, and subsequent release of the soluble content into the extracellular medium. None of these steps has been carefully described biochemically, and no direct biochemical or morphological data exist to demonstrate granule loss due to the TCR-regulated exocytosis in CTL.

Biochemical pathways involved in TCR-mediated triggering of exocytosis in CTL and which couple the TCR crosslinking on the cell surface with biochemical processes in cytoplasm are not yet known. One of the major obstacles in such studies was the absence of a convenient biochemical marker of lymphocyte intracellular granules. It was suggested by the results of a series of studies (4, 14, 15) that both intracellular and cell surface expressed proteases may be involved in CTL effector functions and one such enzyme (BLT esterase) was found to be preferentially expressed in cytotoxic lymphocytes. We recently suggested the use of TCR-triggered secretion of a granule-located trypsin-type serine esterase as a functional assay in CTL activation studies and in biochemical studies of exocytosis in cloned CTL (9). The unique characteristics of this experimental system offer significant advantages in the studies of the molecular mechanisms of CTL activation. The functional effect of mAb to different antigens on the CTL surface, and the effect of specific inhibitors and activators of different intracellular biochemical pathways can now be reevaluated and carefully tested in a simplified short-term assay in the absence of target cells.

[1] *Abbreviations used in this paper:* BLT, *N*-α-benzyloxycarbonyl-L-lysine thiobenzyl ester; BLT-E, BLT esterase; CaM, calmodulin; LDH, lactate dehydrogenase; TFP, trifluoperazine; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

The goal of this study was to provide biochemical evidence for exocytosis of granules in CTL, and to evaluate molecular requirements and early biochemical events involved in the triggering of the secretion of intragranular proteins from cloned murine CTL.

Materials and Methods

CTL Clones. CTL clone 2C (16), OE4 (17), and BM10-37 (18) were maintained as was described earlier (9, 19). CTL clones were isolated from dead cells by Ficoll-Hypaque centrifugation shortly before being used in assays. Clone 2C is specific for H-2L^d, BM10-37 for H-2K^b, and OE4 for H-2K^d.

Monoclonal Antibodies. V_β-specific anti-TCR antibody F23.1 (mouse IgG2a) (20), which reacts with 2C and OE4 CTL clones, was purified by affinity chromatography using a protein A column, and clonotypic anti-TCR mAb 1.B2 (mouse IgG1) (21), which interact with 2C CTL clone was purified by ammonium sulfate precipitation and DEAE-cellulose column chromatography.

Preparation of Wells with Solid-phase mAb. The wells of a 96-well microtiter plate (Immulon or MC-2000; Dynatech Laboratories, Inc., Alexandria, VA) were coated with monoclonal anti-TCR antibodies by incubating 50 μl of antibody solution per well in PBS at 0°C for 30 min. After incubation each well was washed with RPMI 1640/10 mM Hepes/5% FCS. Visual observation of CTL incubated with immobilized anti-TCR mAb under a light microscope confirmed their uniform distribution, because they formed a monolayer of cells occupying the entire surface area where mAb were immobilized; in contrast, CTL formed small pellets in wells where no anti-TCR mAb were immobilized.

Measurements of BLT-E Secretion. The amount of secreted BLT-E from 10^5 CTL was measured in 0.1 ml of RPMI 1640 supplemented with 10 mM Hepes, 5% FCS in a well of 96-well microtiter plate. An incubation time of 4 h was chosen on the basis of time-course studies (9), where we found that a plateau of secretion is reached by CTL after ~4 h of incubation. BLT-E activity was measured using a minor modification (9) of the method of Coleman and Green (22).

Assay for β-Glucuronidase Activity. Activity of β-glucuronidase in CTL supernatants or in Percoll gradient fractions of N_2 cavitation–disrupted CTL was measured using phenolphthalein glucuronic acid as a substrate (23). Briefly, 50 μl of sample solution was mixed with 300 μl of 0.1 M acetate buffer, pH 4.6, which contained 0.04% Triton X-100. The reaction was started by addition of 50 μl of 0.01 M phenolphthalein glucuronic acid, pH 7.0 (Sigma Chemical Co., St. Louis, MO). After 9 h incubation at 37°C the reaction was stopped by adding 1.0 ml of 0.2 M glycine buffer, pH 10.4, 0.2 M NaCl, and absorbance at 550 nm was measured.

Assay for Lactate Dehydrogenase (LDH) Activity. The activity of lactate dehydrogenase, a marker enzyme of the cytosolic compartment, was tested in supernatants of CTL harvested after 4 h incubation of cells in wells as described (24).

Affinity Labeling of Serine Esterases of CTL by [^{3}H]Diisopropylfluorophosphate ([^{3}H]-DIFP). CTL clone OE4 (2.4×10^6 cells) were suspended in 2.4 ml of RPMI 1640, 10 mM Hepes, 0.1 mg/ml BSA (RPMI 1640/Hepes/BSA). The suspension was distributed into 24 wells of a microtiter plate that was precoated with 0.1 μg of anti-TCR mAb F23.1 per well. After 4 h incubation at 37°C, cell suspensions were harvested and spun to isolate supernatant. Cell pellets and cells that adhered to the well were solubilized by 1.2 ml of 0.1% Triton X-100 containing RPMI 1640/Hepes/BSA. Solubilized cells and supernatants (equivalent to 2.0×10^6 cells) were incubated with 25 μCi of 1,3-[^{3}H]-DIFP (1 μCi/μl, 4.7 Ci/mmol, New England Nuclear, Boston, MA) at 37°C for 1 h. The reaction was terminated by addition of 1 mM cold DIFP (Aldrich Chemical Co., Milwaukee, WI), and two volumes of cold acetone (−20°C). After overnight incubation at −20°C, the proteins were precipitated by centrifugation at 12,000 *g* for 15 min and solubilized in 80 μl of SDS-PAGE sample buffer.

SDS-PAGE. After solubilization, samples were split into two parts; one was treated with 5% 2-ME while the other remained unreduced. SDS-PAGE was carried out with 10% polyacrylamide gel and the Laemmli buffer system (26). Gels were fixed in 50%

methanol/7.5% acetic acid, and treated with Autofluor (National Diagnostics, Somerville, NJ) before drying and autoradiography with Kodak XAR-5 film.

Studies of CTL–Target Cell Interactions Using Phase-contrast Microscopy. Cells were observed using phase-contrast optics on a Zeiss ICM 405 microscope (Carl Zeiss, Inc., New York) with a Planapo 100× oil-immersion objective. The specially constructed chambers (total vol, 200 μl, 10^6 CTL per chamber) were used to incubate CTL and simultaneously observe them under the microscope in thin preparations. The number of granule-like subcellular structures in CTL was also counted by applying 15 μl of cell suspensions on clean glass microscopic slides covered with glass coverslip. Such preparations allowed better resolution of granules under the phase-contrast optics needed to enumerate granule-like structures. Cells were incubated in chambers in Hepes-containing incubation medium on the microscope table at 37°C. Temperature (37°C) was maintained with a hot air incubator (Nicholson; Precision Instruments, Bethesda, MD).

