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Effect of thapsigargin on cytoplasmic Ca^{2+} and proliferation of human lymphocytes in relation to AIDS

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The tumour-promoting sesquiterpene lactone, thapsigargin, induced a dose-dependent increase of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in human lymphocytes from a resting level between 100 and 150 nM up to about 1 μM. Half-maximum response was found at about 1 nM of thapsigargin, full response at 100 nM. The effect of thapsigargin on $[Ca^{2+}]_i$ exceeded that of phytohaemagglutinin (PHA) which raised $[Ca^{2+}]_i$ to maximum 300 nM. In combination with phorbol 12-myristate 13-acetate (PMA), thapsigargin stimulated the proliferation of normal lymphocytes to the same extent as did PHA, whereas the thapsigargin/PMA treatment could not restore the defective proliferation of AIDS lymphocytes in spite of the increased $[Ca^{2+}]_i$. Thapsigargin or PMA added separately had no stimulatory effects on cell proliferation. The thapsigargin/PMA treatment caused an increase in the interleukin-2 (IL-2) production of the lymphocytes, which was much higher than that caused by the PHA treatment, even in AIDS lymphocytes. Moreover, the thapsigargin/PMA treatment stimulated the expression of the IL-2 receptors on both normal and AIDS lymphocytes, similar to the effect of PHA. It is concluded that thapsigargin exerts its effects on lymphocyte proliferation by increasing $[Ca^{2+}]_i$, and that the general defect of AIDS lymphocytes, rather than being ascribed to the initiating signal systems, is associated with later events related to DNA synthesis and proliferation.

Introduction

A rise of the cytoplasmic Ca^{2+} concentration above the level in resting cells is one of the important events that precedes cell proliferation induced by mitogens [1], but it is not a sufficient stimulus for the initiation of proliferation. The addition of a Ca^{2+} ionophore (e.g., A23187) to a

Abbreviations: PHA, phytohaemagglutinin; PMA, phorbol 12-myristate 13-acetate; IL-2, interleukin-2; DMSO; dimethylsulphoxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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lymphocyte suspension, which increases the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), has to be combined with the addition of an agent that activates protein kinase C, for instance the tumour-promoting phorbol ester, PMA, in order to mimic the effect of mitogens [2,3]. Therefore, the phosphorylation of certain cellular targets seems to be a prerequisite for the initiation of proliferation.

The cellular Ca^{2+} rise and the kinase C response have been characterized as short-term responses. The full activation of cell proliferation (the long-term responses), however, requires the presence of growth factors, indicating additional signal pathways, possibly including further phosphorylations (for reviews see Refs. 4 and 5).

The sesquiterpene lactone, thapsigargin [6], that activates mast cells and leukocytes [7] and induces cellular phosphorylations in human platelets [8] has recently been shown to raise $[Ca^{2+}]_i$ in platelets to an elevated steady-state level [9], although thapsigargin is not a Ca^{2+} ionophore ([7] and Birthe Foder et al., unpublished results). An elevated level of $[Ca^{2+}]_i$ may explain the tumour-promoting effect of thapsigargin [9], reported recently by Hakii et al. [10].

We have now tested the abilities of thapsigargin to raise $[Ca^{2+}]_i$ and to initiate long-term responses in human lymphocytes, primarily cell proliferation. In order to investigate whether thapsigargin could increase the proliferation of AIDS lymphocytes, we have tested cells from both healthy control persons and AIDS patients.

Materials and Methods

Reagents. The cell-culture medium was RPMI 1640 (Gibco, Grand Island, NY, U.S.A.). Fura2-acetoxymethylester was from Molecular Probes. [*Methyl*-^{3}H]Thymidine was from New England Nuclear (NET-027, specific activity 248 GBq/mmol). Thapsigargin was isolated as previously described [7]. Thapsigargin, the divalent cation ionophore A23187 (Calbiochem), ionomycin (Calbiochem), and phorbol 12-myristate 13-acetate (PMA, Sigma) were all administered dissolved in dimethylsulphoxide (DMSO). The final concentrations of DMSO were 0.1–0.2% in cultures and below 1% during Ca^{2+} measurements. Phytohaemagglutinin P (PHA, Difco) was used in aqueous solution. All other chemicals were, unless otherwise stated, from Merck (pro analysis).

