Inhibition of receptor-mediated calcium influx in T cells by unsaturated non-esterified fatty acids

Sek C. CHOW,*†‡ Ignacio J. ANSOTEGUI* and Mikael JONDAL*

Departments of *Immunology and †Toxicology, Karolinska Institute, Box 60400, Stockholm, Sweden

The effect of $\omega-3$, $\omega-6$ and $\omega-9$ unsaturated fatty acids (UFAs) on receptor-mediated Ca^{2+} entry was investigated in a T-cell line (JURKAT) by using anti-CD3 antibodies (OKT3) to induce intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) increase and Ca^{2+} influx. All the UFAs, as well as Ni^{2+} ions and 12-*O*-tetradecanoylphorbol 13-acetate, decreased the OKT3-induced sustained $[\text{Ca}^{2+}]_i$ increase to basal levels. Although non-esterified fatty acids activate protein kinase C (PKC) [McPhail, Clayton & Snyderman (1984) *Science* **224**, 622–624; Murakami, Chan & Routtenberg (1986) *J. Biol. Chem.* **261**, 15424–15429], studies using H-7 and analysis of the PKC-dependent phosphorylation of 19 and 80 kDa marker substrates ruled out the involvement of PKC in UFA-induced inhibition of Ca^{2+} entry. Flow-cytometry analysis showed that UFAs do not interfere with antibody–receptor binding. BSA (0.2%, w/v) reversed the effect of UFAs after these fatty acids have decreased the OKT3-induced $[\text{Ca}^{2+}]_i$ increase to basal levels. The relevance of these findings and possible mechanisms for inhibition by UFAs of receptor-mediated Ca^{2+} influx were discussed.

INTRODUCTION

One of the earliest events in T-cell activation by T-cell receptor-specific monoclonal antibodies (Weiss *et al.*, 1986) or mitogenic lectins (Gelfand *et al.*, 1987; Hadden, 1988) is the mobilization of intracellular Ca^{2+} (Ca^{2+}). The increase in $[\text{Ca}^{2+}]_i$ is characterized by an initial transient increase owing to release of Ca^{2+} from internal stores, and a second prolonged sustained phase, which appears to be due to Ca^{2+} entry across the plasma membrane, since it requires extracellular Ca^{2+} . The mechanism responsible for the initial transient increase in $[\text{Ca}^{2+}]_i$, now well characterized, involves Ca^{2+} mobilization from intracellular stores by $\text{Ins}(1,4,5)\text{P}_3$ (Irvine *et al.*, 1986; Berridge, 1986; Putney *et al.*, 1989). However, the nature and regulation of Ca^{2+} influx across the plasma membrane during the sustained phase of elevated Ca^{2+} is still not well understood.

One model suggests that Ca^{2+} flows directly into the cytosol through channels in the plasma membrane, which appear to be regulated by $\text{Ins}(1,4,5)\text{P}_3$ (Kuno & Gardner, 1987). Recent evidence (Takemura & Putney, 1989; Putney *et al.*, 1989) suggests that a loss of Ca^{2+} from the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pool activates a pathway of Ca^{2+} re-entry into the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pool from the extracellular milieu. Hence, if $\text{Ins}(1,4,5)\text{P}_3$ is continuously maintained at an elevated level, Ca^{2+} would enter the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pool and subsequently exit through the $\text{Ins}(1,4,5)\text{P}_3$ -mediated channels into the cytosol. This model seems to provide the best explanation for how the sustained phase of $[\text{Ca}^{2+}]_i$ elevation may be maintained.

Recently, we observed that polyunsaturated fatty acids stimulate the discharge of Ca^{2+} from the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pool via a mechanism which can be dissociated from the hydrolysis of inositol lipids (Chow & Jondal, 1990). However, the depletion of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pool by the fatty acids did not induce any influx of Ca^{2+} . This suggested that these fatty acids may be blocking Ca^{2+} entry, as well as mediating Ca^{2+} release from internal stores. In order to study the possible effects of unsaturated fatty acids (UFAs) on agonist-induced Ca^{2+}

influx, we used the anti-CD3 antibody OKT3 to induce $[\text{Ca}^{2+}]_i$ increase and Ca^{2+} influx in JURKAT cells. The effects of OKT3 on Ca^{2+} fluxes in JURKAT cells have been extensively described (Weiss *et al.*, 1986). The present study provides evidence that UFAs reversibly block Ca^{2+} influx in T cells, presumably by acting directly on the receptor-operated Ca^{2+} channels.