Isolation of Intracellular Granules of CTL by Percoll Density Gradient Centrifugation. 11.5 × 10^6 of CTL OE4 were incubated 4 h in 10 ml of RPMI/Hepes/FCS per Petri dish (3003; Falcon Labware, Oxnard, CA), which was precoated with anti-TCR mAb (F23.1; 1.0 μg/dish during immobilization) or without stimuli for 4 h at 37°C. Three dishes were used for both F23.1 stimulating and nonstimulating control system. After incubation, the incubation medium was recovered for measuring secreted enzyme activity. Cells were then harvested, washed with PBS, counted, and suspended in disruption buffer which consisted of 0.25 M sucrose, 10 mM Hepes and 4 mM EGTA, pH 7.4, at 9.3 × 10^6 cells/ml. 2.0 ml of this cell suspension was subjected to the nitrogen cavitation using Kontes' Mini-Bomb (Kontes, Inc., Vineland, NJ) at 450 psi (30 atm) for 20 min at 4°C. The homogenate was spun at 300 *g* for 2 min. 1.5 ml of nuclei-free homogenate was overlayed on top of 10 ml of Percoll solution consisting of 30% Percoll, 10 mM Hepes, 0.25 M sucrose, and 2 mM EGTA, pH 7.4. Using a Beckman 50Ti rotor, this solution was centrifuged at 24,000 rpm for 18 min at 4°C. In a parallel tube Percoll gradient was calibrated using colored density marker beads (Pharmacia Fine Chemicals, Piscataway, NJ) with buoyant density 1.133, 1.112, 1.099, 1.070, 1.050, 1.049, and 1.032 g/ml. The resulting density gradient was fractionated by 0.6 ml per fraction, and used for analysis of enzyme content.

Results

Secretion of BLT-E Is Regulated by Crosslinking of the Antigen Receptor on CTL. Incubation of CTL clones with soluble anti-TCR mAb does not stimulate the secretion of enzyme (9), while solid-phase immobilized anti-TCR mAb trigger strong response. Similar results were obtained with both clonotypic mAb (1.B2) and with F23.1 mAb, while mAb 83-7-2, which do not react with clone 2C (9), did not stimulate BLT-E secretion (Fig. 1*A*). The increase in surface density of immobilized anti-TCR results in the increase of the response until it reaches a plateau (Fig. 1*B*). These data demonstrate that amount of solid-phase anti-TCR mAb per well is a determining factor in triggering BLT-E secretion. To exclude the possibility that BLT-E activity is released as a result of nonspecific damage of CTL during incubation in vitro, we tested supernatants of activated and unstimulated CTL for activity of two granule markers (β-glucuronidase and BLT-E) and the cytoplasmic marker LDH (Fig. 2). Negligible release of LDH (~10%) was detected when CTL were incubated with activating anti-TCR mAb, while significant specific release of β-glucuronidase (~66%) and BLT-E (~50%) was detected in parallel assays. This experiment provides an important control for specificity of secretion of granule-associated enzymes as a result of TCR crosslinking on CTL surface.

It was important to determine if secretion of enzyme is constitutive or results in depletion of the cellular enzyme content, which would indicate regulated

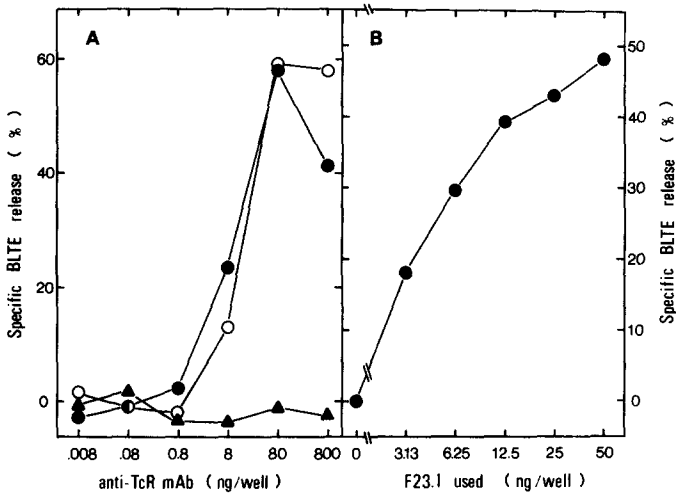

FIGURE 1. Stimulation of BLT-E secretion by immobilized mAb to antigen receptor on the CTL surface. mAb to TCR were immobilized on the surface of the wells of a 96-well microtiter plate by incubating them with different concentrations of purified anti-TCR mAb. Secretion of granule content was expressed as percent of secretion of granule enzyme BLT-E during 4 h incubation of 10^5 CTL with solid-phase anti-TCR mAb as described in Materials and Methods. *A*. Effect of clonotypic (1.B2) and V_β-specific (F23.1) anti-TCR mAb on the secretion of BLT-E by clone 2C. The amount of protein in 40 μl of solution used during immobilization of anti-TCR mAb on the well is indicated on the abscissa, and varied from 0.008 to 800 ng. (○) effect of 1.B2 mAb; (●) effect of F23.1 mAb; (▲) effect of irrelevant mAb (83-7-2) (9) that do not react with 2C CTL clone. *B*. Effect of V_β-specific F23.1 anti-TCR mAb on the secretion of BLT-E by clone 2C. The amount of mAb in 40 μl solution used during their immobilization in the well ranged from 3.13 to 50 ng as indicated on the abscissa.

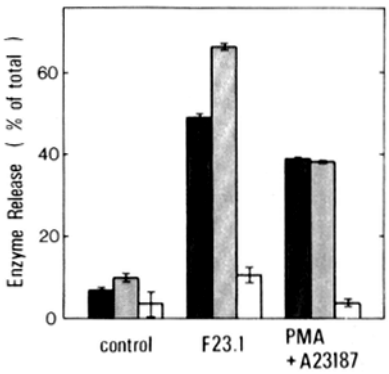

FIGURE 2. Measurement of activity of BLT-E, β-glucuronidase, and LDH in the supernatants of CTLs incubated with immobilized anti-TCR mAb or with mixture of PMA and A23187. CTL OE4 were incubated in the wells in the absence of activating ligands, or in the wells with solid-phase anti-TCR mAb (F23.1, 0.1 μg/well), or with the mixture of PMA (10 ng/ml) and A23187 (0.5 μg/ml). CTL were incubated for 4 h in the CO_2 incubator. Enzyme activities in supernatants were measured as described in Materials and Methods. Open bars, activity of LDH; light-shaded bars, activity of β-glucuronidase; dark-shaded bars, activity of BLT-E.

secretion (13). It is shown in a typical experiment (Fig. 3) that appearance of BLT-E in the supernatant is accompanied by a corresponding decrease in the amount of BLT-E in cell pellets, while the total amount of BLT-E before and after stimulation (cell pellet and supernatant) did not change significantly. Analysis of the amount of BLT-E in Percoll gradient fractions (Fig. 4) confirms these results and reveals dramatic loss of BLT-E and of another marker of granules (β-glucuronidase) in the granule fraction after 4-h incubation of CTL with anti-TCR mAb F23.1.

