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Calcium Influx and Intracellular Calcium Release in Anti-CD3 Antibody-Stimulated and Thapsigargin-Treated Human T Lymphoblasts

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Summary. Jurkat and MOLT-4 cultured T lymphoblasts were loaded with low concentrations (30–50 μM) of indo-1 and with high concentrations (3.5–4.5 mM) of quin-2, respectively, in order to follow the activation of calcium transport pathways after stimulation of the cells by a monoclonal antibody against the T cell antigen receptor (aCD3), or after the addition of thapsigargin, a presumed inhibitor of endoplasmic reticulum calcium pump. In the indo-1 loaded cells the dynamics of the intracellular calcium release and the calcium influx could be studied, while in the quin-2 overloaded cells the changes in cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) were strongly buffered and the rate of calcium influx could be quantitatively determined. We found that in Jurkat lymphoblasts, in the absence of external calcium, both aCD3 and thapsigargin induced a rapid calcium release from internal stores, while upon the readdition of external calcium an increased rate of calcium influx could be observed in both cases. aCD3 and thapsigargin released calcium from the same intracellular pools. The calcium influx induced by either agent was of similar magnitude and had a nonadditive character if the two agents were applied simultaneously. As demonstrated in quin-2 overloaded cells, a significant initial rise in $[Ca^{2+}]_i$ or a pronounced depletion of internal calcium pools was not required to obtain a rapid calcium influx. The activation of protein kinase C by phorbol ester abolished the internal calcium release and the calcium influx induced by aCD3, while having only a small effect on these phenomena when evoked by thapsigargin. Membrane depolarization by gramicidin inhibited the rapid calcium influx in both aCD3- and thapsigargin-treated cells, although it did not affect the internal calcium release produced by either agent. In MOLT-4 cells, which have no functioning antigen receptors, aCD3 was ineffective in inducing a calcium signal, while thapsigargin produced similar internal calcium release and external calcium influx to those observed in Jurkat cells.

Key Words T lymphoblasts · calcium signal · calcium release · calcium influx · thapsigargin · indo-1 · quin-2

Introduction

The uniform cell populations of cultured human lymphoblasts provide immunologically relevant model systems to examine the physiological and pharmacological regulation of various ion transport pathways. The T-type Jurkat lymphoblasts have a functional antigen receptor complex (TCR + aCD3 antigens), and the addition of a monoclonal anti-TCR or anti-CD3 antibody in these cells evokes the activation of membrane phospholipase C, the production of IP_3 and DAG, and a consequent calcium signal which is based on internal calcium release and external calcium influx components (*see* Tsien, Pozzan & Rink, 1982; Hesketh et al., 1983; Imboden, Weiss & Stobo, 1985; Alcover et al., 1987; Gelfand et al., 1987*b*; Imboden & Weiss, 1987; Isakov et al., 1987; Pecht et al., 1987; Gardner, 1989; Grinstein & Klip, 1989; Gupta, 1989). As it has been shown earlier, in T lymphocytes and lymphoblasts the activation of protein kinase C by phorbol esters or by a permeable diacylglycerol downregulates the CD3 receptor (Cantrell et al., 1985; Crumpton et al., 1987) and strongly inhibits the development of the aCD3-induced calcium signal (Tordai et al., 1989; Sarkadi et al., 1990*b*), while membrane depolarization selectively decreases the calcium influx component (Gelfand et al., 1987*a*; Sarkadi, Tordai & Gárdos, 1990*a*). All these effects, observed in other cell types as well (*see* Nishizuka, 1986, 1988; Meldolesi & Pozzan, 1987; Penner, Matthews & Neher, 1988), may have important regulatory functions in the cellular response to various stimuli.

A widely used technique for studying the changes in cytoplasmic free calcium concentrations is to incorporate fluorescent calcium chelators into the cells, based on the method developed by Tsien and his co-workers (*see* Tsien et al., 1982; Tsien, 1983; Grynkiewicz, Poenie & Tsien, 1985). Indo-1 was found to be a highly suitable dye for calcium signal studies in lymphocytes and lymphoblasts, as this calcium indicator has a high fluorescence yield (Grynkiewicz et al., 1985) and its leakage from these

cells, in contrast to that seen with fura-2, is very slow (*see* Grienstein & Klip, 1989; Tordai et al., 1989). In the present experiments the dynamics of the $[Ca^{2+}]_i$ changes were studied by using indo-1-loaded lymphoblasts, while for the quantitative estimation of calcium influx we used higher concentrations of quin-2. In quin-2 overloaded cells, as originally suggested by Tsien et al. (1982) and Tsien (1983), the buffering capacity of the fluorescent dye could be utilized to eliminate rapid changes of $[Ca^{2+}]_i$.

In the present report, by using cultured lymphoblasts, we compared the effects of aCD3 to those evoked by thapsigargin, a plant-derived sesquiterpene lactone (Rasmussen, Christensen & Sandberg, 1978). Thapsigargin has been shown to induce calcium transients independent of physiological stimuli in various cell types (Thastrup, Foder & Scharff, 1987; Thastrup et al., 1989, 1990; Scharff et al., 1988; Brayden et al., 1989). This drug is believed to act predominantly by the inhibition of the endoplasmic reticulum calcium pump (Thastrup et al., 1990), and thus allows the release of calcium from intracellular stores without inositol phosphate generation (Jackson et al., 1988; Takemura et al., 1989; Law et al., 1990; Thastrup et al., 1990), but has also been shown to increase the influx of extracellular calcium (Foder, Scharff & Thastrup, 1989; Takemura et al., 1989; Thastrup et al., 1989; Takemura, Thastrup & Putney, 1990). In order to explain these dual effects, the determining role of stored calcium on the rate of calcium influx as well as the opening of special calcium transport pathways, leading through both external and internal membranes have been postulated (Takemura et al. 1989; Takemura and Putney, 1989; Thastrup et al., 1989). By examining the effects of protein kinase C activation, membrane depolarization, or quenching of the intracellular Ca^{2+} by quin-2, here we compared the receptor-activated calcium fluxes to those evoked by thapsigargin in human lymphoblasts. The cultured T lymphoblasts, designated as MOLT-4, in contrast to Jurkat cells, do not respond to aCD3 or lectin stimulation; thus, we used these cells to examine the effects of thapsigargin in the absence of a physiological receptor-induced calcium-signaling pathway.

Abbreviations

aCD3: monoclonal antibody against the CD3 antigen
$[Ca^{2+}]_i$: cytoplasmic free calcium ion concentration
DAG: 1,2-diacylglycerol
DMSO: dimethyl sulfoxide
diS-C_3-(5): 3,3′-dipropylthiodicarbocyanine
EGTA: ethyleneglycol-bis-(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
HEPES: 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
IL 2: interleukin 2
IP_3: inositol 1,4,5-trisphosphate
indo-1 AM: acetoxy-methylester of indo-1
PIP_2: phosphatidylinositol 4,5-bisphosphate
PKC: protein kinase C
PMA: phorbol 12-myristate, 13-acetate
quin-2 AM: acetoxy-, methylester of quin-2
TG: thapsigargin

Materials and Methods

Reagents and Cells

EGTA, HEPES, gramicidin and valinomycin were purchased from SIGMA; RPMI 1640 cell culture medium and fetal calf serum were from Gibco. Stock solution of indo-1 AM (Calbiochem), 1 mM, and quin-2 AM (Sigma) 20 mM, were prepared in DMSO and stored at −20°C. All the basic chemicals used were of reagent grade. Anti-CD3 monoclonal antibody (OKT3) was obtained from Ortho.

