Two independently regulated Ca^{2+} entry mechanisms coexist in Jurkat T cells during T cell receptor antigen activation

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Receptor-mediated Ca^{2+} influx was studied in the human leukaemic T cell line, Jurkat. Stimulation of these cells through the T cell antigen–receptor complex with OKT3 (an antibody against the CD3 molecules of the T cell antigen–receptor complex), or inhibition of the endoplasmic reticular Ca^{2+} -ATPase with thapsigargin, resulted in Ca^{2+} mobilization from intracellular stores and the activation of Ca^{2+} and Mn^{2+} entry. The rates of thapsigargin-induced Ca^{2+} and Mn^{2+} entry in Jurkat cells were 76% and 64% respectively of those observed after treatment of these cells with OKT3. The combined addition of thapsigargin plus OKT3 to Jurkat cells produced an enhanced effect on the

sustained increase in the cytosolic free Ca^{2+} concentration that was greater than that obtained by addition of thapsigargin or OKT3 alone. The rates of Ca^{2+} and Mn^{2+} entry were increased to 119% and 112% respectively of the OKT3-induced rates. Taken together, these results suggest that the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} -pool-dependent bivalent cation entry only accounts for 57% and 52% respectively of the total OKT3-dependent Ca^{2+} and Mn^{2+} entry, and that the rest is mediated by second messenger(s). Thus two separate pathways coexist in regulating Ca^{2+} entry in Jurkat cells during activation mediated through the T cell receptor.

INTRODUCTION

An early event in cell activation following the binding of hormones or growth factors to their plasma membrane receptors is the rapid hydrolysis of polyphosphoinositides by phospholipase C to give two second messengers, $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol [1–3]. This is followed by an initial transient increase in the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) owing to mobilization of Ca^{2+} from intracellular stores by $\text{Ins}(1,4,5)\text{P}_3$, and a subsequent sustained increase in $[\text{Ca}^{2+}]_i$ due to the transmembrane influx of Ca^{2+} from the extracellular milieu [1,2]. Although the mechanism and regulation of Ca^{2+} influx remain unclear, experimental evidence exists that supports at least three models of receptor-mediated Ca^{2+} entry. These include: (1) receptor-operated Ca^{2+} channels, which involve a direct coupling between the receptor and opening of Ca^{2+} channels [4–6]; (2) second messenger-operated Ca^{2+} channels, where inositol polyphosphates appear to be involved [5,7–9]; and (3) the ‘capacitative’ Ca^{2+} entry mechanism, whereby the emptying of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} store is by itself sufficient to activate the Ca^{2+} entry process [10–12]. Evidence supporting the capacitative model comes mainly from studies using two inhibitors of the endoplasmic reticulum (ER) Ca^{2+} -ATPase, 2,5-di-(tert-butyl)hydroquinone and thapsigargin [12–14]. The inhibition of the ER Ca^{2+} -ATPase by these agents produces a rapid release of Ca^{2+} from both agonist-sensitive and -insensitive Ca^{2+} pools, and is sufficient to stimulate Ca^{2+} entry in the absence of inositol polyphosphate formation [14–18].

Although attractive, the capacitative Ca^{2+} entry mechanism does not operate in all cells. For instance, in bovine chromaffin cells [19] and NG115-401 neuronal cells [20], emptying of the agonist-sensitive Ca^{2+} pool does not stimulate Ca^{2+} entry. These discrepancies suggest that additional mechanisms of regulating Ca^{2+} entry may exist. This contention is further substantiated by recent evidence from our laboratory [21] and from Clementi et al. [17] showing that two independently regulated pathways appear

to coexist during receptor-activated Ca^{2+} entry in isolated hepatocytes and PC12 cells. In addition, our earlier studies with Jurkat cells [22] have provided indications that more than one pathway may be involved in the regulation of Ca^{2+} entry.

In the present studies, the existence of multiple mechanisms of regulating Ca^{2+} entry was investigated in Jurkat cells. Our results show the existence of at least two separate pathways in the regulation of Ca^{2+} entry in these cells during T cell antigen receptor activation.