Lymphocytes. Peripheral blood lymphocytes were isolated by using Lymphoprep (Nygaard, Oslo, Norway) density-gradient centrifugation and suspended to $6 \cdot 10^6$ per ml in freeze medium (15.5 ml RPMI, 2.5 ml heat-inactivated pooled male serum, and 2 ml DMSO). The lymphocytes were either used immediately after isolation for some of the Ca^{2+} measurements (see below) or the lymphocytes were frozen in 1 ml tubes by gradient freezing (Cryoson) to $-80\,^\circ$C and stored in liquid nitrogen. The cells were thawed in a 37 °C water bath and immediately washed twice in RPMI with 15% male serum. The frozen lymphocytes were from nine HIV-1 seronegative controls or from nine homosexual men with AIDS, according to the criteria from Center for Disease Control, Atlanta, U.S.A.

Measurement of cytoplasmic Ca^{2+}. Lymphocytes were loaded with fura2 by incubation for 30 min at 37 °C in RPMI containing 5 μM fura2-acetoxymethyl ester and $5 \cdot 10^6$ cells per ml. After centrifugation, the cells were resuspended to 10^6 cells per ml in Hepes buffer (145 mM NaCl, 5 mM KCl, 0.5 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM NaH_2PO_4, 10 mM glucose, 10 mM Hepes (pH 7.6) at 20 °C). The concentration of cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) was determined by fluorescence measurements (F) at 37 °C with continuous stirring in a Perkin Elmer LS-3B spectrofluorometer with monochromator settings of 340 nm exitation and 500 nm emission, using the equation $[Ca^{2+}]_i = K_d \cdot [(F - F_{min})/(F_{max} - F)]$, in which $K_d = 224$ nM is the apparent dissociation constant for Ca^{2+} and fura2 [11]. Maximum (F_{max}) and minimum (F_{min}) fluorescence were obtained by addition of 1 μM ionomycin and further addition of 10 mM EGTA, respectively.

The shown curves, depicting $[Ca^{2+}]_i$ versus time, were representative of at least three experiments of each type.

Measurement of cell proliferation. Microcultures containing 50 000 responder peripheral blood lymphocytes in 170 μl RPMI, supplemented with antibiotics, glutamine, and 10% fetal calf serum, were prepared in triplicate in microtitre plates (Greiner, Nurtingen, F.R.G.). [^{3}H]thymidine (37

kBq) was added 72 h after stimulation, e.g., by PHA or thapsigargin/PMA. The cells were collected on a semiautomatic harvesting machine (Skatron, Lierbyen, Norway), and the incorporated radioactivity was measured in a liquid scintillation counter (Beckman LS 1800), as described previously [12].

Determination of interleukin-2 activity. Macrocultures containing 10^6 responder peripheral blood lymphocytes in 3.4 ml RPMI were prepared in glass tubes. Test samples, i.e., culture supernatants taken at day 1, when the IL-2 concentration was maximum, were added to an assay culture of IL-2-dependent test cells (murine CTLL 2 cell line, kindly provided by M. Bonneville, M.D., INSERM, Nantes, France). The assay cultures were prepared in triplicate in microtitre plates containing 20 000 washed CTLL 2 cells per well in 150 μl RPMI with 10% fetal calf serum, and test sample was added as 50 μl of four serial dilutions (see also Ref. 13). The standard curve was made by use of purified human IL-2 (Electro-Nucleonics, U.S.A. The unit was equal to the unit of the Biological Response Modifiers Program, National Cancer Institute, NIH, U.S.A.). All determinations were made in one large experiment.

Determination of T lymphocyte subsets. Lymphocyte markers were investigated in cultures sampled at day 3, at which day the expression of IL-2 receptors was highest, on a fluorescence-activated cell analyzer (FACS, Becton-Dickinson), using monoclonal antibodies Leu2 (anti-CD8), Leu3 (anti-CD4), anti-HLA-DR, and anti-IL-2 receptor (anti-CD25) (from Becton-Dickinson) with fluorescence-labelling as described earlier [12]. Two of the AIDS patients with sufficient cell numbers were selected for the subset determinations.

