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Regulation of receptor-mediated calcium influx across the plasma membrane in a human leukemic T-cell line: evidence of its dependence on an initial calcium mobilization from intracellular stores

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It has been repeatedly shown that stimulation of a human leukemic T-cell line, JURKAT, by lectins such as phytohaemagglutinin and anti-T3 antibody (OKT3) leads to an elevation in the concentration of cytosolic free Ca^2. This Ca^{2+} transient results from both an intracellular mobilization and an influx of Ca^{2+} through specific membrane channels. The objective of this study was to investigate the mechanism by which receptor-mediated influx of Ca^{2+} is regulated in JURKAT cells, which demonstrably lack 'voltage-dependent calcium channels'. It was found that upon increased loading with quin2 or 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate (BAPTA) there was a pronounced decline of both phytohaemagglutinin-stimulated and OKT3-stimulated influx of $^{45}Ca^{2+}$. Using 15 μM quin2/AM or 30 μM BAPTA/AM, agonist-stimulated $^{45}Ca^{2+}$ influx was almost totally abolished. At these concentrations of both quin2/AM or BAPTA/AM, phytohaemagglutinin and OKT3 could still induce a rise of cytosolic free Ca^{2+} above 200 nM. In the presence of La^{3+} (200 μM), which completely inhibited the agonist-induced $^{45}Ca^{2+}$ influx, both phytohaemagglutinin and OKT3 were able to raise the concentrations of cytosolic free Ca^{2+} to well above 200 nM by merely mobilizing Ca^{2+} from intracellular stores alone. The data suggest that an agonist-induced increase in the concentration of cytosolic free Ca^{2+}, due to mobilization from intracellular stores, could either directly or indirectly, initiate receptor-mediated Ca^{2+} influx across the plasma membrane in JURKAT cells.

Introduction

In a wide variety of cells, ligand–receptor interactions lead to the activation of a membrane-bound phospholipase C which results in an increased breakdown of PtdIns(4,5)P_2 and the accumulation of diacylglycerol and various inositol phosphates [1–3]. This leads to the activation of protein kinase C and a rise in the concentration of cytosolic free Ca^{2+}, both of which are considered as major components of intracellular signal transduction [1,2,4,5]. A receptor-mediated increase of the cytosolic free Ca^{2+} concentration usually re-

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Abbreviations: PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns, phosphatidylinositol; InsP_3, inositol trisphosphate Ins$P$, inositol monophosphate; InsP_2, inositol bisphosphate; Ins(1,3,4)P_3, inositol 1,3,4-trisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate; PHA, phytohaemagglutinin; Hepes, 4(-2-hydroxyethyl)-1-piperazineethanesulfonic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate; DMSO, dimethyl sulfoxide.

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sults from, both the mobilization of Ca^{2+} from intracellular stores and increased influx of Ca^{2+} across the plasma membrane from the extracellular milieu [6,7].

The mobilization of Ca^{2+} from intracellular stores has been shown in a variety of cell types to be mediated by an increased accumulation of Ins(1,4,5)P_3, which binds to specific receptors on intracellular organelle(s) resulting, in Ca^{2+} release into the cytosol [8,9]. The influx of Ca^{2+} across the plasma membrane is mediated via Ca^{2+} channels, which exhibit different characteristics in different cell types. In excitable cells, 'voltage-gated calcium-channels' are activated following depolarization of the plasma membrane [10,11], whereas in other cell types these channels are not gated by membrane potential, but require a ligand–receptor interaction and are thus referred to as 'receptor-operated calcium channels' [12,13]. It is still unclear whether this type of channel is a component of the receptor complex or if it is physically separated from the receptor. Pertussis toxin has been shown to block agonist-induced Ca^{2+} influx as well as the production of inositol phosphates in human neutrophils and differentiated HL60 cells [14,15]. On this basis, it is tempting to speculate that the increased breakdown of polyphosphoinositide, either directly or indirectly, is involved in the regulation of Ca^{2+} influx through this type of channel. Consequently, it has been suggested that the majority of these 'receptor-operated calcium channels' should be referred to as 'second-messenger calcium channels' [16]. This idea has gained further support by the findings of other workers [17–22].

