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STUDIES ON THE CALCIUM DEPENDENCE OF HUMAN NK CELL KILLING

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Abstract—T lymphocytes and NK cells depend on extracellular Ca^{2+} to mediate cellular cytotoxicity. In the present work, we have used pharmacological tools to analyze the nature of this calcium dependence. Ca^{2+} channel blockers like nifedipine ≥ diltiazem > verapamil > cobalt chloride inhibited NK killing but at concentrations higher than those sufficient to block voltage-operated Ca^{2+} channels. Quercetin and TMB-8 also suppressed killing. Depolarization or NK cells with high K^+ concentration resulted in partial inhibition of lysis in contrast to hyperpolarization with K^+ ionophore valinomycin which had no effect. Depolarization of hyperpolarization in the presence of a protein kinase C activator (phorbol ester, TPA) did not initiate killing of NK resistant target cells. Of the two K^+ channel inhibitors tested, 4-AP and TEA, only 4-AP was inhibitory for NK killing. No release of membrane-bound Ca^{2+} as judged by chlorotetracycline fluorescence could be detected in the NK cell population during binding to target cells although an influx of $^{45}Ca^{2+}$ into the NK cell population was found. Treatment of NK cells with calcium ionophore A23187 did not trigger killing, but lysis could be induced by simultaneous stimulation with A23187 and TPA. The results indicate that NK killing depends on Ca^{2+} channels that are different from voltage operated channels and that intracellular Ca^{2+} may act in concert with protein kinase C activation.

During a cellular immune response, different types of cytotoxic lymphocytes are activated. T cells recognize and kill target cells by genetically rearranged, specific cell surface receptors whereas NK cells attach by undefined non-adaptive receptors [1, 2]. NK cells are believed to form a first line of defence against small numbers of tumor cells and infected host cells whereas adaptive T cells are required for a more efficient and prolonged immunological control.

Ca^{2+}‡ is obligatory in T and NK cell killing for supporting the lethal hit [3, 4]. The exact role for Ca^{2+} in killing has not yet been defined although Martz *et al.* concluded from an earlier study that Ca^{2+} may act primarily extracellularly [3]. However, recent data have shown that activation of various lymphocyte receptors is associated with rapid increases of cytosolic free Ca^{2+} [5–9], which is inhibited by Ca^{2+} channel antagonists [6, 8]. Since NK cells express some of the relevant T cell receptors [10–12], these findings may be relevant for our understanding of the function of Ca^{2+} in NK killing.

In the present work we have used a series of different drugs in an attempt to further elucidate the role of Ca^{2+} in NK killing. The data indicate that an influx of external Ca^{2+} is required for activation of NK killing, but these Ca^{2+} channels in NK cells may differ from those in, for example, smooth muscle. Furthermore, elevated intracellular Ca^{2+} only seems to activate killing when protein kinase C is also activated.

‡ Abbreviations used: Ca^{2+}, calcium; K^+, potassium; Na^+, sodium; Sr^{2+}, strontium; TMB-8,8-(*N*,*N*-diethylamino)oxtyl 3,4,5-trimethoxybenzoate hydrochloride; TPA, 12-O-tetradecanoylphorbol 13-acetate; TEA, tetraethylammonium chloride; 4AP, 4-aminopyridine; NK, natural killer cells.

MATERIALS AND METHODS

Chemicals. Diltiazem, nifedipine, verapamil, quinidine sulphate, cobalt chloride, 8-(*N*,*N*-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8), valinomycin, potassium chloride, veratridine, tetrodotoxin, ouabain, 4-aminopyridine, tetraethylammonium chloride, ionophore A23187, 12-O-tetradecanoylphorbol 13-acetate (TPA) and quercetin were purchased from Sigma Chemical Co. (St Louis, MO). Anti IgM was purchased from Dako-immunoglobulins (Copenhagen, Denmark). OKT3 monoclonal antibody was obtained from Ortho Diagnostic Systems Inc. (Raritan, NJ).