Statistics. The Mann-Whitney test was used for comparison of differences between treatment groups, and the criterion of significance was $P < 0.05$.

Results

Rise of cytoplasmic Ca^{2+} concentration

The addition of thapsigargin to a suspension of human lymphocytes caused an increase of $[Ca^{2+}]_i$ from a resting level between 100 and 150 nM to a steady-state level elevated up to about 1000 nM, dependent on the concentration of thapsigargin (Fig. 1A). The steady-state levels were attained in 2 to 4 min, and the half-maximum response was found at thapsigargin concentrations of about 1 nM. In most experiments the $[Ca^{2+}]_i$ response was higher at 100 than at 1000 nM thapsigargin.

For comparison, the responses dependent on the A23187 concentrations are shown in Fig. 1B. High A23187 concentrations (1 μM and above) resulted in $[Ca^{2+}]_i$ values at which fura2 was saturated, i.e., $[Ca^{2+}]_i$ above 5 μM, which made it impossible to measure higher values of $[Ca^{2+}]_i$.

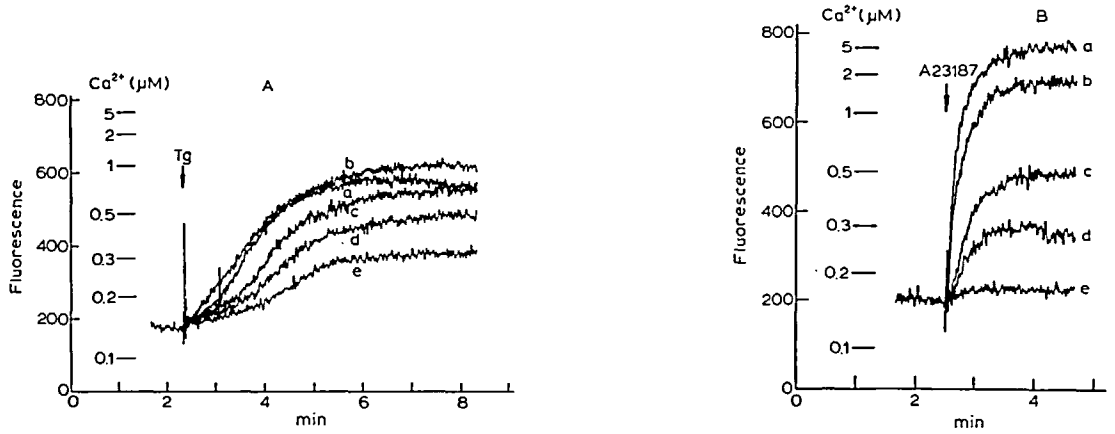

Fig. 1. Cytoplasmic free Ca^{2+} concentration in human lymphocytes monitored as fura2 fluorescence in response to various concentrations of (A) thapsigargin (Tg) and (B) the Ca^{2+} ionophore, A23187. (A) 1000 (a), 100 (b), 1 (c), 0.5 (d) and 0.25 (e) nM. (B) 1000 (a), 100 (b), 10 (c), 5 (d) and 2 (e) nM. The Ca^{2+} concentration of the medium was 1 mM.