The 1,4,5-isomer of InsP_3, which is generally considered to act merely as a mobilizer of Ca^{2+} from intracellular stores, has been demonstrated to activate certain transmembrane Ca^{2+} channels. This type of channel appears to be identical to the recently described mitogen-stimulated, voltage-insensitive Ca^{2+}-permeable channel present in activated T-cells [22]. Rink and Sage [23] recently reported that in human platelets the initial event in the Ca^{2+} transient in cells, stimulated with thrombin, was the influx of Ca^{2+} across the plasma membrane from the external milieu and not the mobilization of Ca^{2+} from intracellular stores. It can be reasoned that if Ins(1,4,5)P_3 is formed in the plasma membrane and if it is responsible for both the influx and the intracellular mobilization of Ca^{2+}, then it is more likely that the Ca^{2+} influx would occur before the mobilization of Ca^{2+} from intracellular stores.

Alternatively, Irvine and Moor [17,18] proposed that Ins(1,3,4,5)P_4 is the second messenger that controls the influx of Ca^{2+} across the plasma membrane. They demonstrated that Ins(1,3,4,5)P_4 in the presence of Ins(1,4,5)P_3 could regulate Ca^{2+} entry following activation of sea-urchin eggs. Another recent study by Imboden and Weiss [19] also demonstrated that Ins(1,3,4,5)P_4 by itself is insufficient to sustain the increased level of cytosolic free Ca^{2+}. They showed that the sustained increase in the concentration of cytosolic free Ca^{2+}, although dependent on extracellular Ca^{2+} influx, is regulated by Ins(1,4,5)P_3-mediated Ca^{2+} mobilization from intracellular stores. von Tscharner and co-workers [20] have reported that in human neutrophils this type of Ca^{2+} channel is regulated by the concentration of cytosolic free Ca^{2+} itself, thus suggesting a direct role for the Ca^{2+} released from intracellular stores, in regulating the subsequent influx of Ca^{2+} across the plasma membrane. It has also been speculated that Ca^{2+} influx into the cells, upon stimulation of its receptors, could be due to the formation of an agonist-receptor complex or transduction mechanisms that are distinct from the intrinsic levels of cytosolic free Ca^{2+} [21].

In the present study, we have examined whether or not the opening of 'second-messenger calcium channels' in JURKAT cells is dependent on an initial mobilization of Ca^{2+} from intracellular stores.

Experimental procedures

Chemicals. OKT3 monoclonal antibody was purchased from Ortho Diagnostic System, NJ, U.S.A. Purified phytohaemagglutinin was from Wellcome Laboratories, U.K. Quin2/AM and Quin2-free acid were obtained from Calbiochem, La Jolla, CA, U.S.A. $^{45}Ca^{2+}$ was obtained from New England Nuclear, Dreieich, F.R.G. *myo*-[^{3}H]Inositol was obtained from Amersham, Amersham, U.K. Lanthanum chloride was

purchased from Sigma Chemical Co. St. Louis, MO, U.S.A. BAPTA/AM was purchased from Molecular Probes (Eugene, OR, U.S.A.). All other reagents used were of analytical grade.

Cell culture. Jurkat cells, a human T-cell acute lymphoblastoid leukemia-derived cell line, were maintained in tissue culture medium (RPMI 1640) supplemented with 10% heat-inactivated fetal calf serum at 37°C in a humidified 5% CO_2/95% air incubator. Penicillin (100 units/ml) and streptomycin (50 μg/ml) were also included in the culture medium [24].

Determination and manipulation of the concentration of cytosolic free Ca^{2+}. The technique of loading cells with quin2 has been described previously [21,24,25]. Certain adjustments of this technique were made in the present study. Cells were loaded with various quin2/AM or BAPTA/AM at a density of $6 \cdot 10^6$ cells/ml (total volume, 5 ml) in their normal culture medium for 60 min at 37°C. After which, the cells were washed and resuspended in their normal culture medium. For the determination of autofluorescence, control cells were incubated with a similar concentration of DMSO. Immediately before use, a sample of the cell suspension was centrifuged and resuspended in medium containing 138 mM NaCl/6 mM KCl/1 mM $MgSO_4$/1.1 mM $CaCl_2$/0.1 mM EGTA/1 mM $NaHPO_4$/5 mM $NaHCO_3$/5.5 mM glucose/20 mM Hepes (pH 7.4). (This medium will be referred to here as 'calcium medium'). For the experiments with $LaCl_3$, this medium was modified by excluding all phosphates [12]. Measurements of fluorescence were performed with a Perkin-Elmer fluorimeter (LS3B). The cuvette holder was thermostated at 37°C and was equipped with a continuous stirring device. Each cuvette contained $(1.5–2.0) \cdot 10^6$ cells/ml in a total volume of 2 ml. Excitation and emission wavelengths were 339 nm and 492 nm, respectively. The intracellular concentration of quin2-free acid was measured by comparing the calcium-dependent fluorescence of a standard solution of quin2-free acid in the presence of unloaded cells, also treated with 0.1% (v/v) Triton X-100 in a calcium-containing medium [26].

