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Functional localization of an exocytosis-triggering G-protein in human cytotoxic T lymphocytes

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SUMMARY

Human cloned CD8$^+$ cytotoxic T lymphocytes permeabilized with α-toxin of *Staphylococcus aureus* can be triggered by the guanosine triphosphate (GTP) analogue GTPγS to release the contents of their granula by exocytosis. To localize the guanosine nucleotide-binding protein (G-protein) activated by GTPγS in the sequence of events after T-lymphocyte triggering we have used several inhibitors of T-cell activation that inhibit early stages in T-cell triggering. The protein kinase C-inhibitor staurosporine, the immunosuppressants cyclosporin A and FK-506 and genistein, an inhibitor of tyrosine kinases, all inhibited esterase release triggered in intact cells by anti-T-cell receptor antibodies but not GTPγS-induced release from permeabilized cells. Cyclosporin A, FK-506 and genistein also blocked exocytosis triggered in intact cells by a combination of phorbolester and the calcium ionophore A23187. In addition, cytochalasin B, an inhibitor of actin polymerization, inhibited exocytosis in intact cells but enhanced exocytosis from permeabilized cells. These data show that the G-protein effecting exocytosis is localized distally in the cascade of events after T-cell activation.

INTRODUCTION

Triggering of the lethal hit in cytotoxic T lymphocytes (CTL) is accompanied by the exocytosis of the contents of cytoplasmic granules. Although granule-independent mechanisms of killing have been described, delivery of the lytic components of the granules is sufficient for destruction of target cells.[1-3] The mechanisms by which antigen recognition by the CTL ultimately leads to granule exocytosis are unclear. The first events after T-cell receptor (TcR) triggering are the phosphorylation of several proteins on tyrosine residues and a breakdown of phosphatidylinositol phosphates to inositol phosphates (IP_3) and diacylglycerol (DAG) by a phospholipase C (PLC). These second messengers cause a rise in cytosolic calcium concentration and an activation of the protein kinase C (PKC).[4,5] Later steps in the cascade of events leading to the exocytotic process are still unclear.

Abbreviations: ATPγS, adenosine-5′-*O*-(thiotriphosphat); BLT, *N*-α-benzoyloxycarbonyl-L-lysine thiobenzylester; CTL, cytotoxic T lymphocytes; CsA, cyclosporin A; DAG, diacylglycerol; DNTB, 5,5′-dithio-*bis*-(2-nitrobenzoic acid); GTP, guanosinetriphosphate; GTPγS, guanosine-5′-*O*-(thiotriphosphate); G-protein, guanosine nucleotide-binding protein; IP_3, inositoltrisphosphate; mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; PLC, phospholipase C; PTK, protein tyrosine kinase; TcR, T-cell receptor.

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It is presently a matter of debate how the PLC is activated to generate IP_3 and DAG. Evidence has been presented by several groups that a guanosine nucleotide-binding protein (G-protein) directly couples the TcR with the PLC,[6-12] other authors favour a direct phosphorylation of PLC by a TcR-associated tyrosine-kinase.[13-15] In addition, it has been suggested that a G-protein may not directly couple TcR and PLC but may modulate TcR–PLC signalling.[16] G-proteins are regulatory proteins linked to a large number of surface membrane receptors. They transduce signals from the receptor molecule to a second messenger system within the cell.

To study the intracellular events following TcR triggering until granule exocytosis we have previously used staphylococcal α-toxin to permeabilize human CTL. α-Toxin selectively induces holes of 2 nm diameter into the plasma membrane of the CTL. These holes allow manipulation of the intracellular milieu of the cells without destroying their structure. The introduction of the compound GTPγS that irreversibly activates G-proteins is sufficient to trigger exocytosis in permeabilized CTL. Surprisingly, after modulation of their TcR such T cells no longer respond to GTPγS.[12] Similarily, TcR-modulated T cells do not respond to the G-protein stimulator aluminium fluoride.[17] These data have indicated that the G-protein stimulated by GTPγS to effect exocytosis might be associated with the TcR. In the present report we have used this system to test the effect of several inhibitors of T-cell activation on GTPγS-induced exocytosis. The data show that the G-protein triggering exocytosis is localized not at the TcR but distally in the cascade of events.