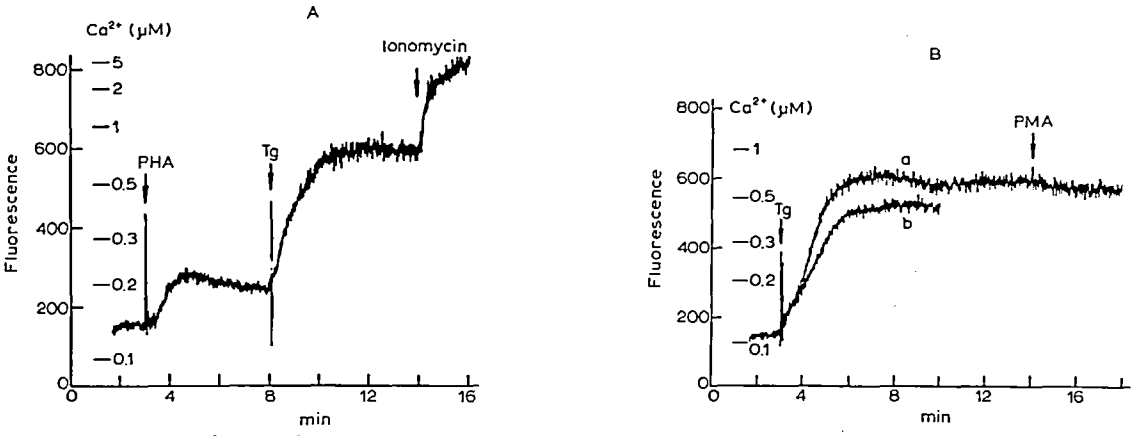

Fig. 2. Cytoplasmic free Ca^{2+} concentration in human lymphocytes monitored as fura2 fluorescence in response to various sequential additions: (A) phytohaemagglutinin PHA (50 μg/ml), thapsigargin (Tg, 100 nM), and ionomycin (1 μM), and (B, a) Tg and then phorbol ester PMA (40 nM), and (B, b) PMA and Tg added together. The Ca^{2+} concentration of the medium was 1 mM.

Half-maximum response could therefore only be estimated to occur at an A23187 concentration that was higher than 100 nM.

The effect of thapsigargin on $[Ca^{2+}]_i$ exceeded that of PHA, also when thapsigargin was added subsequent to PHA either in controls (Figs. 2A) or in AIDS lymphocytes (data not shown). In nine control and nine AIDS lymphocyte preparations the addition of thapsigargin after PHA increased $[Ca^{2+}]_i$ by 1.2 μM (range 0.9–2.5) and 1.5 μM (0.3–3.5) compared to increases of 0.13 μM (0.06–0.22) and 0.11 μM (0.06–0.24), respectively, induced by PHA addition alone (Bo Hofmann et al., unpublished results). There were no significant differences between control and AIDS lymphocytes. The used PHA concentration (50 μg/ml) was near optimum since a 10-times higher concentration of PHA had only a minor additional effect (data not shown).

When the phorbol ester PMA (40 nM) was added together with thapsigargin, the $[Ca^{2+}]_i$ response was delayed and slightly reduced. The PMA addition reduced the thapsigargin-induced rise of fluorescence by $29 \pm 3\%$ ($n = 3$, S.D.). Analo-

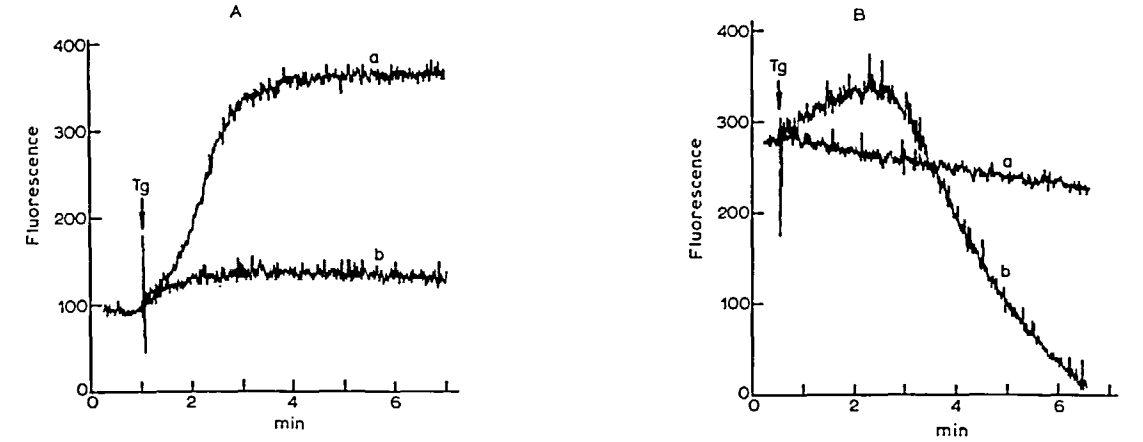

Fig. 3. Effects of extracellularly added EGTA and manganese ions on the fura2 fluorescence. The concentration of thapsigargin (Tg) was 100 nM, the EGTA concentration was 1 mM, and the concentrations of Mn^{2+} and Ca^{2+} were 0.5 and 1 mM. The units of the fluorescence scales are comparable in the two parts of figure (A and B), whereas the points of origin are arbitrary. (A, a) Ca+Tg, (A, b) EGTA+Tg, (B, a) Ca+Mn, and (B, b) Ca+Mn+Tg.