Percoll Gradient Centrifugation Profile of Granule-associated and Cytoplasmic Enzymes after Incubation of CTL with Immobilized Anti-TCR mAb. The major peak

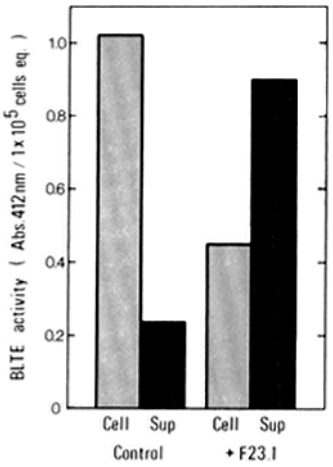

FIGURE 3. Measurement of the amount of released and cell-associated BLT-E after incubation of CTL clone OE4 with stimulating anti-TCR mAb F23.1. Light-shaded bars, activity of cell-associated BLT-E; dark-shaded bars, activity of secreted BLT-E in supernatant. CTL OE4 were incubated 4 h with solid-phase F23.1 mAb, and activity of BLT-E in supernatant and in solubilized CTL was measured as described in Materials and Methods.

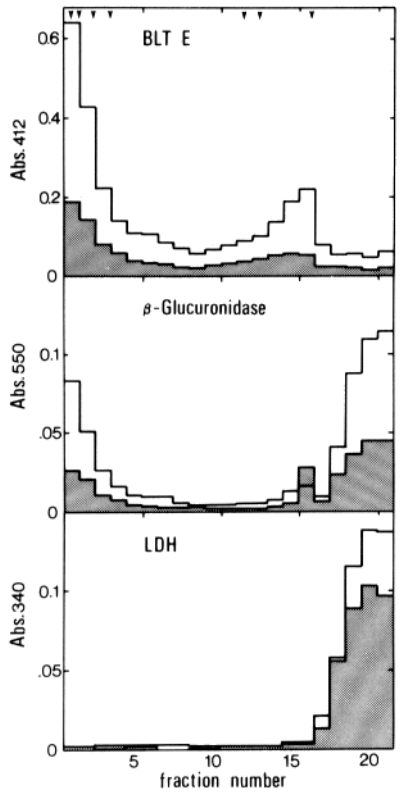

FIGURE 4. Distribution of BLT-E, β-glucuronidase, and LDH enzymatic activities in Percoll gradient fractions of CTL. CTL OE4 were stimulated with solid-phase anti-TCR mAb, F23.1 and disrupted by nitrogen cavitation and cell homogenates were centrifuged in Percoll gradient as described in Materials and Methods. Percoll gradient density markers were centrifuged simultaneously in a parallel tube. Arrowheads on the top of top panel indicate position of density marker beads: from left (bottom of the centrifuge tube) 1.133, 1.112, 1.099, 1.070, 1.050, 1.049, 1.032 g/ml, respectively. Empty bars, activity of enzyme in the Percoll gradient fractions of control CTL; Shaded bars, activity of enzyme in the Percoll gradient fractions of CTL incubated 4 h with solid-phase anti-TCR mAb.

of BLT-E activity was detected in fractions 1–7 at buoyant densities higher than 1.070 g/ml. Additional small peak was found in fractions 14–16, with buoyant density between 1.049 and 1.032 g/ml. The nature of the particulate material with lighter buoyant densities, and which is sedimented near the top of the gradient but below soluble cytoplasmic markers (Fig. 4, LDH panel) is not yet known and awaits further studies. After incubation of CTL with immobilized anti-TCR mAb followed by subcellular fractionation on Percoll gradient (hatched

bars, Fig. 4) significantly less of BLT-E activity could be detected in granule fraction. Similar data, which confirm the exocytic nature of secretion of BLT-E in CTL were obtained when distribution of another granule enzyme β-glucuronidase (8, 23) was studied in parallel experiment (Fig. 4).

Peak of β-glucuronidase activity was found in the same heavy fractions 1–7 with BLT-E and ~62% of β-glucuronidase activity was lost from fractions 1–7 after 4 h incubation (Fig. 4), which correlates well with the 66% of BLT-E loss from these fractions in parallel samples (Fig. 4, BLT-E panel). However, not all β-glucuronidase activity is colocalized with BLT-E in heavy granule fraction; large part of the cellular β-glucuronidase content was detected near the top of the gradient (17–20), where practically all activity of the cytoplasmic marker LDH is found (Fig. 4). These data suggest that β-glucuronidase could be located in at least two different subpopulations of granules: (*a*) in heavy granules with BLT-E, and (*b*) in more fragile intracellular compartments, which may not survive procedure used to disrupt CTL before Percoll gradient centrifugation. The studies of LDH distribution in Percoll gradient helped to identify the areas of the Percoll gradient, where soluble cytoplasmic enzymes are located and to control for nonspecific mechanical CTL damage due to their harvesting from the plates after 4-h incubation with immobilized anti-TCR mAb. In numerous control experiments (e.g., Fig. 2) we were not able to detect specific LDH release that was triggered by stimulating antibody when LDH activity was measured in the same supernatants of CTL, which contain specifically secreted BLT-E and β-glucuronidase. However, ~20% of total cellular content of LDH is lost when CTL were scraped from the plastic with immobilized anti-TCR mAb (Fig. 4) by mechanical harvesting procedure.

Appearance of [^{3}H]DIFP-labeled 29 kD Polypeptide in Supernatants of CTL Incubated with Immobilized Anti-TCR mAb. Another independent evidence for the TCR-regulated secretion of intragranular component is provided by the experiment (Fig. 5), where 29 kD [^{3}H]DIFP labeled polypeptide of CTL (4, 5) is used as a marker of granules.

Using SDS-PAGE analysis under reduced condition, only a 70 kD [^{3}H]DIFP binding protein (polypeptide) was detected in incubation medium of CTL that were incubated without stimuli (Fig. 5, lane *a*), while additional 29 kD band was detected in incubation medium of CTL stimulated with immobilized F23.1 (Fig. 5, lane *b*). The appearance of 29 kD polypeptide in supernatant was accompanied with the decrease in the cell-associated 29 kD polypeptide (Fig. 5, lane *c* and *d*). This apparent correlation between the increase in the amount of cell-associated [^{3}H]DIFP labelled proteins in CTL supernatant and decrease of the amount of cell-associated BLT-E activity is confirmed by gel scanning data (Fig. 5, *A–D*). Integration of the 29 kD peak (Fig. 5, *C* and *D*) allowed us to semiquantitatively estimate that ~40% of [^{3}H]DIFP-labeled 29 kD protein is secreted in the supernatants of CTL. This is in agreement with intensity of secretion of BLT-E by CTL incubated in the same conditions in a parallel control experiment.

