Regulation of T-cell-receptor-stimulated bivalent-cation entry in Jurkat E6 cells: role of protein kinase C

Louise A. CONROY, Janet E. MERRITT* and Trevor J. HALLAM

Roche Research Centre, P.O. Box 8, Welwyn Garden City, AL7 3AY, U.K.

Stimulation of Jurkat E6 cells with anti-CD3 antibody results in a characteristic rise in $[Ca^{2+}]_i$ which is due to both the release of Ca^{2+} from intracellular stores and the entry of external Ca^{2+} . Individual components of the $[Ca^{2+}]_i$ increase were investigated by measuring intracellular Ca^{2+} release in the absence of external Ca^{2+} and determining influx of bivalent cations by following the entry of Mn^{2+} . The increase in $[Ca^{2+}]_i$ induced by anti-CD3 antibody in the presence or absence of extracellular Ca^{2+} could be inhibited by the non-selective kinase inhibitor staurosporine, which also inhibits anti-CD3-stimulated phospholipase C activity. Staurosporine also inhibits the influx of bivalent cations induced by anti-CD3 antibody, but not that induced by depletion of intracellular Ca^{2+} stores using thapsigargin. The effect of staurosporine was compared with that of Ro 31-8425, a potent

and selective inhibitor of protein kinase C (PKC). Ro 31-8425, at concentrations up to $10 \mu M$, has no inhibitory effect on the anti-CD3 antibody-induced $[Ca^{2+}]_i$ increase or phospholipase C activity. These studies are consistent with the concept that augmentation of $[Ca^{2+}]_i$ by stimulated T-cell receptors requires activation of a kinase, probably a tyrosine kinase such as $p56^{lck}$, ZAP-70 or $p59^{lyn}$, and is independent of PKC. Phorbol esters inhibit the anti-CD3-stimulated $[Ca^{2+}]_i$ increase and phospholipase C activity, showing that this can be negatively regulated by PKC. A small potentiation of the anti-CD3 antibody-induced $[Ca^{2+}]_i$ rise in the presence of extracellular Ca^{2+} was detected in the presence of Ro 31-8425; this suggests that T-cell-receptor ligation can also limit the increase in $[Ca^{2+}]_i$ via PKC activation.

INTRODUCTION

Activation of T-cells is the result of stimulation of the T-cell receptor (TCR) (the $\alpha\beta/CD3$ complex) by antigen, which can be mimicked using anti-CD3 antibodies. This induces a series of events beginning with association of tyrosine kinases such as $p56^{lck}$, $p59^{lyn}$ and ZAP-70, and additional signalling factors such as $p21^{ras}$, GAP, phospholipase $C\gamma 1$, phosphatidylinositol 3-kinase, with the TCR complex [reviewed by Abraham et al. (1992)]. Subsequent tyrosine kinase activation induces directly or indirectly tyrosine phosphorylation in phospholipase $C\gamma 1$ which increases its catalytic activity (June et al., 1990; Secrist et al., 1991). Phospholipase $C\gamma 1$ catalyses the cleavage of $PtdInsP_2$ in the inner leaflet of the plasma membrane to yield diacylglycerol and $InsP_3$; diacylglycerol activates protein kinase C (PKC), and $InsP_3$ releases Ca^{2+} from intracellular stores, producing an increase in $[Ca^{2+}]_i$ (Berridge, 1993). These early activation signals converge on a variety of transcription factors, such as NF- κB , AP-1 and NF-AT, which together regulate the induction of interleukin 2 secretion (Crabtree, 1989). The phospholipase $C\gamma 1$ -generated intracellular signals can be mimicked by exogenously applied agents such as Ca^{2+} ionophores and activators of PKC (diacylglycerol or phorbol esters), both of which are required to induce expression of interleukin 2 (Durand et al., 1988). Further proof of the necessity of PKC stimulation during T-cell activation comes from the use of selective PKC inhibitors from the bisindolylmaleimide series including Ro 31-8425 and Ro 32-0432 (Davis et al., 1989; Bit et al., 1993). Ro 31-8425 and Ro 32-0432 inhibit induction of interleukin 2 receptor expression and interleukin 2 secretion in human peripheral blood T-cells and in a human T-cell clone, and inhibit T-cell-mediated inflammation *in vivo* (Hallam, 1993; Birchall et al., 1994).