MATERIALS AND METHODS

Materials

Fura-2/AM was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Thapsigargin, RPMI 1640 medium and fetal calf serum were from Gibco BRL (Gaithersburg, MD, U.S.A.). OKT3 hybridoma was from American Type Tissue Culture (Rockville, MD, U.S.A.), and the antibodies (against the CD3 molecules of the T cell antigen–receptor complex) were purified from hybridoma supernatant using Protein A–Sepharose (Pharmacia, Uppsala, Sweden).

Cell line

Jurkat cells (E6-1 clone) were from American Type Tissue Culture (Rockville, MD, U.S.A.) and were maintained in continuous suspension culture using RPMI 1640 medium supplemented with 7.5% fetal calf serum in a humidified incubator (37 °C) under an atmosphere of 5% CO_2 in air.

Measurement of $[\text{Ca}^{2+}]_i$ and Mn^{2+} influx in Jurkat cells

Jurkat cells (1×10^7 cells/ml) were loaded with 4 μM fura-2/AM in modified Krebs–Henseleit buffer containing 1.3 mM CaCl_2 , 20 mM Hepes, 15 mM glucose and 2% BSA at pH 7.4 (buffer A) for 30 min at room temperature. The cells were then washed free

Abbreviations used: ER, endoplasmic reticulum; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration.

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of fura-2/AM and resuspended in buffer A for a further 15 min, to allow complete hydrolysis of fura-2/AM. The cells were washed again and resuspended in buffer B (as buffer A, but containing 0.2% BSA) until they were used. Measurements of $[Ca^{2+}]_i$ were performed as previously reported [23] in a Sigma ZFP 22 dual-wavelength spectrofluorimeter (excitation wavelengths were at 334 and 366 nm). The fura-2 fluorescence ratio signal was calibrated in terms of $[Ca^{2+}]_i$ as described by Grynkiewicz et al. [24]. To determine the rate of Ca^{2+} entry, fura-2-loaded cells were suspended in nominally Ca^{2+} -free buffer B and stimulated with the Ca^{2+} -mobilizing agents for 6.5 min (until $[Ca^{2+}]_i$ returned to near-basal level). Then $CaCl_2$ (1.3 mM) was reintroduced into the medium, and the ensuing increase in $[Ca^{2+}]_i$ was monitored. Prolonging the stimulation time for more than 6.5 min before addition of $CaCl_2$ had no effect on the rate of Ca^{2+} entry.

The protocol for measuring the rate of Mn^{2+} quenching was the same as for the Ca^{2+} overshoot experiments, except that after 6.5 min of stimulation, 100 μM $MnCl_2$ was added to the medium and the rate of quenching was recorded with a Sigma ZWS II dual-wavelength spectrofluorimeter equipped with a xenon lamp. The fluorescence signals from excitation at 360 nm and 340 nm were monitored separately to distinguish between alterations in Mn^{2+} -induced quenching of the dye and $[Ca^{2+}]_i$. All experiments were performed at 30 °C to minimize dye leakage [25]. Thapsigargin was dissolved in dimethyl sulphoxide, and the final concentration of solvent never exceeded 0.05% (v/v) in the cell suspension.

RESULTS AND DISCUSSION

Ligation of the CD3 molecules of the T cell antigen receptor complex in Jurkat cells with the anti-CD3 antibody OKT3 is known to stimulate the production of inositol phosphates and induce an increase in $[Ca^{2+}]_i$ [23,26,27]. The OKT3-induced $[Ca^{2+}]_i$ increase is typically biphasic and characterized by a rapid release of Ca^{2+} from intracellular stores followed by Ca^{2+} entry across the plasma membrane [15,23]. However, emptying of the intracellular Ca^{2+} stores with thapsigargin [15,28], an ER Ca^{2+} -ATPase inhibitor, or by non-esterified unsaturated fatty acids [22], is sufficient for activation of Ca^{2+} entry in Jurkat cells without any detectable increase in inositol polyphosphates. These observations, therefore, support the capacitative Ca^{2+} entry model previously proposed by Putney [10,11]. The essence of this model is that the Ca^{2+} entry occurring during agonist stimulation is related to the extent of depletion of the intracellular Ca^{2+} pool.

