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Antigen–receptor interaction requirement for conjugate formation and lethal-hit triggering by cytotoxic T lymphocytes can be bypassed by protein kinase C activators and Ca^{2+} ionophores

(target cell lysis/lymphocyte activation)

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ABSTRACT **We show that phorbol esters and Ca^{2+} ionophores can trigger the lysis of nonantigen-bearing target cells by cytotoxic T lymphocytes. This effect obviates the requirement for antigen-receptor-mediated recognition of the antigen; the intensity of lysis is dose and Ca^{2+} dependent and requires contact between cytotoxic T lymphocytes and target cells. Using a fluorescence-activated cell sorter to enumerate cytotoxic T lymphocyte–target cell conjugates, we show that phorbol esters at concentrations that triggered lysis of non-antigen-bearing target cells also increased the number of stable conjugates with these target cells. The results point to the importance of the antigen-nonspecific engagements of cytotoxic T lymphocytes in immunologic surveillance. The data also show that the linkage between the T-cell receptor and antigen is not mandatory for conjugate formation, for the strengthening of conjugates, and for lysis.**

Activation of the cytotoxic T-lymphocyte (CTL) lytic functions is antigen (Ag) specific (for review see ref. 1), which implies involvement of the Ag-recognizing T-cell receptor in the triggering mechanism of the CTL. This assumption is supported by experiments with a monoclonal antibody against the CTL Ag receptor (2–8) and by studies of Ag-nonspecific cytotoxicity (9–11). Results (12) are also consistent with the view that the T-cell receptor is involved in the triggering of CTL. It is not yet clear, however, whether the Ag receptor is involved both in the CTL–target cell "bridge" and in triggering lysis or involved only in triggering lysis.

The biochemical reactions that occur subsequent to the specific binding of T-cell receptor to the Ag on target cells are poorly understood. The identification of the proteins involved in the activation of CTL may come from an approach in which the response of a CTL clone to various characterized chemical stimuli can be studied biochemically in the absence of target cells. One such stimulant may be Ca^{2+} ionophore (13–16), while other possible activators could be phorbol esters, which are known to activate protein kinase C (17, 18) and have been shown to mediate a moderate increase in nonspecific cytotoxicity by the CTL clones (19). The synergistic effect of Ca^{2+} ionophores and phorbol esters in the induction of lymphokine secretion has been shown with murine helper T cells (20) and human T-cell tumors (21).

The results presented here indicate that Ag receptor-mediated activation of CTL lytic functions can be bypassed by Ca^{2+} ionophores and phorbol esters. These reagents were able to trigger lysis of non-Ag-bearing target cells under conditions in which no special effort was made to facilitate conjugate formation between the CTL and the target cells. These data suggest that CTL in so-called "nonspecific" conjugates in the prerecognition step of CTL–target interactions require only an Ag-induced biochemical signal to kill target cells, and data point to the importance of the formation of conjugate with all surrounding cells in CTL surveillance activities, where the role of the Ag-specific receptor is limited to triggering lysis of the Ag-bearing target cells.

MATERIALS AND METHODS

Cells. Several CTL clones were used in these studies. CTL clone 2C was established by M.V.S. in H. Eisen's laboratory (Massachusetts Institute of Technology) by limiting dilution cloning of a long-term CTL line, which had been obtained by immunization of BALB/B mice with P815 ($H\text{-}2^d$) cells. Clone BM10-37 was kindly provided by J. Bluestone (National Cancer Institute, National Institutes of Health), CTL clone G4 was kindly sent by H. Eisen. Clones were maintained as described (22). Tumor cell lines P815 ($H\text{-}2^d$), EL4 ($H\text{-}2^b$), and Con A-stimulated lymphoblasts from C57BL/10 and DBA/2 mice spleens were used as target cells. Clone 2C is specific for $H\text{-}2L^d$; G4 is specific for $H\text{-}2D^d$ (9); BM10-37 is specific for $H\text{-}2K^b$ (23).

Reagents. Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma. Ca^{2+} ionophore A23187 and ionomycin were obtained from Sigma and Calbiochem, respectively. Stock solutions (1 mg/ml) of the reagents in dimethyl sulfoxide were kept at −70°C, and freshly prepared dilutions were used in experiments. Incubation medium was RPMI 1640 supplemented with 10% (vol/vol) fetal calf serum, glutamine, and nonessential amino acids (22).