$^{45}Ca^{2+}$ influx experiments. Jurkat cells ($6 \cdot 10^6$ cells/ml) were incubated with various concentrations of quin2/AM or BAPTA/AM in their normal culture medium for 1 h at 37°C. Control cells were treated in the same manner and were exposed to an equal volume of DMSO. After 1 h incubation, the cells were washed and resuspended in the calcium medium used for quin2 fluorescence studies. For measurements of Ca^{2+} influx, $^{45}CaCl_2$ (1 μCi) was added to each tube of cells and allowed to equilibrate for 5 min at 37°C. OKT3 (1 : 400) monoclonal antibody or phytohaemagglutinin (1 μg/ml) was used to stimulate the cells. When $LaCl_3$ was used, it was added to the cell suspensions 1 min before the stimulation with either agonist. After 5 min, the cells were transferred to ice and the cells were separated from the extracellular medium using a Millipore 12-place filtration apparatus. Cells were subsequently collected on filters (Millipore, type HA; pore size 0.45 μm), washed with 3×5 ml of cold phosphate-buffered saline, dried and counted in 10 ml Aquasol solution. Calcium influx was calculated as percentage of control (unstimulated) cells.

Determination and separation of inositol phosphates. Jurkat cells ($1 \cdot 10^6$ cells/ml) were loaded with *myo*-[^{3}H]inositol (2 μCi/ml) for 24 h in RPMI 1640 minus inositol, supplemented with 10% fetal calf serum at 37°C. After 24 h, the cells were washed three times with phosphate-buffered saline and resuspended in their culture medium at $6 \cdot 10^6$ cells/ml and incubated with 15 mM quin2/AM or 30 μM BAPTA/AM for 1 h at 37°C. The cells were then washed and $1 \cdot 10^7$ cells were resuspended in 250 μl of the Ca^{2+} medium and allowed to equilibrate for 5 min at 37°C. Cells were stimulated with OKT3 (1 : 400) or phytohaemagglutinin (1 μg/ml) for 15 s and terminated by the addition of 250 μl of 30% trichloroacetic acid. The samples were then put on ice for 15 min and centrifuged. The sample supernatants were washed three times with a 5-fold excess of diethyl ether, adjusted to pH 7.5 with Tris buffer (0.2 M), and the inositol phosphates were separated by stepwise elution from Dowex (formate) columns as described previously [26,27]. The radioactivity of the different fractions was determined by liquid scintillation counting with 67% (v/v) Aquasol.

Results

Effect of the buffering capacity of quin2 or BAPTA on agonist-stimulated $^{45}Ca^{2+}$ influx and $InsP_3$ production

As shown in Fig. 1, quin2/AM as well as BAPTA/AM caused a concentration-dependent decrease in $^{45}Ca^{2+}$ influx into the cells when they were stimulated with either OKT3 (1:400) or phytohaemagglutinin (1 μg/ml). At 15–20 μM quin2/AM or 30 μM BAPTA/AM, the $^{45}Ca^{2+}$ uptake, stimulated by either agonist, was totally inhibited. Stimulation with, both OKT3 and phytohaemagglutinin led to a rapid and significant accumulation of $InsP_3$ in JURKAT cells (280% and 250% of basal, respectively). Using concentrations of quin2/AM and BAPTA/AM which abolished agonist-stimulated $^{45}Ca^{2+}$ uptake, neither phytohaemagglutinin- nor OKT3-stimulated formation of $InsP_3$ was significantly affected (data not shown).