The standard incubation media for the fluorescence measurements contained (in mM): 120 NaCl, 5 KCl, 0.4 $MgCl_2$, 0.04 $CaCl_2$, 10 HEPES-Na (pH 7.4), 10 $NaHCO_3$, 10 glucose and 5 Na_2HPO_4. This medium was supplemented during the fluorescence measurements first with 500 μM EGTA and then with the indicated amount of calcium.

The human leukemic T cell lines, Jurkat and MOLT-4, were maintained under standard conditions in RPMI 1640 medium supplemented with 10% fetal calf serum. The cells were harvested in suspensions of 1–2 × 10^6 cells/ml.

$[Ca^{2+}]_i$ Measurements

For loading with the fluorescent calcium indicators the lymphoblasts were resuspended in RPMI + 10% fetal calf serum with a cell number of 2 × 10^6/ml and incubated at 37°C for 30 min with 0.5 μM final concentration of indo-1 AM or for 60 min with 50 μM quin-2 AM. After the loading period the cells were spun down (600 × *g*, 10 min) and resuspended in dye-free RPMI + serum. The cells were kept at room temperature in this medium, and the fluorescence measurements were carried out within 2 hr. The loading procedure was calibrated by using free indo-1 and quin-2 under the normal fluorescence measurement conditions (*see below*), and the intracellular concentration of indo-1 was found to be between 30–50 pmol/10^6 cells, while that of quin-2 was between 3.5–4.5 nmol/10^6 cells. As the mean cell volume was estimated to be 10^{-12} liter, the above concentrations correspond to 30–50 μmol/liter of cells for indo-1 and 3.5–4.5 mmol/liter of cells for quin-2, respectively.

Before each measurement an aliquot of the cell suspension was rapidly spun down (10 sec at 12,000 × *g*) in an Eppendorf microfuge, the pellet rinsed without further centrifugations five times with the standard incubation medium and then the cells were resuspended in 2 ml of the same medium (10^6 cell/ml). Fluorescence was measured in a Hitachi F-4000 fluorescence spectrophotometer at 37°C with continuous gentle stirring. The excitation wavelength for indo-1 and quin-2 were 331 and 337 nm; emissions were measured at 410 and 490 nm, respectively (bandwidth 5 nm). Cytoplasmic free calcium concentration was calculated based on the method described by Grynkiewicz et al.

(1985), that is by lysing the cells in digitonin (20 μg/ml) in the presence of 0.5 mM free calcium in order to obtain maximum fluorescence, and then by quenching the dyes by the removal of Ca^{2+} with 5 mM EGTA at pH 8.5, adjusted with Tris base.

In the experiments the presence of extracellular fluorescence was checked by the addition of 0.5 mM EGTA and then 1 mM calcium to the control cells. In each experiment this 0.5 mM EGTA was added to the media containing initially 40 μM Ca^{2+}, and any further adjustment of the free calcium concentration was based on calculations by using a computer program taking account of all the constituents of the media (*see* Sarkadi et al., 1990*a*). The leakage of indo-1 and quin-2 proved to be very small during a 10–15 min experimental period; however, this leakage was taken into consideration in the calibration procedure. Due to the sufficient buffering capacity in the media, the calcium-EGTA interactions did not produce pH changes exceeding 0.02 pH unit.

For cell stimulation aCD3 was used in these experiments at a concentration of 0.1 μg/ml, which gave a maximum response in the calcium signal development.

The data presented in the figures are representatives of at least six similar experiments carried out with different cell preparations. In this stable cell line the reproducibility of the $[Ca^{2+}]_i$ measurements was extremely good, usually within 5–10% around the mean values.

Calculations

The aim was to calculate the total amount of calcium influx in indo-1 and quin-2 loaded cells. The following fundamental equations were used:

$$K_d = [Ca^{2+}]_{free} \cdot [D]_{free}/[CaD] \quad (1)$$

$$[Ca^{2+}]_{total} = [Ca^{2+}]_{free} + [CaD] \quad (2)$$

$$[D]_{total} = [D]_{free} + [CaD] \quad (3)$$

$$[CaD] = [D]_{total} \cdot f \quad (4)$$

where $[D]$ represents the intracellular concentration of the fluorescent dye (indo-1 and quin-2), $[CaD]$ the chelated calcium concentration, and subscripts refer to total and free concentrations. The last equation was based on the linear calibration assumption used by Tsien et al. (1982) as well as Johansson and Haynes (1988), where f denotes the actual relative fluorescence equal to $(F - F_0)/(F_{max} - F_0)$, F_0 being the fluorescence measured in the cells lysed by digitonin in the absence of free calcium, and F_{max} the fluorescence measured in the lysed cells in the presence of 1 mM calcium.

The K_d values used for indo-1 and quin-2 were 250 and 115 nM, respectively (*see* Grynkiewicz et al., 1985).

By elementary calculations the intracellular calcium concentration can be expressed as

$$[Ca^{2+}]_{total} = K_d \cdot f/(1 - f) + [D]_{total} \cdot f. \quad (5)$$

The desired equation for the calcium influx can be obtained by differentiation Eq. (5).

$$d[Ca^{2+}]_{total}/dt = K_d \cdot (df/dt) \cdot 1/(1 - f)^2 + [D]_{total} \cdot (df/dt). \quad (6)$$

In each experiment the cell number and the absolute value of fluorescence was used to calculate the amount of D_{total}, based on the calibration with the free dyes which gave fluorescence values of 4 U/μM for quin-2 and 130 U/μM for indo-1. For the estimation of the calcium influx rates the initial linear parts of the changes in fluorescence were measured.

Results

Experiments with Indo-1 Loaded Cells

In these experiments we studied the effects of aCD3 monoclonal antibody and TG on the development of calcium signal in indo-1 loaded lymphoblasts and examined the role of protein kinase C activation and membrane depolarization in this process. Figure 1 shows the effect of aCD3 (panels *A* and *B*) and 100 nM TG (panels *C* and *D*) on indo-1 loaded Jurkat lymphoblasts in the presence of 0.5 mM external Ca^{2+}. We found that both agents induced a large increase in $[Ca^{2+}]_i$, with about a similar rate and magnitude. In both cases $[Ca^{2+}]_i$ was stabilized at a plateau level, and this level was practically unchanged within the time limits of our experiments (the reliable measurement period is about 40–60 min under the conditions applied). As shown in Fig. 1*E* and *F*, the effects of aCD3 and TG were not additive; the simultaneous addition of the two agents evoked a similar calcium signal as the separate additions. It is to be mentioned, that TG in concentrations between 50 to 500 nM produced similar calcium signals and already 5 nM TG evoked a measurable increase in $[Ca^{2+}]_i$. The addition of the protein kinase C activator phorbol myristate acetate (PMA, 50 nM) caused a rapid decline in the $[Ca^{2+}]_i$ in aCD3-stimulated cells, while it had only a small effect in TG- or aCD3 + TG-stimulated lymphoblasts. If gramicidin (200 nM) was added to the cells, a rapid decrease in $[Ca^{2+}]_i$ was observed both in the presence of aCD3 and TG.

