CoA and fatty acyl-CoA derivatives mobilize calcium from a liver reticular pool

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The effect of CoA and fatty acyl-CoA esters on Ca^{2+} fluxes has been studied in isolated liver microsomes and in digitonin-permeabilized hepatocytes. When microsomes were loaded with increasing concentrations of Ca^{2+} (6–29 nmol/mg of protein), the extent to which CoA and palmitoyl-CoA released Ca^{2+} increased. At 23 nmol of Ca^{2+}/mg of protein, half-maximal [CoA] and [palmitoyl-CoA] were 35 and 50 μM respectively. Under conditions of minimal Ca^{2+} loading, net release of Ca^{2+} was absent, but Ca^{2+} translocation from a CoA-sensitive to a CoA-insensitive pool took place. The effect of CoA required the presence of fatty acids, probably to form fatty acyl esters. In permeabilized hepatocytes, the pool(s) mobilized by CoA (or by palmitoyl-CoA) appeared to be different from that mobilized by Ins(1,4,5)P_3.

INTRODUCTION

Acyl-CoA esters are potent modulators of the activity of enzymes, receptors and transporters [1], including glucokinase [2,3], protein kinase C [4,5], adenine nucleotide translocase [6], carnitine palmitoyltransferase I [7] and the nuclear thyroid hormone receptor [8]. Fatty acyl-CoA derivatives are required for the acylation of both resident [9] and secreted [10] proteins of the endoplasmic reticulum (ER), a reaction that appears to play a role in intracellular membrane trafficking [11,12].

Three recent reports indicate that acyl-CoA derivatives are involved also in the intracellular handling of Ca^{2+}. Deeney et al. [13] showed that long-chain acyl-CoA derivatives stimulate ATP-dependent Ca^{2+} accumulation in a reticular pool of clonal β-cells. Comerford and Dawson [14] showed that CoA (and its fatty acyl derivatives) suppresses GTP-induced Ca^{2+} release from liver microsomes. Fulceri et al. [15] showed that palmitoyl-CoA releases Ca^{2+} selectively from the terminal cisternae subfraction of sarcoplasmic reticulum. In an extension of our work on the control of Ca^{2+} uptake and release by liver microsomes [16–18], we initiated a programme of experiments to determine the extent to which acyl-CoA derivatives and CoA might influence Ca^{2+} uptake and release in liver microsomes.

We show that CoA and its fatty acyl derivatives do mobilize Ca^{2+} from a liver intracellular (reticular) pool which appears to be insensitive to the action of the second messenger Ins(1,4,5)P_3.

EXPERIMENTAL

Materials

ATP, phosphocreatine, creatine kinase (Type III), CoA, acyl-CoA esters, thapsigargin, Fluo 3 (free acid), A23187 and digitonin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Collagenase, fatty-acid-free BSA and Ins(1,4,5)P_3 were obtained from Boehringer, Mannheim, Germany. All other chemicals were of analytical grade. Ca^{2+} electrodes were purchased from Ionetics Inc., Palo Alto, CA, USA. Male Sprague–Dawley rats weighing 180–220 g were purchased from Nossan, Milan, Italy.

Preparation of liver microsomal fractions and digitonin-permeabilized hepatocytes

Rat liver microsomes were prepared as previously reported [17]. Rough and smooth microsomal subfractions were prepared by centrifugation on discontinuous sucrose gradients [16]. The microsomal fractions were resuspended (approx. 100 mg of protein/ml) in a medium of the following composition (mM): KCl, 100; NaCl, 20; $MgCl_2$, 3.5; Mops, 20, pH 7.2. The suspensions were frozen and maintained under liquid N_2 until used.

Hepatocytes were isolated by collagenase perfusion as reported previously [19]. Cells were permeabilized by incubation with digitonin (80 μg/ml, 5 min at 37 °C) in the KCl/Mops medium as above, containing 5 mM NaN_3. After permeabilization, cells were washed and resuspended (approx. 300 mg of protein/ml) in the KCl/Mops containing 5 mM NaN_3. Permeabilized cells were maintained at 0–4 °C and used within 3 h.

Measurement of Ca^{2+} fluxes with the Ca^{2+} electrode

Microsomes (2 mg of protein/ml) or permeabilized cells (6–8 mg of protein/ml) were incubated in a thermostatistically controlled (37 °C) Plexiglass vessel in which a Ca^{2+} electrode and a reference electrode (Radiometer K4040) were immersed. The incubation medium (volume 1 ml) consisted of (mM): KCl, 100; NaCl, 20; $MgCl_2$, 3.5; Mops, 20 (pH 7.2); ATP, 3; phosphocreatine, 10; NaN_3, 5. Creatine kinase (10 units/ml) was also present. The amount of Ca^{2+} present in the incubation medium (as a contaminant of routine solutions) ranged between 10 and 15 nmol/ml as measured by atomic-absorption spectroscopy. Different amounts of $CaCl_2$ (up to 50 nmol/ml) were added to the medium in order to load microsomal vesicles with different amounts of Ca^{2+}. The amount of Ca^{2+} accumulated or released by various agents was quantified by adding several pulses of $CaCl_2$ (5 μM each) to parallel reaction media. By using trace amounts of $^{45}Ca^{2+}$, it was verified that free Ca^{2+} variations were indeed reflecting Ca^{2+} movements under any of the experimental circumstances used. The Ca^{2+} electrodes were calibrated as described by others [20]; in any experiment, the Ca^{2+} electrode used gave

Abbreviation used: ER, endoplasmic reticulum.