MATERIALS AND METHODS

Materials

Fura-2/AM (the acetoxymethyl ester), α -linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DCHA), oleic acid (OA), linoleic acid (LA), arachidonic acid (AA), leupeptin, phenylmethanesulphonyl fluoride, H-7 [1-(5-isoquinolylsulphonyl)-2'-methylpiperazine] and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) were purchased from Sigma (St. Louis, MO, U.S.A.). Carrier-free ^{32}P was obtained from New England Nuclear (Stockholm, Sweden). Molecular-mass markers were from Pharmacia, Uppsala, Sweden. BSA (fraction V) was from Boehringer (Mannheim, Germany). Fluorescein isothiocyanate (Fic)-labelled rabbit anti-mouse IgG was from Dakopatts, Glostrup, Denmark. DE-52 ion-exchange DEAE-cellulose was from Whatman, Maidstone, Kent, U.K. RPMI 1640 and fetal-calf serum (FCS) were from Gibco, Glostrup, U.K. All other reagents used were of analytical grade. OKT3 hybridoma was from the American Type Culture Collection (Rockville, MD, U.S.A.). OKT3 monoclonal antibodies were purified from culture supernatant on a Protein A–Sepharose column (Pharmacia, Stockholm, Sweden).

Cell line

JURKAT cells (T-cell leukaemia) were maintained in continuous suspension culture in RPMI 1640 medium supplemented with 7.5% (v/v) FCS and antibiotics (100 units of penicillin/ml and 100 μg of streptomycin/ml) in a humidified incubator (37 °C) under an atmosphere of 5% CO_2 in air.

Abbreviations used: UFA, unsaturated fatty acid; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concn.; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; ALA, α -linolenic acid; EPA, eicosapentaenoic acid; DCHA, docosahexaenoic acid; LA, linoleic acid; AA, arachidonic acid; OA, oleic acid; FCS, fetal-calf serum; AM, acetoxymethyl ester.

† To whom all correspondence and reprint requests should be addressed, in the Department of Immunology.

Measurement of $[Ca^{2+}]_i$

JURKAT cells (1×10^7 cells/ml) were loaded with 5 μ M-Fura-2/AM in RPMI (with 7.5% FCS) for 30 min at room temperature. Cells were then washed free of extracellular Fura-2/AM and resuspended in modified Krebs-Henseleit buffer (1.3 mM- $CaCl_2$) supplemented with 5.5 mM-glucose and 10 mM-Hepes, pH 7.2. The cells were maintained in the modified Krebs-Henseleit buffer until use. Just before $[Ca^{2+}]_i$ measurement, portions of the cell suspension (3×10^6 cells) were again washed and resuspended in 2 ml of modified Krebs-Henseleit buffer in a quartz cuvette maintained at 35 °C in a Sigma ZFP 22 dual-wavelength spectrofluorimeter (Sigma Instruments, West Berlin, Germany) with excitation wavelengths 334 and 366 nm (emission was measured with a 500 nm-cut-off filter). Because Fura-2 leakage is temperature-dependent, 35 °C was used instead of 37 °C to minimize leakage (Treves *et al.*, 1987). This procedure decreased Fura-2 leakage to a minimum over the time course of the experiments. Changes in $[Ca^{2+}]_i$ were monitored by recording changes in the fura-2 fluorescence-ratio signal, calibrated in terms of $[Ca^{2+}]_i$ as described by Grynkiewicz *et al.* (1985). The maximal fluorescence was obtained by lysing the cells with 10% (v/v) Nonidet P40. EGTA (final concn. 500 mM in 2 M-Tris/HCl, pH 8.4) was then added to obtain the minimum fluorescence. A value of 225 nm was used for the K_d of Fura-2, as previously reported (Treves *et al.*, 1987; Grynkiewicz *et al.*, 1985).

