Phorbol ester regulation of Ca^{2+} flux during natural, lectin and antibody-dependent killing

M. JONDAL, J. NG, M. PATARROYO & P.-A. BROLIDEN *Karolinska Institute, Department of Immunology, Stockholm, Sweden*

SUMMARY

Phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C, suppresses natural, lectin and antibody-dependent killing by normal human lymphocytes in short-term radioisotope release assays. Fifty percent inhibition of killing of lymphoid target cells was seen at approximately 5 ng/ml TPA and inhibition was further potentiated by the presence of monocytic cells. In contrast, TPA increased killing of K-562 erythroleukaemic cells by non-adherent NK cells with optimal activity around 1 ng/ml. Two anti-estrogenic drugs, tamoxifen and clomiphene, known to inhibit protein kinase C, gave near to complete inhibition of NK killing at concentrations 12 μM and 30 μM, respectively. Retinal, another protein kinase C inhibitor, inhibited both antibody-dependent killing and lectin-dependent killing. An influx of $^{45}Ca^{2+}$ into the effector population was found during effector-target cell conjugation and this flux was suppressed at TPA concentrations similar to those that suppressed killing. The results suggest that killing depends on a co-ordinated activation of protein kinase C together with a channel-dependent calcium influx. TPA may suppress killing by a negative feedback effect of protein kinase C on the hydrolysis of inositol phospholipids, as demonstrated in many other systems, or through the down-regulation of cell surface receptors required for triggering of lysis.

INTRODUCTION

Human lymphocytes mediate strong natural killing of leukaemic cells and some normal target cells (Herberman, 1982). Addition of target specific IgG or mitogenic lectins further increases the lytic potential (Jondal & Targan, 1978; Berke, 1983). The initiation of killing requires transmembrane signals that eventually may lead to the degranulation of a lytic end product (Henkart & Martz, 1985). Although natural killing shares characteristics of 'stimulus-secretion' coupling (Trinchieri & Perussia, 1984) many events in the triggering process remain undefined.

In lymphocytes, as in other cells, two major transduction pathways are defined, namely a suppressive pathway that involves cyclic AMP and an inductive pathway that uses a combination of calcium ions and diacylglycerol (DAG) as second messengers (Nishizuka, 1984). Calcium as the main cellular regulator can be mobilized either from internal stores or from the outside of the cell by an uptake through specific membrane channels (Carafoli & Penniston, 1985). A variety of

cellular functions can be triggered by the combined treatment of cells with calcium ionophore and protein kinase C activators such as phorbol esters (Eisenberg, Lieman & Pecht, 1985; Kaibuchi, Takai & Mishizuka, 1985; Truneh *et al.*, 1985; Albert & Tashijian, 1985; Wolf *et al.*, 1985).

Phorbol ester treatment of cytotoxic effector cells either increases or decreases activity. Abrams, Bray & Brahmi (1983) demonstrated that TPA treatment of human NK cells suppressed killing of K-562 target cells, but did not influence target cell binding. Seaman *et al.* (1981) reported that this suppression was greatly reduced when adherent monocytes were removed from the effector cell population. Phorbol ester also selectively inactivates the lytic triggering machinery of cytolytic T lymphocytes (Russell, 1985) and this suppression is reversible when analysed on clonal effector cells (Orosz & Roopenian, 1985; Orosz, Roopenian & Bach, 1983). Orosz *et al.* (1985) reported that the cytolytic function of PMA-treated T-cell clones can be directly restored by incubation with highly purified interleukin-2 (IL-2) demonstrating that IL-2 not only regulates T-cell proliferation but also the expression of cytolytic function. Trinchieri *et al.* (1984) found increased killing of K-562 after phorbol ester treatment of the effector cell population and Ramos *et al.* (1983) reported a similar effect after long-term treatment together with the observation that this activation could be counteracted by the suppressive functions of monocytes.

Abbreviations; cAMP, cyclic adenosine monophosphate; Con A, concanavalin A; DAG, diacylgylcerol; IP_3, inositoltrisphosphate; NK cells, natural killer cells; PIP_2, phosphotidylinositol 4,5-biphosphate; TPA, 12-O-tetradecanoylphorbol-13-acetate.

Correspondence: Dr M. Jondal, Karolinska Institute, Dept. of Immunology, 104 01 Stockholm, Sweden.