Because the 70 kD [^{3}H]DIFP binding peptide was detected even in the absence of cells (Fig. 5*e*), this band is most likely derived from BSA preparation (Sigma Chemical Co.; fraction V) which was used as a carrier protein for protein precipitation procedure. This [^{3}H]DIFP binding protein and secreted or cell-

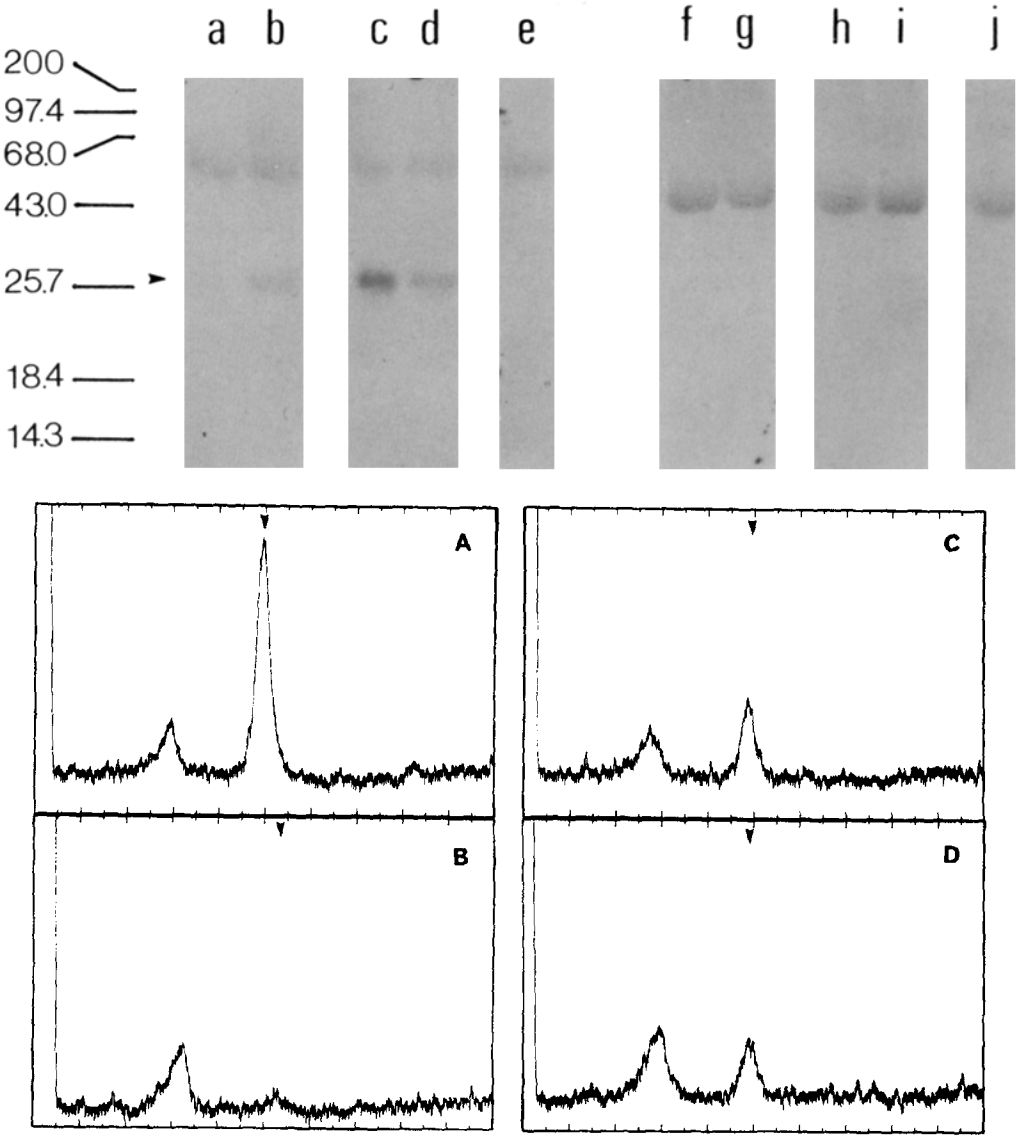

FIGURE 5. Release of [^{3}H]DIFP labelled proteins by CTL incubated with immobilized anti-TCR mAb. Labelling of protein in solubilized cells (CTL clone OE4) and cell supernatants with [^{3}H]DIFP, precipitation of protein and preparation of samples for SDS-PAGE are described in Materials and Methods. Specific BLT-E secretion determined in a parallel experiment was 40%. 15 μl of each sample (3.2×10^6 cell equivalent) was used for each lane. SDS-PAGE and autofluorography were carried out as described in Materials and Methods. Lanes *a–e*, SDS-PAGE of [^{3}H]DIFP-labeled proteins in reducing conditions. Lane *a*, supernatant of CTL incubated in the absence of stimuli ligand; lane *b*, supernatant of CTL incubated with immobilized F23.1 mAb; lane *c*, cell-associated [^{3}H]DIFP-binding proteins of CTL, which were incubated in the absence of activating mAb; lane *d*, cell-associated [^{3}H]DIFP-binding proteins of CTL, which were incubated with immobilized F23.1 mAb; lane *e*, no CTL, only BSA-containing incubation media was present in the tube during [^{3}H]DIFP labeling. Lanes *f–j*, SDS-PAGE of [^{3}H]DIFP-labeled proteins in nonreducing conditions. Lane *f*, the same as in lane *b*; lane *g*, the same as in lane *a*; lane *h*, the same as in lane *d*; lane *i*, the same as in lane *c*; lane *j*, the same as in lane *e*. Numbers on the left side indicate the molecular mass of the standard proteins run in the same gel. The arrowhead near lane *a* indicates the position of [^{3}H]DIFP-labeled protein of 29 kD. Panels *A–D* represent a densitometry scan of lanes *c*, *a*, *d*, and *b*, respectively. Relative signal intensities of 29 kD band (position of 29 kD band is indicated by the arrows on the panels A–D) estimated by densitometry were 6:4 between lane *c* (CTL pellets after secretion) and lane *d* (CTL supernatants with secreted material), which is in agreement with parallel assay of BLT-E release from CTL.

associated [^{3}H]DFP binding protein were superimposed to give one diffused band under nonreducing condition (Fig. 5, *f–j*).

Taken together, the results described in Figs. 1–5 provide biochemical evidence for the TCR-regulated exocytosis in CTL and demonstrate that TCR triggered activation of CTL results in appearance in the supernatants of granule-associated enzymatic activities, of a granule-associated [^{3}H]DIFP binding protein, and this release of granule components into the media is accompanied by their disappearance from the granules as detected by analysis of CTL by Percoll gradient centrifugation.

Morphological Studies of Granules During CTL–Target Cell Interactions. Secretory granules were implicated in the CTL's lethal hit delivery on the basis of earlier morphological data, where deposition of acid phosphatase (7) and osmophilic staining material (27) was detected in CTL–target cell contact areas. Using high-resolution cinematography, a population of cytoplasmic granules was visualized in cloned CTL (28). Because the time course of detected granule reorientation and fusion correlated well with the time course of programming for lysis, the authors suggested (28) that at least some of the observed granules are the same as cytolytic granules described by others (1, 2, 8). In our phase-contrast microscopic studies we found that CTL that are in contact with antigen-bearing target cells undergo profound morphological changes in shape (they become rounded) and in organization of granules. Our studies confirm observation of Yannelli et al. (28) and suggest that granule reorganization in CTL is antigen-specific. Because we have shown that TCR-triggered CTL activation results in significant loss of granule-associated enzymes (Figs. 1–5) we expected to confirm the loss of granules in visual observations (Table I). It was found that after 4 h of incubation of CTL OE4 with antigen-specific target cells P815 less granules could be detected in comparison with CTL OE4, which were incubated 4 h with antigen-nonbearing EL4 target cells (Table I). CTL incubated with antigen-nonspecific target cell EL4 had elongated shape, while most CTL had a rounded shape when incubated with P815. This may reflect the TCR-mediated shape change in CTL, and such interesting phenomenon warrants further investigation. Similar results were obtained when CTL were analyzed after incubation with PMA/A23187. However, the decrease in the number of granules per CTL was less profound with PMA/A23187 than with antigen-bearing target cells (Table I). This is consistent with the results of BLT-E secretion assay of CTL OE4; PMA/A23187 treatment was less efficient than target cells in BLT-E secretion triggering in numerous experiments. These morphological data are in agreement with our secretion studies, where TCR-crosslinking with antigen on the target cell surface (9) or by anti-TCR mAb (Figs. 1–5) results in the loss of granule-associated material and its appearance in the supernatant.