In order to separate the intracellular calcium release and the calcium influx phenomena in the stimulated cells we used a previously described method (Sarkadi et al., 1990*a,b*), that is, the induction of the calcium signal in the absence of external Ca^{2+} and the rapid repletion of $[Ca^{2+}]_o$ after the internal calcium stores had been emptied. As demonstrated in Fig. 1, in the control Jurkat lymphoblasts the addition of external EGTA and readdition of calcium did not have much effect on the indo-1 fluorescence. Figure 2 shows that, when aCD3 was added to the cells in the presence of excess EGTA in the medium (panel *A*), a $[Ca^{2+}]_i$ spike developed, reflecting the calcium release from the intracellular stores. After the restoration of the low $[Ca^{2+}]_i$ level, the addition of extracellular calcium (final concentration about 0.5 mM), due to the rapid calcium influx, produced an increase in $[Ca^{2+}]_i$ again

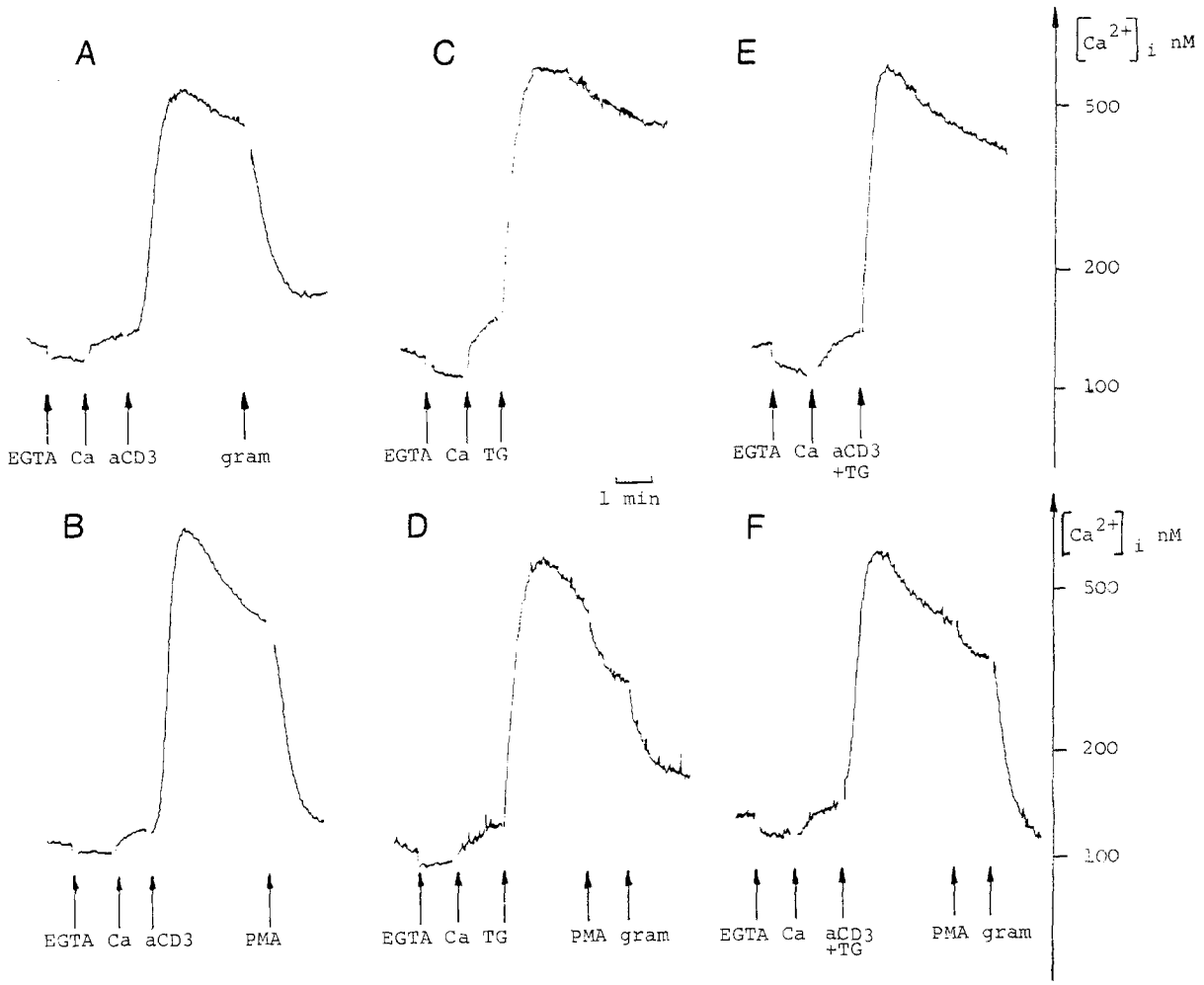

Fig. 1. Effects of aCD3 and thapsigargin (TG) on the intracellular calcium concentration in indo-1 loaded Jurkat lymphoblasts. Indo-1 fluorescence was measured, and $[Ca^{2+}]_i$ and indo-1 concentrations were calibrated as described in Materials and Methods. The lymphoblasts were incubated in the standard medium and then 0.5 mM EGTA, 1.0 mM $CaCl_2$ (final $[Ca^{2+}]_o$ about 0.54 mM), 0.1 μg/ml aCD3 or 100 nM TG and 50 nM PMA or 200 nM gramicidin (*gram*) were added at the times indicated by the arrows. The upper panels show the changes in $[Ca^{2+}]_i$ in the control experiments (on *A* they also represents the $[Ca^{2+}]_i$ values after gramicidin addition). Internal indo-1 concentration was 35 μM in the experiment shown in this figure

and a similar plateau phase as observed in the experiments shown in Fig. 1. Addition of 50 nM PMA to the aCD3-stimulated cells strongly decreased this plateau and gramicidin (200 nM) had a similar effect. As shown earlier, the effect of PMA correlates with its PKC-activating effect (Tordai et al., 1989), while gramicidin acts through membrane depolarization (Sarkadi et al., 1990*a*).

When, under similar conditions, 100 nM TG was added to the Jurkat cells (Fig. 2*C* and *D*), a calcium release from the internal stores could also be observed, although its development was somewhat more prolonged than that seen with aCD3. The calcium release curve was similar at TG concentrations between 50–500 nM. Upon readdition of external calcium a rapid calcium influx was observed, similar to that seen in Fig. 2*A* and *B*, with aCD3. In this case the addition of PMA caused only a slight decrease in the level of $[Ca^{2+}]_i$, while gramicidin produced a rapid restoration of $[Ca^{2+}]_i$ near to its baseline level.