Cytotoxicity Assay. Ficoll-purified CTL clones were mixed with ^{51}Cr-labeled target cells as described in the standard assay (24). Briefly, 5×10^3 or 10^4 ^{51}Cr-labeled target cells were added to the cloned CTL in U-bottom-well plates (96-well "Costar" plates) containing medium and phorbol ester, Ca^{2+} ionophore, or both reagents. The plate was centrifuged at $150 \times g$ for 2 min. Spontaneous ^{51}Cr release from the target cells did not exceed 15%. After a 6-hr incubation, the cells were pelleted by centrifugation, supernatants were assayed for radioactivity, and the percent of specific ^{51}Cr release was calculated as $100 \times [(a - b)/(t - b)]$, where a is ^{51}Cr release in the presence of CTL, b is spontaneous release from labeled target cells in the absence of CTL, and t is the maximum ^{51}Cr release from the target cells.

Conjugate Formation Assay. A modification (25) of a conjugate enumeration assay (26, 27) using a two-color fluorescence-activated cell sorter was also used. The enu-

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Abbreviations: CTL, cytotoxic T lymphocyte; E/T, effector to target; Ag, antigen; PMA, 4β-phorbol 12-myristate 13-acetate.

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meration of conjugates was also assessed visually using a fluorescence microscope (27, 28).

RESULTS

Combined Effects of Ca^{2+} Ionophores and Phorbol Esters in the Triggering of CTL-Mediated Lysis of Non-Ag-Bearing Target Cells. Different CTL clones were mixed with Ag-nonspecific ^{51}Cr-labeled target cells in the presence of various concentrations of phorbol esters and Ca^{2+} ionophores in a standard ^{51}Cr-release cytotoxicity assay. Fig. 1 *A–E* shows that PMA and Ca^{2+} ionophores synergistically induce a dose-dependent activation of CTL clones, resulting in the lysis of the nonspecific target cells. Release of ^{51}Cr from the target cell was detected only when both PMA and A23187 or ionomycin were added to the mixture of CTLs and target cells. The effect of the Ca^{2+} ionophores on ^{51}Cr release was small or negligible. When various concentrations of PMA were tested, it was found that concentrations of PMA as low as 5.0 nM act synergistically with 0.1 μM A23187 (Fig. 1*D*) and induced 18% of ^{51}Cr release at 24:1 effector/target (E/T) ratio. The Ca^{2+} ionophores A23187 and ionomycin were similar in their effect on CTL-mediated lysis (Fig. 2 *A* and *B*). The optimal combinations of PMA and A23187 or PMA and ionomycin for synergistic induction of nonspecific cytotoxicity were 15 nM PMA with 1.0 μM A23187 or 15 nM PMA with 0.3 μM ionomycin. These concentrations of reagents were optimal for activation of at least two different CTL clones (Fig. 2*C*). However, we were unable to induce nonspecific cytotoxicity with one CTL clone, G4, in four separate experiments (data not shown). CTL clones 2C and BM10-37 were chosen for further detailed studies. The PMA/A23187-induced cytotoxic activity against nonspecific target cells was always lower than the activity of the same CTL clone against the Ag-specific target cells (Fig. 1*F*) and lower than the level of cytotoxicity of a given CTL clone against the nonspecific target cells in the presence of a lectin (data not shown). The extent of PMA- and A23187-induced nonspecific target cell lysis varied in the different experiments. In most experiments PMA alone induced a modest increase in nonspecific cytotoxicity in agreement with the report of Russell (19) but occasionally PMA-induced, CTL-mediated ^{51}Cr release from the target cell was high even in the absence of Ca^{2+} ionophores. In such experiments combined effects of PMA and A23187 could not be considered synergistic.

The synergistic effect of PMA and A23187 was observed not only with tumor target cells but also with Con A-stimulated lymphoblasts from DBA/2 or C57BL/10 murine spleen cells with both CTL clones BM37 and 2C (data not shown). In control experiments we have shown that treatment with PMA or Ca^{2+} ionophores is not toxic for CTL and target cells (in the lactate dehydrogenase release test, in the trypan blue exclusion test, and in ^{51}Cr-release assays). We have also demonstrated that incubation of ^{51}Cr-labeled target cells with PMA and A23187 did not result in the significant increase of spontaneous ^{51}Cr release (e.g., Fig. 2) and that pretreatment of target cells with these reagents did not increase their susceptibility to nonspecific lysis by CTL clones (data not shown).