MATERIALS AND METHODS

Cells

Human CTL clones were generated from peripheral blood mononuclear cells (PBMC) by limiting dilution. T-cell blasts after stimulation with antigen or mitogen were incubated at a density of 1 cell/well in 20 μl containing 10^4 irradiated PBMC and 50% phytohaemagglutin PHA-conditioned medium in microtest plates. Growing colonies were expanded using feeder cells and phytohaemagglutin (PHA) in interleukin-2 (IL-2) containing medium. They were propagated as described previously.[18] The cells had the $CD2^+$ $CD3^+$ $CD4^-$ $CD8^+$ phenotype, were $CD25^+$, $CD26^+$ and $HLA\text{-}DR^+$ and grew in culture for several months.

Reagents

Staurosporine was obtained from Boehringer Mannheim, (Mannheim, Germany), and genistein from GIBCO-BRL (Eggenstein, Germany). These compounds were dissolved in DMSO. Cyclosporin A (CsA) was a gift from Dr B. Ryffel (Sandoz AG, Basel, Switzerland) and FK-506 a gift from Fujisawa Pharmaceutical Co. (Osaka, Japan). These compounds were dissolved in ethanol. All experiments included solvent controls. In no case was an effect of DMSO or ethanol observed (data not shown). All other chemicals were obtained from Sigma Chemical Co. (Munich, Germany). Monoclonal antibodies (mAb) to a constant part of the αβ TcR (BMA031) were a kind gift from Dr R. Kurrle (Behring Werke AG, Marburg, Germany). α-Toxin from *Staphylococcus aureus* was purified from culture supernatants as described.[19] One unit is defined as the amount of toxin required to cause half-maximal lysis of 2·5% rabbit erythrocytes in phosphate-buffered saline (PBS).

Permeabilization

CTL were permeabilized in a buffered medium (assay medium) consisting of 150 mM potassium glutamate, 5 mM nitriloacetic acid, 10 mM Pipes, 0·5 mM EGTA, and 0·1% bovine serum albumin (BSA), pH 6·9, adjusted with KOH.[20] This medium contained 50 μM free Ca^{2+} and 1 mM ATP. The exact free Ca^{2+} concentration in the medium was calculated by means of a computer program using the stability constants given.[21] CTL were permeabilized with α-toxin from *S. aureus* by adding 30 U toxin/10^6 cells directly to the assay.

Measurement of serine esterase release

$CD8^+$ CTL (usually $0{\cdot}8 \times 10^5$ cells/well) were incubated in U-shaped 96-well microtiter plates (Nunc, Wiesbaden, Germany) at 37°. In some experiments, the wells had been precoated with goat anti-mouse IgG antibodies and subsequently with the anti-αβ TcR BMA031. After 45 min of incubation for permeabilized cells or after 3–4 hr for intact cells, the plates were centrifuged at 100 *g* for 3 min, and 25 μl samples of supernatant were harvested. Activity of serinesterase in the supernatant was measured by a modification of the method of Coleman and Green.[22] Briefly, 25 μl samples of supernatants were mixed with 225 μl of substrate solution (0·2 mM BLT and 0·22 mM DTNB in 200 mM Tris-HCl, pH 8·05). The mixture was incubated at 37° for 20 min. Absorbance at 405 nm was measured in comparison with a blank solution that was treated exactly as experimental. Maximal release was determined by solubilizing the cells using 0·1% saponin. Spontaneous release was determined in supernatants of unstimulated non-permeabilized cells. Data are given as per cent esterase release: (experimental release − spontaneous release) : (maximal release − spontaneous release) × 100. Data represent mean of triplicates; SD was usually below 10% and is not given. Triplicates were used throughout. Each experiment was performed at least three times and different T-cell lines were used.

Table 1. Exocytotic response of intact and permeabilized cytotoxic T lymphocytes to activation of protein kinase C

Stimulus	% esterase release from CTL
PMA (3×10^{-7} M)	2
A23187 (50 ng/ml)	5
PMA + A23187	31
α-Toxin	5
α-Toxin + PMA	21
α-Toxin + GTPγS (100 μM)	27
α-Toxin + GTPγS + PMA	65

Cloned cytotoxic T lymphocytes were treated with the reagents indicated and esterase release was measured after 3 h from intact cells and after 45 min from cells treated with α-toxin.