The increase in $[Ca^{2+}]_i$ during T-cell activation is thought to be necessary primarily to activate a Ca^{2+} -dependent phosphatase, calcineurin. Calcineurin activates NF-AT, a transcription factor required for induction of the interleukin 2 gene; this pathway is blocked by immunosuppressants such as cyclosporin (Clipstone and Crabtree, 1992). The time course for stimulation of NF-AT activity, assessed by electrophoretic mobility-shift assays, is of the order of 1–2 h (D. H. Williams, unpublished work). Thus the mechanism by which the TCR is able to maintain stimulation of Ca^{2+} -activated processes over prolonged periods is fundamental to T-cell activation, and is as yet uncharacterized. One potential mechanism for driving Ca^{2+} -activated processes in an energy-efficient manner is the periodic elevation of $[Ca^{2+}]_i$ (Berridge, 1993). Measurements of $[Ca^{2+}]_i$ oscillations in single T-cells have been reported (Donnadieu et al., 1992a,b). Although $InsP_3$ regulates the initial release of Ca^{2+} from agonist-sensitive intracellular stores (Berridge, 1993), the entry of extracellular Ca^{2+} is necessary to sustain the increase in intracellular $[Ca^{2+}]_i$ (Imboden and Weiss, 1987; Donnadieu et al., 1992a,b). The mechanisms that regulate Ca^{2+} entry are not clearly understood, but are thought to depend on the concentration of Ca^{2+} in the intracellular stores, a concept referred to as the capacitative theory (Putney, 1990). Capacitative Ca^{2+} entry, in the absence of receptor occupation by agonists, can be stimulated by thapsigargin. Thapsigargin inhibits the endoplasmic-reticulum Ca^{2+} -ATPase and so prevents resequestration of Ca^{2+} into intracellular stores, which eventually results in their depletion and activation of capacitative Ca^{2+} entry (Sarkadi et al., 1991). The mechanics of the communication between the stores and the plasma-membrane channel are currently under debate (Meldolesi et al., 1991; Putney and Bird, 1993; Randriamampita and Tsien, 1993; Parekh et al., 1993).

Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TCR, T-cell receptor; Ro 31-8425, 3-(8-(aminomethyl)-6,7,8,9-tetrahydropyrido[1,2-a]indol-10-yl)-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione hydrochloride.

* To whom correspondence should be addressed.

Figure 1 Effect of anti-CD3 antibody on $[Ca^{2+}]_i$

Fura-2-loaded Jurkat E6 cells were stimulated by the addition of 1 μ g/ml anti-CD3 antibody (aCD3) in the presence of 1 mM $CaCl_2$ (a), 1 mM EGTA (b), or 100 μ M $MnCl_2$ (c, d). In (a) and (b) $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence at 340 and 380 nm. In (c) and (d), actual fluorescence is shown at excitation wavelengths of 340 nm (c) and 360 nm (d). The dotted trace represents basal unstimulated cells (i.e. with 100 μ M $MnCl_2$ but no anti-CD3 antibody added).

In the present study we have examined the effects of a selective PKC inhibitor, Ro 31-8425, and a non-selective kinase inhibitor, staurosporine, on TCR-regulated $[Ca^{2+}]_i$ to determine whether PKC or other protein kinases are involved. We have compared this with capacitative Ca^{2+} entry stimulated by thapsigargin.

EXPERIMENTAL

Cells and reagents

Jurkat E6.1 cells were obtained from ECACC (ECACC No. 88042803), and were maintained in RPMI 1640 medium supplemented with penicillin, streptomycin and 10% (v/v) fetal calf serum. Phorbol 12-myristate 13-acetate (PMA), other phorbol esters, staurosporine, ionomycin and thapsigargin were obtained from Calbiochem. Anti-CD3 antibody (UCH-T1) was from Sera-Lab; Fura-2 acetoxymethyl ester was from Molecular Probes Inc. (Eugene, OR, U.S.A.); Dowex anion-exchange resin (AG-X18, formate form) was from Bio-Rad; [3H]inositol was from Amersham. Ro 31-8425 was synthesized by Dr. C. Hill (Department of Medicinal Chemistry, Roche Products Ltd.).