Purification of lymphocytes. Peripheral blood lymphocytes were obtained from normal healthy blood donors. Lymphocytes were collected by buoyant density centrifugation on Ficoll-Isopaque [13] and washed three times in phosphate-buffered saline. After the final wash, the cells were resuspended in RPMI 1640 supplemented with 10% fetal calf serum. Adherent cells were removed by passage through a nylon wool column after a preincubation period of 30 min at 37°.

Selection of OKT3-cells by the panning method. The obtained lymphocytes were fractionated into OKT3+ and OKT3− populations according to the method described by Ullberg and Jondal [13]. Briefly, cells were treated with mouse anti-OKT3 monoclonal antibodies and the OKT3 positive population of cells was adsorbed onto plastic petri dishes

coated with immunosorbent purified rabbit anti-mouse IgG. The free floating OKT3 negative population was removed by decanting and the cells were twice with phosphate buffered saline to remove remaining antibody.

Cell lines. Target cell lines were maintained in tissue culture in RPMI 1640 containing 10% fetal calf serum and antibiotics. K-562 line is a cell line from a patient with chronic myeloic leukaemia [14] and Molt-4 is a T-cell line derived from a patient with acute lymphocytic leukaemia [15]. Daudi and Raji cell lines are both B cell lines from Burkitt lymphoma [16, 17].

Labelling of target cells. Fifty micro Curies of $Na(^{51}Cr)_2O_7$ were added to approximately 2×10^6 cells in 200 μl and were incubated at 37° for an hour. The cells were then washed three times and a further incubation for 20 min to lower the spontaneous release. After this incubation, the cells were washed twice and diluted to the required concentration.

Determination of NK cell activity. Cytotoxicity assays were carried out in duplicates using 96 wells V-shaped microtitre plates. The total volume of each well was 150 μl. A 20:1 effector cell to target cell ratio were used. The effector cells were preincubated with the various drugs tested at the different concentrations used for 20 min at room temperature. Thereafter, the target cells were added to the wells for a 4 hr incubation period at 37°. Fifty microlitres of the supernatant from each well was harvested and counted in a LKB gamma counter. Percentage of killed target cells was calculated as

$$100 \times \frac{\text{test release} - \text{spontaneous release}}{80\% \text{ of total label} - \text{spontaneous release}}$$

In assays where results are given as ^{51}Cr-release (%), the values are obtained from the above equation. When results are stated as ^{51}Cr-release (% of control), the values are calculated using the data from percentage of killed target cells by effector cells that were not treated with any drugs as control.

Binding assay in agarose. The target binding assay used to study conjugates at the single cell level was carried out as described by Ullberg and Jondal [13].

$^{45}Ca^{2+}$ 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% fetal calf serum in 5 ml test tubes. Five microCuries of $^{45}CaCl_2$ were added to test tube. After incubation at 37° for 30 min, varying concentrations of target cells used were added except for a set of duplicate tubes which serves as control for the effector cell fraction as well as tubes with target cells alone. In some tests Daudi cells were used after pretreatment with anti-IgM before addition to the effector cell population. Effector-target cells were spun for 1 min to ensure contact between the two populations of cells. All tubes were incubated at 37° for another 15 min and the assay was stopped by addition of 3 ml ice-cold RPMI 1640 medium containing 2 mM of lanthanum chloride and the tubes were left at 4° for an hour to remove extracellular Ca^{2+}. After 1 hr, the cells were washed three times with ice-cold medium. Care was taken to remove excess medium from tubes. Two blank control tubes were also treated in the same way. Traces of $^{45}CaCl_2$ in the control tubes were subtracted from the test counts. When the cell pellet was ready, 500 μl of water was added to each tube, including the blank control. This volume was then transferred to plastic vials and 4 ml of scintillation fluid was added to each. The amount of radioactivity was counted using LKB beta counter.

Labelling of lymphocytes with chlorotetracycline. OKT3− cells at 10×10^6 cells per ml were treated with 50 μM of chlorotetracycline for 20 min at 37°. The cells were washed three times with RPMI 1640 medium supplemented with 5% fetal calf serum. OKT3− cells were then spun down together with target cells at a ratio of 1:1. The cell pellets were resuspended lightly and incubated for 10 min at 37°. Fluorescence studies were carried out using a standard u.v. microscope.