Since TPA act as a DAG analogue and activate protein kinase C, we have further analysed the participation and role of protein kinase C in killing as reflected by the effect of TPA in different systems. Our results indicate that a cytotoxic cellular response depends on a co-ordinated stimulation of intracellular calcium and protein kinase C activity for the regulation of a membrane associated calcium channel. Both inhibitors and activators of protein kinase C seem to prevent a trigger influx of calcium into the effector cell population as has earlier been demonstrated in similar systems (Eisenberg *et al.*, 1985).

MATERIALS AND METHODS

Isolation of effector lymphocytes

Peripheral blood cells were obtained form healthy donors using density centrifugation on Ficoll-Isopaque (Jondal & Targan, 1978). The obtained cell population contained lymphocytes and monocytes and is referred to as whole mononuclear cell fraction. Most adherent cells were removed by passage through a nylon wool column after 30 min of preincubation at 37 °. The collected fraction of cells was called nylon wool passed cells. Monocytes were isolated using the plastic adherence method by incubation in large petri-dishes in RPMI-1640 medium supplemented with 20% fetal calf serum (FCS) for 1 hr at 37°. Thereafter, non-adherent cells were removed by washing the dishes several times and the remaining monocytes were removed by gentle scrubbing with a sterile rubber policeman. Monocyte fraction had a purity of above 90% as determined by morphology under light microscope.

Target cell lines

Target cell lines were maintained in tissue culture in RPMI-1640 medium supplemented with 10% FCS and penicillin and streptomycin.

Labelling of target cells

Fifty μCi of sodium-[51]chromate were added to approximately 20×10^6 cells in 200 μl and this incubated at 37 ° for 1 hr. Thereafter, the cells were washed three times and further incubated for 20 min to lower the spontaneous release. After the last incubation, the cells were washed again and diluted to the appropriate concentration required.

[51]Cr-release assay—natural killing, lectin and antibody-dependent cytotoxicity assays

Natural killing (NK) activity was tested by the standard microplate method using an effector:target cell ratio of 20:1 in a total volume of 150 μl of RPMI-1640 medium containing 10% FCS. The assay time was 3 hr and cytotoxicity was calculated by the standard formula (Jondal & Targan, 1978).

Lectin- and antibody-dependent cytotoxicity assays were carried out using Con A and rabbit IgG anti-human IgM antibody, respectively. The target cell line, Daudi, was pre-coated with either Con A or the antibody for 45 min at 37 °. Con A was used at 100 μg/ml as the starting concentration with subsequent concentrations diluted at 1:3. The antibody was diluted from 1:100 with eight 1:4 dilutions.

Inhibition of killing by TPA

In these studies, both whole mononuclear cell and nylon wool passed cell fractions were tested. These two populations of effector cells were treated with TPA, starting concentration of 100 ng/ml with 1:4 dilutions for the next eight concentrations. After 30 min at room temperature, these effector cells were tested for NK activity on the different target cell lines used.

[45]Ca^{2+} uptake studies

In the [45]Ca^{2+} uptake studies, different ratios of effector to target cells were used. Nylon wool passed cells were suspended in 250 μl RPMI medium with 10% FCS in 5 ml test tubes. Five μCi of [45]CaCl$_2$ were added to each tube. After incubation at 37° for 30 min, varying concentrations of target cells were added to all tubes except for a set of duplicate tubes that served as control for the effector cell fraction. The cells were spun down for 1 min and resuspended. This step ensured contact between the two populations of cells. Duplicate tubes of target cells alone were also set up and 5 μCi of [45]CaCl$_2$ were added. All tubes were incubated for another 15 min at 37°. The assay was stopped by adding 3 ml of ice-cold RPMI-1640 medium containing 2 mM of lanthanum chloride and was left at 4° for 1 hr to remove extracellular calcium. After 1 hr, the cells were washed three times with ice-cold medium. After the final washing, care was taken to remove excess medium from the tubes. Two blank control tubes were also treated in the same way. Traces of [45]CaCl$_2$ in the control tubes were subtracted form the test counts. When the cell pellet was ready, 500 μl of water were added to each tube, including the blank control. This volume was then transferred to plastic vials and 4 ml of scintillation fluid were added to each. The amount of radioactivity was counted using LKB beta counter.