It is most likely that the effect of PMA is mediated by the protein kinase C (17, 18). In our experiments (Fig. 3), we have shown that PMA and 4β-phorbol 12,13-didecanoate, which activate protein kinase C *in vitro* [100% and 80%, respectively (17)], also activate CTL either alone (Fig. 3*A*) or in synergy with Ca^{2+} ionophore (Fig. 3*B*). In contrast 4α-phorbol 12,13-didecanoate, which does not bind and does not activate this enzyme, has only a small effect on CTL-

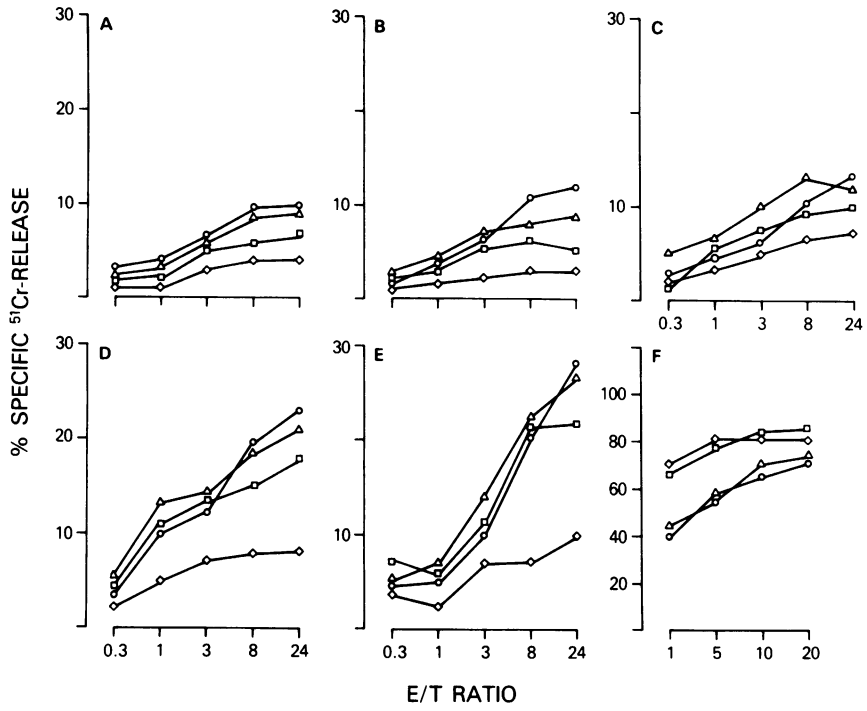

FIG. 1. Effect of phorbol ester and Ca^{2+} ionophore on the lytic activity of CTL clones toward the Ag-nonbearing and Ag-specific target cells. (*A–E*) Target cells (EL4, H-2^b) were incubated with CTL clone 2C (anti-H-$2L^d$) in the presence of different combinations of PMA and A23187. (*A*) ◇, medium only; □, 0.1 μM A23187; △, 0.3 μM A23187; ○, 1.0 μM A23187. (*B*) ◇, 0.5 nM PMA; □, 0.5 nM PMA and 0.1 μM A23187; △, 0.5 nM PMA and 0.3 μM A23187; ○, 0.5 nM PMA and 1.0 μM A23187. (*C*) ◇, 1.6 nM PMA; □, 1.6 nM PMA and 0.1 μM A23187; △, 1.6 nM PMA and 0.3 μM A23187; ○, 1.6 nM PMA and 1.0 μM A23187. (*D*) ◇, 5 nM PMA; □, 5 nM PMA and 0.1 μM A23187; △, 5 nM PMA and 0.3 μM A23187; ○, 5 nM PMA and 1.0 μM A23187. (*E*) ◇, 15 nM PMA; □, 15 nM PMA and 0.1 μM A23187; △, 15 nM PMA and 0.3 μM A23187; ○, 15 nM PMA and 1.0 μM A23187. EL4 target cell ^{51}Cr spontaneous release was 13.4%. (*F*) Ag-specific target cell P815 (H-2^d) were incubated with CTL clone 2C (anti-H-$2L^d$). □, CTL 2C, P815; ○, CTL 2C, P815, 15 nM PMA and 1.0 μM A23187; △, CTL 2C, P815, 15 nM PMA; ◇, CTL 2C, 1.0 μM A23187. P815 target cell spontaneous release was 12.8%.

