Modulation of calcium fluxes in Jurkat T cells by myristic acid

Inhibition is independent of membrane potential and intracellular pH

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Treatment of T lymphocytes with mitogenic antibodies against the T-cell receptor/CD3 complex induces within seconds a rise in the concentration of intracellular free Ca^{2+} . We recently reported that free myristic acid, but not its methyl ester, inhibits both the anti-CD3-induced Ca^{2+} influx across the cell membrane and the Ca^{2+} release from intracellular stores in Jurkat T cells. Here we show that myristic acid induced a rapid hyperpolarization of the cell membrane potential and a decrease in intracellular pH in Jurkat cells. Lauric acid and palmitic acid caused minor hyperpolarization, whereas other saturated non-esterified fatty acids tested were without effect. Hyperpolarization of the membrane potential in Jurkat cells with valinomycin did not, however, inhibit the anti-CD3-induced Ca^{2+} signal, and the blocking effect on the Ca^{2+} signal in myristic acid-treated Jurkat cells was not reversed after normalization of the cell membrane potential by treatment with gramicidin. The inhibitory effect of myristic acid on the Ca^{2+} fluxes thus cannot be explained by changes in membrane potential. We also present evidence that the blocking effect of myristic acid on the receptor-operated Ca^{2+} flux is not due to the myristic acid-induced decrease in intracellular pH. Moreover, we demonstrate that myristic acid does not prevent the release of Ca^{2+} triggered by inositol 1,4,5-trisphosphate from intracellular pools in permeabilized cells. Our findings indicate that myristic acid blocks anti-CD3-induced Ca^{2+} traffic in Jurkat cells by interfering with the regulation of Ca^{2+} mobilization, apparently by blocking an early step in signal transduction from the T-cell-antigen receptor/CD3 complex.

INTRODUCTION

Rapid alterations in membrane potential [1,2], intracellular pH (pH_i) [3,4] and intracellular concentration of free Ca^{2+} ($[\text{Ca}^{2+}]_i$) [2,5] are among the earliest events associated with mitogen treatment of quiescent T cells. In particular, the increase in free cytoplasmic Ca^{2+} occurring after T-cell-antigen receptor stimulation with mitogenic lectins or monoclonal antibodies has been postulated to be important for the initiation of cell proliferation. This Ca^{2+} signal is composed of a release from intracellular stores and a sustained influx of Ca^{2+} through the cell membrane [6]. Ca^{2+} release from intracellular stores is generally believed to be mediated by the inositol trisphosphate (InsP_3) that is rapidly formed when phospholipase C is activated [7]. The nature and regulation of Ca^{2+} channels through which the influx occurs are still poorly understood.

In T cells, mitogenic ligands have been reported to depolarize [1] or hyperpolarize [2] the cell membrane potential, and changes in membrane potential has been reported to influence ligand-induced Ca^{2+} signalling [8,9]. However, the precise relationship between membrane potential and Ca^{2+} traffic remains elusive. In Jurkat T cells mitogen-induced Ca^{2+} influx has been shown to occur via a membrane-potential-sensitive (but not voltage-gated) transport system [9]. Thus, in contrast with voltage-dependent Ca^{2+} channels in neural and muscle cells, Ca^{2+} channels in T cells cannot be opened by membrane depolarization. However, already-open Ca^{2+} channels are rapidly closed by depolarization [9].

Unsaturated non-esterified fatty acids have been reported to modulate Ca^{2+} signals in T-cell lines [10,11]. We recently reported that non-esterified myristic acid, a saturated fatty acid, inhibits the OKT3-induced increase in $[\text{Ca}^{2+}]_i$ in Jurkat T cells [12]. Myristic acid inhibited both the OKT3-induced Ca^{2+} release from intracellular stores and the influx of Ca^{2+} through the cell

membrane. The inhibitory effect on the Ca^{2+} signal occurred within 1 min of treatment and was completely reversed by addition of fatty-acid-free albumin [12]. Other saturated fatty acids with shorter or longer carbon chains and the methyl ester of myristic acid had negligible effects on the Ca^{2+} signal.