RESULTS

Effect of TPA on NK killing of K562 and Jurkat target cells

Two different effector cell populations, the whole mononuclear cell fraction and nylon wool passed cell fraction were treated with different concentrations of TPA for 30 min at room temperature and tested for killing of target cell lines K562 or Jurkat (Fig. 1). The erythroleukaemic K562 is a standard NK cell target and Jurkat, a T-cell line from acute lymphocytic leukaemia (Orosz *et al.*, 1985). With monocyte containing effector cells, complete suppression of killing was seen at 8 ng/ml of TPA and with non-adherent effector cells similar suppression was seen with Jurkat cells although higher TPA concentrations
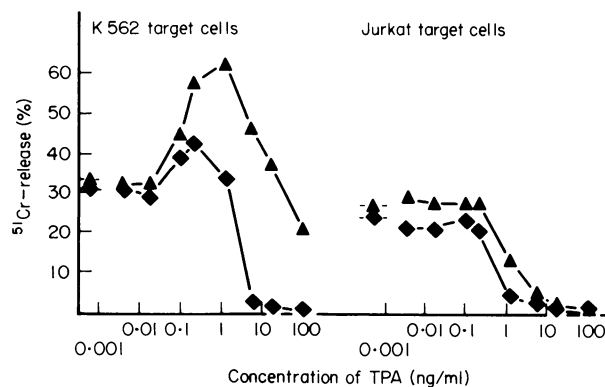

Figure 1. Effect of phorbol ester, TPA, on NK cell killing of two target cell lines, K562 and Jurkat cells: (▲—▲) nylon wool passed cells; (♦—♦) whole mononuclear cells; (-▲-) and (-♦-) control killing for respective cell fraction.
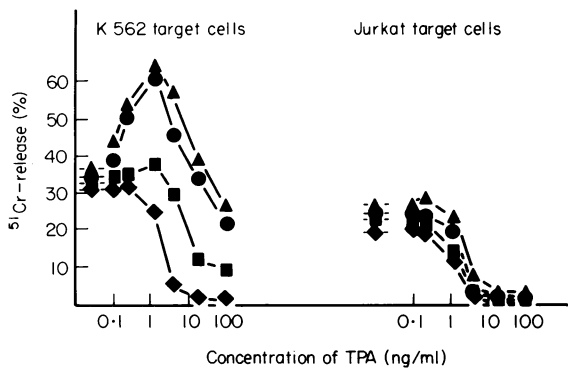
Figure 2. Mixed cell populations were tested for NK killing with varying concentrations of TPA on K562 and Jurkat target cells. Non-adherent NK cells alone (●————●); NK cells+NK cells (▲————▲); NK cells+whole mononuclear cell fraction (■————■); NK cells+monocytes (♦————♦).
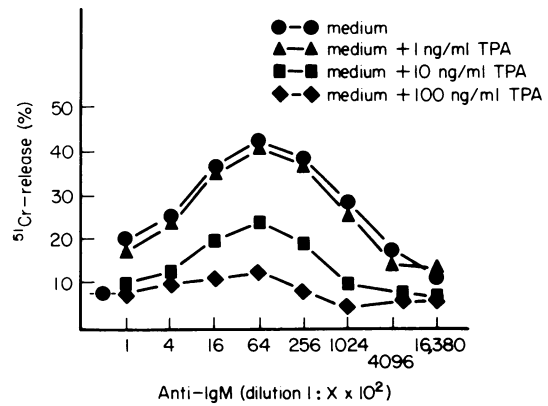

Figure 3. Influence of TPA on antibody-dependent killing. Daudi target cells were preteated with the various dilutions of anti-IgM and cytotoxicity assay was then carried out in the presence or absence of TPA.

were needed for similar levels of suppression. With K562 cells, there was a strong induction of killing by non-adherent cells at an optimal TPA concentration of 3 ng/ml. The TPA induction was most prominent against K562 cells and rarely seen against lymphoid target cells including a variety of normal Epstein-Barr virus transformed lymphoblastoid cell lines, Burkitt lymphoma derived cell lines and several other T-cell lines (data not shown).

TPA induced suppression was further investigated by mixing different effector cell populations. Non-adherent effector cells were tested against K562 and Jurkat when mixed with either non-adherent cells, the whole mononuclear cell fraction, or isolated monocytes (Fig. 2). Induction of K562 killing was seen with non-adherent cells and addition of the whole mononuclear cell fraction suppressed this induction. The addition of purified monocytes resulted in NK cell suppression similar to that seen against Jurkat target cell. With Jurkat cells the presence of monocytic cells resulted in a somewhat stronger TPA suppression as compared to that seen in Fig. 1.