Effect of the buffering capacity of quin2 on agonist-stimulated increase in cytosolic free Ca^{2+}

JURKAT cells, loaded with increasing concentrations of quin2, were stimulated with either OKT3 or phytohaemagglutinin and changes in the concentration of cytosolic free Ca^{2+} were monitored by spectrofluorometry (Fig. 2). There was a rapid drop in the cytosolic free Ca^{2+} level when the quin2/AM concentration was increased from 5 to 20 μM. But when the concentration of quin2/AM was further increased to 60 μM there was a less pronounced decrease. The concentration of cytosolic free Ca^{2+} in resting cells was taken as 100%. All agonist-induced increases in

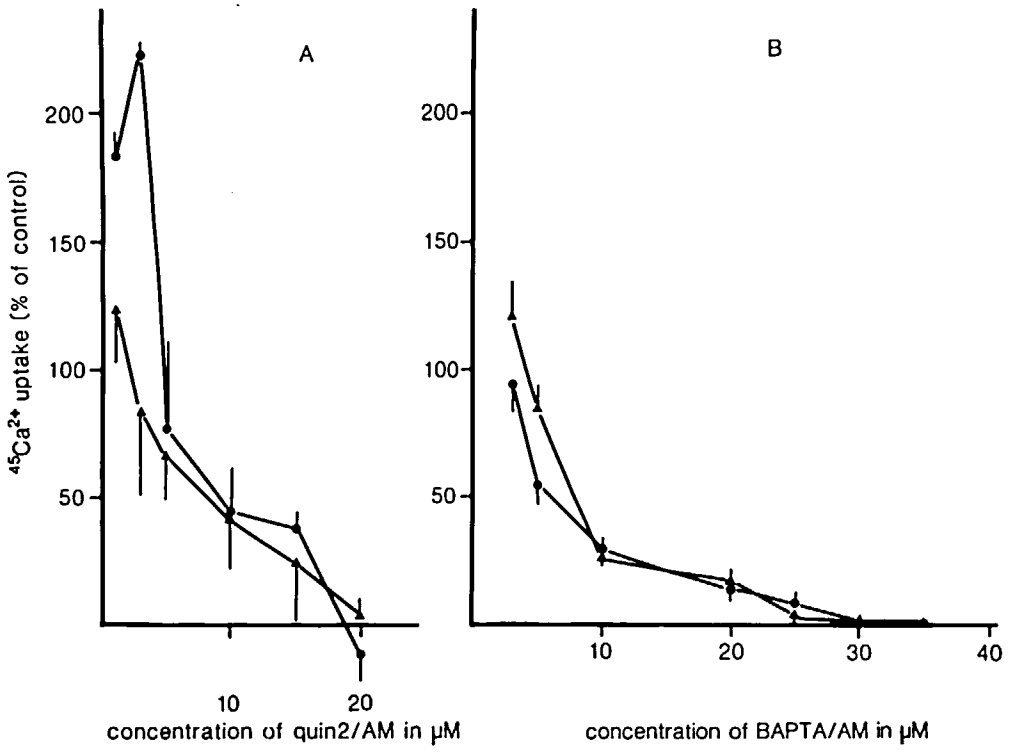

Fig. 1. Effect of increased quin2 or BAPTA buffering on agonist-stimulated uptake of $^{45}Ca^{2+}$. JURKAT cells ($6 \cdot 10^6$) were loaded with various concentrations of quin2 for 1 h at 37°C, washed and resuspended in the calcium-containing medium. $^{45}Ca^{2+}$ (1 μCi) was added to each sample 5 min before stimulation with either OKT3 (1:400; ▲) or phytohaemagglutinin. 5 min after stimulation, the cells were harvested as described in Materials and Methods. The agonist-stimulated uptake of $^{45}Ca^{2+}$ is expressed as a percentage of the value obtained from non-loaded cells. In panel A, the activity in non-loaded cells were $(1.9 \pm 0.2) \cdot 10^5$ cpm and $(2.4 \pm 0.9) \cdot 10^5$ cpm for OKT3 and phytohaemagglutinin, respectively. In panel B, the activity in non-loaded cells were $(5.4 \pm 0.3) \cdot 10^4$ cpm and $(6.6 \pm 0.6) \cdot 10^4$ cpm for OKT3 and phytohaemagglutinin, respectively. Values are mean ± S.E. and n = 4–6.