gously, the addition of PMA subsequent to thapsigargin lowered the steady-state level of $[Ca^{2+}]_i$ a little in most experiments (Fig. 2B). The low concentration of PMA (0.5 nM) used in the proliferation experiments had only a slight effect on $[Ca^{2+}]_i$ (data not shown). It appears from Fig. 2A that the addition of ionomycin subsequent to thapsigargin resulted in a further increase of $[Ca^{2+}]_i$.

In the presence of extracellular EGTA, thapsigargin was still able to raise $[Ca^{2+}]_i$, indicating that thapsigargin may act on intracellular Ca^{2+} stores. However, the $[Ca^{2+}]_i$ response was much lower in the presence than in the absence of EGTA (Fig. 3A), suggesting that thapsigargin in human lymphocytes exerts its main effect on $[Ca^{2+}]_i$ by increasing the Ca^{2+} net flux into the cells from the outside.

This concept was supported by the experiments with addition of manganese ions to the fura2-loaded lymphocytes (Fig. 3B). As shown by Grynkiewicz et al. [11], Mn^{2+} binds to fura2 with high affinity and quenches the fura2 fluorescence. The addition of 0.5 mM Mn^{2+} alone to the lymphocyte suspension containing 1 mM Ca^{2+} caused a slowly progressive decrease in the fluorescence due to influx of Mn^{2+}, probably via the same transport mechanisms that were responsible for Ca^{2+} influx, leading to Mn^{2+}-induced quenching of the intracellular fura2 (see Ref. 14). If thapsigargin was added subsequent to Mn^{2+}, the drug-induced increase of fluorescence was inhibited (Fig. 3B, cf. Fig. 3A) and the succeeding decrease of fluorescence due to quenching was much faster, indicating that thapsigargin increased the rate of Mn^{2+} influx from the outside.

Lymphocyte proliferation

In preliminary experiments we investigated the concentration ranges of thapsigargin and the phorbol ester PMA that were able to stimulate lymphocyte proliferation, determined as [^{3}H] thymidine incorporation. We found that the responses increased from day 1 to day 3 and that maximum responses were obtained with 50 to 100 nM of thapsigargin combined with 0.2 to 1 nM of PMA (data not shown). Similar responses were obtained with 0.3 to 0.6 μM of the Ca^{2+} ionophore, A23187, combined with PMA (Bo Hofmann et al., unpublished results).

The effective concentrations of A23187 in the cell culture experiments are lower than the nominal concentrations stated, due to binding of A23187 to the albumin present in the added serum (cf. Materials and Methods) and therefore not direct comparable to the A23187 concentrations in Fig. 1B.

In Table I the effect of thapsigargin was compared with the effects of PHA and A23187 in the presence or absence of PMA. It appears that thapsigargin per se, just like A23187 alone, had no effect on the proliferative response, whereas the lymphocytes responded in the presence of PHA alone. In the presence of PMA, however, both thapsigargin and A23187 caused proliferative responses of the same magnitude as those provoked by PHA. Apart from the much smaller responses

TABLE I

Proliferative response of control and AIDS lymphocytes stimulated with PHA, thapsigargin (Tg) or A23187 in various combinations with PMA. Entries are [^{3}H]thymidine incorporation expressed as cpm$\times 10^{-3}$. Unstimulated cells and cells exposed to PMA alone did not incorporate significant amounts of [^{3}H]thymidine (data not shown). The concentrations were 50 μg/ml (PHA), 100 nM (Tg), 500 nM (A23187), and 0.5 nM (PMA).