Measurement of intracellular $[Ca^{2+}]$ using Fura-2

Jurkat E6.1 cells, grown at a density of 0.5×10^6 /ml, were incubated for 45 min with 2 μ M Fura-2 acetoxymethyl ester in normal growth medium at 37 °C. Cells were then washed, resuspended (3×10^6 cells/ml) in HEPES buffer (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, pH 7.4) containing 1 mM $CaCl_2$ and 1% (v/v) fetal calf serum, and stored at room temperature until use. Fura-2 fluorescence was measured at 37 °C in a dual-excitation-wavelength fluorimeter (Spex) with excitation wavelengths set to 340 and 380 nm, and emission read at 505 nm. Portions of cells were resuspended in fresh HEPES buffer and equilibrated at 37 °C. For experiments in the presence of extracellular Ca^{2+} , 1 mM $CaCl_2$ was added. For experiments in the absence of extracellular Ca^{2+} , 1 mM EGTA was added. Autofluorescence was determined after every run by the addition of 2 μ M ionomycin and 5 mM $MnCl_2$. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al. (1985). To study the entry of extracellular bivalent cations, Mn^{2+} was used as a surrogate for Ca^{2+} (Merritt et al., 1989). $MnCl_2$ (100 μ M) was included, and fluorescence was measured at excitation wavelengths of 340 nm and 360 nm.

Measurement of inositol phosphates

Jurkat E6.1 cells were grown in inositol-free RPMI medium supplemented with 2 $\mu\text{Ci}/\text{ml}$ [^3H]inositol for 4–5 days before use and then washed to remove extracellular [^3H]inositol. They were resuspended (0.5 ml portions at 10^6 cells/ml) in normal RPMI medium containing 10 mM LiCl, and incubated at 37 °C for 5 min. Inhibitors or vehicle control were added 5 min before the addition of anti-CD3 antibody. After 15 min at 37 °C, reactions were terminated by the addition of ice-cold 5% (w/v) perchloric acid. The supernatants, containing the water-soluble inositol phosphates, were neutralized with KOH solution (60 mM EDTA, 75 mM Hepes, 1.2 M KOH), and total inositol phosphates were separated from free inositol on Dowex columns (Berridge et al., 1983; Kirk et al., 1990). Total inositol phosphates were eluted with 2.0 M ammonium formate in 0.4 M formic acid.

RESULTS

Characterization of the $[\text{Ca}^{2+}]_i$ increase stimulated by anti-CD3 antibody

Figure 1 shows the effect of anti-CD3 antibody (1 $\mu\text{g}/\text{ml}$) on $[\text{Ca}^{2+}]_i$ in Fura-2-loaded Jurkat E6 cells. In the presence of external Ca^{2+} (Figure 1(a)), basal $[\text{Ca}^{2+}]_i$ was 150 nM, and addition of anti-CD3 antibody increased it after a lag of about 20 s; this resulted in a peak of 700 nM, which then fell to a sustained level of 400 nM after about 3 min. In the absence of external Ca^{2+} (external Ca^{2+} chelated by the presence of 1 mM EGTA), anti-CD3 antibody induced an increase in $[\text{Ca}^{2+}]_i$ from basal (50 nM) to a peak of 100 nM, which then declined to the basal level after 3 min (Figure 1(b)). To examine the influx of bivalent cations, Fura-2-loaded cells were incubated without added Ca^{2+} or EGTA and stimulated with anti-CD3 antibody in the presence of 100 μM extracellular MnCl_2 . Figure 1(c) and (d) shows that the addition of anti-CD3 antibody causes sim-

ultaneous discharge of Ca^{2+} stores (measured by increased fluorescence at 340 nm) and influx of Mn^{2+} across the membrane (measured by quenching of the fluorescence signal at 360 nm) after an approx. 20 s lag time.

Effect of protein kinase activators and inhibitors on the $[\text{Ca}^{2+}]_i$ increase stimulated by anti-CD3 antibody

Figure 2 shows the effect of preincubation (3 min) with staurosporine, PMA or Ro 31-8425 on the subsequent increase in $[\text{Ca}^{2+}]_i$ stimulated by anti-CD3 antibody in the presence of external Ca^{2+} . Preincubation with staurosporine (1 μM) inhibited the subsequent anti-CD3 antibody-stimulated $[\text{Ca}^{2+}]_i$ increase. The more selective PKC inhibitor, Ro 31-8425 (1 μM), had no

Figure 2 Effects of staurosporine, Ro 31-8425 and PMA on anti-CD3 antibody-induced increase in $[\text{Ca}^{2+}]_i$

Fura-2-loaded Jurkat E6 cells were stimulated with 1 $\mu\text{g}/\text{ml}$ anti-CD3 antibody (aCD3) in the presence of external Ca^{2+} after a 3 min preincubation with staurosporine (1 μM), Ro 31-8425 (1 μM) or PMA (1 μM) as shown (arrow A).