Effect of TPA on lectin and antibody-dependent killing

Nylon wool passed effector lymphocytes were pretreated with TPA and tested for antibody-dependent killing of Daudi target cells (Fig. 3). Daudi cells express a large amount of surface-bound IgM and are killed by human lymphocytes in the presence of rabbit IgG anti-human IgM. Antibody-dependent killing usually demonstrated a pro-zone effect with an optimal induction at the intermediate antibody concentrations (1000–6400 in Fig. 3). Suppression of antibody-dependent killing against the Daudi cells was complete at 100 ng/ml and approximately 50% at 10 ng/ml. Lectin-dependent killing induced against the same target cells with Con A demonstrated an optimal activity at 33 ng/ml (Fig. 4). As with antibody-dependent killing, Con A-induced killing was almost completely suppressed at a TPA concentration of 100 ng/ml and 50% at 10 ng/ml.

Effect of retinal, tamoxifen and clomiphene on NK, lectin and antibody-dependent killing

The vitamin A analogue, retinal, is a tumour promoter and

protein kinase C inhibitor (Taffet, Greenfield & Haddox, 1983). The effect of retinal (20 μM) on NK, lectin and antibody-dependent killing was tested against different target cells (Table 1). Effector cells were pretreated for 30 min at room temperature with retinal and then tested for NK activity against the Burkitt lymphoma derived Daudi cells, Epstein-Barr virus transformed lymphoblastoid cell line LCL-1, T-cell line Molt-4 and K562 (Table 1). Lectin-dependent and antibody-dependent killing was tested as above against Daudi cells. In all test systems retinal was found to strongly suppress cytotoxicity. As with TPA suppression, NK cell activity was somewhat more suppressed than lectin- and antibody-dependent killing. The anti-estrogenic drugs tamoxifen and clomiphene, which have been found to inhibit protein kinase C from rat brain, ovary and muscle (Su *et al.*, 1985), were tested for inhibition of NK killing (Fig. 5). Near to complete inhibition was found at non-toxic concentrations of 12 μM and 30 μM for tamoxifen and clomiphene, respectively.
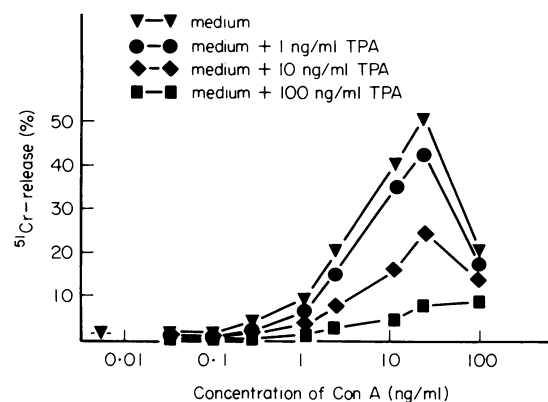

Figure 4. Influence of TPA on lectin-dependent killing of Daudi target cell line. Daudi cells were preteated with Con A at various concentrations and cytotoxicity assay was carried out in the presence or absence of TPA.

**Table 1.** Inhibition of natural, lectin and antibody-dependent killing by retinal

Experiment	Target cell	Cytotoxicity (^{51}Cr-release)		Inhibition (%)
		Control	Retinal	
1	Daudi	14·3	3·2	78
	Daudi/anti-IgM	31·4	14·6	53
	Daudi/Con A	46·7	22·7	51
	LCL-1	0	—	
	LCL-1/Con A	33·1	17·2	48
2	Molt-4	47·8	4·6	90
	K562	43·1	14·2	67

Effector cells were pretreated with retinal (20 μM) for 30 min at room temperature before the addition of target cells in a 3 hr ^{51}Cr-release assay. Target cells were also treated with anti-IgM for antibody-dependent killing and Con A for lectin-dependent killing.
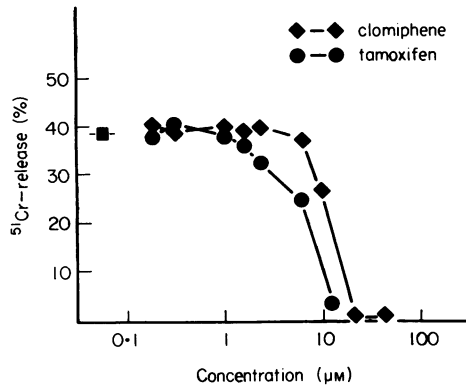
Figure 5. Effect of clomiphene and tamoxifen on NK killing with K562 target cells. Effector cells were pretreated with the two anti-estrogenic drugs at various concentrations.