Figure 3 shows experiments in which the Jurkat lymphoblasts were pretreated with 50 nM PMA or with 200 nM gramicidin for 2 min before the addition of aCD3 or TG. As shown in Fig. 3*A*, in the presence of PMA, aCD3 was unable to induce a significant calcium signal either in the absence or after the readdition of $[Ca^{2+}]_o$, while TG (Fig. 3*B*) produced a qualitatively similar internal calcium release and external calcium influx as seen in the absence of PMA. Compiled data from five similar experiments showed

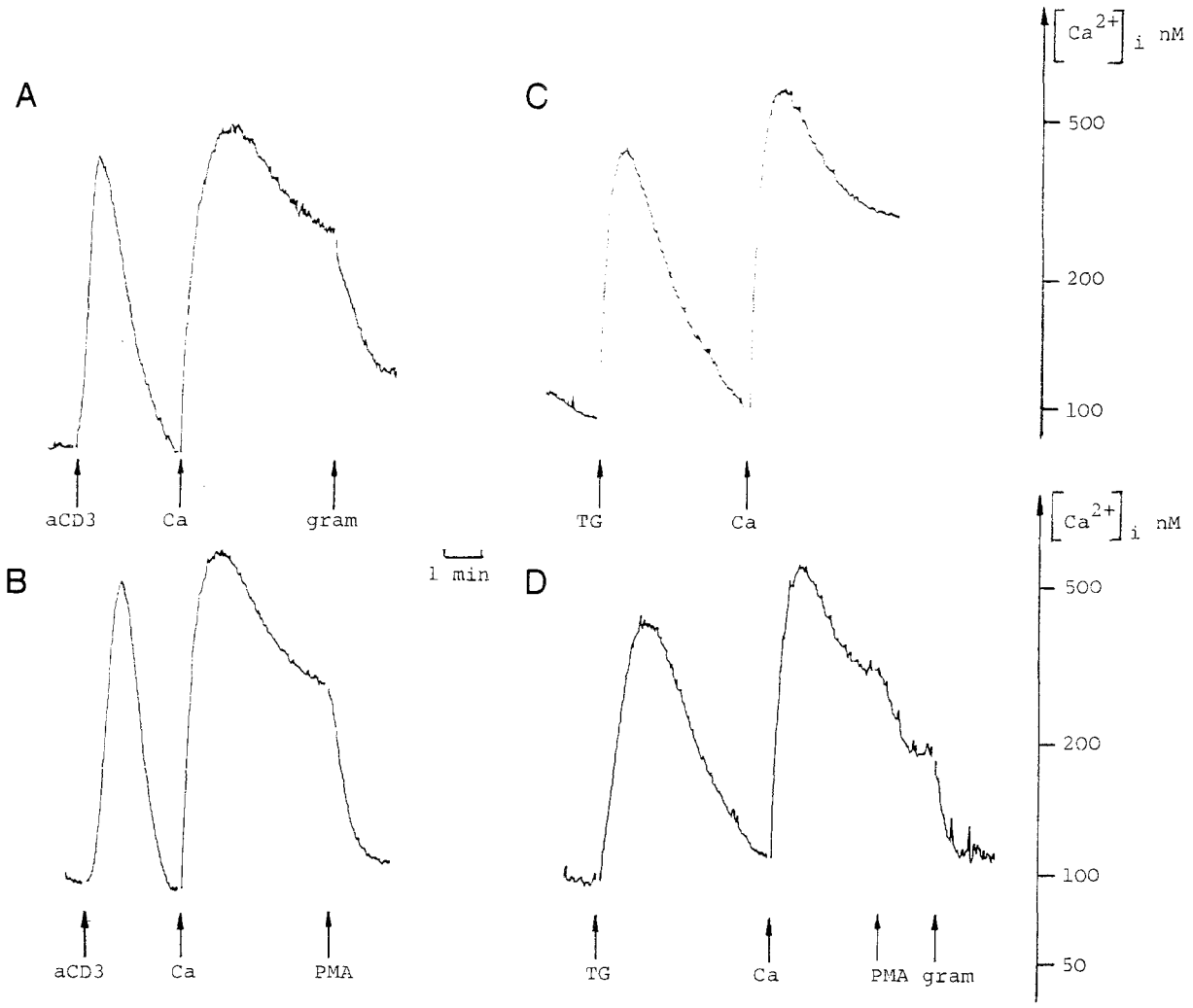

Fig. 2. Effects of aCD3 and TG on intracellular calcium release and external calcium influx in indo-1 loaded Jurkat lymphoblasts. Jurkat lymphoblasts (internal indo-1 = 35 μM) were incubated in standard medium + 0.5 mM EGTA ($[Ca^{2+}]_o$ less than 0.1 μM). At the times indicated by the arrows 0.1 μg/ml aCD3 or 100 nM TG and 50 nM PMA or 200 nM gramicidin (*gram*) were added to the media. The arrow marked *Ca* indicates the addition of 1 mM final concentration of $CaCl_2$, that is, the adjustment of $[Ca^{2+}]_o$ to about 0.54 mM. The upper panels show the changes in $[Ca^{2+}]_i$ in the control experiments, and on *A* they represent the $[Ca^{2+}]_i$ values after gramicidin addition

a 10–15% decrease in the peak levels of the TG-induced calcium signals in PMA-pretreated cells as compared to that in the control cells. As shown in Fig. 3*C*, the membrane-depolarizing ionophore, gramicidin, did not influence the TG-induced calcium release, but inhibited the development of a rapid calcium influx. A similar effect of gramicidin was demonstrated earlier in aCD3-treated Jurkat cells (Sarkadi et al., 1990*a*).

In the following experiments we examined the relationship between the cellular calcium stores affected by aCD3 and TG, respectively. In these experiments the extracellular free calcium concentration was kept below 0.1 μM by EGTA. As shown in Fig. 4*A*, after the calcium release induced by a maximum effective dose of aCD3, TG had no significant calcium releasing activity and even ionomycin (1 μM) produced only a small rise in $[Ca^{2+}]_i$. As demonstrated in Fig. 4*B*, this nonadditive effect was true in the reversed order of additions as well; after a TG-induced calcium release aCD3 was unable to produce any further calcium spike. These experiments indicate that the two agents act on the same internal calcium stores in the Jurkat lymphoblasts. Figure 4*C* shows the effect of ionomycin (1 μM), which causes a rapid and short rise in $[Ca^{2+}]_i$ by emptying the cellular calcium stores, thereafter which neither aCD3 nor TG could produce any further calcium release.