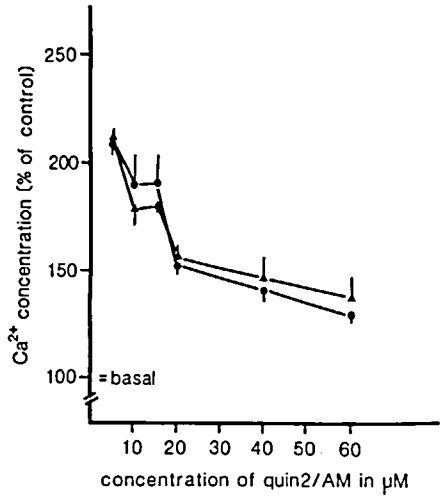

Fig. 2. Effect of increased quin2-buffering on agonist-stimulated increase in cytosolic free Ca^{2+}. JURKAT cells were loaded with various concentrations of quin2 for 1 h at 37 °C. Prior to measuring quin2 fluorescence, $2 \cdot 10^6$ cells were centrifuged and resuspended in 2 ml of the calcium containing medium. After a stable base line was attained, the cells were stimulated with 1:400 OKT3 (▲) or 1 μg/ml phytohaemagglutinin (●). Cytosolic free Ca^{2+} concentrations are expressed as a percentage of the basal level of resting cells prior to stimulation. The basal levels of cytosolic free Ca^{2+} were 121 ± 36 nM and 103 ± 18 nM for OKT3 and phytohaemagglutinin, respectively. Values shown are mean ± S.E. of n where $n = 3$.

the cytosolic free Ca^{2+} concentration were expressed as a percentage of the resting level with the same quin2 loading. In cells which had been incubated with 15 μM quin2/AM (a concentration which inhibits most of the $^{45}Ca^{2+}$ uptake) for 1 h, the agonists could still raise the cytosolic free Ca^{2+} concentration to 231 ± 26 nM ($n = 3$) and 182 ± 11 ($n = 3$) for OKT3 and phytohaemagglutinin, respectively.

Effect of the buffering capacity of BAPTA/AM on agonist-stimulated increase in cytosolic free Ca^{2+}

JURKAT cells, incubated with 5 μM quin2/AM and 30 μM BAPTA/AM, were stimulated with either OKT3 or phytohaemagglutinin and changes in the concentration of cytosolic free Ca^{2+} were followed in a spectrofluorometer. In Fig. 3, using a BAPTA/AM concentration (30 μM) which totally inhibited agonist-induced $^{45}Ca^{2+}$ influx (see Fig. 1), both OKT3 and phytohaemagglutinin could still raise the peak level of cytosolic free Ca^{2+} to well above 200 nM (245 ± 4 nM; $n = 6$ and 210 ± 11 nM; $n = 6$, respectively).

Effect of La^{3+} on agonist-stimulated $^{45}Ca^{2+}$ influx

Addition of increasing concentrations of La^{3+} led to a corresponding decrease in agonist-stimulated uptake of $^{45}Ca^{2+}$ (Fig. 4). The addition of La^{3+} to the cells, after equilibration and just before agonist-stimulation (1 min), did not alter the basal uptake of $^{45}Ca^{2+}$ (not shown). We found that 200 μM La^{3+} could totally block, both, OKT3 and phytohaemagglutinin-stimulated influx of $^{45}Ca^{2+}$. We then used 200 μM La^{3+} for studies on agonist-induced mobilization of intracellular Ca^{2+}.