Studies on the effect of UFAs and OKT3 on Ca^{2+} influx into JURKAT cells

Bivalent-cation influx into JURKAT cells was monitored by the Mn^{2+} quenching technique described by Merritt *et al.* (1989); this method is based on the ability of Mn^{2+} to displace Ca^{2+} and quench Fura-2 fluorescence, and the fact that stimulation of receptor-mediated Ca^{2+} entry results in concomitant Mn^{2+} influx. Fura-2-loaded cells (3×10^6 cells) were incubated in a cuvette in the presence of 50 μ M- $MnCl_2$, and after 1 min agents were added to the cells as described in the Figure legends. The fluorescence of Fura-2 was monitored continuously as outlined above, except that the two excitation wavelengths were recorded separately.

Phosphorylation studies

JURKAT cells (3×10^6 cells/ml) were labelled with carrier-free $[^{32}P]P_i$ (100 μ Ci/ml) in phosphate-free RPMI medium without FCS or BSA for 5 h at 37 °C. Then 1 ml portions of cell suspension were placed in 1.5 ml Eppendorf tubes containing the various stimulating agents. The cells were incubated for 20 min at 37 °C. Stimulation was terminated by rapidly centrifuging and washing with ice-cold PBS (phosphate-buffered saline). The washed cell pellets were resuspended in 200 μ l of lysis buffer (20 mM-Tris/HCl, 150 mM-NaCl, 2.5 mM-EDTA, 10 mM-NaF, 1 mM-phenylmethanesulphonyl fluoride, 5 μ g of leupeptin/ml and 1% Triton X-100, pH 7.2) and placed on ice for 15 min, with regular vortex-mixing every 5 min. The lysates and proteins were processed as described by Brattsand *et al.* (1989), except that the CM-Sepharose absorption step was omitted. In brief, the lysates were separated from nuclei by centrifugation (9000 g) and boiled for 10 min. The precipitated proteins were spun down (9000 g), and 100 μ l portions of the supernatants were placed in tubes containing pre-equilibrated DE-52 ion-exchange DEAE-cellulose. After 20 min of constant mixing, the supernatants were removed and the proteins were precipitated overnight with 66% (v/v) acetone at -20 °C. Proteins were then analysed by reducing SDS/PAGE (11% acrylamide). Gels were treated with alkali (0.9 M-KOH) for 4 h, washed for 6 h before drying and autoradiographed.

Studies on the effect of UFAs on OKT3 binding to CD3 receptors

The method was essentially the same as reported by Schols *et al.* (1989). JURKAT cells (2×10^6) were incubated in 50 μ l of PBS containing optimum concentration of OKT3 antibodies in the presence of different UFAs (15 μ M) at 4 °C for 30 min. The cells were then washed twice and incubated with a second antibody (Fits-conjugated rabbit anti-mouse IgG) for another 30 min at 4 °C. After washing, cells were fixed in 0.5% formaldehyde and analysed with a fluorescence-activated cell sorter equipped with an argon laser (FACSCAN, Becton and Dickinson).

Other methods

Fatty acids were dissolved in ethanol and stored under N_2 at -20 °C. Just before use, the fatty acids in ethanol were converted into their sodium salts by dissolving in 100 mM- Na_2CO_3 . The final concentration of ethanol in all experiments was less than 0.05%. Fura-2/AM was dissolved in dimethyl sulphoxide to give a 1 mM stock solution and kept in the dark at -20 °C until used.

RESULTS

Inhibition of the OKT3-induced sustained increase in $[Ca^{2+}]_i$ by TPA, Ni^{2+} and UFAs