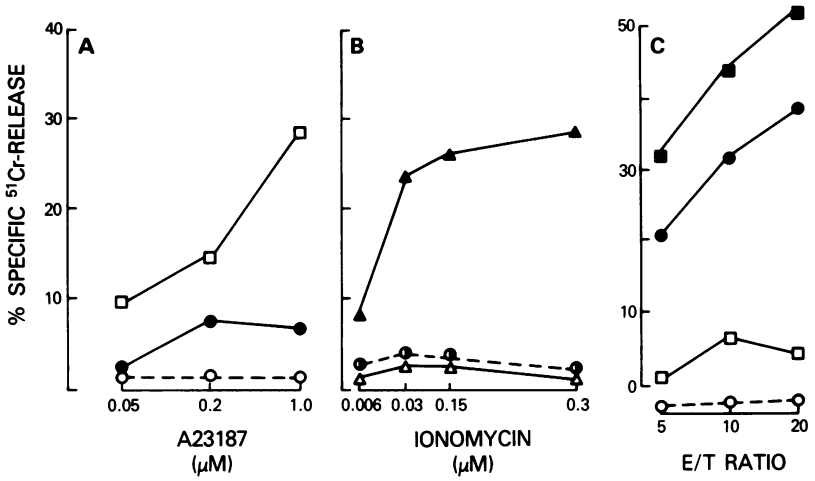

FIG. 2. Triggering of the lytic activity of the CTL clones by the Ca^{2+} ionophores and phorbol esters. (*A*) Effect of different combinations of PMA and Ca^{2+} ionophore A23187 on the specific ^{51}Cr release from EL4 (H-2^b) target cells in the absence and presence of CTL clone 2C (anti-H-2^d); E/T, 10:1. ○, CTLs, target cells, A23187, no PMA added; ●, target cells, A23187, and PMA (15 nM), no CTL added; □, CTLs, target cells, both PMA and A23187 added. (*B*) Effect of different combinations of PMA and Ca^{2+} ionophore ionomycin on the ^{51}Cr release from EL4 target cells in the presence and absence of CTL clone 2C (E/T, 10:1). ◐, CTLs, target cells, ionomycin, no PMA added; △, target cells, ionomycin, and PMA (15 nM); no CTL added; ▲, CTLs, target cells, ionomycin, and PMA (15 nM) added. (*C*) Comparison of the effect of PMA and A23187 on the lytic activity of 2C and BM10-37 CTL clones against Ag-nonspecific target cells in the ^{51}Cr-release assay. ○, CTL clone BM37 (anti-H-2^b) and P815 (H-2^d) target cell; ●, CTL clone BM10-37, P815 target cells, PMA (15 nM), A23187 (1 μM); □, CTL clone 2C, EL4 target cell; ■, CTL clone 2C, EL4 target cell, PMA (15 nM); A23187 (1 μM).

mediated nonspecific lysis (Fig. 3). While PMA is the most potent activator of CTL when added alone (Fig. 3*A*), it has the same potency as phorbol 12,13-didecanoate when added with Ca^{2+} ionophores (Fig. 3*B*).

The calcium dependence of the effect of PMA and A23187 on CTL was revealed in experiments where addition of 2 mM EGTA inhibited PMA plus A23187-induced nonspecific target cytotoxicity. Addition of excess Ca^{2+}, but not Mg^{2+}, reversed the inhibition by EGTA (Table 1). The data presented in Figs. 1, 2, and 3 and Table 1 suggest that Ag-specific activation of CTL-mediated target cell lysis can be bypassed by Ca^{2+} ionophores and phorbol esters that are acting in synergy. Effect of these agents is most likely mediated through the activation of protein kinase C followed by an increase in the intracellular Ca^{2+} concentration. The presence of CTL is necessary to observe PMA- and A23187-induced nonspecific target cell lysis in a ^{51}Cr-release assay. We could not detect lysis of ^{51}Cr-labeled target cells incubated in the supernatants of the CTL clones that were preincubated with PMA and A23187 for 3 or 6 hr. This suggests the requirement for CTL–target cell contact in triggering PMA/A23187-induced Ag-nonspecific lysis, as has been established for Ag-specific lysis.