Figure 3 Inhibition by staurosporine, Ro 31-8425 and PMA of anti-CD3 antibody-induced release of Ca^{2+} from intracellular stores (a) and rate of Mn^{2+} influx (b)

(a) Fura-2-loaded Jurkat E6 cells were resuspended in medium containing 1 mM EGTA, and stimulated with anti-CD3 antibody (1 $\mu\text{g}/\text{ml}$) after preincubation for 5 min with various concentrations of staurosporine ($\blacktriangle$), Ro 31-8425 ($\blacksquare$) or PMA ($\bullet$). The peak $[\text{Ca}^{2+}]_i$ reached (above basal control) was measured. (b) The rate of quench of fluorescence at 360 nm by Mn^{2+} was measured. Staurosporine ($\blacktriangle$), Ro 31-8425 ($\blacksquare$) or PMA ($\bullet$) was added 5 min before the addition of MnCl_2 (100 μM) and anti-CD3 antibody (1 $\mu\text{g}/\text{ml}$). The control rate of quench (in the absence of anti-CD3 antibody) was subtracted from the experimental results. Data are expressed as means $\pm$ S.E.M. ($n = 3$).

Figure 4 Ability of Ro 31-8425 to relieve the inhibition of the anti-CD3 antibody-induced increase in $[Ca^{2+}]_i$ by PMA

$[Ca^{2+}]_i$ was measured in the presence of external Ca^{2+} . Various concentrations, as indicated, of Ro 31-8425 (Ro) were added; PMA ($1 \mu M$) was then added after 3 min. Cells were stimulated with anti-CD3 antibody (aCD3; $1 \mu g/ml$) 3 min later. Controls were samples treated with anti-CD3 antibody only (no PMA).

Figure 5 Effects of staurosporine, PMA and Ro 31-8425 on anti-CD3 antibody-stimulated accumulation of inositol phosphates

$[^3H]$ Inositol-labelled Jurkat E6 cells were preincubated for 5 min with staurosporine (concentrations as shown), Ro 31-8425 ($1 \mu M$) or PMA ($1 \mu M$) before the addition of anti-CD3 antibody ($1 \mu g/ml$). Accumulation of inositol phosphates was determined by dividing total inositol phosphates in anti-CD3 antibody-stimulated samples by that in basal control samples (i.e. basal = 1.0). Data are expressed as means $\pm$ S.E.M. ($n = 3$) from one of three representative experiments.

inhibitory effect and in fact caused consistent enhanced $[Ca^{2+}]_i$ responses. Activation of PKC by PMA ($1 \mu M$) inhibited the increased $[Ca^{2+}]_i$ induced by anti-CD3 antibody, and PMA also reduced resting $[Ca^{2+}]_i$ by $50 nM$.

Figure 6 Increase in $[Ca^{2+}]_i$ induced by anti-CD3 antibody compared with that induced by thapsigargin

The effects of anti-CD3 antibody (aCD3; $1 \mu g/ml$) or thapsigargin (Tg; $1 \mu M$) on $[Ca^{2+}]_i$ were measured in the presence of external Ca^{2+} .

Figure 3 shows the effects of staurosporine, Ro 31-8425 and PMA on anti-CD3 antibody-stimulated Ca^{2+} release from intracellular stores and Mn^{2+} influx. Staurosporine inhibited both release of Ca^{2+} from intracellular stores and Mn^{2+} influx with an IC_{50} of $80 nM$. Neither the release of Ca^{2+} from intracellular stores nor Mn^{2+} influx were inhibited by Ro 31-8425. Potentiation by higher concentrations of Ro 31-8425 was scarcely evident on Ca^{2+} release of Mn^{2+} influx (Figure 3), but was clearly evident on the response to anti-CD3 antibody in the presence of Ca^{2+} (Figure 2). PMA inhibited both Ca^{2+} release from intracellular stores and Mn^{2+} influx. Another phorbol ester, phorbol dibutyrate, gave similar results to PMA, whereas 4α -phorbol didecanoate, a phorbol ester that is not able to activate PKC, had no effect on the response to anti-CD3 antibody (results not shown).