The described system of cloned CTL interacting with immobilized anti-TCR mAb (Figs. 1–5) allows us to manipulate the intensity of CTL response (exocytosis) and study the molecular requirements for exocytosis triggering in carefully controlled experimental conditions.

Synergy Between Protein Kinase C Activators and Calcium Ionophores in Triggering Exocytosis in CTL. The results, described in Fig. 1, demonstrate the strict requirement for TCR crosslinking in CTL exocytosis triggering. We and others (16, 29) demonstrated recently that such requirement for the "lethal hit" trig-

TABLE I

Changes in Number of Intracellular Granular Structures in CTL Clone after Incubation with Target Cells

Exp.	Incubation of CTL in observation chamber	Number of granules per CTL			
		0–10	10–20	20–30	30–40
1	OE4 + EL4*	11	59	55	25
	OE4 + P815*	68	62	15	5
2	OE4 alone‡	23	76	40	11
	OE4 + PMA/A23187‡	56	56	30	8

CTL with or without target cells were introduced into the observation chambers, incubated at 37°C, and were monitored by microscopic observation. After 4 h, the number of granule-like structures per each individual CTL was determined using a phase-contrast microscope in different focal planes. Total number of CTL evaluated per sample was 150.

* CTL OE4 were mixed with target cells (E/T ratio, 1:1), spun down to promote conjugate formation, resuspended, and introduced into the observation chamber as described in Materials and Methods.

‡ CTL OE4 were incubated in the observation chamber with 10 ng/ml PMA, 0.5 μg/ml A23187, or with an equivalent volume of DMSO (0.05% of total volume) for 4 h at 37°C, and the number of granules in CTL was evaluated under phase-contrast microscopy.

gering in CTL can be bypassed by the synergistic action of protein kinase C activators and calcium ionophores when they are added to the mixture of CTL and ^{51}Cr-labelled antigen-nonbearing target cells. None of the tested protein kinase C (30) activators (phorbol esters or bryostatin I and bryostatin II [31]) nor Ca^{2+} ionophore had a significant effect on BLT-E release from CTL when added alone (Figs. 6 and 7).

However, when added together, Ca^{2+} ionophore and protein kinase C activators exerted a synergistic effect in triggering secretion (Figs. 6 and 7, *A–D*). A maximum response, similar to its magnitude to that elicited by target cells or by immobilized anti-TCR mAb was produced by 10 ng/ml PMA in the presence of 0.5 μg/ml of A23187 (Fig. 1*A* and *B*; Figs. 6 and 7). A similar effect of PMA and A23187 was observed when release of [^{3}H]DIFP-labelled proteins was analyzed (data not shown). In some CTL clones (e.g., 2C) PMA/A23187 treatment was as effective as incubation with immobilized anti-TCR mAb in triggering the secretion, while other CTL clones (e.g., OE4) were more efficiently triggered to secrete BLT-E by solid-phase anti-TCR mAb than by PMA/A23187; CTL clone BM10-37, on the other hand, was more efficiently triggered to secrete BLT-E by PMA/A23187 than by clonotypic anti-TCR mAb (data not shown). Incubation with PMA/A23187 was not toxic for CTL both by visual observations, by trypan blue exclusion assay and by LDH assay (Fig. 2).

The data described in Figs. 6 and 7, *B–D* suggest that protein kinase C activation is involved in CTL exocytosis triggering. Experimental results described in Fig. 7, *A*, *B*, *E*, and *F*, support such interpretation. When different phorbol esters that have different potency of protein kinase C activation in vitro (32) were tested in CTL activation and BLT-E secretion assay, the same hierarchy was found in their ability to stimulate exocytosis: PMA was the most potent (Fig. 7*B*), while the less potent 4-β-phorbol-12,13-didecanoate (4-β-PDD) still induced

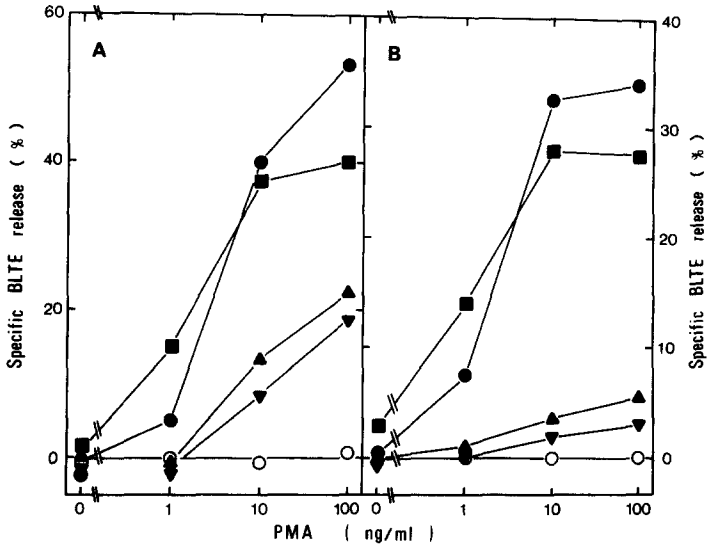

FIGURE 6. Effect of phorbol esters and Ca^{2+} ionophores on BLT-E release from CTL clones. CTLs were incubated at 10^5 cells/well with different combinations of PMA and A23187 for 4 h and released BLT-E was tested in the supernatant as described in Materials and Methods. *A*, CTL clone 2C. *B*, CTL clone BM 10-37. CTLs were incubated with different concentrations of PMA (▼); different concentrations of PMA in the presence of 0.05 μg/ml A23187 (▲) or 0.5 μg/ml A23187 (●) or 5.0 μg/ml A23187 (■); medium and dimethylsulfoxide only (○). At optimal combinations of PMA (10 ng/ml) and A23187 (0.5 μg/ml) for BLT-E release, only 7.4% of specific release of LDH was detected after 4 h incubation in a parallel control experiment. None of the tested agents interfered with the enzymatic assay.

strong exocytosis (Fig. 7*F*). 4-α-PDD, which does not activate protein kinase C in vitro, also does not have any activity in triggering of the exocytosis in CTL (Fig. 7*E*).

Role of Extracellular Ca^{2+} in TCR-triggered Exocytosis. Addition of 2.5 mM EGTA or EDTA alone or in different combinations with 3.0 mM Ca^{2+} or 3.0 mM Mg^{2+} did not affect the basal level of secretion (Fig. 8*A*). However, secretion of BLT-E triggered by immobilized anti-TCR mAb is inhibited by 2.5 mM EGTA or EDTA (Fig. 8). Addition of excess of Ca^{2+} (3.0 mM), but not Mg^{2+} (3.0 mM) reversed inhibition by EGTA and EDTA almost completely (Fig. 8*B*). These results reflect the necessity of extracellular Ca^{2+} in exocytosis, which is triggered by the interaction of solid-phase anti-TCR mAb with TCR on the surface of CTL.