^{45}Ca^{2+} flux during NK cell killing

The influx of ^{45}Ca^{2+} into the effector cell population was tested during the conjugation and killing of K562 target cells (Fig. 6). Influx of ^{45}Ca^{2+} was measured on isolated effector cells and target cells and on an effector–target-cell mixture during conditions allowing active killing, as described in the legend to Fig. 6. After background subtraction, there was a modest uptake of ^{45}Ca^{2+} in isolated effector cells and a larger uptake in K562 cells. During the first 15 min of contact between lymphocytes and K-562 there was a substantial influx of ^{45}Ca^{2+} into this combined cell population using an effector target ratio of 5:1 (Fig. 6). If the number of target cells was doubled, there was a corresponding uptake in isolated cells, although the specific uptake remained essentially the same (Panel B in Fig. 6). If the number of effector cells was reduced by 50% there was an approximate similar reduction of specific uptake, demonstrating that the influx of ^{45}Ca^{2+} occurred in the effector cell population. Several repeat experiments with K562 were per-
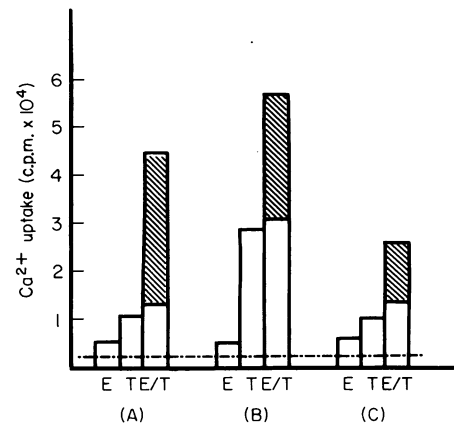
Figure 6. The influx of ^{45}Ca^{2+} was studied during effector-target cell conjugation. Panel A, the amount of ^{45}Ca^{2+} into effector cells alone (5 × 10^6), target cells alone (1 × 10^6), effector-target cell (5:1) conjugated population. Panel B, ^{45}Ca^{2+} into effector cells alone (5 × 10^6), target cells alone (2 × 10^6), effector-target cell conjugated population (2·5:1). Panel C, ^{45}Ca^{2+} influx into effector cells alone (2·5 × 10^6), target cells alone (1 × 10^6), effector-target cell conjugated population (2·5:1). E = effector cells. T = target cells. Shaded areas indicates specific influx of ^{45}Ca^{2+} into effector cell population.

formed, giving similar results. Uptake of ^{45}Ca^{2+} was also tested during NK killing of other target cell lines (Table 2). Specific uptake was most extensive during killing of K562, lower with Molt-4 cells and barely detectable during low degree of killing of Raji cells. Resistant Daudi and LCL-cells induced no ^{45}Ca^{2+} influx. The results are representative of several repeat experiments, always demonstrating a strong influx of ^{45}Ca^{2+} into lymphocytes killing K562 cells and a more variable response with other target cell lines. There was also a correlation between influx of ^{45}Ca^{2+} and degree of NK killing between different cell population as lymphocytes from low reactive blood donors demonstrated a much lower increase in ^{45}Ca^{2+}, as compared to high reactive donors (data not shown).

Table 2. Correlation between ^{45}Ca^{2+} uptake and ^{51}Cr-release during killing of different target cell lines with different NK cell susceptibility

Target cell	^{45}Ca^{2+}-uptake* (c.p.m.)	Cytotoxicity (^{51}Cr-release)
K562	36,423	23·4
Molt-4	14,096	31·2
Raji	5643	6·1
Daudi	0	0·8
LCL-Lars	0	0

The various target cell lines were studied for ^{45}Ca^{2+} uptake during NK cell killing. Parallel tests with ^{51}Cr-release were done with the same batch of effector cells.

* Specific uptake measured as described in the Methods and Materials section and the legend to Fig. 6.