Here we studied the effects of free myristic acid on the cell membrane potential and intracellular pH in Jurkat T cells. We also examine the possible relationship between these and the inhibition of Ca^{2+} mobilization by myristic acid and further elucidate the site of inhibition.

MATERIALS AND METHODS

Cells

Jurkat cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 5% (v/v) fetal-calf serum (Gibco).

Reagents

Fura-2 acetoxymethyl ester (fura-2/AM), bis-(1,3-diethylthiobarbiturate)trimethineoxonol (bis-oxonol) and bis(carboxyethyl)-carboxyfluorescein (BCECF) were from Molecular Probes (Eugene, OR, U.S.A.) and were dissolved in dimethyl sulphoxide and stored at -70°C until use. All fatty acids, ionophores and $\text{Ins}(1,4,5)\text{P}_3$ were from Sigma (St. Louis, MO, U.S.A.). OKT3, a monoclonal antibody against the CD3 ϵ -chain, was used as a 1:5000 dilution of ascites produced in Pristane-treated BALB/c mice by using hybridoma cells obtained from the American Type Culture Collection (Rockville, MD, U.S.A.).

Measurement of $[\text{Ca}^{2+}]_i$ with fura-2

$[\text{Ca}^{2+}]_i$ was measured essentially as described by Tsien *et al.* [2] by using the fluorescent Ca^{2+} indicator fura-2. The cells were

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; pH_i , intracellular pH; BCECF, bis(carboxyethyl)-carboxyfluorescein.

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loaded in 3 μM of the membrane-permeant fura-2/AM for 20 min. After washing, the cells were resuspended in 100 mM-NaCl/10 mM-Hepes/5.4 mM-KCl/1.2 mM-MgCl₂/0.44 mM-KH₂PO₄/4.2 mM-NaHCO₃/0.4 mM-CaCl₂/5 mM-glucose. Fluorescence was monitored at excitation wavelength 340 nm and emission wavelength 510 nm. During measurements the temperature of the cell suspension was kept at 37 °C. After each experiment the intracellularly trapped fura-2 was released with 1% Triton X-100, and fluorescence at 0% (F_{min}) and 100% (F_{max}) Ca²⁺ saturation was measured. [Ca²⁺]_i was calculated from the equation:

$$[\text{Ca}^{2+}]_i = K_d (F - F_{\text{min}}) / (F_{\text{max}} - F)$$

where K_d for fura-2 (at 37 °C) is 200 nM and F is the observed fluorescence from the intracellular fura-2.

Measurement of membrane potential

Membrane potential was monitored with the lipophilic fluorescent dye bis-oxonol as previously described [13].

Measurement of pH_i

pH_i was determined fluorimetrically by using BCECF as previously described [14]. Calibration of fluorescence versus pH_i was performed by the K⁺/nigericin method of Thomas *et al.* [15].

⁸⁶Rb-efflux assay

For the efflux assay, 30 × 10⁶ cells were loaded with 20 μCi of ⁸⁶Rb/ml in RPMI 1640 medium (containing 20 mM-Hepes) for 2 h at 37 °C. The cells were pelleted and washed once with RPMI medium. The cells were then suspended in RPMI medium (20 mM-Hepes), divided into batches and kept at 37 °C. After addition of the compounds to be tested, triplicate samples were withdrawn at indicated time points and pipetted into polyethylene micro-centrifuge tubes containing 20 μl of 1% HClO₄ in phosphate-buffered saline (137 mM-NaCl/8 mM-Na₂HPO₄/1.4 mM-KH₂PO₄/2.6 mM-KCl, pH 7.2) overlaid with 80 μl of oil (butyl phthalate/dinonyl phthalate, 3:6, v/v) and 100 μl of RPMI 1640. The tubes were immediately centrifuged at 15000 g for 1 min, frozen at -70 °C, and the tips of the tubes containing the cell pellet were cut off and counted for radioactivity in liquid-scintillation counter.