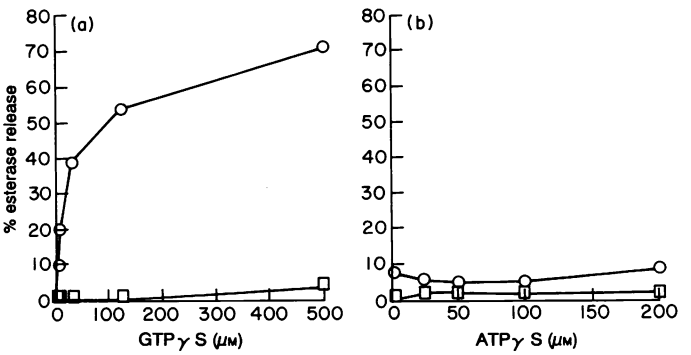

Figure 1. Triggering of exocytosis in permeabilized CTL by the GTP-analogue GTPγS. Cells were permeabilized with α-toxin or left untreated and then incubated with GTPγS (a) or ATPγS (b) at the concentrations indicated. Permeabilized CTL (○); CTL without α-toxin (□).

Assay for cytotoxic activity

A standard ^{51}chromium release assay was performed in triplicates in V-shaped microtitre plates. CTL were incubated with target cells (5×10^3/well) at an effector to target cell ratio of 3 for 4 hr.

RESULTS

To determine the localization of the G-protein triggering exocytosis upon activation with GTPγS we used several inhibitors of T-cell activation with defined action on steps in the sequence of events occurring after TcR triggering. We compared the effects of these compounds on triggering of intact and on permeabilized cells. For permeabilization cloned $CD8^+$ CTL

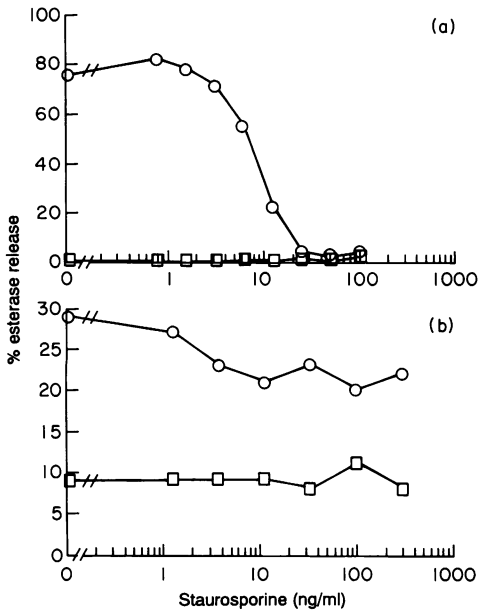

Figure 2. Effect of staurosporine on granula exocytosis triggered via TcR or GTPγS. CTL were triggered with solid phase bound anti-TcR mAb BMA031 (a) or with 100 μM GTPγS (b). Stimulated CTL (○); CTL without stimulus (□). Staurosporine was added at the concentrations indicated: it did not cause enhanced spontaneous release.

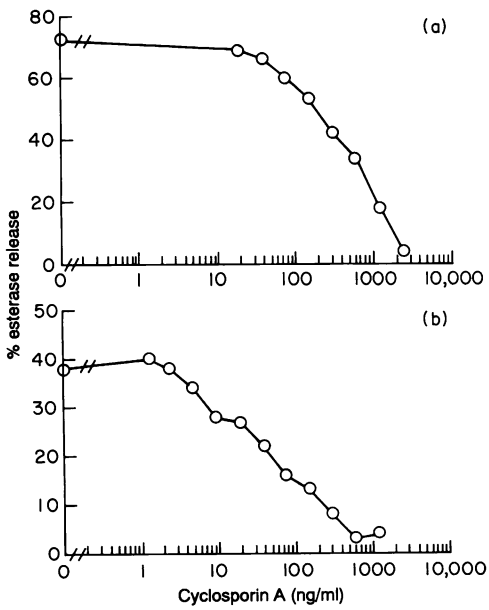

Figure 3. Inhibition of esterase release from intact CTL with cyclosporin A. CTL were incubated with solid phase bound mAb BMA031 (a) or with 5×10^7 M PMA and 100 ng/ml A23187 (b).