Interaction of the antibody OKT3 with CD3 receptors of the T-cell receptor complex is known to generate inositol phosphates and induce $[Ca^{2+}]_i$ increase (Imboden & Stobo, 1985; Weiss *et al.*, 1986; Chow & Jondal, 1990). In JURKAT cells loaded with the Ca^{2+} -indicator dye Fura-2, the OKT3-induced $[Ca^{2+}]_i$ increase was transient, and returned to a sustained level higher than the basal (Fig. 1a). Ni^{2+} ions (5 mM), which block divalent-cation entry via plasma-membrane channels (Merritt *et al.*, 1989), decreased $[Ca^{2+}]_i$ to near-basal level when added during the sustained phase of Ca^{2+} flux (Fig. 1b). On its own, Ni^{2+} does not affect the basal $[Ca^{2+}]_i$ (results not shown). Long-chain UFAs, i.e. ALA ($C_{18:3}$, $\omega-3$), EPA ($C_{20:5}$, $\omega-3$), DCHA ($C_{22:6}$, $\omega-3$), LA ($C_{18:2}$, $\omega-6$) and AA ($C_{20:4}$, $\omega-6$), which mobilize intracellular Ca^{2+} in JURKAT cells, and the non- Ca^{2+} -mobilizing OA ($C_{18:1}$, $\omega-9$) (Chow & Jondal, 1990), also decreased $[Ca^{2+}]_i$ to basal level when added during the sustained phase (Figs. 1c and d; only traces for OA and AA are shown). Of the six UFAs, OA was the most effective, requiring only 3 μ M to give complete reversal of the OKT3-induced sustained phase of $[Ca^{2+}]_i$ elevation (Fig. 1c). Other fatty acids required at least twice the effective concentration of OA to produce the same effect (e.g. AA in Fig. 1d). Except for OA, all other unsaturated fatty acids still induce a small increase in $[Ca^{2+}]_i$ at 6 μ M (results not shown). Activation of protein kinase C (PKC) by TPA during the sustained phase of Ca^{2+} flux had essentially the same effect as Ni^{2+} and UFAs, although a time lag of 10–15 s was observed in some experiments. The maximum effect obtained with TPA was observed at 100 ng/ml (Fig. 1e).

Effect of H-7 on both TPA- and UFA-induced inhibition of the OKT3-induced sustained $[Ca^{2+}]_i$ increase in JURKAT cells

Since the activation of PKC by TPA produces results similar to those of UFAs in blocking Ca^{2+} entry, it is possible that UFAs are activating PKC. It has been reported that non-esterified fatty acids can activate PKC in cell-free systems (McPhail *et al.*, 1984; Murakami *et al.*, 1986). However, nothing has been reported on whole cells. To see if PKC was activated by UFAs, various concentrations of the PKC inhibitor H-7 (Hidaka *et al.*, 1984; Nishikawa *et al.*, 1986) were added to the JURKAT cells 1 min before OKT3 stimulation. At 100 μ M-H-7, inhibition by TPA

Fig. 1. Effect of Ni^{2+} , UFAs and TPA on OKT3-induced sustained $[\text{Ca}^{2+}]_i$ increase in JURKAT cells

Changes in $[\text{Ca}^{2+}]_i$ were monitored with the Ca^{2+} -indicator dye Fura-2 as outlined in the Materials and methods section. OKT3 (10 $\mu\text{g}/\text{ml}$) was added when a steady baseline was obtained. When the sustained phase of $[\text{Ca}^{2+}]_i$ was reached, 5 mM- NiCl_2 (Ni), OA (3 μM), AA (3 μM each addition) and TPA (three traces superimposed on to one, where trace 1 was 10 ng/ml, trace 2 50 ng/ml and trace 3 100 ng/ml) was added as indicated. All the other UFAs tested (ALA, LA, EPA and DCHA) showed similar results.

(100 ng/ml) was abolished (Fig. 2a). The same concentration of H-7 (100 μM) had no effect on the UFA-induced decrease in $[\text{Ca}^{2+}]_i$ (Fig. 2b; only trace for OA shown). H-7 on its own had no effect on the Ca^{2+} transient owing to OKT3 activation (Figs. 2a and 2b).

UFAs do not affect PKC-dependent phosphorylations in JURKAT cells

Although H-7 has no effect on the UFA-induced $[\text{Ca}^{2+}]_i$ decrease, we cannot positively rule out PKC involvement in the UFA effect, owing to lack of specificity of H-7 in inhibiting PKC (Ruegg & Burgess, 1989; Watson *et al.*, 1988). To be certain that UFAs do not activate PKC, two PKC-dependent phosphorylation marker substrates in JURKAT cells (19 and 80 kDa) were analysed (Friedrich *et al.*, 1988; Alexander & Cantrell, 1989). As shown in Fig. 3, UFAs administered together with OKT3 did not induce any increase in phosphorylation of the 19 and 80 kDa proteins [only the results with LA (lane 3) and OA (lane 4) are shown], compared with OKT3 alone (lane 2). In contrast, TPA (20 ng/ml) caused a considerable increase in phosphorylation of these two markers, as expected (lane 5). These results provide clear evidence that PKC is not involved in the UFA-induced $[\text{Ca}^{2+}]_i$ decrease.