RESULTS

Inhibition of NK cell cytotoxicity by Ca^{2+} antagonists

Several different compounds, which represent a chemically heterogenous group and have been described as Ca^{2+} channel antagonists, Ca^{2+} entry blockers or slow channel blockers [18] were tested. All inhibitors, including verapamil, cobalt chloride, diltiazem, nifedipine suppressed NK killing (Fig. 1). Nifedipine gave a 50% inhibition at approximately 3 μM whereas diltiazem and verapamil gave a comparable inhibition at doses between 5 μM and 50 μM, respectively. Cobalt chloride gave a similar inhibition at approximately 150 μM. Inhibition of both Ca^{2+} and Na^+/K^+ ATPase with quercetin suppressed lysis (Fig. 2) whereas inhibition of Na^+/K^+ ATPases by ouabain (1 mM) was ineffective (data not shown). TMB-8, which has been suggested to stabilize Ca^{2+} binding to cellular stores [19] and to inhibit receptor-operated Ca^{2+} channels [20], also inhibited NK cell lysis with 50% inhibition found at 50 μM (Fig. 2). Some of the Ca^{2+} channel inhibitors, quercetin, TMB-8 and the calmodulin inhibitors were also tested in the agarose single cell assay for inhibition of target cell conjugation or inhibition of the lytic

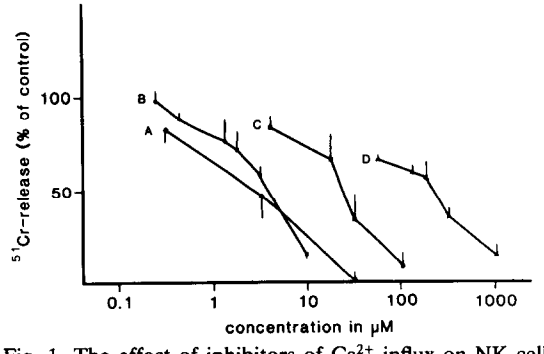

Fig. 1. The effect of inhibitors of Ca^{2+} influx on NK cell killing. Effector cells were pretreated with the various drugs for 30 min at room temperature before the addition of K562 target cells for a 4 hour NK cytotoxicity assay. Cytotoxicity is given as percentage of control killing in medium. A = nifedipine, B = diltiazem, C = verapamil and D = cobalt chloride. Values are given as mean + SEM from 3 experiments.

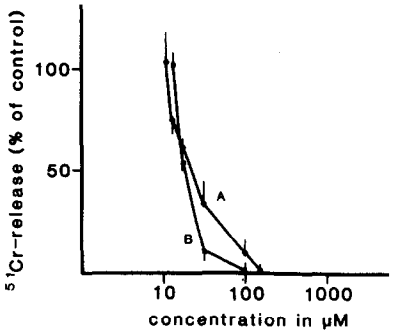

Fig. 2. The effect of two inhibitors of Ca^{2+} utilization on NK cell killing. The experiments were carried out with conditions similar to those as described in Fig. 1. Cytotoxicity values are calculated as percentage of control killing in medium. A = quercetin and B = TMB-8. Values are given as mean + SEM from 3 experiments.

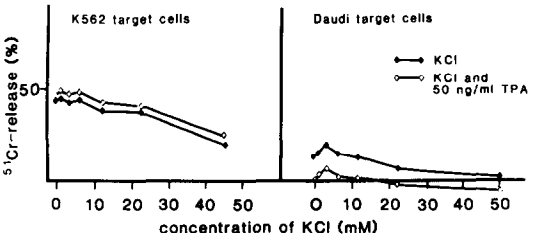

Fig. 4. High K^+ concentrations cause a partial inhibition of NK lysis. In this set of experiments, Hanks' Balanced Salt Solution with one modification were used. KCl was omitted in the preparation of the medium. Effector cells were suspended in this balanced salt solution with increasing concentration of KCl for 30 min at room temperature before the addition of either K562 cells or Daudi cells as target cells in a 4 hr NK cytotoxicity assay. The combined effect of KCl and TPA was also studied. Cytoxocity is given as percentage of specific ^{51}Cr-release. The results were reproducible in two other experiments.

triggering at the post-target cell binding stage. None of the inhibitors influenced target cell binding by enriched NK cell population.