⁴⁵Ca²⁺-release experiments with permeabilized cells

For these, 60 × 10⁶ cells were loaded with 20 μCi of ⁴⁵Ca/ml in 10 ml of Ca²⁺-free Hanks Balanced Salt Solution, with 20 mM-Hepes, pH 7.2, for 40 min at room temperature. The cells were pelleted and suspended in 5 ml of intracellular buffer (20 mM-Hepes/20 mM-NaCl/100 mM-KCl/5 mM-MgSO₄/1 mM-EGTA/200 μM -CaCl₂). These concentrations of EGTA and CaCl₂ were calculated to give a 100 nM concentration of free Ca²⁺. To permeabilize the cells, saponin was added to 60 $\mu\text{g}/\text{ml}$ and the cells were incubated for 5 min. The cells were pelleted, resuspended in 3 ml of the intracellular buffer without saponin, and divided into 1 ml portions. After addition of the compounds to be tested, 150 μl samples were withdrawn at indicated time points, and pipetted on to Whatman no. 3 filter discs. The filters were washed with intracellular buffer twice and counted for radioactivity in a liquid-scintillation counter.

Uptake of myristic acid

This was measured by adding 0.7 μCi of [³H]myristic acid/ml to 2 × 10⁶/ml of Jurkat cells, removing samples at indicated times and counting the cells for radioactivity after three washes in myristic acid-free medium. Membrane and cytosol fractions were prepared as described previously [16].

RESULTS

Uptake of myristic acid: kinetics and subcellular distribution

Myristic acid was rapidly taken up by Jurkat cells. Equilibrium was reached within 3–4 min (results not shown). Subcellular fractionation of the cells showed that 44% of the labelled myristic acid was found in a membrane fraction, whereas 22% was cytosolic and 34% was found in a nuclear fraction. The last of these might also have contained some intact cells and large membrane fragments

Effects of myristic acid on the membrane potential

Changes in plasma-membrane potential of Jurkat T cells was monitored by using the fluorescent lipophilic dye bis-oxonol [13]. When Jurkat T cells, equilibrated with bis-oxonol, were treated with 20 μM -myristic acid, the detected fluorescence started to decrease after a lag of 5–10 s and reached a new lower plateau after approx. 1 min (Fig. 1a). This decline in fluorescence represents a hyperpolarization of the cell membrane. The methyl ester of myristic acid was without effect (Fig. 1b). Of the other saturated fatty acids tested, palmitic acid and lauric acid had minor effects (Figs. 1d and 1e), whereas decanoic acid and stearic acid were without effect (Figs. 1c and 1f). Thus myristic acid hyperpolarized the cell membrane within the 1 min preincubation required for inhibition of the OKT3-induced Ca²⁺ signal [12]. Again, the length of the carbon chain and the free carboxyl group seem to be crucial.

In comparison, when Jurkat cells were treated with OKT3 in the presence of bis-oxonol, the detected fluorescence increased slightly within seconds (Fig. 2a), indicating a barely detectable depolarization of the cell membrane. The Ca²⁺-mobilizing drug thapsigargin (2 μM) did not cause any significant alteration in membrane potential (Fig. 2b). OKT3 and thapsigargin at these concentrations caused similar increases in [Ca²⁺]_i in fura-2-loaded Jurkat cells (Figs. 2a and 2b). Thus Ca²⁺ mobilization does not itself cause any major change in membrane potential.

How does myristic acid cause hyperpolarization?