Fig. 2. Effect of the PKC inhibitor H-7 on the inhibition of OKT3-induced sustained $[\text{Ca}^{2+}]_i$ increase in JURKAT cells by TPA and OA

The changes in $[\text{Ca}^{2+}]_i$ were monitored with Fura-2 as described in the Materials and methods section. In (a), different concentrations of H-7 were added 1 min before OKT3 addition. TPA (100 ng/ml) was then added when the sustained phase of $[\text{Ca}^{2+}]_i$ was reached. The traces (1, without H-7; 2, 20 μM -H-7; 3, 40 μM -H-7; 4, 100 μM -H-7) were then superimposed on to one. In (b), 100 μM -H-7 was added 1 min before OKT3 and, when the sustained phase was reached, 3 μM -OA was added.

Fig. 3. Effects of UFAs on PKC-dependent phosphorylation of both 19 and 80 kDa proteins

An autoradiogram of a one-dimensional gel of ^{32}P -labelled proteins from JURKAT cells incubated with carrier buffer (lane 1), 10 μg of OKT3/ml (lane 2), 10 μg of OKT3/ml + 15 μM of UFAs (lane 3, LA; lane 4, OA) and 20 ng of TPA/ml (lane 5) for 20 min at 37 $^{\circ}\text{C}$. Other UFAs (AA, ALA, EPA and DCHA) gave essentially the same results. Cell lysates and proteins were prepared as described in the Materials and methods section. Similar results were obtained in another experiment with a different batch of cells.

Effect of Ni^{2+} , TPA and UFAs on the $[\text{Ca}^{2+}]_i$ of JURKAT cells when added simultaneously with OKT3

The rapid UFA-induced decrease in $[\text{Ca}^{2+}]_i$ observed during the sustained phase of $[\text{Ca}^{2+}]_i$ elevation suggests that UFAs might be acting directly on receptor-operated Ca^{2+} channels.

Fig. 4. Effect of Ni^{2+} , TPA and UFAs on JURKAT-cell $[\text{Ca}^{2+}]_i$ status when added together with OKT3

The changes in $[\text{Ca}^{2+}]_i$ were monitored with Fura-2 as outlined in the Materials and methods section. 10 mM- NiCl_2 (Ni), TPA (100 ng/ml), OA (6 μM) and AA (12 μM) were added together with OKT3 (10 $\mu\text{g}/\text{ml}$) as indicated. Only traces for OA and AA are shown; the other UFAs tested (ALA, LA, EPA and DCHA) gave similar results.

When Ni^{2+} (which blocks bivalent-cation entry) was added together with OKT3 to the JURKAT cells, the elevated $[\text{Ca}^{2+}]_i$ returned to basal level within 4 min (Fig. 4a). TPA, which can down-regulate the T-cell receptor, did not decrease $[\text{Ca}^{2+}]_i$ totally to basal levels when added together with OKT3, but the amplitude of the OKT3-induced $[\text{Ca}^{2+}]_i$ peak was slightly decreased (Fig. 4b). When UFAs were added together with OKT3, the normal transient increase in $[\text{Ca}^{2+}]_i$ was observed; however, $[\text{Ca}^{2+}]_i$ rapidly returned to basal level (Figs. 4c and 4d; only traces for OA and AA are shown). The time for $[\text{Ca}^{2+}]_i$ to reach a peak and return to basal levels in the presence of UFAs was only half of that when Ni^{2+} was present.

Effect of Ni^{2+} , UFAs and TPA on OKT3-induced bivalent-cation influx

JURKAT cells loaded with Fura-2 were incubated in Krebs-Henseleit buffer supplemented with 50 μM - MnCl_2 . Assuming that there is no specificity for both bivalent-cation (Mn^{2+} and Ca^{2+}) entry via the Ca^{2+} channels, stimulation of Ca^{2+} influx will simultaneously allow Mn^{2+} to enter the cells and quench the Fura-2 signal (Merritt *et al.*, 1989). OKT3 stimulated a rapid decrease in the Fura-2 fluorescence intensity (Fig. 5, trace 6) compared with the control cells (Fig. 5, trace 1), indicating that bivalent-cation influx is activated. Fura-2 fluorescence was completely quenched within 4 min after OKT3 addition. If Ni^{2+} (Fig. 5, trace 3), OA (Fig. 5, trace 4) or TPA (Fig. 5, trace 5) was added 2 min after OKT3 addition, the rapid decrease in fluorescence was interrupted and the normal rate of slow quenching (i.e., as in control cells) of the fluorescence resumes. This demonstrates that Ni^{2+} , UFAs and TPA inhibit the OKT3-induced influx of bivalent cations. On their own, UFAs did not induce any bivalent-cation entry (only OA shown; Fig. 5, trace 2).