	PHA	Tg	Tg/PMA	A23187	A23187/PMA
Control ($n = 9$)					
median	17.3	0.6	18.6	0.5	13.0
range	(7.5–25.0)	(0.3–2.1)	(4.1–34.3)	(0.3–1.3)	(3.5–22.5)
AIDS ($n = 9$)					
median	1.9	0.3	2.6	0.3	2.6
range	(0.3–6.6)	(0.2–0.6)	(0.4–11.1)	(0.2–0.9)	(0.3–3.9)

TABLE II

Production of IL-2 in control and AIDS lymphocytes after 24 h in culture in the presence of PHA alone and thapsigargin (Tg) or A23187 in combination with PMA. For the IL-2 units see Materials and Methods. Concentrations as in Table I.

	PHA	Tg/PMA	A23187/PMA
Control ($n = 9$)			
median	354	7000 *	11680 *
range	(100–2214)	(2560–8192)	(6606–17071)
AIDS ($n = 9$)			
median	111 *	732	508
range	(90–275)	(356–1107)	(196–1638)

* Significantly different from PHA controls ($P < 0.02$).

of the AIDS lymphocytes, the pattern of the agonist effects was the same as that of the controls.

Interleukin-2 production

In combination with PMA, the thapsigargin stimulated strongly the production of interleukin-2 in macrocultures of peripheral blood lymphocytes from controls and to a less extent in AIDS lymphocytes, compared to the effect of PHA (Table II). This is in accordance with the findings that the stimulatory effect of A23187 combined with PMA was much higher than the effect of PHA alone (Bo Hofmann et al., unpublished results, see Table II). Surprisingly, the IL-2 production induced by A23187/PMA in AIDS lymphocytes was not less than that induced by PHA in the control lymphocytes, and the same relation was apparent for the IL-2 production induced by thapsigargin/PMA in AIDS lymphocytes (Table II).

However, since the purpose of this work primarily was to investigate the effect of thapsigargin on the proliferation of lymphocytes, we have not analyzed the interactions between the various agents more detailed, neither of the IL-2 production nor of the expression of IL-2 receptors (see below).

Expression of interleukin-2 receptors

The percentage of the peripheral blood lymphocytes expressing the IL-2 receptors after stimulation with thapsigargin/PMA was of the same magnitude as that obtained by stimulation with PHA alone in both normal and AIDS lymphocytes (Table III), in accordance with the findings of identical effects on the IL-2 receptor expression of either A23187/PMA or PHA additions (Bo Hofmann et al., unpublished results).

The thapsigargin/PMA-induced stimulation had no effect on the percentage of CD8 and HLA-DR markers in the cell population but, as an intriguing feature, thapsigargin/PMA decreased the proportion of CD4 markers, also in AIDS lymphocytes (Table III). These effects on the CD4

TABLE III

The activation markers, IL-2 receptors (CD25) and HLA-DR, and the T lymphocyte subsets, CD4 and CD8, before and after stimulation of peripheral blood lymphocytes from control subjects and patients with AIDS. Entries are percentage of cell population. Concentrations as in Table I.

Marker	Unstimulated		PHA-stimulated		Tg/PMA-stimulated	
	control	AIDS	control	AIDS	control	AIDS
	($n = 8$)	($n = 2$)	($n = 8$)	($n = 2$)	($n = 8$)	($n = 2$)
CD25	1 *	2 *	40	22	41	14
	(0–2)	(1–3)	(18–49)	(17–26)	(30–49)	(9–18)
HLA-DR	34	36	45	47	38	37
	(28–43)	(35–37)	(41–59)	(45–49)	(23–53)	(34–39)
CD4	38	30	35	24	11 *	4 *
	(20–67)	(25–35)	(20–47)	(20–28)	(5–16)	(3–4)
CD8	21	38	30	24	18	15
	(12–36)	(321–55)	(14–40)	(20–28)	(14–41)	(11–18)

* Significantly different from corresponding PHA controls ($P < 0.05$).

percentage were not found by stimulation with A23187/PMA (Bo Hofmann et al., unpublished results).