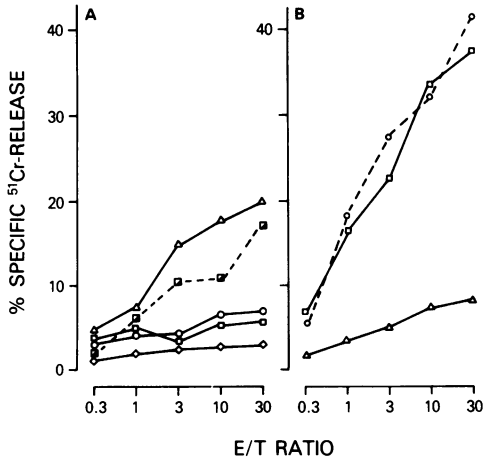

FIG. 3. Effect of different phorbol esters and Ca^{2+} ionophore on the lysis of the Ag-nonbearing target cells (EL4, H-2^b) by CTL clone (2C, anti-H-2L^d). (*A*) CTL 2C and target cells EL4 were incubated with the following agents: ◇, medium alone; ○, 1.0 μM A23187; △, 15 nM PMA; □, 15 nM, 4α-phorbol 12,13-didecanoate; ◩, 15 nM 4β-phorbol 12,13-didecanoate. (*B*) ○, 15 nM PMA and 1.0 μM A23187; △, 15 nM 4α-phorbol 12,13-didecanoate, and 1.0 μM A23187; □, 15 nM 4β-phorbol 12,13-didecanoate and 1.0 μM A23187.

Effect of Phorbol Ester and Ca^{2+} Ionophore on Conjugate Formation Between CTL Clones and Non-Ag-Bearing Target Cell. It has been demonstrated (29, 30) that engagement of CTL with an Ag-specific target cell is accompanied by the accumulation of some cytoskeletal proteins near the contact area of the CTL. One of these proteins, talin, was shown (31) to participate in transmembrane complexes formed between cytoplasmic proteins, integral membrane proteins (surface antigens), and extracellular proteins. It is possible that similar interactions between CTL surface proteins and the underlying membrane matrix are regulated by the specific Ag receptor and form the basis for mechanical activities of CTL. As a result of such activities strong, shearing-force resistant conjugates can be formed. Therefore, we expected that PMA and Ca ionophores would increase the number of antigen-independent conjugates between the CTL and target cells.

To test this prediction, two different conjugate-formation assays were used. The representative results of these experiments are shown on Fig. 4 and Table 2. Inspection of the contours and the computer-assisted estimation of the number of CTL in conjugates reveal an increase in the number of CTL in stable conjugates with Ag-nonspecific target cells when PMA was included in the incubation medium (Fig. 4 *A–D* and Table 2). Addition of PMA to CTL clones 2C and BM10-37 increased the number of nonspecific conjugates as measured by flow cytometry and microscopic assays. No effect of PMA and A23187 on the number of conjugates with Ag-specific target cells was detected. The number of CTLs in conjugates with Ag-nonspecific target cells estimated by conventional microscopic assay was also higher in the presence of both PMA and A23187. The number of conjugates was 2.4 times higher on average in four separate experiments where 200 CTLs were counted on each slide (range, from 1.7- to 3.1-fold increase). It is obvious that PMA alone induces as much

Table 1. Effect of Ca^{2+} and Mg^{2+} chelators on the PMA- and A23187-induced nonspecific target cell lysis