Addition of thapsigargin (100 nM) together with OKT3 (10 $\mu g/ml$) to fura-2-loaded Jurkat cells resulted in a rapid sustained increase in $[Ca^{2+}]_i$ (Figure 1a) which was greater than that induced by either OKT3 or thapsigargin alone (Figures 1b and 1c). Upon addition of excess EGTA (5 mM), the sustained increase in $[Ca^{2+}]_i$ rapidly declined to near-basal levels, suggesting that an increase in Ca^{2+} influx was involved. The enhanced effects of the two agents on the increase in Jurkat cell $[Ca^{2+}]_i$ were most evident when thapsigargin was added to the cells during the sustained phase of Ca^{2+} entry caused by OKT3, or vice versa (Figures 1b and 1c). It is unlikely that this enhanced effect was due to inhibition of Ca^{2+} re-uptake into the intracellular stores by thapsigargin, since the $[Ca^{2+}]_i$ increase in Jurkat cells after thapsigargin treatment alone never reached this level. The additive response of the $[Ca^{2+}]_i$ increase observed in Jurkat cells after exposure to thapsigargin and OKT3 is in direct contrast with the findings in parotid acinar cells [29], the rat pancreatoma cell line AR4-2J [30] and rat thymocytes [31], where the addition

of both agonist and thapsigargin together did not produce any further increase in Ca^{2+} entry. The concentration of thapsigargin used in the present study was optimal in depleting the Jurkat cell intracellular Ca^{2+} pools, and increasing the thapsigargin concentration to 500 nM did not affect the extra increase in $[Ca^{2+}]_i$ due to OKT3 (results not shown). Although the sustained increase in $[Ca^{2+}]_i$ elicited by thapsigargin or OKT3 (Figures 1b and 1c) was further elevated to a higher level following the addition of the second Ca^{2+} -mobilizing agent, the kinetics were different. Addition of thapsigargin to Jurkat cells during the OKT3-induced sustained phase produced an immediate further increase in $[Ca^{2+}]_i$ (Figure 1b). However, when OKT3 was added during the thapsigargin-induced sustained $[Ca^{2+}]_i$ increase, there was a lag phase of about 30–60 s before any further change in $[Ca^{2+}]_i$ was observed. In some experiments a very small transient increase in $[Ca^{2+}]_i$ was observed during the lag phase (results not shown). After the lag phase, the $[Ca^{2+}]_i$ first declined slowly and then rose to the same level that was reached when both Ca^{2+} -mobilizing

Figure 1 Effect of simultaneous and sequential addition of OKT3 and thapsigargin on $[Ca^{2+}]_i$ in Jurkat cells

Changes in $[Ca^{2+}]_i$ were monitored using the fluorescence Ca^{2+} indicator dye fura-2, as outlined in the Materials and methods section. Where indicated by the arrow, Jurkat cells were treated with a combination of 10 $\mu g/ml$ OKT3 and 100 nM thapsigargin (TG) followed by 5 mM EGTA (E; indicated by the broken trace) (a), or by sequential addition of OKT3 and thapsigargin (b) or vice versa (c). Each trace is representative of at least three experiments with different batches of cells.

Table 1 Stimulation of Ca^{2+} and Mn^{2+} influx by OKT3 and thapsigargin

Fura-2-loaded Jurkat cells were suspended in nominally Ca^{2+} -free buffer B and then stimulated with OKT3 (10 $\mu\text{g}/\text{ml}$), thapsigargin (100 nM) or both compounds together. When $[\text{Ca}^{2+}]_i$ had returned to near-basal levels, 1.3 mM Ca^{2+} or 100 μM Mn^{2+} was added to the cells and the ensuing $[\text{Ca}^{2+}]_i$ increase or quenching of the fura-2 signal by Mn^{2+} were monitored as outlined in the Materials and methods section. The data are actual stimulated rates (after subtracting from control rates) calculated from the changes in the fluorescence ratio at 334/366 nm excitation for Ca^{2+} , and quenching of the fura-2 signal at 360 nm for Mn^{2+} . All values are the means $\pm$ S.E.M. from eight separate batches of cells. Control rates for Ca^{2+} and Mn^{2+} were 0 nM/s and 0.17 ± 0.014 arbitrary units/s respectively. *Significantly different from OKT3-stimulated Ca^{2+} or Mn^{2+} entry as analysed by Student's unpaired *t* test ($P < 0.05$).