EGTA and EDTA also completely blocked secretion of BLT-E triggered by the synergistic action of PMA and A23187 (Fig. 8*C*). Addition of excess Ca^{2+}, but not Mg^{2+} reversed the inhibitory effect of EDTA to a large degree, and the inhibitory effect of EGTA was partially reversed by Ca^{2+}. Thus, the presence of Ca^{2+} in incubation buffers is obligatory for the triggering of the exocytosis in CTL.

This conclusion is supported by experimental results, where Mn^{2+} inhibited PMA + A23187–induced exocytosis (Fig. 8*D*). Compound A23187 is not accurately characterized by the common designation Ca^{2+} ionophore, since it is able to transport various metal cations in exchange for protons (33). Therefore, we tested if the effect of A23187 on CTL is due to calcium transport by using the

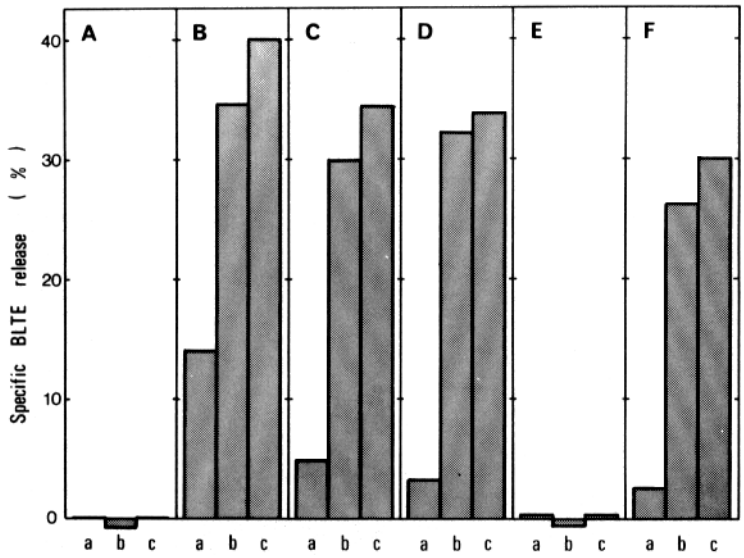

FIGURE 7. Effect of different protein kinase C activators and Ca^{2+} ionophores on BLT-E secretion by CTL clone 2C. Cells were incubated for 4 h in wells in the presence of different combinations of activating agents. In each panel, bars *a*, *b*, and *c* indicate the addition of dimethylsulfoxide as control, ionomycin (0.7 μg/ml = 1 μM), and A23187 (0.5 μg/ml = 1 μM), respectively. Protein kinase C activators were: *A*, dimethylsulfoxide control; *B*, PMA (10 ng/ml); *C*, bryostatin I (10 ng/ml); *D*, bryostatin II (10 ng/ml); *E*, 4α-phorbol-12,13-didecanoate (10 ng/ml); *F*, 4β-phorbol-12,13-didecanoate (10 ng/ml). None of the tested agents interfered with enzymatic assay as demonstrated in a control experiment.

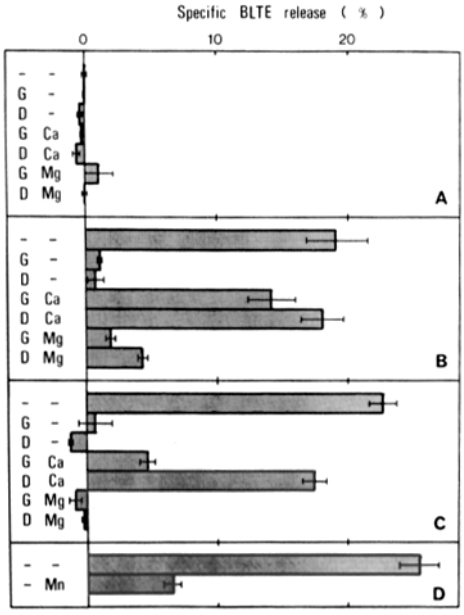

FIGURE 8. Effect of Ca^{2+} and Mg^{2+} chelators and Mn^{2+} on BLT-E secretion in CTL. CTL clone OE4 was incubated with stimulating agents in the presence of different combinations of EDTA (*D*), EGTA (*G*), Ca^{2+}, Mg^{2+}, and Mn^{2+}. *A*, Effect of Ca^{2+} and Mg^{2+} chelators on the basal level of BLT-E release. *G*, *D*, *Ca*, and *Mg* indicate addition of 2.5 mM EGTA, 2.5 mM EDTA, 3 mM Ca^{2+}, and 3 mM Mg^{2+}, respectively. *B*, Effect of Ca^{2+} and Mg^{2+} chelators on the secretion of BLT-E triggered by solid-phase mAb against the TCR on CTL clone. Bar markings are the same as in *A*. *C*, Effect of Ca^{2+} and Mg^{2+} chelators on the secretion of BLT-E, triggered by a synergistic action of PMA (10 ng/ml) and A23187 (0.5 μg/ml). Bar markings are the same as in *A*. *D*, Effect of Mn^{2+} (40 μM) on PMA (10 ng/ml) plus A23187 (0.5 μg/ml)–induced BLT-E secretion. None of the tested agents interfered with the BLT-E activity assay, as demonstrated in a control experiment.

ability of Mn^{2+} to block Ca^{2+} binding to A23187 (32, 33). It is known that Mn^{2+} has ~100 times higher affinity for A23187 than Ca^{2+}; therefore, it successfully competes with Ca^{2+} for A23187 and inhibits translocation of Ca^{2+} through the plasma membrane. It was reported that some effects of A23187 on lymphocyte