In order to compare the receptor-ligand

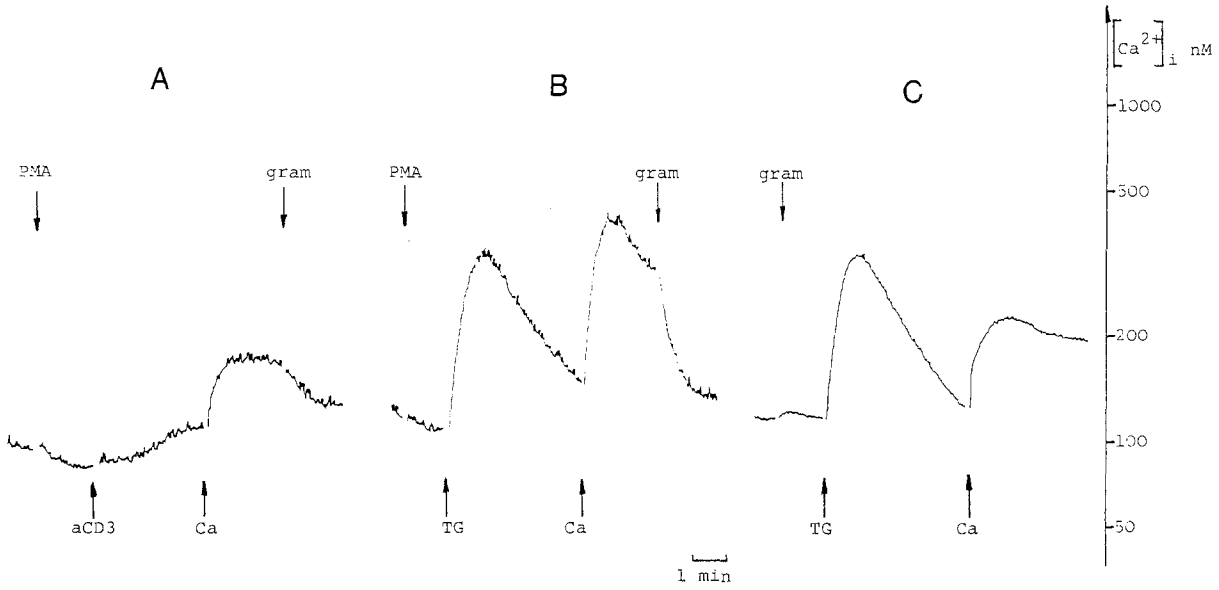

Fig. 3. Effects of PMA and gramicidin on the changes in intracellular calcium concentration evoked by aCD3 or TG in indo-1 loaded Jurkat lymphoblasts. Jurkat lymphoblasts (internal indo-1 = 45 μM) were incubated in standard medium + 0.5 mM EGTA ($[Ca^{2+}]_o$ less than 0.1 μM). At the times indicated by the arrows 50 nM PMA, 200 nM gramicidin (*gram*) 0.1 μg/ml aCD3 or 100 nM TG was added to the media. The arrow marked *Ca* indicates the addition of 1 mM final concentration of $CaCl_2$, that is, the adjustment of $[Ca^{2+}]_o$ to about 0.54 mM

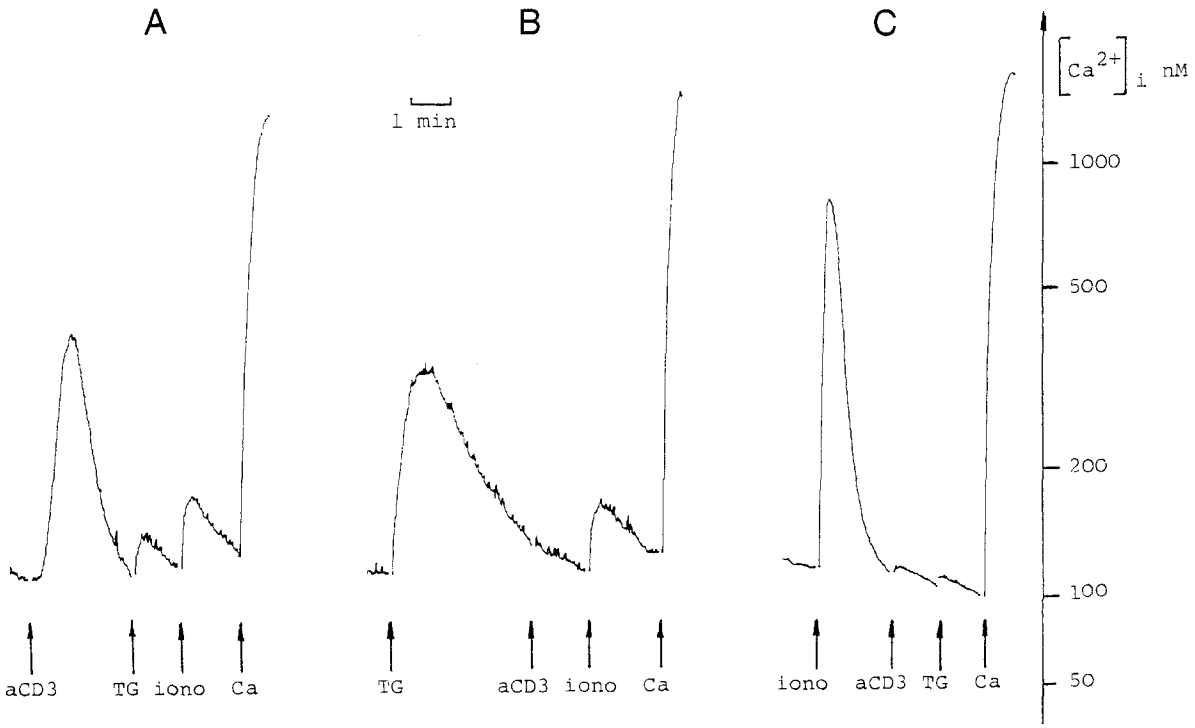

Fig. 4. Intracellular calcium release evoked by aCD3, TG or ionomycin in indo-1 loaded Jurkat lymphoblasts. Jurkat lymphoblasts (internal indo-1 = 40 μM) were incubated in the standard medium + 0.5 mM EGTA ($[Ca^{2+}]_o$ less than 0.1 μM). At the times indicated by the arrows 0.1 μg/ml aCD3, 100 nM TG, or 500 nM ionomycin (*iono*) was added to the media. The arrow marked *Ca* indicates the addition of 1 mM final concentration of $CaCl_2$, that is, the adjustment of $[Ca^{2+}]_o$ to about 0.54 mM

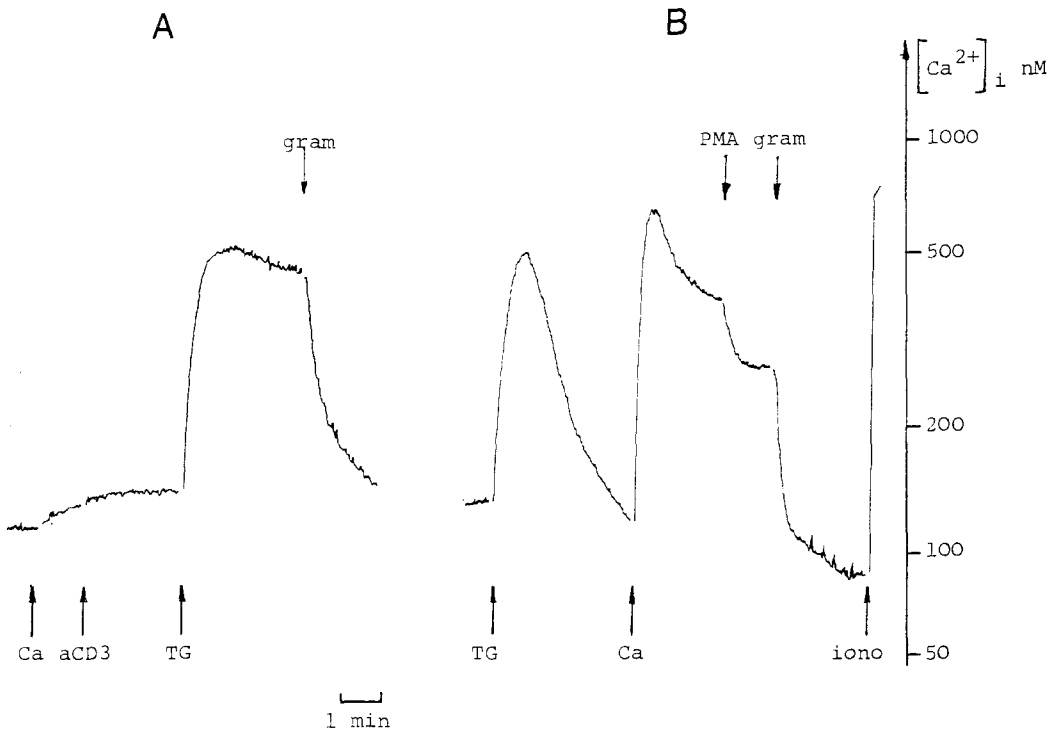

Fig. 5. Effects of aCD3 and TG on the intracellular calcium concentation in indo-1 loaded MOLT-4 lymphoblasts. MOLT-4 lymphoblasts (internal indo-1 = 40 μM) were incubated in the standard medium + 0.5 mM EGTA ($[Ca^{2+}]_o$ less than 0.1 μM). At the times indicated by the arrows 0.1 μg/ml aCD3, 100 nM TG, 50 nM PMA, 200 nM gramicidin (*gram*), or 500 nM ionomycin (*iono*) was added to the media. The arrow marked *Ca* indicates the addition of 1 mM final concentration of $CaCl_2$, that is, the adjustment of $[Ca^{2+}]_o$ to about 0.54 mM