Addition	Ca^{2+} influx	
	(nM/s)	(%)
OKT3	101.8 ± 5.6	100
Thapsigargin	$77.3 \pm 4.1^*$	76
OKT3 + thapsigargin	$120.9 \pm 7.3^*$	119

Addition	Mn^{2+} influx	
	(arbitrary units/s)	(%)
OKT3	2.32 ± 0.15	100
Thapsigargin	$1.49 \pm 0.08^*$	64
OKT3 + thapsigargin	2.59 ± 0.19	112

Figure 2 Stimulation of Ca^{2+} and Mn^{2+} entry by OKT3 and thapsigargin in Jurkat cells

Fura-2-loaded Jurkat cells were suspended in nominally Ca^{2+} -free buffer B. Where indicated, 10 $\mu\text{g}/\text{ml}$ OKT3 (A), 100 nM thapsigargin (B), both compounds together (C) or carrier solvent (D) was added to the cells at 0 min. After 6.5 min, 1.3 mM CaCl_2 or 100 μM Mn^{2+} was added to the cells and the ensuing increase in $[\text{Ca}^{2+}]_i$ (a) or quenching of fura-2 fluorescence (b) was monitored. Note that after 6.5 min, the scale of the x-axis was increased. Each trace is representative of at least three experiments from different batches of cells.

agents were added either together (Figure 1a) or sequentially (Figure 1b). One interpretation of this observation is that OKT3 stimulates both Ca^{2+} efflux (decline in $[\text{Ca}^{2+}]_i$) and influx (increase in $[\text{Ca}^{2+}]_i$) in Jurkat cells, as previously observed in hepatocytes and pancreatic acinar cells during agonist stimulation [32,33]. The rate of Ca^{2+} entry is presumably high and eventually exceeds that of efflux, hence a net increase in $[\text{Ca}^{2+}]_i$ is seen after the transient delay.

Figure 3 Effects of thapsigargin and OKT3 on $[\text{Ca}^{2+}]_i$ of Jurkat cells in the absence of extracellular Ca^{2+}

Fura-2-loaded Jurkat cells were suspended in nominally Ca^{2+} -free buffer B. (a) Following the addition of 0.5 mM EGTA (E) the cells were first stimulated with 10 $\mu\text{g}/\text{ml}$ OKT3; when $[\text{Ca}^{2+}]_i$ had returned to near-basal levels, 100 nM thapsigargin (TG) was added to the cells. (b) As (a), except that thapsigargin was added before OKT3. Each trace is representative of at least three experiments with different batches of cells.

Figure 4 Concentration-dependence of thapsigargin- and OKT3-stimulated Ca^{2+} and Mn^{2+} entry

Fura-2-loaded Jurkat cells were suspended in nominally Ca^{2+} -free buffer B. Different concentrations of OKT3 (A, 10 $\mu\text{g}/\text{ml}$; B, 1 $\mu\text{g}/\text{ml}$; C, 0.1 $\mu\text{g}/\text{ml}$; D, 0 $\mu\text{g}/\text{ml}$) in (a) and (b), or thapsigargin (A, 100 nM; B, 10 nM; C, 1 nM; D, 0.1 nM; E, 0 nM) in (c) and (d), were added to the cells (0 min). After 6.5 min, 1.3 mM CaCl_2 (a and c) or 100 μM MnCl_2 (b and d) was added to the medium. Note that after 6.5 min the scale of the x-axis is increased. Each trace is representative of at least two experiments with different batches of cells.