Discussion

Thapsigargin added in nanomolar concentrations has the ability to increase the cytoplasmic Ca^{2+} concentration in human lymphocytes, according to the results shown above. Since thapsigargin increases $[Ca^{2+}]_i$ in the presence of 1 mM EGTA in the medium, the drug apparently can mobilize Ca^{2+} from the intracellular stores, as shown earlier for human platelets [8,9].

The Ca^{2+} responses of the human lymphocytes differ, however, in some respects from those of human platelets. The lymphocytes seem to be more sensitive to thapsigargin, and the rise of $[Ca^{2+}]_i$ in lymphocytes is apparently more dependent on the presence of extracellular Ca^{2+} (compare Fig. 3A and Ref. 8). Some of the differences between the two types of cell may originate from the relative sizes of their intracellular Ca^{2+} stores, the dense tubular system of the platelets making up a higher proportion of the cell volume than the sparsely developed endoplasmic reticulum of lymphocytes [15].

The effects of thapsigargin on cell proliferation, interleukin-2 production, and expression of IL-2 receptors in human lymphocytes (cf. Results) are very similar to the effects of the Ca^{2+} ionophore A23187 on these lymphocyte reactions. Since thapsigargin is not a Ca^{2+} ionophore, the mechanisms of action of the two drugs are different. Their common effects, therefore, can probably be ascribed solely to the raised $[Ca^{2+}]_i$ which in combination with the PMA-induced activation of the protein kinase C initiates the various responses.

Thapsigargin has the advantage over A23187 of exerting a more moderate effect on $[Ca^{2+}]_i$, which prevents Ca^{2+} overloading of the cells. This difference between the drugs may be ascribed to the kinetics of the A23187-facilitated Ca^{2+} net flux into the cells. In red blood cells this flux has been shown to be proportional to the A23187 concentration raised to a power of about 1.5, rather than showing a linear dependence of the A23187 concentration [16,17].

The much higher IL-2 production induced by thapsigargin/PMA (or A23187/PMA) compared to that induced by PHA (Table II) is in accordance with the findings of Yamamoto et al. [18]. These authors found increasing effects on the IL-2 production of the treatments PHA < PHA/PMA < A23187/PMA, but no effect of either A23187 or PMA per se (see also Ref. 2). At the much higher level of $[Ca^{2+}]_i$ caused by thapsigargin (or A23187) compared to that caused by PHA (Figs. 1 and 2), the cellular calmodulin is closer to be saturated with Ca^{2+}. If the Ca^{2+}/calmodulin system is involved in the IL-2 production, as suggested by Yamamoto et al. [18], the level of $[Ca^{2+}]_i$ might be of significance for the effect on the IL-2 production of the tested Ca^{2+}-mobilizing agents acting synergetically in combination with PMA which probably activates the protein C kinase. Similar mechanisms may cause the surprisingly high IL-2 production of the AIDS cells treated with thapsigargin/PMA or A231287/PMA (Table II).

Both the cell proliferation (Table I) and the expression of IL-2 receptors (Table III) responded equally to the treatment with either PHA or thapsigargin/PMA. Possibly, these two lymphocyte responses are elicited at lower levels of $[Ca^{2+}]_i$ than the IL-2 production.

The intriguing down-regulation of the CD4 markers induced by thapsigargin/PMA in the peripheral blood lymphocytes (Table III) may be analogous to the down-regulation of CD4 caused by ionomycin/PMA and to a lesser extent by PMA alone, reported recently by Isakov et al. [19] who, in addition, found down-regulation of CD8 and CD3 markers. On the other hand, we found no down-regulation due to treatment with A23187/PMA (Bo Hofmann et al., unpublished results). Further studies are required to clarify this point.

Neither thapsigargin nor A23187 in combination with PMA could restore the defective proliferation of AIDS lymphocytes, although both treatments stimulated the infected cells to IL-2 production and expression of IL-2 receptors. This suggests that the general defect of AIDS lymphocytes, rather than being related to the initiating signal systems, is associated with inhibited activation of the major enzymes leading to DNA

synthesis and proliferation, as suggested by Bo Hofmann et al. (unpublished results).

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