(aCD3–CD3) interaction-dependent calcium signal development to that induced by TG we studied a T cell line (MOLT-4) which has no functional antigen receptors (CD3 negative), and thus, cannot be stimulated by aCD3. We hypothesized that TG should produce a calcium signal also in these lymphoblasts. As shown in Fig. 5*A*, indo-1 loaded MOLT-4 lymphoblasts did not show a calcium signal upon the addition of aCD3 (this was true either in the absence or presence of $[Ca^{2+}]_o$, *data not shown*), while TG induced a rapid increase in $[Ca^{2+}]_i$. As demonstrated in Fig. 5*B*, TG had a similar calcium mobilizing effect in MOLT-4 lymphoblasts as in Jurkat cells; in the absence of $[Ca^{2+}]_o$ a calcium release signal, upon readdition of external calcium a rapid calcium influx was observed. Just as in the Jurkat cells, the elevated level of $[Ca^{2+}]_i$ after TG in MOLT-4 was only slightly sensitive to PMA, while a strong decrease in cytoplasmic calcium was seen upon the addition of gramicidin.

Experiments with Quin-2 Loaded Cells

In order to assess the quantitative characteristics of the calcium influx into the aCD3- or TG-stimulated Jurkat lymphoblasts, the cells were loaded with relatively high concentrations (3.5–4.5 mM) of quin-2. This amount of intracellular chelator (as shown in Fig. 6*A* and *B*) in the absence of extracellular Ca^{2+} practically eliminated the rise in $[Ca^{2+}]_i$, caused by the release of calcium from internal stores. However, if external calcium was present, a rapid influx of calcium occurred in the presence of aCD3 (Fig. 6*A* and *C*) or of TG (Fig. 6*B* and *D*). Since in quin-2 loaded cells the changes in $[Ca^{2+}]_i$ were relatively small, the initial rate of fluorescence increase probably closely correlated with the functioning of the calcium influx pathways (*see* Discussion). The slope of the increase in $[Ca^{2+}]_i$ induced by aCD3 and TG were similar (*see* Table), and the simultaneous addition of aCD3 and TG produces the same rate of calcium influx (*data not shown*). In TG-treated cells this increase did not show a lag phase whether the calcium influx was initiated by the addition of TG (Fig. 6*D*) or by external calcium (Fig. 6*B*), while in the case of aCD3 a short lag phase could be seen if the reaction was started by a CD3 (Fig. 6*C*).

Figure 7 shows the effects of PMA and gramicidin on the calcium influx in quin-2 loaded Jurkat lymphoblasts after the addition of aCD3 (panels *A* and *B*) or TG (panel *C*). In accordance with the

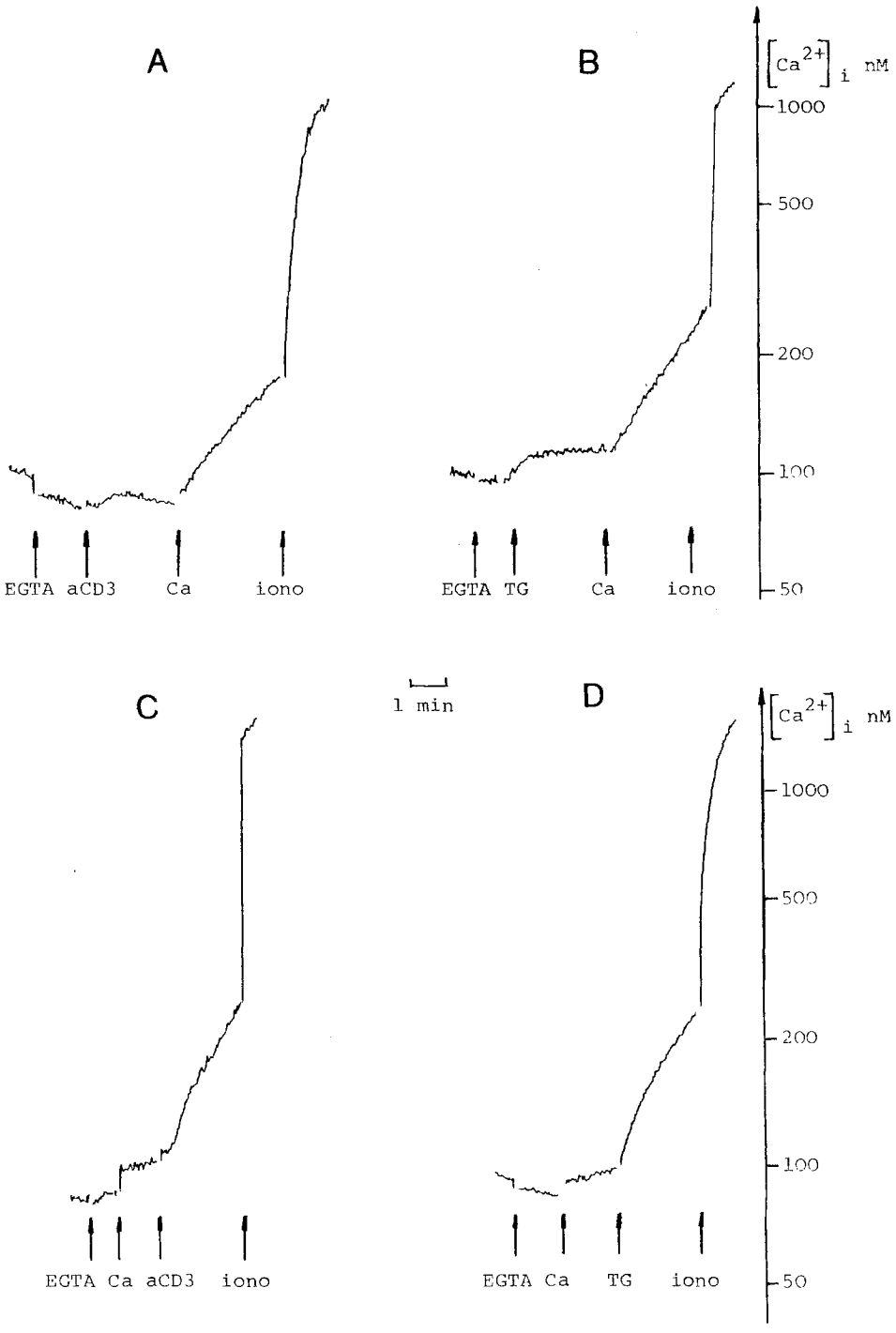

Fig. 6. Effects of aCD3 and TG on the intracellular calcium concentration in quin-2 loaded Jurkat lymphoblasts. For the description of quin-2 loading and fluorescence measurements *see* Materials and Methods. Intracellular quin-2 in these experiments was 3.9 mM. The cells were incubated in the standard medium, and at the times indicated by the arrows 0.5 mM EGTA ($[Ca^{2+}]_o$ less than 0.1 μM), 0.1 μg/ml aCD3, 100 nM TG, or 500 nM ionomycin (*iono*) was added to the media. The arrow marked *Ca* indicates the addition of 1 mM final concentration of $CaCl_2$, that is, the adjustment of $[Ca^{2+}]_o$ to about 0.54 mM

data obtained with into-1 loaded cells, in the case of aCD3, both gramicidin (Fig. 7*A*) and PMA (Fig. 7*B*) strongly reduced the calcium influx, while in the case of TG (Fig. 7*C*), only gramicidin inhibited this calcium movement.