Three obvious mechanisms could be suggested for myristic acid-induced hyperpolarization: transport of negative charge

Fig. 1. Effect of saturated non-esterified fatty acids on the membrane potential in Jurkat T cells

Where indicated by arrows, bis-oxonol-pre-equilibrated Jurkat cells were treated with: (a) 20 μM -myristic acid (Myr); (b) 20 μM -myristic acid methyl ester (Myr-CH₃); (c) 20 μM -palmitic acid (Palm); (d) 20 μM -lauric acid (Lau); (e) 20 μM -decanoic acid (Dec); (f) 20 μM -stearic acid (Stea). Similar results were obtained in at least five independent experiments.

Fig. 2. Effect of OKT3 and thapsigargin on $[Ca^{2+}]_i$ and membrane potential in Jurkat T cells

Where indicated by arrows, fura-2-loaded (upper panels) or bis-oxonol-pre-equilibrated (lower panels) Jurkat cells were treated with (a) OKT3 or (b) $2 \mu M$ -thapsigargin (TG). One representative experiment, out of five conducted, is shown.

Fig. 3. Myristic acid does not cause ^{86}Rb efflux from Jurkat T cells

^{86}Rb -loaded cells were treated with medium (○), $20 \mu M$ -myristic acid (●) or $5 \mu g$ of nigericin/ml (▲). The results are given as percentage of radioactivity remaining in the cells. The data shown represent means $\pm$ S.D. of triplicate determinations from a single experiment. Similar results were obtained in three independent experiments.

into the cells, activation of the plasma-membrane Na^+/K^+ -ATPase, or stimulation of K^+ efflux. In fact, enhanced extrusion of K^+ has been reported to occur in cardiac cells and muscle cells in response to certain fatty acids, including myristic acid [17,18].

To study the effects of myristic acid on K^+ efflux, we performed experiments with the rubidium isotope ^{86}Rb , which behaves like potassium in biological systems. When Jurkat T cells were loaded with ^{86}Rb , washed and suspended in normal medium, they lost radioactivity at a slow basal rate. This rate was not affected by $20 \mu M$ -myristic acid, whereas $5 \mu g$ of nigericin (a known H^+/K^+ ionophore)/ml caused a very rapid efflux of the isotope (Fig. 3). Nigericin did not affect the basal efflux of $^{45}Ca^{2+}$ from similarly $^{45}Ca^{2+}$ -loaded Jurkat cells (results not shown).

These findings indicate that the hyperpolarization seen after the treatment with myristic acid is not caused by redistribution of K^+ ions. The possibility that myristic acid hyperpolarized the

Fig. 4. OKT3-induced increase in $[Ca^{2+}]_i$ in Jurkat cells in the presence of valinomycin-induced hyperpolarization

Where indicated by arrows, fura-2-loaded (upper panels) or bis-oxonol-pre-equilibrated (lower panels) cells were treated with (a) $1 \mu g$ of valinomycin (VAL)/ml followed by OKT3, or (b) OKT3 and 1.5 min later $1 \mu g$ of valinomycin/ml. One representative experiment, out of five conducted, is shown.

membrane potential by directly stimulating the Na^+/K^+ -ATPase pump at the cell membrane was excluded by rubidium-uptake experiments (results not shown).

Effect of changes in membrane potential on OKT3-induced Ca^{2+} mobilization

The impact of hyperpolarization on ligand-induced Ca^{2+} mobilization is controversial. In some lymphoid cells hyperpolarization seems to inhibit the receptor-triggered increase in $[Ca^{2+}]_i$ [19,20]. In contrast with these findings, Virgilio *et al.* [21] showed that hyperpolarization by valinomycin potentiated fMet-Leu-Phe-induced changes in $[Ca^{2+}]_i$ in human neutrophils. Sarkadi *et al.* [9] used Jurkat T cells and reported that hyperpolarization of the cell membrane by valinomycin did not influence the OKT3-induced Ca^{2+} signal.