To confirm that Ro 31-8425 is able to inhibit PKC completely in these cells, we tested its ability to suppress the inhibition by PMA of the anti-CD3 antibody-induced $[Ca^{2+}]_i$ increase. Figure 4 shows that preincubation with Ro 31-8425 before addition of PMA ($1 \mu M$) suppressed the inhibitory effect of PMA; full restoration of the anti-CD3 antibody response was seen at $5 \mu M$ Ro 31-8425. Ro 31-8425 also prevented the decrease in basal $[Ca^{2+}]_i$ induced by PMA.

Effects of protein kinase activators and inhibitors on phospholipase C activity stimulated by anti-CD3 antibody

Figure 5 shows the effects of staurosporine, PMA and Ro 31-8425 on anti-CD3 antibody-induced accumulation of inositol phosphates in the presence of LiCl ($10 mM$). Li^+ inhibits the breakdown of inositol phosphates to free inositol, and therefore allows the measurement of total inositol phosphates as an

Figure 7 Influx of Ca^{2+} stimulated by prior addition of (a) thapsigargin or (b) anti-CD3 antibody

Cells incubated in the absence of added CaCl_2 or EGTA were stimulated with either (a) thapsigargin (Tg; $1 \mu\text{M}$) or (b) anti-CD3 antibody (aCD3; $1 \mu\text{g/ml}$). Staurosporine ($1 \mu\text{M}$), Ro 31-8425 ($1 \mu\text{M}$) or PMA ($1 \mu\text{M}$) was added 5 min later (arrow A). After a further 5 min, CaCl_2 (Ca ; 1 mM) was added.

indication of phospholipase C activity. Staurosporine inhibited phospholipase C activity induced by anti-CD3 antibody in a concentration-dependent manner, which is similar to that obtained for the inhibition of the $[\text{Ca}^{2+}]_i$ response (Figure 3). PMA ($1 \mu\text{M}$) also inhibited anti-CD3 antibody-stimulated phospholipase C activation, whereas Ro 31-8425 ($1 \mu\text{M}$) had no effect.

Regulation of bivalent-cation entry

Figure 6 shows that, in the presence of extracellular Ca^{2+} , thapsigargin ($1 \mu\text{g/ml}$) causes an increase in $[\text{Ca}^{2+}]_i$, as a result of capacitative Ca^{2+} entry, comparable with that induced by anti-CD3 antibody. Our results (not shown) and those of Llopis et al. (1991) demonstrate that both agents release Ca^{2+} from the same intracellular pool. Thapsigargin does not affect phospholipase C activity significantly (present work; Guoy et al., 1990). Figure 7 shows the effects of protein kinase activators and inhibitors on the $[\text{Ca}^{2+}]_i$ rise caused by adding back extracellular Ca^{2+} after store depletion by thapsigargin or anti-CD3 antibody. The addition of thapsigargin or anti-CD3 antibody in the absence of extracellular Ca^{2+} induced a small transient elevation in $[\text{Ca}^{2+}]_i$

which then returned to near-basal levels; this is due to discharge of Ca^{2+} from intracellular stores. Subsequent addition of extracellular Ca^{2+} caused a large increase in $[\text{Ca}^{2+}]_i$ which peaked around 800–900 nM then declined. The responses were similar with both thapsigargin and anti-CD3 antibody.

The addition of Ro 31-8425 ($1 \mu\text{M}$) 5 min before replacement of extracellular Ca^{2+} had no inhibitory effect on the subsequent $[\text{Ca}^{2+}]_i$ increase in the presence of thapsigargin or anti-CD3 antibody. The $[\text{Ca}^{2+}]_i$ increases in the presence of anti-CD3 antibody or thapsigargin were potentiated by Ro 31-8425. Addition of PMA ($1 \mu\text{M}$) or staurosporine ($1 \mu\text{M}$) 5 min before CaCl_2 inhibited the subsequent $[\text{Ca}^{2+}]_i$ increase in the presence of anti-CD3 antibody (Figure 7b) with a concentration-dependence similar to that previously seen for inhibition of anti-CD3 antibody-stimulated release of Ca^{2+} stores and Mn^{2+} influx. However, addition of PMA ($1 \mu\text{M}$) or staurosporine ($1 \mu\text{M}$) 5 min before CaCl_2 had no effect on the subsequent $[\text{Ca}^{2+}]_i$ increase in the presence of thapsigargin (Figure 7a), under conditions in which anti-CD3 antibody-stimulated Ca^{2+} influx was totally inhibited (Figure 7b).