Effect of membrane hyperpolarization and depolarization on NK cell cytotoxicity

NK cells were treated with the K^+ ionophore, valinomycin (Fig. 3) and by high external K^+ concentration (Fig. 4) in the presence and absence of protein kinase C activator TPA. Valinomycin did not influence killing of NK susceptible K562 cells or induce killing of NK resistant Raji cells. Simultaneous protein kinase C activation only resulted in some inhibition of lysis as found earlier [21]. Depolarization resulted in partial inhibition of K562 killing but did not induce any substantial amount of killing of target cell Raji even in the presence of TPA.

Effect of K^+ and K^+/Na^+ channel inhibitors on NK cell cytotoxicity

The Na^+ channel activator veratridine suppressed NK lysis by 50% at approximately 12 μM (Fig. 5). However, the inhibitor of voltage-dependent Na^+ channels tetrodotoxin, did not influence lysis by itself and did not neutralize the veratridine inhibition when it was used at a concentration sufficient to neutralize the action of veratridine. The veratridine suppression was probably dependent on a depolarizing effect or the effect mimicks that obtained by high K^+. The K^+/Na^+ channel inhibitor ouabain (data not shown) and the K^+ channel inhibitor TEA did not influence killing (Fig. 6), although 4-AP gave a 50% inhibition at concentrations slightly above 1 mM (Fig. 6).

Induction of killing against NK resistant target cells with calcium ionophore A23187 and TPA

Ca^{2+} ionophore A23187 was added to unfractionated or NK cell enriched population when tested against resistant Raji cells (Table 1). No induction of lysis was obtained when the ionophore was used in the concentration range over 5 μM and 0.16 μM. When ionophore treatment was combined with TPA stimulation (50 ng/ml), a reproducible induction of lysis occurred both in unfractionated, non-adherent lymphocytes and in the NK cell-enriched population [22].

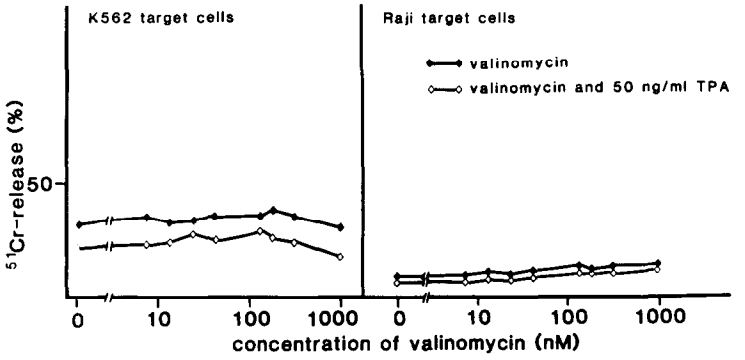

Fig. 3. Valinomycin does not influence NK killing. The influence of valinomycin was used to study NK cell cytotoxicity on both an NK sensitive target cell line (K562) and an NK resistant cell line (Raji) in a 4 hour NK cytotoxicity assay. The combined effect of valinomycin and activation of protein kinase C by TPA was also studied. Cytotoxicity is given as percentage of specific ^{51}Cr-release. Similar results were obtained in another 2 experiments.