Effect of TPA on ⁴⁵Ca²⁺ flux during NK-killing of K562 target cells

Non-adherent effector lymphocytes were pretreated with TPA and tested for $^{45}Ca^{2+}$ flux during killing of K562 cells (Table 3). TPA at 100 ng/ml gave a strong reduction in killing and also a similar strong inhibition of uptake of $^{45}Ca^{2+}$. TPA at 5 ng/ml did not reduce NK activity and only modestly reduced uptake of $^{45}Ca^{2+}$. The correlation between inhibition of NK lysis and inhibition of $^{45}Ca^{2+}$ was found in several other experiments using different lymphocyte donors.

Table 3. Suppression of $^{45}Ca^{2+}$-uptake and ^{51}Cr-release by TPA during NK cell killing of target cell line K562

	$^{45}Ca^{2+}$ uptake*		^{51}Cr-release	
Condition	(C.p.m.)	Inhibition (%)	% of control	Inhibition (%)
Medium	72,124	0	40·2	0
TPA 100 ng/ml	13,216	81·9	5·9	85·3
TPA 5 ng/ml	64,447	11·1	44·1	0

$^{45}Ca^{2+}$ uptake and ^{51}Cr-release assay were done using the same batch of effector target cells. TPA at 100 ng/ml and 5 ng/ml were used to pretreat the effectors cells in the same test.

* Specific uptake as measured in the Methods and Materials section and in the legend to Fig. 6.

DISCUSSION

Phorbol esters that were originally described as tumour promoters induce a multitude of cellular functions, such as DNA synthesis, phagocytosis, secretion and differentiation (Kolata, 1983) and influence the activation and regulation of cytotoxic effector cells when used in long term assays systems. The present results are similar to earlier data on the short term effect of TPA on cytotoxic NK cells and T cells and further demonstrate that these effects depend on the type of both the effector and the target cell used (Kabelitz, 1985). In agreement with Seaman et al. (1981) we find that suppression of K562 killing depends on monocytic cells, although the induction reported here, with non-monocytic cells, was not described. The TPA mediated induction of K562 killing suggests that the regulation of NK killing of these target cells is different as compared to other target cells. With lymphoid target cells, TPA was found to be purely suppressive although suppression was potentiated by monocytes.

The inhibitory effect of TPA on NK-killing could be explained in at least two different ways. As TPA is known to rapidly down-regulate cell surface receptor structures, such as CD4 and Fc-receptors for IgG (CD16), and as such down-regulation is complete within 1 hr (Trinchieri et al., 1984; Tatsumi et al., 1984), it is possible that down-regulation of Fc-receptors explains suppression of antibody-dependent killing.

However, as suppression is seen of both natural, lectin and antibody-dependent killing, down-regulation of Fc-receptors cannot explain the suppression in all these different systems. Another possibility is the described effect of TPA on negative feedback inhibition of phosphatidylinositol 4,5-biphosphate (PIP₂) hydrolysis (Corvera & Garcia-Sainz, 1984; Watson & Lapetina, 1985; Mellors & Stalmach & Cohen, 1985; Baraban et al., 1985). Eisenberg et al. (1985) reported that TPA completely block the increase of intracellular calcium induced by antigen in IgE sensitized basophilic leukaemic cells. Mellors et al. (1985) showed that TPA suppressed the phosphatidylinositol response in lymphocytes during early mitogenesis, and Baraban et al. (1985) reported that phorbol esters block contractions in guinea-pig and rat uterus produced by neurotransmitters. In addition, Watson et al. (1985) showed that phorbol esters inhibit agonist induced formation of inositol phosphates in human platelets. The inhibitory effect of TPA could thus be a consequence of an uncoordinated protein kinase C stimulation in the absence of IP₃ formation and an intracellular calcium release.

The direct inhibition of protein kinase C, with both retinal and the anti-estrogens tamoxifen and clomiphene, clearly demonstrates the dependence of NK killing on some protein kinase C activity.

Consequently NK killing in the other systems depends on an intact, coordinated protein kinase C activation. This would mean that NK cells depend on IP₃ and DAG as second messengers to express lytic activity as has recently been demonstrated for the T3-T cell receptor complex in T-cell triggering (Imboden & Stobo, 1985).