The presence of a lag phase suggests that second messengers may be responsible for the additional increase in $[Ca^{2+}]_i$ upon stimulation by OKT3. One likely candidate is $Ins(1,4,5)P_3$, which is capable of activating Ca^{2+} entry in Jurkat cells [7], most probably via its receptors that have recently been identified on the plasma membranes of these cells [34]. Another possible messenger is inositol 1,3,4,5-tetrakisphosphate, which induces Ca^{2+} entry in lacrimal and endothelial cells [8,9] and binds to the $Ins(1,4,5)P_3$ receptors in Jurkat cell plasma membranes [35]. Thus it is clear that an additional regulatory mechanism is activated in Jurkat cells during OKT3 stimulation in addition to emptying the $Ins(1,4,5)P_3$ -sensitive Ca^{2+} pool, although the second messenger(s) involved in maintaining the elevated sustained increase in Jurkat cell $[Ca^{2+}]_i$ remain to be determined.

Using an alternative approach to measure receptor-mediated Ca^{2+} entry, fura-2-loaded Jurkat cells were treated with thapsigargin or OKT3 in nominally Ca^{2+} -free medium. When the $[Ca^{2+}]_i$ had returned to a near-basal level (after 6.5 min), the extracellular Ca^{2+} concentration was then restored to 1.3 mM and the ensuing rates of Ca^{2+} entry were measured. In order to obtain a quantitative estimate of Ca^{2+} entry, the linear rate of increase in the $[Ca^{2+}]_i$ during the initial 10 s after restoration of the extracellular Ca^{2+} level was monitored. In addition, Ca^{2+} entry was also measured using Mn^{2+} (100 μ M) as a surrogate ion for Ca^{2+} [36], by monitoring the rate of fura-2 quenching due to the binding of Mn^{2+} . The results in Table 1 and Figure 2 show that emptying of the intracellular Ca^{2+} pools with thapsigargin resulted in a slower rate of Ca^{2+} entry (76%) compared with the rate of OKT3-stimulated Ca^{2+} entry (100%). When OKT3 and thapsigargin were added together to Jurkat cells, the rate of Ca^{2+} influx was increased to 119%. The rate of Mn^{2+} entry after addition of thapsigargin was only 64% compared with the rate obtained with OKT3 (100%), whereas when both Ca^{2+} -mobilizing agents were added together, the rate of fura-2 quenching was increased to 112%. From the data in Table 1 it can be calculated that the $Ins(1,4,5)P_3$ -sensitive Ca^{2+} -pool-dependent bivalent cation entry accounted for about 57% and 52% respectively of the total OKT3-mediated Ca^{2+} and Mn^{2+} influxes, whereas second messenger(s) presumably accounted for the remainder (43% and 48% for Ca^{2+} and Mn^{2+} respectively). The small increases in the rates of Ca^{2+} and Mn^{2+} influx (19% and 12% respectively) seen when cells were treated with OKT3 and thapsigargin together may be due to the emptying of an additional, $Ins(1,4,5)P_3$ -insensitive, Ca^{2+} pool(s) by thapsigargin (Figure 3).

Unlike in hepatocytes and anterior pituitary cells, the Ca^{2+} channels in Jurkat cells appear to have little selectivity in conducting Ca^{2+} over Mn^{2+} ions [21,37]. This is further illustrated by the similar rates and magnitudes of both Ca^{2+} and Mn^{2+} influx, which were proportional to the extent of Ca^{2+} mobilization by the different concentrations of thapsigargin (Figures 4a and 4c). Similar results were also obtained when lower concentrations of OKT3 were used to stimulate the Jurkat cells (Figures 4b and 4d). One possible explanation for this is that in hepatocytes and anterior pituitary cells different mechanisms may be involved in the regulation of Ca^{2+} entry through different subsets of Ca^{2+} channels, some of which are impermeable to Mn^{2+} ions.

In summary, at least two separate pathways regulating Ca^{2+} entry during T cell receptor stimulation are present in T cells. One of these pathways is activated by the emptying of the $Ins(1,4,5)P_3$ -sensitive Ca^{2+} pool, whereas the second pathway is receptor-dependent and appears to be mediated by second

messenger(s). The existence of these two different mechanisms for the regulation of bivalent cation entry may help to explain and reconcile some of the conflicting observations on receptor-mediated Ca^{2+} entry in non-excitable cells.

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