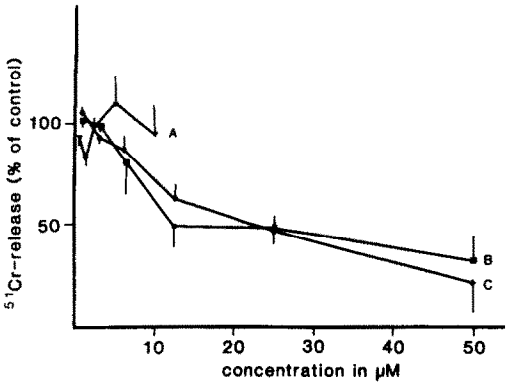

Fig. 5. Effect of drugs affecting Na^+ channel. Effector cells were pretreated with either drugs alone or in combination for 30 min at room temperature before the addition of K562 target cells for a 4 hr NK cytotoxicity assay. A = tetrodotoxin, B = veratridine and C = veratridine and tetrodotoxin (1 μM). Values are given as percentage of killing in control medium, mean + SEM from 3 experiments.

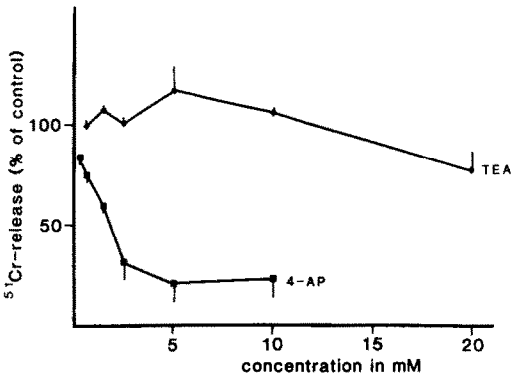

Fig. 6. Effect of drugs affecting K^+-channel. Effector cells were pretreated with either drugs alone or in combination for 30 min before the addition of K562 target cells for a 4hr NK cytotoxicity assay. Cytotoxicity is given as percentage of killing in control medium. 4-AP = 4-aminopyridine and TEA = tetraethylammonium chloride. Values are given as mean + SEM from 3 experiments.

No release of intracellular, membrane bound Ca^{2+} *during NK cell binding to target cells*

No adherent peripheral lymphocytes were fractionated into OKT3+ and OKT3− fractions by the panning method [13]. The OKT3− fraction is enriched in NK cells as detected by morphology, number of target binding cells and lytic activity. OKT3− cells were labelled with chlorotetracycline and a distinctive fluorescent pattern was seen in almost all cells using excitation and emission wavelengths of 380 nm and 520 nm, respectively. This fluorescence reflects the intracellular content of membrane bound Ca^{2+} and is rapidly lost when Ca^{2+} is mobilized from the membrane pool as shown by stimulation of human neutrophils [23]. Highly NK active, chlorotetracycline-labelled OKT3− cells were then conjugated to different target cells and the percentage of chlorotetracycline positive cells in the target-bound and unbound fractions estimated in UV light (Table 2). Several different effector cell populations were tested at different time points. No loss of chlorotetracycline fluorescence was seen in the target-bound fraction which comprise of at least 50% of active killer cells against Molt-4 target cells [24].

Uptake of $^{45}Ca^{2+}$ *suring effector cell conjugation to different target cells*

Non-adherent peripheral lymphocytes were conjugated to K-562 target cells and to Daudi target

Table 1. Induction of killing of NK resistant target cell line Raji by treatment with Ca^{2+} ionophore A23187 and phorbol ester TPA

Ionophore (μM)	TPA (ng/ml)	Target cell	Cytotoxicity unfractionated exp 1	Cytotoxicity unfractionated exp 2	(^{51}Cr-release) OKT3− exp 1	(^{51}Cr-release) OKT3− Exp 2
5	50	Raji	31.2	13.6	28.4	11.9
2.5	50	Raji	28.2	12.4	27.1	8.3
1.25	50	Raji	22.1	10.1	24.6	7.8
0.63	50	Raji	19.3	8.9	21.3	5.0
0.31	50	Raji	14.6	5.8	12.5	3.8
0.16	50	Raji	6.7	3.0	7.3	2.1
5	—	Raji	2.0	1.9	6.7	0.1
2.5	—	Raji	1.9	0.2	4.2	0.5
1.25	—	Raji	0.8	1.0	2.9	0.0
0.63	—	Raji	0.1	1.2	1.7	0.2
0.31	—	Raji	0.9	0.5	1.5	0.0
0.16	—	Raji	0.8	0.6	0.9	0.3
—	50	Raji	1.2	0.4	0.1	0.5
—	—	Raji	2.5	1.2	2.1	1.2
—	—	K-562	55.2	15.6	66.7	17.4

Combined effect of phorbol ester, TPA and Ca^{2+} ionophore A23187 on NK killing of a resistant target cell line, Raji.