Effect of UFAs on the binding of OKT3 to CD3 receptors

Exposure of JURKAT cells to OKT3 antibodies in the presence of different UFAs resulted in no change in the percentage of

Fig. 5. Effect of Ni^{2+} , TPA and OA on OKT3-induced bivalent-cation influx into JURKAT cells

Fura-2-loaded cells were incubated with 50 μM - MnCl_2 in a cuvette. After 1 min, 0.05% ethanol (trace 1), 3 μM -OA (trace 2) and 10 μg of OKT3/ml (traces 3, 4, 5 and 6) were added as indicated (vertical arrow). For traces 3, 4 and 5, 5 mM- NiCl_2 (Ni), OA (3 μM) and TPA (100 ng/ml) respectively were added 2 min after adding OKT3. The fluorescence was monitored at 334 nm. Only traces for OA are shown; other UFAs (AA, ALA, LA, EPA and DCHA) tested gave similar results.

Table 1. Effect of UFAs on CD3 expression in JURKAT cells

JURKAT cells were incubated with optimum concentration of OKT3 antibodies, either alone (no treatment) or with 15 μM of UFAs (OA, ALA, AA, EPA and DCHA) as described in the methods section. Second antibody used was FITC-conjugated rabbit anti-mouse IgG. Measurements were based on the percentage of cells showing fluorescence intensity greater than the control (second antibody only). Results are means $\pm$ S.E.M. from three separate experiments using different batches of cells.

Treatment	CD3 expression (%)
Control	2.51 $\pm$ 0.33
No treatment	89.98 $\pm$ 0.41
OA	90.37 $\pm$ 0.63
LA	89.96 $\pm$ 0.90
AA	90.44 $\pm$ 1.88
ALA	90.29 $\pm$ 1.07
EPA	89.88 $\pm$ 0.70
DCHA	90.04 $\pm$ 1.00

positive cells expressing CD3 receptors (Table 1). The results indicate that the inhibition of OKT3-induced sustained $[\text{Ca}^{2+}]_i$ by UFAs is not due to displacement of OKT3 antibodies from the CD3 receptors.

BSA reversal of OA-induced inhibition of the sustained $[\text{Ca}^{2+}]_i$ in JURKAT cells

When BSA (final concn. 0.2% w/v) was added to the cells after

Fig. 6. Effect of BSA on the inhibition of OKT3-induced sustained $[\text{Ca}^{2+}]_i$ increase by OA in JURKAT cells

Changes in $[\text{Ca}^{2+}]_i$ were monitored as described in the Materials and methods section. Where indicated, OKT3 (10 $\mu\text{g}/\text{ml}$), OA (3 μM), BSA (final concn. 0.2%, w/v) and TPA (100 ng/ml) were added to JURKAT cells in the cuvette. Only the trace for OA is shown; the other UFAs gave similar results.

OA had decreased $[\text{Ca}^{2+}]_i$ to basal levels, there was a sustained increase in $[\text{Ca}^{2+}]_i$ (Fig. 6). As observed previously (Fig. 2), TPA (100 ng/ml, added 5 min after BSA) decreased $[\text{Ca}^{2+}]_i$ to near basal levels. These results suggest that the fatty acids block Ca^{2+} entry by acting on the outside of the plasma membrane, and addition of BSA reverses the effects of UFAs by binding the fatty acids.