Addition						% specific ^{51}Cr release			
PMA	A23187	EGTA	EDTA	$MgCl_2$	$CaCl_2$	20:1	10:1	5:1	2.5:1
−	−	−	−	−	−	1.6	1.2	0.2	1.6
+	−	−	−	−	−	19.4	17.4	13.8	6.9
−	+	−	−	−	−	7.0	5.7	2.8	1.7
+	+	−	−	−	−	40.7	32.5	27.5	17.8
+	−	+	−	−	−	9.0	7.5	3.5	1.6
+	−	+	−	+	−	12.9	9.1	6.4	3.0
+	−	+	−	−	+	15.3	14.9	12.4	7.2
+	−	−	+	−	−	9.5	7.7	2.7	1.9
+	−	−	+	+	−	16.7	14.8	10.6	4.1
+	−	−	+	−	+	19.4	17.6	13.2	5.5
+	+	+	−	−	−	11.7	7.3	6.0	1.3
+	+	+	−	+	−	16.6	13.4	6.3	2.2
+	+	+	−	−	+	34.7	33.0	21.7	11.0
+	+	−	+	−	−	13.3	8.7	5.4	3.0
+	+	−	+	+	−	14.9	10.9	6.0	2.9
+	+	−	+	−	+	33.1	29.1	25.6	13.7

The % specific ^{51}Cr release at four E/T ratios is presented. Concentrations of the added reagents were as follows: 15 nM PMA; 1.0 μM A23187; 1.5 mM EGTA; 1.5 mM EDTA; 2 mM $MgCl_2$; 2 mM $CaCl_2$ in RPMI 1640. +, Presence of the compound in the incubation medium; −, absence of the compound.

increase conjugate formation as PMA plus A23187, while the ionophore alone has little or no effect (Table 2). EGTA partially inhibited the PMA/A23187-induced conjugate formation, implicating Ca^{2+} in PMA-induced CTL–target cell conjugate formation. Pretreatment of CTL with PMA and A23187 (Table 2) did not substantially affect conjugate formation when compared to the addition of PMA and A23187 after mixing CTL with target cells. Inhibition of PMA/A23187-induced conjugate formation (Table 2) as well as lethal hit delivery (data not shown) by anti-LFA-1 monoclonal antibodies implicates the same accessory surface molecules in both Ag-specific and -nonspecific interactions. The ability of PMA (at 50 ng/ml) to stimulate leukocyte adhesion and the requirement for LFA-1 antigen in such homotypic aggregation were demonstrated by Rothlein and Springer (33).

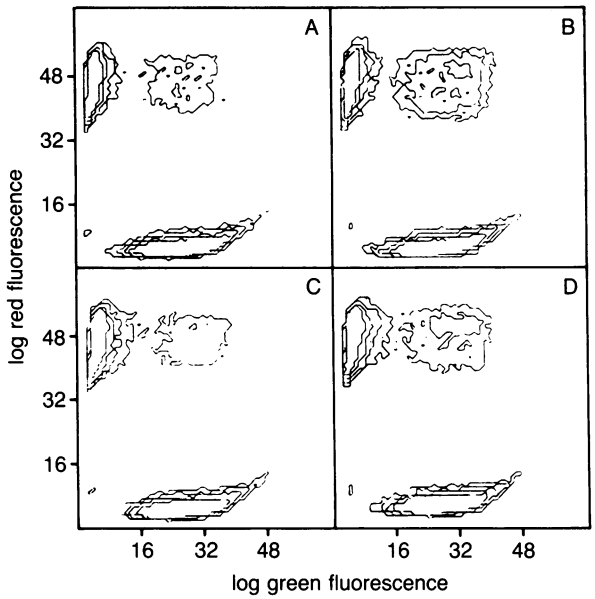

FIG. 4. Effect of phorbol ester and Ca^{2+} ionophore on the conjugate formation between CTL clone and Ag-nonspecific target cell. Representative results of two-color cell sorter conjugate enumeration assay. Hydroethidine-labeled (red) EL4 target cells were mixed with sulfofluorescein diacetate-labeled CTL (green) clone 2C cells and were analyzed by flow cytometry. Cell suspensions were Vortex mixed before the flow cytometry analysis. Contour plots were drawn through points containing 10, 20, and 40 particles; 5×10^4 particles were analyzed in each sample. Fluorescence was measured in arbitrary units. (*A*) CTLs and target cells; (*B*) CTLs and target cells mixed in the presence of 15 nM PMA; (*C*) CTL and target cells mixed in the presence of 1.0 μM A23187; (*D*) CTL and target cells mixed in the presence of both 15 nM PMA and 1.0 μM A23187. Computer-assisted estimation of the number of CTL in conjugates with target cells in this assay was as follows: (*A*) 6.9%; (*B*) 20.8%; (*C*) 9.9%; (*D*) 15.1%.