A large amount of earlier data clearly indicate the importance of calcium in cell-mediated killing (Imboden & Stobo, 1985; Martz et al., 1982). Calcium may act both externally and internally and data so far do not clearly discriminate whether an active, channel operated influx is required for triggering. In other cells, such as granulocytes, it is clear that a modest increase of intracellular calcium occurs from internal stores through IP₃ generation in the absence of extracellular calcium (Andersson et al., submitted for publication). However, such a modest increase may not in itself suffice to generate a cytotoxic response but only to regulate the opening of a membrane associated calcium channel for a more sustained increase of calcium. Our results (Fig. 6, Table 2 and 3) demonstrate the influx of calcium into the effector cell population during killing of K562 target cells. The kinetics of this influx is slower than an IP₃ induced calcium increase which suggests a channel-dependent mechanism. From the $^{45}Ca^{2+}$ influx data it is also clear that K562 cells induce a comparatively large influx and that such a strong response was not always detectable during killing of other susceptible target cells using this particular methodology. Preliminary experiments with the intracellular calcium fluorochrome probe quin2 also demonstrated an increase of intracellular calcium during conjugation to K562 cells (to be published). Such a method, especially using a more potent fluorochrome such as fura2, may be adapted for a more precise measurement on the single cell level. Furthermore, our data demonstrate a correlation between TPA suppression of NK lysis and TPA suppression of $^{45}Ca^{2+}$ uptake. A similar inhibitory effect of TPA on calcium influx has been demonstrated in other systems (Eisenberg et al., 1985).

In conclusion, the present data suggest the importance of a co-ordinated protein kinase C/IP₃ response for triggering of killing and also clearly demonstrate the regulatory role for

M. *Jondal* et al.

monocytic cells as suggested earlier (Seaman *et al.*, 1981). Monocyte-dependent TPA suppression may depend on the generation of prostaglandin-type molecules and induction of suppressive levels of cyclic AMP. Further experiments are needed in order to assess these mechanisms and the function of protein kinase C in cell-mediated cytotoxicity.

ACKNOWLEDGMENTS

REFERENCES

ABRAMS S.I., BRAY R.A. & BRAHMI Z. (1983) Mechanism of action of phorbol myristate acetate on human natural killer cells activity. *Cell. Immunol.* **80**, 230.

ALBERT P.R. & TASHJIAN JR. A.H. (1985) Dual Actions of phorbol esters on cytosolic free Ca^{2+} concentration and reconstitution with ionomycin of acute thyrotropine-releasing hormone response. *J. biol. Chem.* **260**, 8746.

BARABAN J.M., GOULD R.J., PEROUTKA S.J. & SYNDER S.H. (1985) Phorbol ester effects on neurotransmission: Interaction with neurotransmitters and calcium in smooth muscle. *Proc. natl. Acad. Sci. U.S.A.* **82**, 604.

BERKE G. (1983) Cytotoxic T-lymphocyte. How do they function? *Immunol. Rev.* **72**, 5.

CARAFOLI E. & PENNISTON J.T. (1985) The calcium signal. *Scientific American*, **253**, 50.

CORVERA S. & GARCIA-SINZ J.A. (1984) Phorbol esters inhibit alphal adrenergic stimulation of glycogenolysis in isolated rat hepatocytes. *Biochim. Biophys. Res. Comm.* **119**, 1128.

EISENBERG R.S., LIEMAN H. & PECHT I. (1985) Protein kinase C regulation of the receptor-coupled calcium signal in histamine-secreting rat basophilic leukaemic cells. *Nature (Lond.)*, **313**, 59.

HENKART P. & MARTZ E. (eds) (1985) Mechanism of cell-mediated cytotoxicity. *Adv. expt. Med. and Biol.* **184**.

HERBERMAN R.B. (ed) (1982) *NK Cells and Other Natural Effector Cells.* Academic Press, New York.

IMBODEN J.B. & STOBO J.D. (1985) Transmission signalling by the T cell antigen receptor. Perturbation of the T3-antigen receptor complex generates inositol phosphates and releases calcium ions from intracellular stores. *J. exp. Med.* **161**, 446.

JONDAL M. & TARGAN S. (1978) Spontaneous and lectin-dependent cellular cytotoxicity by lymphocyte subpopulation against cell lines susceptible to or resistant to spontaneous cytotoxicity. *Clin. exp. Immunol.* **33**, 121.

KABELITZ D. (1985) Modulation of natural killing by tumor promoters: the regulatory influence of adherent cells varies with the type of target cell. *Immunobiol.* **169**, 436.

KAIBUCHI K., TAKAI Y. & NISHIZUKA Y. (1985) Protein kinase C and calcium ion in mitogenic response of macrophage-depleted human peripheral lymphocytes. *J. biol. Chem.* **260**, 1366.