DISCUSSION

The effect of UFAs on receptor-mediated Ca^{2+} entry was studied by using the anti-CD3 antibody OKT3 to induce an increase in $[\text{Ca}^{2+}]_i$ and Ca^{2+} influx in a T-cell line. Changes in $[\text{Ca}^{2+}]_i$ were monitored in Fura-2-loaded JURKAT cells, and Ca^{2+} influx was detected by the quenching of Fura-2 fluorescence owing to concomitant influx of Mn^{2+} added to the extracellular medium. The present results provide evidence that UFAs block Ca^{2+} entry through receptor-operated channels. TPA induced results similar to those with the UFAs, and, since fatty acids have been reported to activate PKC (McPhail *et al.*, 1984; Murakami *et al.*, 1986), it was suggestive that the UFA-induced $[\text{Ca}^{2+}]_i$ decrease might operate through PKC. However, by using the PKC inhibitor H-7 (Fig. 2) and analysis of PKC-dependent phosphorylation of the 19 and 80 kDa marker substrates (Fig. 3) (Friedrich *et al.*, 1988; Alexander & Cantrell, 1989), the involvement of PKC activation was ruled out.

Recent studies (Takemura & Putney, 1989; Hughes *et al.*, 1988) have shown that atropine, a muscarinic-receptor antagonist, can displace metacholine from its receptor and rapidly shut down the Ca^{2+} influx. The effect of atropine appears to be due to a rapid decline in the cellular concentration of $\text{Ins}(1,4,5)\text{P}_3$. By flow-cytometry analysis (Schols *et al.*, 1989), UFAs were found not to interfere with the binding of the anti-CD3 antibodies to the CD3 receptors (Table 1). Therefore, UFA-induced decreased in $[\text{Ca}^{2+}]_i$ must be due to a mechanism other than displacement of OKT3 antibodies from the CD3 receptors.

Previous studies (Chow & Jondal, 1990; Kolesnick & Gershengorn, 1985) have shown that BSA, which has high affinity for fatty acids (Purdon & Rao, 1989), can abolish the Ca^{2+} -mobilizing effects of UFAs when added to cell suspensions before fatty acids. The present results show that BSA can also remove, and thereby reverse, the effect of UFAs after these fatty acids have decreased the OKT3-induced sustained $[\text{Ca}^{2+}]_i$ increase to basal levels (Fig. 6). This suggests that the UFAs exert their inhibitory effect on the outer membrane, presumably by binding to surface proteins such as the Ca^{2+} channel itself or some component(s) of the channel.

The dual effects of UFAs (i.e. Ca^{2+} release from internal stores and inhibition of Ca^{2+} influx) can be explained by the fact that UFAs are hydrophobic and have a high affinity for binding to proteins. When JURKAT cells are exposed to UFAs, the fatty acids will bind to proteins on the membrane surface, and some will permeate across the membrane into the cytosol. Once inside the cell, they induced Ca^{2+} release from the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pool. The mechanism involved is still undefined. Those fatty acids bound to the membrane surface proteins seem to affect the receptor-mediated Ca^{2+} channel and prevent Ca^{2+} entry. Studies by Chan & Turk (1987) demonstrated that AA inhibits the uptake of Ca^{2+} in liver microsomes. It is unlikely that the mechanism of inhibition by fatty acids is the same for both the microsomes and whole cells, since Ca^{2+} uptake in microsomes is ATP-dependent.

Compared with Ni^{2+} (5 mM), only 6 μM -UFA is required to block Ca^{2+} entry (Fig. 1). The time for $[\text{Ca}^{2+}]_i$ to reach a peak and then to return to basal levels is shorter in the presence of UFAs than in the presence of Ni^{2+} (Fig. 4). Of all the UFAs tested, only OA seems to be useful as a tool to study Ca^{2+} influx, since it does not mobilize Ca^{2+} in JURKAT cells. In contrast, other UFAs still mobilize intracellular Ca^{2+} at 3–6 μM (Chow & Jondal, 1990).

Previous studies of fatty acid-induced Ca^{2+} mobilization have only looked at Ca^{2+} efflux (Wolf *et al.*, 1986; Kolesnick *et al.*, 1984). Fatty acids such as AA have been shown to block agonist-induced $[\text{Ca}^{2+}]_i$ increase in platelets (Kowalska *et al.*, 1988) and GH3 pituitary cells (Kolesnick & Gershengorn, 1985); however, no attempt has been made to explain or to characterize the effect.

The present results demonstrate that UFAs can potentially inhibit the Ca^{2+} influx into cells. Such an effect would be expected to be harmful to cells, owing to disruption of normal Ca^{2+} homeostasis. Thus the present results support for a protective role for albumin in the plasma, i.e. providing a buffer for removing any non-esterified fatty acids which might affect the blood cells.