DISCUSSION

We have shown that stimulation of the TCR by anti-CD3 antibody increases phospholipase C activity and causes an increase in $[\text{Ca}^{2+}]_i$ through discharge of intracellular stores and by stimulating bivalent-cation influx, as also shown previously (Imboden and Weiss, 1987; Inokuchi and Imboden, 1990). Staurosporine and H7 have previously been used as PKC inhibitors and have been shown to inhibit TCR activation (Ward et al., 1988; Kubbies et al., 1989; Hengel et al., 1991); interpretation of these data led to the assumption that PKC was directly involved in the TCR-stimulated increase in $[\text{Ca}^{2+}]_i$. However, staurosporine is now known to be a non-selective inhibitor of protein kinases (Bit et al., 1993). Therefore, we have used Ro 31-8425, which is a selective PKC inhibitor (Bit et al., 1993), to define the role of PKC in TCR-stimulated increases in $[\text{Ca}^{2+}]_i$. The increase in $[\text{Ca}^{2+}]_i$ and phospholipase C activity induced by anti-CD3 antibody could not be inhibited by Ro 31-8425, showing that activation of PKC is not required for stimulation of this pathway by the TCR. The inhibition observed with staurosporine is therefore not due to an effect on PKC. Staurosporine has been shown to inhibit p56^{lck} *in vitro* with an IC_{50} of $2 \mu\text{M}$ (Bit et al., 1993), and we have previously shown that it reduces the level of protein tyrosine phosphorylation induced by anti-CD3 antibody in Jurkat cells (Conroy et al., 1993). Inhibition of the TCR-stimulated $[\text{Ca}^{2+}]_i$ rise and phospholipase C activity by staurosporine is therefore probably due to inhibition of protein tyrosine kinases, as ligation of the TCR results in activation of phospholipase $\text{C}\gamma 1$ via tyrosine kinases including p56^{lck} and p59^{lyn} (June et al., 1990).

Activation of PKC via PMA inhibits the anti-CD3 antibody-induced $[\text{Ca}^{2+}]_i$ rise, and Ro 31-8425 inhibits this effect of PMA. (This confirms that Ro 31-8425 is able to inhibit PKC in these cells, despite its lack of inhibitory effect on the anti-CD3 antibody response.) PKC activation therefore inhibits TCR-mediated signalling rather than being required for signalling, as was initially thought from experiments using staurosporine (Ward et al., 1988; Kubbies et al., 1989; Hengel et al., 1991). A major target of PKC in T-cells is phospholipase $\text{C}\gamma 1$, which is phosphorylated on serine, while TCR-stimulated tyrosine phosphorylation is inhibited (Granja et al., 1991; Park et al., 1992). This is consistent with our observations that PMA inhibits anti-CD3 antibody-stimulated phospholipase C as well as the rise in $[\text{Ca}^{2+}]_i$. PKC has also been shown to phosphorylate and

promote internalization of receptors on T-cells, including CD3, CD4 and CD8, but this is slow and not very sensitive to PMA (Cantrell et al., 1985), so is unlikely to be the full explanation for our observations. The role of PKC in negative-feedback effects on TCR signalling may be more complex in different types of T-cells as PMA did not inhibit TCR-stimulated phospholipase C in T-lymphoblasts (Ward and Cantrell, 1990).

PMA also reduced basal $[Ca^{2+}]_i$, which could be reversed by Ro 31-8425, and is probably due to PKC-induced Ca^{2+} efflux. The ability of PKC to regulate plasma-membrane Ca^{2+} -ATPase has been shown in other cells (Lagast et al., 1984; Pollock et al., 1987).