KOLATA G. (1983) Clues to cell growth and differentiation. *Science*, **220**, 291.

MARTZ E., PARKER W.L., GATELY M.K. & TSOUKAS C.D. (1982) The role of calcium in the lethal hit of T lymphocyte-mediated cytolysis. *Adv. exp. Med and Biol.* **146**, 121.

MELLORS A., STALMACH M.E. & COHEN A. (1985) Co-mitogenic tumor promoters suppress the phosphatidylinositol response in lymphocytes during early mitogenesis. *Biochim. Biophys. Acta*, **833**, 183.

NISHIZUKA Y. (1984) The role of protein kinase C in cell surface signal transduction and tumor production. *Nature (Lond.)*, **308**, 693.

OROSZ C.G., ROOPENIAN D.C. & BACH F.H. (1983) Phorbol esters mediates reversible reduction of cloned T lymphocytes cytolysis. *J. Immunol.* **130**, 2499.

OROSZ C.G. & ROOPENIAN D.C. (1985) Phorbol myristate acetate and *in vitro* T lymphocyte function. III. Selective impairment by PMA of lethal hit delivery by cloned CTL. *Transplantation*, **39**, 411.

OROSZ C.G., SCOOT, J.W., GILLIS S. & FINKE J.H. (1985) Reversal of phorbol ester-mediated reduction of cloned T lymphocyte cytolysis by Interleukin-2. *J. Immunol.* **134**, 324.

RAMOS O.F., MASSUCI M.G., BEJARANO M.T. & KLEIN E. (1983) The tumor promoter phorbol-12,13-dibutyrate (P(Bu)$_2$) stimulates cytotoxic activity of human blood lymphocytes. *Immunobiol.* **165**, 403.

RUSSELL J.H. (1985) Phorbol esters inactivate the cyclic apparatus of cytotoxic T lymphocytes. *J. Immunol.* **133**, 907.

SEAMAN W., GUIDHART T.D., BLACKMAN M.A., DALAL B., TALAL N. & WERB Z. (1981) Natural killing of tumor cells by human peripheral blood cells—suppression of killing in vitro by tumor-promoting phorbol esters. *J. clin. Invest.* **67**, 1324.

SU H.D., MAZZEI G.J., VOGLER W.R. & KUO J.F. (1985) Effect of tamoxifen, a non-steroidal antiestrogen, on phospholipid/calcium-dependent protein kinase C and phosphorylation of its endogenous substrate proteins from the rat brain and ovary. *Biochem. Pharmacol.* **34**, 3649.

TAFFET S.M., GREENFIELD A.R.L. & HADDOX M.K. (1983) Retinal inhibits TPA activated, calcium-dependent phospholipid-dependent protein kinase (C kinase). *Biochem. Biophys Res. Comm.* **114**, 1194.

TATSUMI E., PIONTEK C., SUGIMOTO T., MINATO K. & MINOWADA J. (1984) Selective loss of the expression of OKT4 or Leu 3A defined antigen by 12-O-tetradecanoylphorbol-13-acetate (TPA) in human lymphoid cells. *Am. J. Hematol.* **17**.

TRINCHIERI G. & PERUSSIA B. (1984) Biology of disease human natural killer cells: biologic and pathologic aspects. *Lab. Invest.* **50**, 480.

TRINCHIERI G., O'BRIEN T., SHADE M. & PERUSSIA B. (1984) Phorbol esters enhance spontaneous cytotoxicity of human lymphocyte, abrogate Fc receptor expression and inhibit antibody-dependent lymphocyte-mediated cytotoxicity. *J. Immunol.* **133**, 1869.

TRUNEH A., ALBERT F., GOLDSTEIN, P. & SCHMITT-VERHULST A. (1985) Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol esters. *Nature (Lond.)*, **313**, 318.

WATSON S.P. & LAPETINA E.G. (1985) 1-2-diacylglycerol and phorbol ester inhibit agonist-induced formation of inositol phosphates in human platelets: possible implications for negative feedback regulation of inositol phospholipid hydrolysis. *Proc. natl. Acad. Sci. U.S.A.* **82**, 2623.

WOLF M., LEVINE H, STRATFORD MAY W., CUATRECASAS P. & SAHYOUN N. (1985) A model for intracellular translocation of protein kinase C involving synergism between Ca^{2+} and phorbol esters. *Nature (Lond.)*, **317**, 546.