We thank Professor Sten Orrenius and Dr. S. K. Duddy for helpful advice and for reading the manuscript. This investigation was supported by grants from the Swedish Cancer Association and the Karolinska Institute.

REFERENCES

- Alexander, D. & Cantrell, D. A. (1989) *Immunol. Today* **10**, 200–205
- Berridge, M. J. (1986) in *Phosphoinositides and Receptor Mechanisms* (Putney, J. W., Jr., ed.), pp. 25–46, Alan R. Liss, New York
- Brattsand, G., Friedrich, B. & Gullberg, M. (1989) *Scand. J. Immunol.* **30**, 233–240
- Chan, K. M. & Turk, J. (1987) *Biochim. Biophys. Acta* **928**, 186–193
- Chow, S. C. & Jondal, M. (1990) *J. Biol. Chem.* **265**, 902–907
- Friedrich, B., Noreus, K., Cantrell, D. A. & Gullberg, M. (1988) *Immunobiology* **176**, 465–470
- Gelfand, E. W., Mills, G. B., Cheung, R. K., Lee, J. W. W. & Grinstein, S. (1987) *Immunol. Rev.* **95**, 59–87
- Gryniewicz, G., Boemie, M. & Tsien, R. J. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Hadden, J. W. (1988) *Mol. Immunol.* **25**, 1105–1112
- Hidaka, H., Inakagi, M., Kawamoto, S. & Sakai, Y. (1984) *Biochemistry* **23**, 5036–5041
- Hughes, A. R., Takemura, H. & Putney, J. W., Jr. (1988) *J. Biol. Chem.* **263**, 10314–10319
- Imboden, J. B. & Stobo, J. D. (1985) *J. Exp. Med.* **161**, 446–456
- Irvine, R. F., Letcher, A. J., Lander, D. J. & Berridge, M. J. (1986) *Biochem. J.* **240**, 301–304
- Kolesnick, R. N. & Gershengorn, M. C. (1985) *J. Biol. Chem.* **260**, 707–713
- Kolesnick, R. N., Musacchio, I., Thaw, C. & Gershengorn, M. C. (1984) *Am. J. Physiol.* **246**, E458–E462

- Kowalska, M. A., Rao, K. & Disa, J. (1988) *Biochem. J.* **253**, 255–262
- Kuno, N. & Gardner, P. (1987) *Nature (London)* **326**, 301–304
- McPhail, L. C., Clayton, C. C. & Snyderman, R. (1984) *Science* **224**, 622–624
- Merritt, J. E., Jacob, R. & Hallam, T. J. (1989) *J. Biol. Chem.* **264**, 1522–1527
- Murakami, K., Chan, S. Y. & Routtenberg, A. (1986) *J. Biol. Chem.* **261**, 15424–15429
- Nishikawa, M., Uemura, Y., Hidaka, H. & Shirikawa, S. (1986) *Life Sci.* **39**, 1101–1107
- Purdon, A. D. & Rao, A. K. (1989) *Prostaglandins, Leukotrienes Essent. Fatty Acids* **35**, 213–218
- Putney, J. W., Jr., Takemura, H., Hughes, A. R., Horstman, D. A. & Thastrup, O. (1989) *FASEB J.* **3**, 1899–1905
- Ruegg, U. T. & Burgess, G. M. (1989) *Trends Pharmacol. Sci.* **10**, 218–220
- Schols, D., Baba, M., Paywels, R., Desmyter, J. & Clercq, E. D. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3322–3326
- Takemura, H. & Putney, J. W., Jr. (1989) *J. Biol. Chem.* **258**, 409–421
- Treves, S., DiVirgilio, F., Cerundolo, V., Zanovello, P., Collavo, D. & Pozzan, T. (1987) *J. Exp. Med.* **166**, 23–32
- Watson, S. P., McNally, J., Shipman, L. J. & Godfrey, P. P. (1988) *Biochem. J.* **249**, 345–350
- Weiss, A., Imboden, J., Hardy, K., Manger, B., Terhorst, C. & Stobo, J. (1986) *Annu. Rev. Immunol.* **4**, 593–620
- Wolf, B. A., Turk, J., Sherman, W. R. & McDaniel, M. L. (1986) *J. Biol. Chem.* **261**, 3501–3511

Received 2 October 1989/30 November 1989; accepted 10 January 1990