The Table summarizes the above findings and provides quantitative figures obtained for the rate of calcium influx in quin-2 loaded Jurkat lymphoblasts. The values for the rate of aCD3- and TG-induced calcium influx were not found to be significantly different. PMA inhibited only the aCD3-induced calcium influx, while gramicidin strongly decreased this influx both in the presence of aCD3 and TG. If the aCD3- or TG-induced calcium influx rates were calculated from the indo-1 experiments (*data not shown* in detail), these values were 20–30% smaller than those obtained with the quin-2 loaded cells.

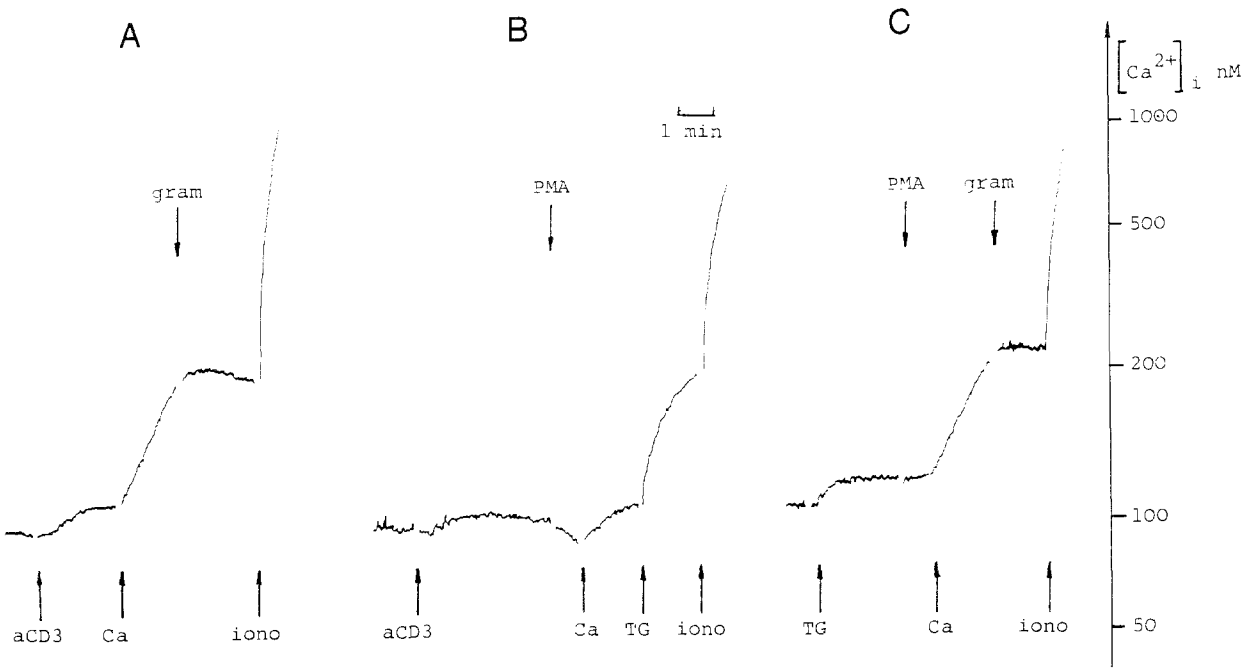

Fig. 7. Modulation of the calcium influx in quin-2 loaded Jurkat lymphoblasts. Quin-2 loaded cells (internal quin-2 = 4.2 mM) were incubated in the standard medium + 0.5 mM EGTA ($[Ca^{2+}]_o$ less than 0.1 μM). At the times indicated by the arrows 0.1 μg/ml aCD3, 100 nM TG, 50 nM PMA, 200 nM gramicidin (*gram*), or 500 nM ionomycin (*iono*) was added to the media. The arrow marked *Ca* indicates the addition of 1 mM final concentration of $CaCl_2$, that is, the adjustment of $[Ca^{2+}]_o$ to about 0.54 mM

Table. Calculated rates of calcium influx into aCD3- or TG-stimulated quin-2 loaded Jurkat lymphoblasts

Pretreatment	Calcium influx (μmol · liter of cells^{-1} · min^{-1})		
Addition:	None	aCD3	TG
0.2% Ethanol	23 ± 3	266 ± 12	282 ± 18
50 nM PMA	5 ± 2	55 ± 6	248 ± 16
200 nM Gramicidin	18 ± 4	42 ± 8	46 ± 4

Quin-2 loaded Jurkat lymphoblasts (internal quin-2 between 3.5–4.5 mM) were incubated in standard media + 0.5 mM EGTA ($[Ca^{2+}]_o$ less than 0.1 μM). Four min after the addition of aCD3 (0.1 μg/ml) or TG (100 nM), calcium influx was initiated by the addition of $CaCl_2$ (final $[Ca^{2+}]_o$ in the medium about 0.54 mM). Before the addition of $CaCl_2$ the cells were pretreated for 2 min with 0.2% ethanol (solvent for PMA and gramicidin), 50 nM PMA, or 200 nM gramicidin. Calcium influx was computed as described in Materials and Methods. Number of experiments = 5; the calculated means ± SD values are shown.

Discussion

In the experiments presented above we have studied the calcium signal development in lymphoblasts by using two different calcium indicators, indo-1 and quin-2, respectively. In the first case indo-1 is introduced into the cells in low concentrations (30–50 μM) in order to study the dynamics of calcium signaling. Since there is no significant intracellular calcium buffering by the dye, this technique allows the separation of intracellular calcium release from the external calcium influx (*see* Magócsi et al., 1989; Sarkadi et al., 1990*a*). In contrast, in the quin-2 overloaded cells, when the concentration of the fluorescent calcium chelator is intentionally increased to millimolar levels (*see* Fewtrell & Sherman, 1987; Johansson & Haynes, 1988; Owen, 1988; Crofts & Barritt, 1989), calcium buffering counteracts the rapid changes in $[Ca^{2+}]_i$, and moreover, eliminates the calcium signal produced by intracellular calcium release in the absence of external calcium. In quin-2 loaded cells calcium influx causes only a small and practically linear increase in $[Ca^{2+}]_i$; thus, the activation of ATP-dependent calcium pumps and other calcium-dependent systems may not immediately occur at a significant level.