Table 2. No release of membrane bound Ca^{2+} in NK cells during conjugation to susceptible target cells detectable by loss of chlorotetracycline fluorescence

Target cell	TBC*	CTC positive cells† TBC	CTC positive cells† non-TBC	NK lysis 20:1 (% specific ^{51}Cr-release)	NK lysis 10:1 (% specific ^{51}Cr-release)
K562	29.6	92.1	95.1	65.2	48.4
Molt-4	27.3	95.2	98.5	55.3	42.8
Raji	32.6	89.2	88.2	7.1	3.2

* TBC = target bound lymphocytes as percentage of whole OKT3 negative cell fraction.
† Chlorotetracycline positive cells as percentage of either the TBC fraction or the non-TBC fraction.
NK lytsis was tested in a standard 3-hr assay with OKT3− effector cells, fractionated by the panning method (see Materials and Methods section).

cells pretreated with anti-IgM or concanavalin A as described in the Materials and Methods section. Untreated Daudi cells are comparatively resistant to NK whereas Daudi cells pretreated with rabbit IgG anti-human IgM are readily killed in an anti-body-dependent cellular cytotoxicity reaction. Target and effector cells incubated alone with $^{45}Ca^{2+}$ have a background uptake reflecting the normal turnover rate (Table 3). When K-562 was used in the test, a strong uptake was found in contrast to Daudi cells which did not give any specific uptake above background values. When Daudi cells were pretreated with anti-IgM antibodies, a strong uptake of $^{45}Ca^{2+}$ was found in parallel with a strong cytotoxicity reaction. The specific $^{45}Ca^{2+}$ uptake was detectable within minutes and reproducibly found with several different target cells as will be presented in more detail elsewhere [22].

DISCUSSION

The present data indicate that NK killing depends on an uptake Ca^{2+} into the effector cells. Thus, at least some of the well-known [3, 4] calcium dependency of the killing may be due to uptake of calcium. Our results also indicate that the Ca^{2+}-influx is not through the classical voltage-operated channels even though it is blocked by compounds such as nifedipine, diltiazem, verapamil and cobalt chloride [25]. These compounds are also effective in suppressing mast cell release of histamine [26]. They are less potent in inhibiting histamine release and in suppressing NK killing than in relaxing smooth muscle [25]. For example, we found nifedipine to block NK cell killing with an EC_{50} close to 3 μM (Fig. 1), whereas the EC_{50} for relaxation of potassium depolarized smooth muscle is usually between 3 and 20 nM [27]. Such quantitative comparisons indicate that the mechanism by which these drugs inhibit histamine release or affects NK cell killing, is different from the main function for which they are used clinically, i.e. inhibition of voltage- dependent Ca^{2+} channels. Also, no voltage-dependent Ca^{2+} channels have been found in NK cells using patch-clamp techniques [28, 29].

TMB-8 inhibited NK cell killing at concentrations that have been shown to block calcium entry and contractile responses in smooth muscle [19]. Simi-

Table 3. Uptake of $^{45}Ca^{2+}$ during effector cell conjugation to K-562 target cells and to Daudi cells pretreated with anti-IgM

Target* cell	Effector cell added	Cytoxicity (% ^{51}Cr-release)	$^{45}Ca^{2+}$-uptake (cpm)†
K-562	—	—	13503
Daudi	—	—	4098
Daudi/anti IgM	—	—	3344
—	+	—	10879
K-562	+	43.2	86596(+62124)
Daudi	+	2.1	16344(+1367)
Daudi/anti Ig-M	+	63.8	213844(+199621)