were treated with α-toxin to selectively induce holes of 2–3 nm into the plasma membrane.[23] This toxin is an ideal permeabilizing agent since upon contact with the target lipid bilayer the α-toxin monomers self-associate to form small transmembrane channels.[23] These pores represent a homogeneous population of ring structured hexamers with a defined pore diameter and do

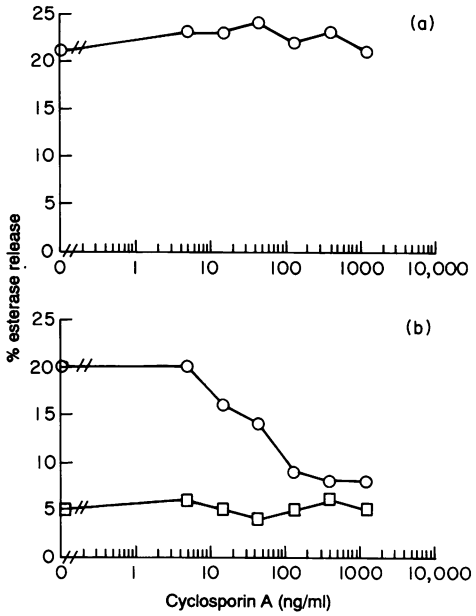

Figure 4. Effect of cyclosporin A on esterase release from permeabilized cells. CTL were permeabilized and stimulated with 100 μM GTPγS (a) or 5×10^{-7} M PMA (b). CsA was added at the concentrations indicated. Release from CTL in the absence of a stimulus (□).

not allow passage of molecules with a MW > 4000. Since α-toxin monomers cannot pass through these pores, the action of the toxin is strictly confined to the plasma membrane. This system allows the introduction of membrane-impermeable substances such as GTPγS or ATPγS into cell without inducing release of the macromolecular content from the cytoplasm. Figure 1 shows that GTPγS triggers exocytosis only in permeabilized cells already at at 20 μM (a) and that the related compound ATPγS had no effect even at much higher concentrations (b) on permeabilized T cells. Furthermore, we tested the release of the cytosolic enzyme lactate dehydrogenase from permeabilized cells: no lactate dehydrogenase was released from the CTL with GTPγS even at 10-fold higher α-toxin concentrations (data not shown).

Effects of inhibition of PKC

Activation of intact $CD8^+$ CTL by phorbol 12-myristate 13-acetate (PMA) and the ionophore A23187 lead to a strong release of BLT esterase (Table 1). PMA alone had no stimulatory effects. In permeabilized cells, in a medium containing 100 μM free Ca^{2+}, PMA leads to BLT esterase release. This release could be substantially enhanced by the addition of GTPγS (Table 1). Exocytosis of BLT esterase from intact cells triggered with the anti-TcR mAb BM031 could be completely inhibited by staurosporine, an inhibitor of PKC,[24] with 5–10 ng/ml causing half-maximal inhibition (Fig. 2). In contrast, staurosporine affected only marginally the GTPγS triggered exocytosis from permeabilized cells (Fig. 2).

Effects of cyclosporin A and FK-506

The immunosuppressants CsA and FK-506 are inhibitors of the peptidyl-prolyl-*cis*-trans-isomerase activities of the immunophilins.[25,26] They inhibit proliferation and lymphokine release of