Equation (6) (*see* Materials and Methods) applied here for the estimation of the transmembrane calcium flux is based on the calculation of the initial rate of a net increase in the total amount of cytoplasmic calcium. In principle, this method can be used for both types of indicators, as it considers the changes both in the free and complexed forms of this

ion. However, the changes in fluorescence are much too rapid in indo-1 loaded Jurkat lymphoblasts to find a quasi-linear portion under the measurement conditions applied, and also, the activation of various calcium transport pathways may significantly counteract the actual calcium influx. These problems explain the finding that in aCD3-stimulated, indo-1 loaded Jurkat lymphoblasts the estimated rate of calcium influx was considerably smaller than in quin-2 loaded cells. The rate of calcium influx was found to be similar between intracellular quin-2 concentrations of 3–5 mM; although, in contrast to that seen by Ng et al. (1988), at quin-2 concentrations above 6 mM, a considerable increase in the inward leakage of calcium and a decrease in the rate of stimulus-induced calcium influx was observed. This may be due to a variable toxic effect of higher concentrations of quin-2 or the formaldehyde liberation during the loading phase (*see* Tsien, 1983).

By using the above techniques we have demonstrated that TG, presumably a specific inhibitor of the endoplasmic reticulum calcium pump (Thastrup et al., 1990), which does not activate phospholipase C and does not increase IP_3 or DAG levels (Jackson et al., 1988; Takemura et al., 1989; Thastrup et al., 1989), similar to that found in several cell types (*see* Thastrup et al., 1989), liberates intracellular calcium and concomitantly increases the rate of calcium influx both in Jurkat and MOLT-4 lymphoblasts. In Jurkat cells these changes in calcium movements qualitatively and also quantitatively (*see* Table) were similar to those observed after the stimulation through the antigen receptor by the addition of an anti-CD3 monoclonal antibody. Although in the TG-stimulated cells the release in internal calcium was somewhat prolonged, in the presence of external calcium a sustained increase in $[Ca^{2+}]_i$ was just as apparent as in aCD3-stimulated ones. The role of the antigen receptor structure in the TG-induced calcium fluxes is clearly excluded by the finding that MOLT-4 lymphoblasts, which do not bind aCD3 and lack a functional antigen receptor, showed a similar response to TG as the Jurkat cells.

In the indo-1 loaded Jurkat lymphoblasts it could be demonstrated that the effects of aCD3 and TG on the release of intracellular calcium were mutually exclusive and involved most of the calcium stores in these cells (*see* Fig. 4—even ionomycin, a calcium ionophore could not liberate a significant amount of calcium after the action of aCD3 or TG). Thus in Jurkat lymphoblast the receptor-induced, most probably IP_3-mediated (*see* Imboden & Weiss, 1987; Gelfand et al., 1987*b*) calcium release reaction seems to occur from the same internal pools in which the high calcium level is ensured by a TG-sensitive calcium pumping.

An intriguing problem is the appearance of an increased calcium influx in TG-treated cells, similar to that seen after antigen receptor stimulation. It has been shown that TG has no calcium ionophore characteristics and does not induce calcium leakage in red cell membrane vesicles (*see* Thastrup et al., 1989). One explanation for the rapid calcium influx is a direct activating effect of TG on plasma membrane calcium channels, which is unlikely in the light of the experiments of Thastrup et al. (1990), showing no such effect in isolated hepatocyte plasma membranes. Another possibility is the induction of plasma membrane calcium channels in intact cells, whenever the cellular calcium is released (*see* Putney, 1986; Putney et al., 1989; Takemura et al., 1989; Takemura & Putney, 1989; Sage, Reast & Rink, 1990). In the light of the present experiments the role of an initial calcium signal, based on internal calcium release, in the opening of plasma membrane calcium channels (as advocated by Ng et al., 1988) seems to be unlikely, as the rapid calcium influx develops without a significant increase in $[Ca^{2+}]_i$ in the quin-2 loaded lymphoblast (*see* Fig. 6). The suggestions for an inductive role of the emptying of cellular stores in calcium entry (*see* Putney et al., 1989; Thastrup et al., 1989) are also not supported by the present findings; TG-induced calcium influx in lymphoblasts started immediately with a high rate, without a substantial internal calcium release (*see* Figs. 1*C* and 6*D*). These experiments, however, cannot exclude the role of small and/or local changes in free cytoplasmic or storage calcium concentrations in the induction of calcium entry.

It is important to note that aCD3- and TG-induced calcium influx pathways share several characteristics. They carry calcium ions with about the same transport rate (*see* Table), are not additive in allowing calcium transport, and are both sensitive to plasma membrane depolarization by gramicidin. Gramicidin has no effect on aCD3- or TG-induced internal calcium release (Fig. 3*C*), or on the aCD3-induced calcium influx in choline-Cl media, where this ionophore cannot depolarize the cells (Sarkadi et al., 1990*a*). The same is true for TG-treated cells (*data not shown*). The finding that the receptor-operated and the TG-induced calcium channels are both sensitive to the changes in membrane potential may be due either to a direct effect of the membrane potential on these pathways or to the decreased driving force for Ca^{2+} ions, moving through conductive channels (for the modulatory role of membrane potential on receptor-operated calcium channels in various cell types (*see* Kuno et al., 1986; DiVirgilio et al., 1987; Gelfand et al., 1987*a*; Gray et al., 1987; Meldolesi & Pozzan, 1987; Merritt & Rink, 1987; Mohr & Fewtrell, 1987; Lewis & Cahalan,

1989; Savage, Biffen & Martin, 1989; Kovács et al., 1990; Laskey et al., 1990). A direct effect of membrane potential on the receptor-activated calcium channels is supported by our earlier data obtained in aCD3-stimulated Jurkat lymphoblasts (Sarkadi et al., 1990*a*).

A major difference was found between the phorbol ester sensitivity of the calcium signal of the aCD3- and the TG- stimulated Jurkat lymphoblasts, respectively. In the former case the activation of the protein kinase C by a short (2-min) PMA pretreatment abolishes both the intracellular calcium release and the increased calcium influx. A similar inhibitory effect of receptor-stimulated cell activation has been found in various cell types (Cantrell et al., 1985; MacIntyre, McNicol & Drummond; 1985; Mellors, Stalmach & Cohen, 1985; Naccache et al., 1985; Harnett & Klaus, 1988; Aboolian, Vander Molen & Nord, 1989; Tordai et al., 1989; Willems et al., 1989). As shown in Figs. 1 and 2, the aCD3-induced calcium influx even after its full development could be inhibited by PMA. In contrast, in TG-treated cells neither the calcium release nor the increased calcium influx was blocked by PMA (though a slight decrease in the calcium influx rate was observed). These findings are in good agreement with the data of Scharff et al. (1988), showing only a modest effect of PMA in thapsigargin-treated lymphocytes. In contrast, in neutrophils the thapsigargin-induced Mn^{2+} entry was found to be PMA sensitive (Foder et al., 1989).

Since in the Jurkat lymphoblasts the calcium signal induced by the lectin Concanavalin A is also much less sensitive to PKC activation than the aCD3-induced signal (Tordai et al., 1989), the action of protein kinase C is probably quite specific for the antigen receptor-mediated changes in the calcium metabolism. This notion is supported by the finding that in MOLT-4 lymphoblasts the TG-induced calcium fluxes are also insensitive to PMA (although depolarization by gramicidin blocks the increased calcium influx). These findings indicate that the decreased calcium signal seen in PMA-pretreated, aCD3-activated Jurkat lymphoblasts is not only a nonspecific consequence of the activation of the plasma membrane calcium pump by protein kinase C, which has been suggested to occur as well (Lagast et al., 1984; Pollock, Sage & Rink, 1987; Smallwood, Gugi & Rasmussen, 1988).

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