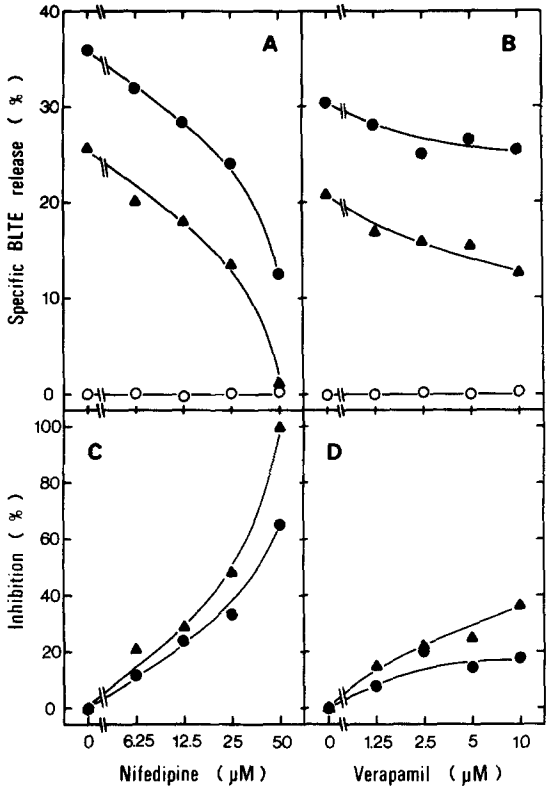

FIGURE 9. Effect of Ca $^{2+}$ channel blockers on BLT-E secretion from CTL clone. CTL clone OE4 was incubated with solid-phase F23.1 mAb, and effect of different concentrations of nifedipine (Sigma Chemical Co.; 5 mM stock solution in dimethylsulfoxide) and verapamil hydrochloride (Sigma Chemical Co.; 5 mM stock solution in distilled water) on secretion was tested. *A*, Effect of nifedipine on the basal level of BLT-E release (○) and on secretion of BLT-E triggered by F23.1 anti-TCR mAb, which were immobilized using a solution of mAb at 12.5 ng/well (●) or 3.13 ng/well (▲). *B*, Effect of verapamil on the basal level of BLT-E release (○), and on BLT-E secretion triggered by F23.1 mAb, which were immobilized using solution of mAb at 12.5 ng/well (●) or 3.13 ng/well (▲). *C*, Calculated inhibition values by nifedipine from data presented in *A*. *D*, Calculated inhibition values by verapamil from data presented in *B*. The concentrations of nifedipine and verapamil used here are the highest that are nontoxic, since at higher tested concentrations they affected the basal level of BLT-E release.

activation may be due to membrane perturbing properties of A23187 (34). In CTL, however, A23187 is most likely involved in extracellular Ca^{2+} translocation, and its activating effects are not due to membrane-perturbing properties, since addition of 40 μM of Mn^{2+} (Fig. 8*D*) did inhibit PMA/A23187 induced exocytosis. The results of this experiment support the view that A23187 participates in CTL activation by translocating extracellular Ca^{2+} into CTL. Demonstration of the obligatory requirements in extracellular Ca^{2+} for TCR-triggered exocytosis presented here complements and extends the results of stopped-flow fluorimetry in studies of the early transmembrane events in CTL activation (35).

Effect of Ca^{2+} Channel Blockers on TCR-triggered Exocytosis in CTL. The results we presented in Figs. 6–8 implicate extracellular Ca^{2+} in TCR-triggered exocytosis in CTL, and suggest that extracellular Ca^{2+} may be transported through the plasma membrane. Little is known about the role of plasma membrane Ca^{2+} channels in CTL function. To investigate the possibility that Ca^{2+} channels are involved in TCR-regulated exocytosis, we used two known Ca^{2+} channel blockers, nifedipine and verapamil (36) (Fig. 9). Neither Ca^{2+} antagonist exerted an effect on the basal level of secretion in CTL, but both inhibited TCR-triggered secretion (Fig. 9, *A* and *B*). A higher level of exocytosis was less inhibited by both Ca^{2+} antagonists, so that at 50 μM of nifedipine practically complete inhibition was observed when anti-TCR mAb triggered 26% BLT-E secretion, and only 64% inhibition occurred when anti-TCR mAb triggered 36% BLT-E secretion (Fig. 9, *A* and *C*). 50 μM nifedipine and 10 μM verapamil were maximum

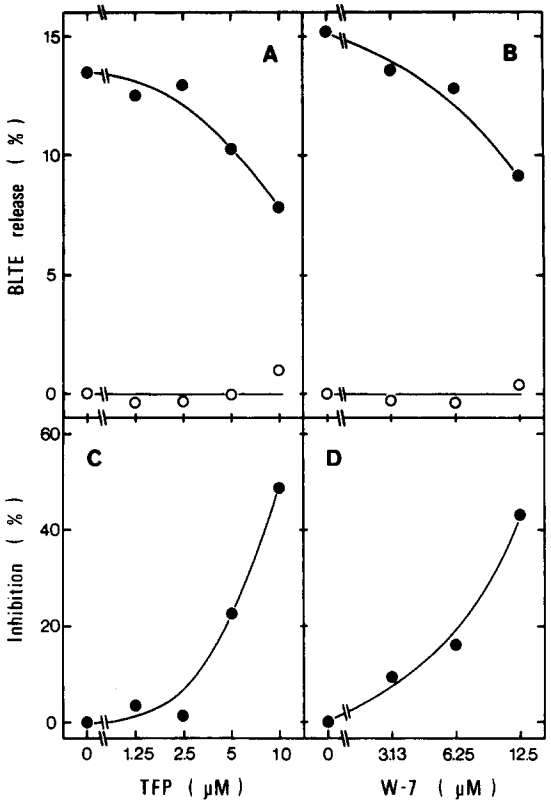

FIGURE 10. Inhibition of anti-TCR mAb–triggered exocytosis in CTL by calmodulin antagonists. CTL clone OE4 was incubated with immobilized F23.1 mAb and effect of different concentrations of TFP (Sigma Chemical Co.), and W-7 (Seikagaku America, Inc., St. Petersburg, FL), on the secretion was tested. *A*, (○), effect of TFP on the basal level of secretion; (●), effect of TFP on the anti-TCR-triggered secretion. *B*, (○) effect of W-7 on the basal level of secretion; (●), effect of W-7 on the anti-TCR-triggered secretion. *C*, Calculated inhibition value by TFP from data presented in *A*. *D*, Calculated inhibition value by W-7 from data presented in *B*.

nontoxic doses. The concentrations of Ca^{2+} channel blockers we used in these experiments are in the same range as used in studies of heart cells (36), and are similar to used in studies of early transmembrane events in CTL activation by stopped-flow fluorimetry (35). Results of experiments described in Fig. 9 suggest that Ca^{2+} channels are involved in TCR-triggered exocytosis in CTL.

Role of Calmodulin (CaM) and CaM-binding Proteins in TCR-triggered Exocytosis in CTL. The obligatory role of extracellular Ca^{2+} in TCR-triggered exocytosis (Fig. 8) and well documented increases in concentration of intracellular Ca^{2+} in activated T lymphocytes (37) and CTL (35, 38) suggest that Ca^{2+} and Ca^{2+}-dependent proteins are directly involved in CTL activation. Many Ca^{2+}-dependent reactions in eukaryotic cells are mediated by CaM, which is considered to be the main intracellular acceptor of Ca^{2+} (40). To investigate the possibility that CaM is involved in the exocytosis of granules from CTL we tested the effect of CaM antagonists on TCR-triggered secretion (Fig. 10). To exclude side effects of the reagents on TCR-triggered exocytosis, two CaM antagonists were used in studying CaM involvement in exocytosis in CTL. Trifluoperazine (TFP) (40), which prevents Ca^{2+} binding to CaM, and an unrelated CaM antagonist, *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7) (41), were used in experiments described in Fig. 10. We found that both TFP and W-7 were efficient in blocking anti-TCR mAb–triggered exocytosis. The data described in Fig. 10 implicate CaM and CaM-binding proteins in the exocytosis of granules in cyto-

toxic T lymphocytes, as was demonstrated earlier using CaM antagonists in the studies of exocytosis in other cells (42).

Discussion

The secretory granules in lymphocytes could be strategically important organelles in antigen receptor–mediated responses as concentrating and storage vesicles for cytotoxic proteins, enzymes (1–8), and for other yet-undiscovered biologically active molecules. It is therefore possible that antigen receptor–regulated exocytosis of granules is an important functional response of CTL on its own.