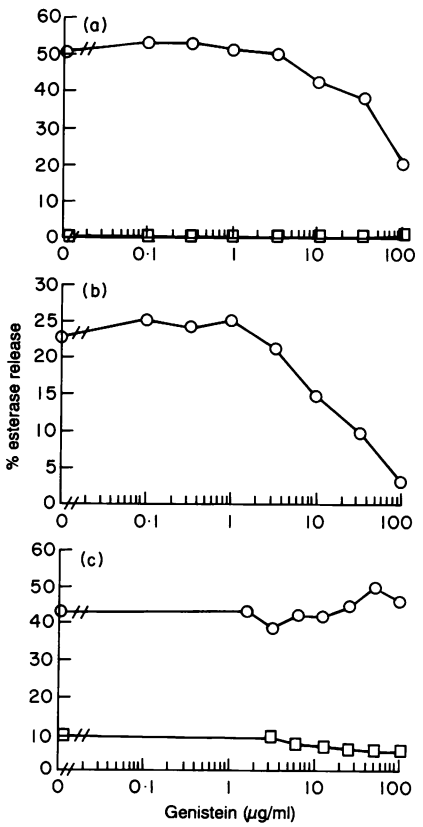

Figure 5. Effect of genistein on esterase release from CTL. (a) Esterase release triggered by solid phase anti-TcR mAb in intact cells. (b) Esterase release induced by 5×10^{-7} M PMA and 100 ng/ml A23187 in intact cells. (c) Esterase release triggered by 100 μM GTPγS in permeabilized cells. Spontaneous release from intact or permeabilized cells (□).

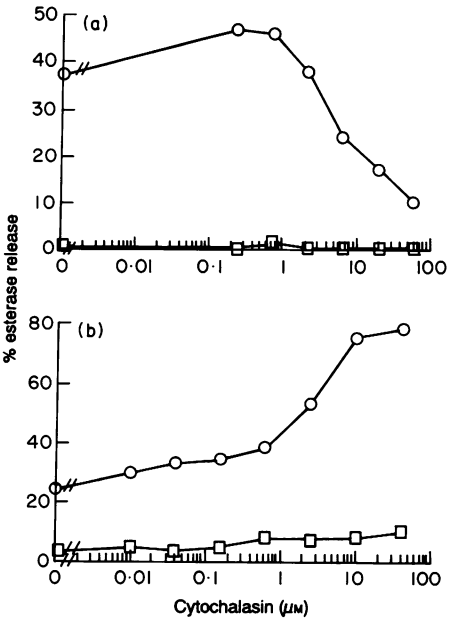

Figure 6. Effect of cytochalasin B on esterase release from CTL. (a) Esterase release triggered by solid phase anti-TcR mAb in intact cells. (b) Esterase release from permeabilized cells after triggering with 100 μM GTPγS. Spontaneous release from intact or permeabilized cells (□).

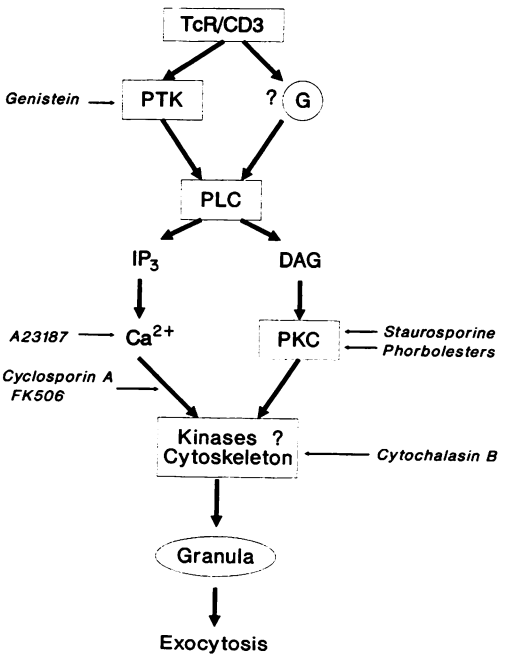

Figure 7. Flow diagram of T-cell activation showing the putative loci of action of the inhibitors used.

T cells. The precise mechanism of action of these substances is still unknown but there is evidence that CsA and FK-506 have an action in the early events in T-cell activation. We therefore tested the exocytotic response of CTL in the presence or absence of these compounds. In intact, non-permeabilized CTL triggered by either the anti-TcR mAb BMA031 or by PMA plus A23187, CsA inhibited exocytosis of serine esterases. Half-maximal inhibition was found at 400 nM or 40 nM, respectively (Figure 3). This indicates that CsA blocks a step after the action of PKC in CTL. In permeabilized cells triggering of exocytosis by PMA could be inhibited by CsA at a similar concentration as in intact cells. In contrast, triggering of exocytosis by GTPγS was not affected (Fig. 4).