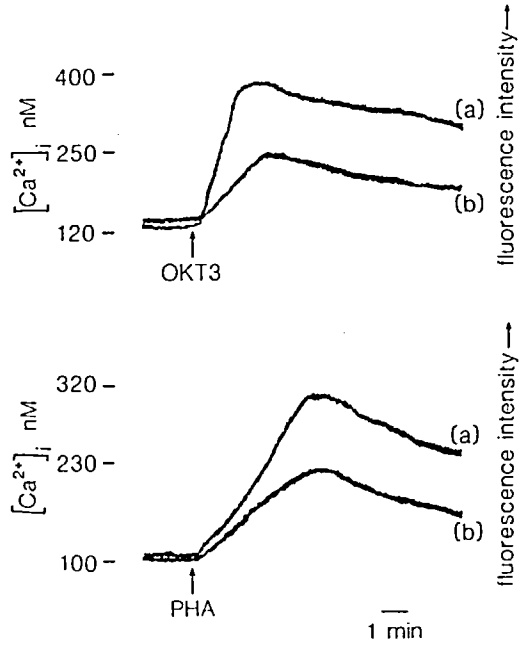

Fig. 3. Effect of BAPTA-buffering on agonist-stimulated increase in cytosolic free Ca^{2+}. JURKAT cells were loaded with 5 μM quin2/AM and 30 μM BAPTA/AM for 1 h at 37 °C. Prior to measuring quin2 fluorescence, $2 \cdot 10^6$ cell were centrifuged and resuspended in 2 ml of the calcium-containing medium. After a stable base line was attained, the cells were stimulated with either 1:400 OKT3 or 1 μg/ml phytohaemagglutinin. Trace (a) represents cells that is loaded with quin2 alone and trace (b) represents cells loaded with both quin2 and BAPTA. The traces shown are representative of six similar experiments.

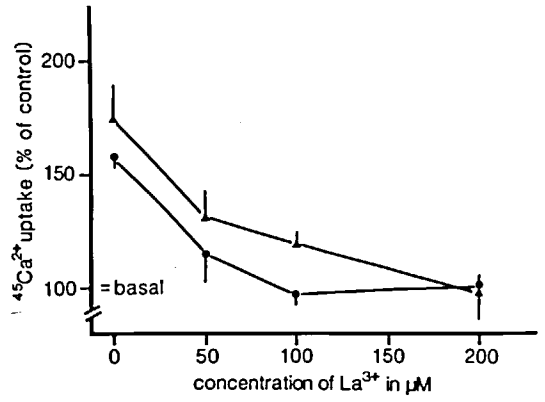

Fig. 4. Effect of La^{3+} on agonist-stimulated uptake of $^{45}Ca^{2+}$. JURKAT cells were incubated with $^{45}Ca^{2+}$ (1 μCi/ml) for 5 min at 37°C in calcium-containing medium lacking phosphate prior to stimulation with either 1:400 OKT3 (▲) or 1 μg/ml phytohaemagglutinin (●). Where included, La^{3+} was added 1 min prior to stimulation with either agonist. The cells were harvested 5 min after agonist stimulation as described in the legend to Fig. 1 and in Materials and Methods. Agonist-stimulated $^{45}Ca^{2+}$ uptake is expressed as a percentage of the control values of unstimulated cells. The activities in control cells were $(1.3 \pm 0.3) \cdot 10^5$ cpm and $(1.4 \pm 0.3) \cdot 10^5$ cpm for OKT3 and phytohaemagglutinin, respectively. Values are means ± S.E. where $n = 4$ experiments.

Agonist-stimulated mobilization of intracellular Ca^{2+}

Fig. 5 shows that addition of La^{3+} (200 μM) to the cells 1 min before stimulation significantly reduced the cytosolic free Ca^{2+} concentration induced by either agonist (lower and upper panels). In the presence of La^{3+}, the Ca^{2+} transient peaked at approx. 300 nM and exhibited much shorter duration (less than 5 min). In the absence of La^{3+} the duration of the agonist-induced cytosolic free Ca^{2+} transient was approx. 26 for both agonists.

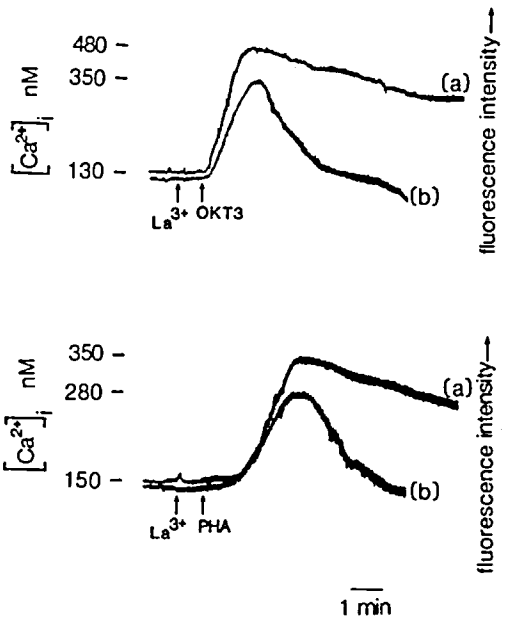

Fig. 5. Agonist-stimulated mobilization of intracellular Ca^{2+}. JURKAT cells were incubated with quin2/AM (5 μM) for 1 h at 37°C. Cells ($2 \cdot 10^6$) were spun down and resuspended in a calcium-containing medium lacking phosphate ions. The cells were stimulated with either OKT3 (1:400; top panel) or phytohaemagglutinin (1 μg/ml; lower panel); (a) represents cells stimulated with either OKT3 or phytohaemagglutinin alone, while (b) represents cells pretreated with La^{3+} (200 μM) for 1 min prior to stimulation with either OKT3 or PHA. The traces shown are representative of six similar experiments.