Inhibition of PKC by Ro 31-8425 potentiated the anti-CD3 antibody-induced elevation of $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} . This suggests that there is a physiological feedback mechanism acting via PKC to limit the TCR-stimulated $[Ca^{2+}]_i$ rise. Negative-feedback regulation of the TCR-stimulated $[Ca^{2+}]_i$ increase is likely to be important in maintaining the long-term oscillations in $[Ca^{2+}]_i$ increases required for T-cell activation. For example, phorbol esters inhibit agonist-induced $[Ca^{2+}]_i$ oscillations in hepatocytes (Woods et al., 1987). The effect of TCR-activated PKC is small compared with the inhibition caused by PMA. Activation of PKC driven by phorbol ester is greater both in magnitude and duration than that driven by physiological stimulation by diacylglycerol produced by receptor-stimulated phospholipase C (Berry and Nishizuka, 1990). Potentiation by Ro 31-8425 was only seen for the TCR-stimulated $[Ca^{2+}]_i$ rise in the presence of extracellular Ca^{2+} , and was not apparent for stimulated release from intracellular stores, unidirectional Mn^{2+} influx or phospholipase C activity. This suggests that Ro 31-8425 inhibits a TCR-derived diacylglycerol-activated PKC that stimulates Ca^{2+} efflux by a Ca^{2+} -ATPase. However, PMA-stimulated PKC appears to inhibit phospholipase C activity as well as Ca^{2+} efflux.

Thapsigargin was used to deplete intracellular Ca^{2+} stores, in the absence of external Ca^{2+} , and replacement of external Ca^{2+} resulted in an increase in $[Ca^{2+}]_i$. This capacitative Ca^{2+} entry was not inhibited by staurosporine, PMA or Ro 31-8425. The response was potentiated by Ro 31-8425, again suggesting an effect of PKC on Ca^{2+} efflux. In contrast, when anti-CD3 antibody was used instead of thapsigargin, the receptor-induced Ca^{2+} influx was inhibited by staurosporine and PMA, but not by Ro 31-8425. This result differs from that in platelets, where tyrosine kinase inhibitors, including genistein, inhibited both ADP- and thapsigargin-stimulated Ca^{2+} influx (Sargeant et al., 1993), and neutrophils, where phorbol esters inhibited thapsigargin-stimulated Ca^{2+} influx (Montero et al., 1993).

TCR ligation and store-regulated Ca^{2+} entry (capacitative entry) therefore appear to be regulated by two independent mechanisms. Capacitative entry appears to be independent of protein kinase inhibition (tyrosine kinases or PKC) or PKC activation. Continuous activation of the TCR stimulates Ca^{2+} entry, which is dependent on activation of protein kinases (tyrosine kinases such as $p56^{lck}$ and $p59^{fyn}$) and inhibited by activation of PKC. TCR-stimulated phospholipase C is also dependent on activation of tyrosine kinases and is inhibited by activation of PKC. This suggests that TCR-stimulated phospholipase C-dependent $InsP_3$ production is required to keep the intracellular Ca^{2+} stores empty to promote capacitative Ca^{2+} entry. Alternatively, stimulation of the TCR promotes Ca^{2+} entry by a different mechanism, which may be dependent on or independent of $InsP_3$. An $Ins(1,4,5)P_3$ receptor has been located in the T-cell plasma membrane and reported to co-cap with the TCR on activation of the cell (Khan et al., 1992), and caged $Ins(1,4,5)P_3$ has been found to activate a plasma-membrane Ca^{2+}

current in human T-cells (McDonald et al., 1991). There is also evidence for a role for tyrosine kinases in the regulation of $[Ca^{2+}]_i$ independent of inositol phosphate metabolism; Niklinska et al. (1992) have reported a T-cell hybridoma, expressing a $v-src^+$ construct, which exhibits elevated basal intracellular Ca^{2+} levels and greater TCR-stimulated $[Ca^{2+}]_i$ increases, whereas inositol phosphate metabolism is equivalent to that of wild-type cells.

Activation of the TCR elevates $[Ca^{2+}]_i$ by a tyrosine kinase-dependent mechanism that is inhibited by PKC. Use of a selective PKC inhibitor has allowed clear definition of the role of PKC. Negative regulation by PKC appears to be an important physiological feedback mechanism to limit the TCR-stimulated increase in $[Ca^{2+}]_i$. This is likely to be important in maintaining the long-term oscillating $[Ca^{2+}]_i$ rises required for T-cell activation.

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