To mimic the myristic acid-induced hyperpolarization we used valinomycin, a K^+ -carrying transport antibiotic that is known to hyperpolarize the cell membrane rapidly in various cell types [19,21]. In Jurkat cells $1 \mu g$ of valinomycin/ml caused a rapid hyperpolarization, comparable with that induced by $20 \mu M$ -myristic acid (Fig. 4a). Valinomycin, however, did not affect the OKT3-induced increase in $[Ca^{2+}]_i$ or block the ongoing influx of Ca^{2+} when added after OKT3 at a time when maximal $[Ca^{2+}]_i$ levels had already been reached (Figs. 4a and 4b).

Myristic acid blocks Ca^{2+} mobilization independently of its effects on membrane potential

To determine whether the myristic acid-induced hyperpolarization described above is causally related to the inhibition of the OKT3-induced elevation in $[Ca^{2+}]_i$, we used gramicidin to normalize the membrane potential in myristic acid-treated cells. Gramicidin alone, however, altered the Ca^{2+} response (Fig. 5a), as also described by Sarkadi *et al.* [9]. In the presence of gramicidin the OKT3-induced Ca^{2+} response was transient, suggesting that Ca^{2+} influx was blocked, but Ca^{2+} release from intracellular stores remained intact. Gramicidin rapidly terminated the ongoing Ca^{2+} influx maintaining the sustained increase in $[Ca^{2+}]_i$ after OKT3 stimulation (results not shown). In Na^+ -free medium ($NaCl$ replaced by choline chloride) gramicidin had no effect (results not shown).

When 100 nM -gramicidin was added to Jurkat cells previously hyperpolarized by $20 \mu M$ -myristic acid, the membrane potential

Fig. 5. Effects of membrane potential on the OKT3-induced increase in $[Ca^{2+}]_i$ in Jurkat cells

Fura-2-loaded (upper panels) or bis-oxonol-pre-equilibrated (lower panels) Jurkat cells were treated with: (a) 100 nM-gramicidin (GRAM) followed by OKT3; (b) 20 μ M-myristic acid (MYR) followed by 100 nM-gramicidin (titrated to neutralize the myristic acid-induced hyperpolarization exactly) and then OKT3; (c) same concentrations of myristic acid and gramicidin added simultaneously, followed by OKT3; (d) 1 μ g of valinomycin (VAL)/ml followed by 100 nM-gramicidin and then OKT3. One representative experiment, out of five conducted, is shown.

returned to normal values within 1 min (Fig. 5b). However, when fura-2-loaded Jurkat cells were treated with myristic acid and gramicidin and stimulated with OKT3, the Ca^{2+} signal was still completely blocked (Fig. 5b). When gramicidin and myristic acid were added simultaneously the membrane potential remained unchanged (Fig. 5c), but OKT3 was still unable to induce any elevation in $[Ca^{2+}]_i$ (Fig. 5c). In contrast, when the hyperpolarization caused by 1 μ g of valinomycin/ml was counteracted by addition of 100 nM-gramicidin, the OKT3-induced Ca^{2+} signal was identical with that seen in the presence of gramicidin alone (Fig. 5d).

Effects of myristic acid on pH_i

An early response of quiescent T cells stimulated with mitogens or phorbol esters is an increase in pH_i within minutes [22,23]. This alkalization is apparently caused by a protein kinase C-stimulated Na^+/H^+ exchanger [22], and seems to be required for entry into the S phase of the cell cycle [3].

The pH_i was measured with the fluorescent pH indicator BCECF. Jurkat cells display a pH_i of 7.4–7.5 (Fig. 6). This is higher than that found in quiescent T cells and similar to that seen in mitogen-activated normal T cells. Treatment of Jurkat cells with 20 μ M-myristic acid induced a decrease in pH_i from 7.4 to 7.1 within 1 min (Fig. 6a), and it remained at this level for at least 4 min. Myristic acid methyl ester or decanoic acid (20 μ M) did not induce any changes in pH_i (results not shown). Treatment with 50 ng of nigericin/ml, a known H^+/K^+ ionophore, induced a decrease in the pH_i of the same magnitude as 20 μ M-myristic acid (Fig. 6b).