Discussion

Activation of JURKAT cells by lectins or anti-T3 antibody leads to a rapid increase in the concentration of cytosolic free Ca^{2+} [19,24]. This increase in the concentration of cytosolic free Ca^{2+} is believed to result from both intracellular mobilization and influx of Ca^{2+} across the plasma membrane [28]. In contrast to excitable tissues, such as muscle, nerves and adrenal glomerulosa cells, JURKAT cells have been shown to lack 'voltage-gated Ca^{2+} channels' [29]. Instead they possess what might be generally referred to as 'second-messenger Ca^{2+} channels' [16].

In the present study, we have used the human T-leukemic cell line (JURKAT) to test the hypothesis that changes in the concentration of cytosolic free Ca^{2+} would be involved in the regulation of Ca^{2+} entry across the plasma membrane [20]. The ability of quin2 and BAPTA to act as Ca^{2+} chelators [21,30–32] was used to test this hypothesis. Increasing concentrations of both chelators were able to inhibit $^{45}Ca^{2+}$ influx when the cells were stimulated with either OKT3 or phytohaemagglutinin. Our results also demonstrate that an initial increase in quin2 load (up to 20 μM quin2/AM) led to a more rapid decline of the cytosolic free Ca^{2+} transient. A further increase in quin2/AM concentration caused a less pronounced effect. This indicated that apart from acting as a 'calcium sink', an initial increase of

quin2 concentration in the cytosol would also affect other important element(s) involved in the regulation of calcium homeostasis in these cells. Since the same concentrations of quin2 also blocked the agonist-induced uptake of $^{45}Ca^{2+}$, our data strongly suggest that the initial rapid decrease in cytosolic free Ca^{2+} can be explained by the inhibition of agonist-induced Ca^{2+} influx.

We found that at a certain concentration of quin2, both agonists were still able to increase the cytosolic free Ca^{2+} concentration to above 200 nM, even though Ca^{2+} influx across the plasma membrane was abolished. This indicates that for the activation of Ca^{2+}-influx, it is first necessary to raise the cytosolic free Ca^{2+} level to about 200 nM. This interpretation is further supported by the data obtained using a BAPTA/AM concentration (30 μM), which inhibited agonist-induced $^{45}Ca^{2+}$ influx. At this concentration of BAPTA/AM, both agonists could still raise the cytosolic free Ca^{2+} level to above 200 nM. Using a concentration of La^{3+} that totally blocked the agonist-induced uptake of $^{45}Ca^{2+}$, it was possible to demonstrate that, both OKT3 and phytohaemagglutinin were able to raise the concentration of cytosolic free Ca^{2+} to well above 200 nM. Thus, mobilization of Ca^{2+} from intracellular stores alone can elevate the concentration of cytosolic free Ca^{2+} well above 200 nM.

Although incubation of JURKAT cells with quin2/AM or BAPTA/AM affected neither OKT3 nor phytohaemagglutinin stimulated InsP_3 production, we do not know whether the reduction of the Ca^{2+} transient might lower the level of a putative inositol phosphate formed, which could initiate the influx of Ca^{2+} in these cells. In fact, it has been shown that a reduction of the concentration of cytosolic free Ca^{2+} lowers the activity of Ins(1,4,5)P_3 3-kinase thus decreasing the levels of Ins(1,3,4,5)P_4 and subsequently also the levels of Ins(1,3,5)P_3 [33–35]. Consequently, our findings that a reduction of the concentration of cytosolic free Ca^{2+} regulates the influx of Ca^{2+} could occur via reduced levels of inositol phosphates. However, the present data strongly indicate that the direct rate-limiting control is exerted by Ca^{2+} released from intracellular stores.

In conclusion, in the JURKAT cell line the initial agonist-induced increase of the cytosolic free Ca^{2+} concentration, due to mobilization of Ca^{2+} from intracellular stores, is the critical rate-limiting factor which directly or indirectly regulates the subsequent entry of Ca^{2+} into these cells.

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