The same results were obtained when we tested FK-506 for inhibitory activity. It inhibited release from intact CTL triggered with anti-TcR mAb or PMA plus A23187 with approximately 100-fold higher potency than CsA. It had no effect on triggering via GTPγS (data not shown). It should be noted that the concentrations of both CsA and FK-506 required to inhibit exocytosis were similar to those inhibiting T-cell proliferation.

Effects of an inhibitor of tyrosine phosphorylation

T-cell triggering is accompanied by a rapid phosphorylation of several proteins on tyrosine residues. It has been suggested that activation of phospholipase C proceeds via tyrosine phosphorylation because inhibitors of tyrosine kinases block the generation of second messengers after TcR activation.[13-15] We used genistein, an inhibitor of tyrosine kinases, in our system. Genistein blocked exocytosis of CTL triggered via TcR or by the

addition of PMA and ionophore (Fig. 5). It also blocked IL-2 production and IL-2-dependent proliferation by T cells (not shown). Triggering of exocytosis by GTPγS, however, was not inhibited even at 100 μg/ml.

Effects of cytochalasine B

Cytochalasine, an inhibitor of polymerization of actin filaments, inhibited PHA-induced cytotoxicity of $CD8^+$ CTL against several target cells (not shown) and inhibited the esterase release triggered by anti-TcR mAb in intact, non-permeabilized cells (Fig. 6). The exocytosis of BLT esterase from permeabilized cells by GTPγS was not inhibited but rather enhanced (Fig. 6).

DISCUSSION

GTPγS triggers exocytosis in permeabilized human cytotoxic T lymphocytes. That the response was only seen after introduction of GTPγS but not of ATPγS suggests that indeed a G-protein is mediating this exocytotic response. In this report we have used several inhibitors of T-cell activation to localize the G-protein mediating exocytosis. The loci of action of these compounds in the putative sequence of events after TcR triggering are shown in Fig. 7. All these inhibitors block exocytosis after TcR triggering of intact cells. However, genistein, that blocks the early events in T-cell activation, staurosporine, an inhibitor of PKC, cyclosporin A or FK-506 that block still not clearly defined later steps in T-cell activation, do not have an inhibitory effect on GTPγS-induced exocytosis. Not even cytochalasine B that blocks actin polymerization is able to inhibit exocytosis in permeabilized cells. It had, in contrast, reproducibly an enhancing activity.

We conclude from these experiments that the G-protein that is activated by GTPγS to effect exocytosis is localized distally in the cascade of events. It is possible that this G-protein is directly involved in the exocytotic process as has been postulated for the degranulation of neutrophils[27] and mast cells.[28] It may be related to the small G-proteins of 20,000–25,000 MW that control vesicle trafficking.[29] The enhancing activity of cytochalasin B could indicate that actin is involved in preventing vesicles from spontaneous fusion and that actin depolymerization leads to a higher sensitivity to the fusion promoting G-protein.

We have previously, based on our own data and on the results of others, suggested that a G-protein is mediating TcR–PLC coupling.[12] This suggestion was based on the findings that GTPγS was inhibitory for signalling via the TcR, that preincubation with PKC to deplete PKC leads to a reduction in the response to GTPγS and, most importantly, that modulation of the TcR/CD3 complex by antibodies leads to an unresponsiveness to GTPγS[12] and to AlF_4^-, an activator of G-proteins.[17] The results of the present study do not allow any conclusion about the mechanism of TcR–PLC coupling. Recent results from several laboratories make it likely that this coupling is mediated via direct tyrosine phosphorylations[13–15,30] and that G-proteins may only influence TcR signalling.[16] Why antibody-induced TcR modulation leads to an inhibition of the exocytotic response to GTPγS is still unclear. This inhibition is apparently unrelated to the refractoriness of alternative pathways after TcR triggering.[31] Modulation of the TcR complex, that requires some activation of the T cell,[32] possibly has consequences beyond the refractory state that affects early events in T-cell activation.

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