Fig. 6. Effects of changes in pH_i on the OKT3-induced increase in $[Ca^{2+}]_i$ in Jurkat cells

Fura-2-loaded (upper panels) or bis-oxonol-pre-equilibrated (lower panels) Jurkat cells were treated with: (a) 20 μ M-myristic acid (MYR) followed by OKT3, (b) 50 ng of nigericin (NIG)/ml followed by OKT3; (c) 5 μ M-monensin (MON) followed by OKT3; (d) 20 μ M-myristic acid followed by 5 μ M-monensin and the OKT3. One representative experiment, out of five conducted, is shown.

The effect of myristic acid on intracellular pH_i is compatible with the notion that myristic acid is rapidly taken up by the cells, and suggests that protons are co-transported.

Effect of changes in pH_i on OKT3-induced Ca^{2+} fluxes in Jurkat T cells

To determine whether the myristic acid-induced decrease in pH_i might interfere with Ca^{2+} mobilization, we acidified the cytosol with nigericin at doses that mimicked the effect of 20 μ M-myristic acid. Addition of 50 ng of nigericin/ml to fura-2-loaded Jurkat cells neither altered the basal $[Ca^{2+}]_i$ nor affected the OKT3-induced Ca^{2+} signal (Fig. 6b). Likewise, elevation of pH_i by addition of monensin, a Na^+/H^+ exchanger, neither changed the basal $[Ca^{2+}]_i$ nor affected the OKT3-induced Ca^{2+} signal (Fig. 6c). The lower pH_i in myristic acid-treated Jurkat cells could be normalized by addition of 5 μ M-monensin (Fig. 6d). This treatment did not, however, reverse the blocking effect of myristic acid on the OKT3-induced Ca^{2+} signal (Fig. 6d). Apparently, the blocking effect of myristic acid on the Ca^{2+} signal cannot be attributed to the observed decrease in intracellular pH_i .

Effects of myristic acid on $InsP_3$ -induced Ca^{2+} release

We have previously reported [12] that myristic acid blocks OKT3-induced mobilization of Ca^{2+} from intracellular stores as well as the OKT3-induced Ca^{2+} influx. To determine whether the inhibition of intracellular release is caused by direct inhibition of $InsP_3$ -operated Ca^{2+} channels on intracellular Ca^{2+} stores, we tested the effects of myristic acid on the release of Ca^{2+} from $^{45}Ca^{2+}$ -loaded and permeabilized cells. When optimally 3 μ M- $InsP_3$ was added to the cells, approx. 25% of the total cell-associated $^{45}Ca^{2+}$ was liberated within 2 min (Fig. 7). Thereafter

Fig. 7. Effects of myristic acid on $^{45}\text{Ca}^{2+}$ release from saponin-permeabilized Jurkat T cells in response to InsP_3 .

$^{45}\text{Ca}^{2+}$ -loaded and subsequently saponin-permeabilized cells were treated with medium (○), 3 μM - InsP_3 (●), or 20 μM -myristic acid followed by 3 μM - InsP_3 (△). The results are given as percentages of radioactivity remaining in the cells. The data shown represent means $\pm$ S.D. of three separate experiments.

a slow re-uptake of released $^{45}\text{Ca}^{2+}$ occurred. Ca^{2+} ionophore A23187 (5 μM) released approx. 75 % of the total cell-associated $^{45}\text{Ca}^{2+}$ (results not shown). Thus InsP_3 was able to liberate approx. 30 % of the Ca^{2+} contained within intracellular organelles. Myristic acid (20 μM) added to the cells 1 min before the addition of InsP_3 had no effect on the subsequent InsP_3 -induced release of $^{45}\text{Ca}^{2+}$ (Fig. 7).