DISCUSSION

The results presented here support the view (34, 35) that during the nonspecific adhesion of CTL to target cells almost all of the requirements for triggering lysis are met and that the principal role of Ag recognition is simply to trigger a biochemical cascade, that includes protein kinase C and Ca^{2+}-dependent reactions (Figs. 1, 2, and 3).

Table 2. Effect of phorbol ester (PMA) and Ca^{2+} ionophore (A23187) on the conjugate formation between CTL clone 2C and nonspecific target cells (EL4) (% of CTL in conjugates)

Addition	Flow cytometry assay	
	Before	After
None	15	11
15 nM PMA	28	20
1.0 μM A23187	17	16
15 nM PMA/1.0 μM A23187	28	23
15 nM PMA/1.0 μM A23187/2 mM EGTA	22	16
15 nM PMA/1.0 μM A23187/anti-LFA-1 mAb	13	14

The percentages of CTLs in conjugates were determined from the flow cytometry data as described (25, 26). Number of conjugates between CTL 2C and antigen-specific target P815 in this experiment was 41% (5×10^4 CTLs were analyzed in each flow cytometry experiment). Only 2.2% of CTL were detected in aggregates when CTL 2C were mixed with EL4 immediately before analysis on the cell sorter. Before, CTLs were pretreated with agents before mixing with target cells. After, agents were added after mixing CTLs with target cells. Monoclonal antibodies to LFA-1 antigen (FD 4.4.1.8) were described (32) and were used at the concentration that caused 80% inhibition of Ag-specific CTL 2C-mediated cytolysis (G. Trenn, personal communication).

The foregoing explanation of cytolysis of nonspecific target cells by CTL in the presence of PMA and Ca^{2+} ionophore assumes that CTL immunological function requires them to engage in contact (nonspecific engagement) with every cell to be able to distinguish Ag-bearing, specific target cells from the surrounding syngeneic cells.

The ability of PMA to promote conjugate formation with Ag-nonbearing target cells (Fig. 4) excludes the necessity for Ag-receptor–ligand links in the formation and strengthening of the CTL–target cell conjugate. These results also emphasize the importance of accessory cell surface molecules in CTL–target cell contact interactions (35–37). It would be of interest in future studies to correlate the effects of Ag recognition and different combinations of protein kinase C activators and Ca^{2+} ionophores on the organization of the cytoskeleton and contractile system of CTL. It is important to point out the similarities between activation of lymphokine secretion by PMA and A23187 from cloned helper T cells (38) and the triggering lysis by CTL (Fig. 1). This may indicate that lysis by CTL also involves a secretory process as was proposed earlier in the granule–exocytosis model of cytotoxicity (1). In fact we have shown that protein kinase C activators act synergistically with Ca^{2+} ionophores in the triggering of the exocytosis of the intracellular granules from CTL clones (H.T. and M.V.S., unpublished data).

Synergistic effects of PMA and Ca^{2+} ionophores in the induction of nonspecific cytotoxicity were independently found by Lancki *et al.* (39). They noted that neither PMA nor Ca^{2+} ionophore alone induced efficient lysis of non-Ag-bearing target cells. In our experiments we always observed effect of the PMA and in some of our experiments effect of the PMA alone was not much lower than the effect of PMA and A23187 combined. We believe that such discrepancies are best explained by the differences between the CTL clones. It is also possible that the outcome of the combined addition of PMA and A23187 on lysis by CTL could partly depend on the cell cycle of the CTL, since translocation of protein kinase C from cytoplasm to plasma membrane could be regulated by the binding of interleukin 2 to the interleukin 2 receptors on T-lymphocyte plasma membranes (40).

Besides providing a triggering signal to CTL, ligand binding to the antigen receptor on the CTL surface could participate in the formation and strengthening of CTL–target cell conjugate. Although the latter are possibilities, the data presented here suggests that Ag receptor occupancy is required only for triggering the CTL lytic function and is not a mandatory requirement for conjugate formation. Published experiments using T-helper cells (20, 38) suggest that the same may be true for interactions between T-helper cells and Ag-presenting cells.

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