Data described in Figs. 1, 3, 4, and 5 demonstrate that the intensity of exocytosis in CTL is dependent on the surface density of activating anti-TCR mAb (or, by implication, of surface antigen) and suggest that TCR-triggered exocytosis is not constitutive, but is regulated by TCR crosslinking.

Observations of CTL under phase-contrast microscopy allowed us to correlate direct biochemical studies of secretion of several intragranular enzymes (Figs. 1–5) during TCR-regulated CTL activation with semiquantitative morphological data. It is not yet proven, however, that granule-like structures we observed are the same granules that contain BLT-E, β-glucuronidase, cytolysin, and [^{3}H]DIFP labeled 29 kD polypeptide. Nevertheless, these observations do provide a circumstantial evidence that supports and correlates with direct biochemical studies of the granule exocytosis in CTL.

The presence of Ca^{2+} in the incubation medium is found to be obligatory for TCR-triggered exocytosis (Figs. 7 and 8) and protein kinase C and increases of Ca^{2+} concentration are implicated in the exocytosis of granules in CTL by the synergistic effects of different protein kinase C activators and Ca^{2+} ionophores (Figs. 6–8). Secretion of intragranular material from the mast cells is one of the best documented models of Ca^{2+}-mediated exocytosis (6, 12, 43, 44). Despite obvious similarities between granule exocytosis in mast cells and CTL, one striking difference must be pointed out. The exocytosis of granules from CTL is much slower, which possibly reflects differences in localization of the granules in CTL and mast cells. While granules in mast cells are believed to be prepositioned near the plasma membrane, thereby facilitating their fast fusion with the plasma membrane (10), no preferential intracellular localization of granules has yet been reported in CTL in the absence of target cells. Use of "compound" exocytosis was suggested to account for the accleration of granule release in mast cells (10). We consistently were not able to trigger exocytosis in CTL by adding ionophore A23187 or ionomycin alone (Figs. 3 and 4). The documented ability of mast cells to respond by granule exocytosis to the increases in intracellular or extracellular Ca^{2+} or to the addition of Ca^{2+} ionophore (43) while CTL require both PMA and Ca^{2+} ionophore for exocytosis triggering may reflect different requirements for exocytosis of granules in these cells. Activation of contractile apparatus and cytoskeletal elements in CTL is most likely needed for translocation of granules toward the fusion site with plasma membrane. In contrast to our observations using normal CTL clones and secretion of granule enzymes as a measure of the exocytosis, Young and coauthors (45) suggested that stimulation of mouse CTLL-A11 and CTLL-R8 lines with A23187 alone resulted in the release of pore-forming protein into the extracellular medium.

The strict dependence of both TCR-triggered and PMA/A23187-triggered

exocytosis in CTL on extracellular Ca^{2+} (Fig. 8) suggests that sustained increase of $[Ca^{2+}]_i$ must be obligatory for the exocytosis maintenance. Such sustained $[Ca^{2+}]_i$ increase achieved by transmembrane signalling through TCR may be maintained through plasma membrane Ca^{2+} channels, since the Ca^{2+} channel inhibitors nifedipine and verapamil block TCR-mediated exocytosis (Fig. 9). Ca^{2+} channels were implicated in mechanisms of stimulation of human T lymphoma cells by mAb to T3/TCR complex (46). Data presented here provide missing functional evidence for the obligatory requirement and involvement of external Ca^{2+} translocation through the CTL plasma membrane Ca^{2+} channels in activation of CTL function. It remains to be seen in future studies if Ca^{2+} channels are involved in early events of CTL activation or in the latter stages of exocytosis, or both. The obligatory requirement in external Ca^{2+} for both the TCR-triggered and for the PMA/A23187-triggered exocytosis suggest that inositol triphosphate–induced release of calcium from intracellular stores may not be sufficient to support activation of CTL effector functions.

Data presented in Fig. 10 implicate CaM and CaM-binding proteins in CTL exocytosis. Changes in cellular Ca^{2+} levels might also control subcellular CaM distribution in CTL, as was demonstrated in chromaffin cells (47). It is of interest to investigate in future studies whether granules in CTL possess CaM-binding proteins, and if binding of CaM to the granules is Ca^{2+}-dependent. The stages of the transmembrane signalling pathway and exocytosis of granules that depend on CaM in CTL are not yet determined. When a CaM antagonist (TFP) was studied in other cells (48) it was found to inhibit the secretory response at a step distal from Ca^{2+} entry.

The experiments described in this paper establish the main features of the TCR-regulated exocytosis in CTL. They form a basis for a hypothetical model of CTL exocytosis activation through crosslinking of TCR and triggering of the phosphoinositide/Ca^{2+} pathway, where sustained increase of intracellular levels of Ca^{2+} is maintained by the translocation of extracellular Ca^{2+} through Ca^{2+} channels in the plasma membrane. Additional detailed studies are needed to understand the role of CaM and CaM-binding proteins in CTL activation and exocytosis. Discovery of synergistic properties of Ca^{2+} ionophores and protein kinase C activators, which have different structure and pattern of induced protein phosphorylation in triggering exocytosis in CTL provide a convenient and simplified experimental system in which further biochemical studies will help to identify cytosolic, membrane, and cytoskeletal proteins that are involved in the regulation of these complex, TCR-triggered mechano-biochemical activities of CTL.

Summary

We demonstrate here that T cell receptor for antigen (TCR)-triggered exocytosis in cytotoxic T lymphocytes (CTL) is not constitutive and is regulated through crosslinking of the TCR by antigen or monoclonal anti-TCR antibodies. Morphological and biochemical data using three different biochemical markers of granules and Percoll gradient fractionation analysis are presented, suggesting that TCR-triggered exocytosis is accompanied by the loss of granules from CTL and appearance of intragranular proteins and enzymatic activities in the incubation medium. The strict requirement for crosslinking of the TCR in exocy-

tosis triggering could be bypassed by protein kinase C activators (phorbol esters or bryostatin I and II) acting in synergy with Ca^{2+} ionophores. It is shown that external Ca^{2+} is obligatory for both the TCR-triggered and for the PMA/A23187-triggered exocytosis, since Ca^{2+} chelators and divalent cations that compete with Ca^{2+} for A23187 can inhibit exocytosis of granules. These data suggest that Ca^{2+} from intracellular stores is not sufficient to support exocytosis in CTL. Ca^{2+} channel blockers and calmodulin antagonists significantly inhibited TCR-triggered exocytosis without affecting the basal level of secretion. The described results are consistent with a model in which exocytosis of granules in CTL is triggered by the crosslinking of TCR, transmembrane protein kinase C activation, and external Ca^{2+} translocation through CTL plasma membrane Ca^{2+} channels and modulation of activity of Ca^{2+}, calmodulin-dependent enzymes, and cytoskeletal proteins.

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Note added in proof: The absolute requirement for extracellular Ca^{2+} for exocytosis of granules reported here and the ability of CTL to kill certain target cells in the absence of Ca^{2+} suggested the possibility of dissociating exocytosis of granules and lethal-hit triggering. Results of direct experiments performed in the presence of EGTA or in a calcium-free medium and designed to address this issue (G. Trenn, H. Takayama, and M. Sitkovsky, manuscript submitted for publication) support this conclusion and suggest that TCR-triggered exocytosis of cytolytic granules may not be required for target cell lysis by cytotoxic T lymphocytes.

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