DISCUSSION

In a previous report we showed that free myristic acid inhibited the OKT3-induced increase in $[\text{Ca}^{2+}]_i$ in Jurkat T cells [12]. Myristic acid blocked both enhanced influx of Ca^{2+} from the external medium and the mobilization of Ca^{2+} from intracellular stores ('calciosomes'). The inhibitory effect of myristic acid was seen less than 1 min after addition, and the inhibition was completely reversed by treating cells with fatty-acid-free albumin [12]. The unsaturated fatty acid oleic acid had similar effects on Ca^{2+} mobilization in Jurkat cells (T. Nordström, J. Mustelin, T. Pessa-Morikawa & L. C. Andersson, unpublished work). Casabiell *et al.* [24] recently reported that oleic acid inhibits epidermal-growth-factor-triggered Ca^{2+} mobilization, and they also showed that the fatty acid blocked growth-factor-induced production of InsP_3 . In our previous study [12], we measured inositol phosphate formation 15 min after addition of stimulus and found it to be unaffected by myristic acid. We cannot, however, exclude the possibility that production of InsP_3 was reduced at earlier time points. In fact, Richieri & Kleinfeld [10] found that oleic acid inhibited inositol phosphate formation at 2 min, but not at 15 min. Our finding that myristic acid did not block mobilization of Ca^{2+} from intracellular stores by the endoplasmic-reticulum Ca^{2+} -ATPase inhibitor thapsigargin [12] indicates that this intracellular pool of Ca^{2+} is intact. In addition, the lack of effect of myristic acid on the InsP_3 -mediated release of Ca^{2+} from this pool (Fig. 7) indicates that the function of the Ca^{2+} channel on this organelle is unaffected by myristic acid. Since addition of myristic acid prevents subsequent OKT3-induced Ca^{2+} release from this pool in fura-2-loaded cells, it seems likely that the initial production of InsP_3 is insufficient, as suggested by Casabiell *et al.* [24] and Richieri & Kleinfeld [10].

We have also found that myristic acid within 1 min both hyperpolarizes the cell membrane potential and decreases pH_i in Jurkat T cells, but that neither of these effects is causally related

to its effect on Ca^{2+} mobilization. Thus, even if myristic acid rapidly both blocks the Ca^{2+} signal and hyperpolarizes the cell membrane, our results demonstrate that hyperpolarization in itself is not responsible for inhibition of Ca^{2+} signal. First, concentrations of valinomycin that caused a similar hyperpolarization did not impair the OKT3-induced Ca^{2+} signal. Second, when the membrane potential in myristic acid-treated cells was normalized by gramicidin, the Ca^{2+} response was still blocked. In contrast with gramicidin, the inhibitory effect of myristic acid was unchanged when the extracellular Na^+ was replaced by choline⁺ (results not shown). Thus the effect of gramicidin on Ca^{2+} influx, but not on intracellular Ca^{2+} release, is dependent on extracellular Na^+ , whereas myristic acid blocked both events independently of Na^+ .

The rapid effects of myristic acid on membrane potential and pH_i suggest that the fatty acid is rapidly taken up by the cells. This was confirmed by direct measurements. Interestingly, a large proportion of labelled myristic acid was recovered in a membrane fraction of the cells. These findings are compatible with the possibility that myristic acid exerts its biological effects at an intracellular membrane-bound location, such as the cytoplasmic face of the plasma membrane. If the acid is taken up in its protonated form [25], this could cause the intracellular acidification.

In conclusion, our data indicate that myristic acid blocks Ca^{2+} mobilization from intracellular stores and the external medium, not by directly blocking Ca^{2+} channels, but by interfering with signal transduction from the T-cell-antigen receptor/CD3 complex at a step before opening of these channels. We have excluded hyperpolarization and intracellular acidification as the mechanism for this inhibition. Apparently, myristic acid directly blocks a critical second-messenger pathway regulating InsP_3 formation and Ca^{2+} fluxes, such as tyrosine phosphorylation of phospholipase C [16] or some other regulatory enzyme.

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