* K-562, Daudi and anti-IgM labelled Daudi cells were used as target cells. NK lysis was tested in a three hour standard assay.
† $^{45}Ca^{2+}$-uptake was tested as described in M&M section. Values in the parenthesis indicate above controls target and effector cells alone. In order to assess that the calcium flux is into the effector cell population in the conjugation assays, varying ratio of effector: target cell were carried out. If the number of target cells was doubled, there was a corresponding uptake of calcium in the isolated cells but the specific uptake remained essentially the same. If the number of effector cells was reduced by 50%, there was a corresponding decrease in the specific uptake of calcium.

larly quercetin was effective in concentration similar to those shown to block histamine release from mast cells induced by a variety of different agents [30]. While the exact mechanism behind the action of these two agents is unclear, their inhibitory effects further emphasize the importance of increases in intracellular calcium for NK cell killing.

Recently voltage-gated K^+ channels were found in NK cells [27, 28]. DeCoursey *et al.* [1985] pointed out that these are inhibited by Ca^{2+} channel antagonists and that therefore an effect on lymphocyte function by these compounds cannot be taken as evidence for a role of Ca^{2+} channels [28]. However, the order of potency of these compounds to block K^+ channels is verapamil > nifedipine > diltiazem [31], which is quite different from the order of potency to block NK cell killing that we observed. Therefore, we favor the interpretation of Birx *et al.* [32] rather than Chandy *et al.* [27, 31], i.e. that the results with Ca^{2+} antagonists indicate a role of Ca^{2+} channels in combination with K^+ channels.

To further investigate the involvement of changes in membrane potential or K^+ channel conductance in NK killing, we tried to induce potential alterations by valinomycin (hyperpolarization) or high external K^+ concentrations and veratridine (depolarization) and found that only high K^+ concentration caused a partial inhibition of lysis even in the presence of the protein kinase C activator, TPA. Interestingly, valinomycin and high K^+ concentrations were found by Oettgen *et al.* to affect anti-CD3 induced Ca^{2+} uptake by Jurkat cells the initial step in the activation of IL-2 production [8]. Therefore, the triggering of the NK cell to cause lysis seems to differ from the triggering by antibody of the Jurkat cell to cause IL-2 production. Possible reasons for this difference are subject of other studies.

Most K^+ channels are blocked both by TEA and 4AP. The potassium channels in lymphocytes are blocked by 4AP (0.2 mM) and TEA (10 nM) [29]. We found that 4AP did block NK cell killing, but TEA did not. The low potency of 4AP and the ineffectiveness of TEA could be taken as evidence against a role of K^+ channels. However, it is known that some K^+ channels require high amounts of TEA for blockade [33] and it is possible that a substantial number of the K^+ channels need to be blocked in order for killing to be inhibited. Thus, it seems more likely that the positive results with 4AP depend upon the presence of important K^+ channels, but that these are somewhat different from K^+ channels in neurons which are blocked by lower concentrations of the antagonists.

The induction of Raji cells killing with the combined treatment of Ca^{2+} ionophore and phorbol ester demonstrates that a Ca^{2+} influx alone is not sufficient to induce killing as earlier noted by Martz *et al.* [4]. The Ca^{2+} and TPA induced killing was a reproducible finding in several repeated experiments and was also detectable in the T cell fraction of cells expressing the T3 marker. The induction of lysis in both NK and non-NK cells suggests either that this stimulation triggers multiple effector cell populations or that it is unrelated to the normal lytic machinery.

In summary, the present results indicate that an increase in intracellular Ca^{2+} is required for NK lysis, but it may not be sufficient, and killing may be triggered simultaneous stimulation with Ca^{2+} ionophore and a protein kinase C activation. No gross release of intracellular membrane-bound Ca^{2+} was detected during NK cell conjugation to target cells, although a prompt NK uptake of $^{45}Ca^{2+}$ was seen during killing of K562 target cells. A reasonable interpretation of the findings is that NK cell lysis depends on receptor-operated Ca^{2+} channels. For example, it is possible that conjugation of effector cells with their target leads to the formation of inositol phosphates which open up calcium channels [34].

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