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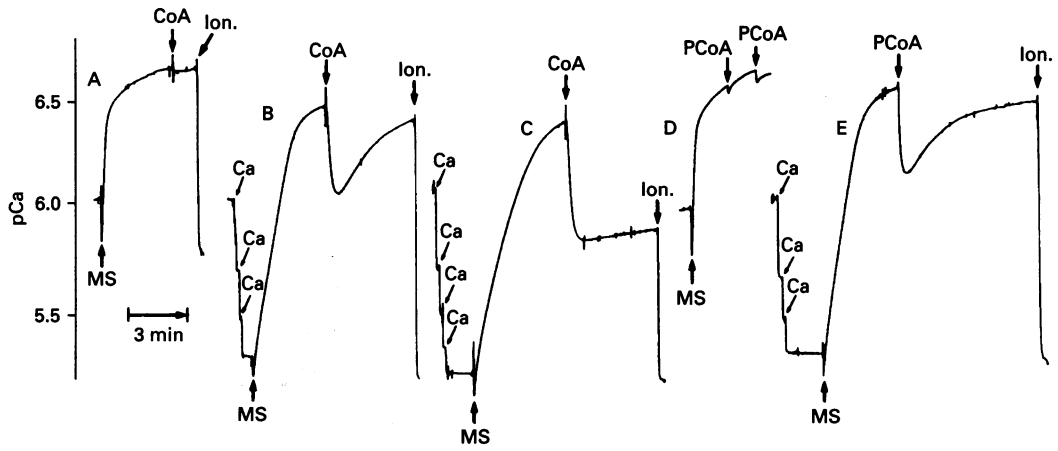

Figure 1 Ca^{2+}-releasing effect of CoA and of palmitoyl-CoA in liver microsomes loaded with different concentrations of Ca^{2+}

The volume of the reaction mixture was 1 ml; different concentrations of Ca^{2+} (Ca, 10 nmol for each arrow) were added and the incubation was started by adding 2 mg of microsomal protein (MS). Ca^{2+} fluxes were measured with a Ca^{2+} electrode as detailed in the Experimental section. CoA and pamitoyl-CoA (PCoA) were added at a final concentration of 50 μM. Microsomes were loaded with 6, 23, 29, 7 and 24 nmol of Ca^{2+}/mg of protein in traces A, B, C, D and E respectively. Other addition: Ion., 3 μM A23187. Traces are representative of numerous experiments undertaken.

linear mV responses as the pCa value of the medium was gradually varied between 7 and 5. The Ca^{2+} electrode mV signal (via a pH/ion meter model ION 83; Radiometer) was fed to MacLab hardware (AD Instruments) equipped with a computer and Chart v3.2.5. software (Macintosh). Continuous traces (mV versus time) were observed on the monitor and stored for printing and calculations.

Measurement of Ca^{2+} fluxes with the Ca^{2+} indicator Fluo 3

The incubation medium (composed as above) also included 1 μM Fluo 3 (free acid) and microsomes were added at a final concentration of 1 mg of protein/ml. Fluo 3 fluorescence was measured in a Perkin–Elmer model LS-3B fluorimeter (excitation wavelength 506 nm, emission wavelength 526 nm) equipped with a temperature-controlled cuvette holder (37 °C) and a magnetic stirrer. Under the present experimental conditions a K_d of 680 nM (at 37 °C) for the Fluo 3–Ca^{2+} complex was determined by using the Ca^{2+} electrode. Free Ca^{2+} concentrations of the assay medium were determined on the basis of this K_d value. The fluorescence mV signal was fed to MacLab hardware (see above), and continuous traces (mV versus time) were observed on the computer monitor and stored for printing and calculations.

Other assays

Ca^{2+}- and Mg^{2+}-dependent-ATPase activities of liver microsomes were measured as reported in detail elsewhere [21]. Protein was determined as reported by others [22], with BSA as standard.

RESULTS

CoA and fatty acyl-CoA derivatives release Ca^{2+} from liver microsomes

Figure 1 shows the Ca^{2+}-releasing effect of CoA (50 μM) in liver microsomes preloaded with different amounts of Ca^{2+} in the presence of MgATP. At the lower concentration of loaded Ca^{2+} (6 nmol/mg of protein; trace A), CoA did not release appreciable amounts of this ion; the releasing effect became more evident, however, as the microsomal Ca^{2+} load was increased (to 23 and 29 nmol/mg of protein; traces B and C respectively). Similarly to CoA, fatty acyl-CoA derivatives (palmitoyl-CoA, 50 μM) released Ca^{2+} from microsomes loaded with relatively high amounts of Ca^{2+} (Figure 1, trace E), whereas it released little Ca^{2+} in microsomes loaded with low amounts of Ca^{2+} (Figure 1, trace D).

Ca^{2+} released by CoA or palmitoyl-CoA was re-accumulated (see, e.g., Figure 1, traces B and E), provided that microsomes were pre-loaded with Ca^{2+} concentrations lower than their maximal capacity (as verified by the further uptake of Ca^{2+} added at the steady state). In microsomes loaded with Ca^{2+} concentrations up to their maximal loading capacity, CoA (see, e.g., Figure 1, trace C) or palmitoyl-CoA (results not shown) caused a release of Ca^{2+} which was followed by little or no re-uptake.

The lack of Ca^{2+} release by either CoA and/or its palmitoyl ester in microsomes loaded with low Ca^{2+} amounts (far below their maximal capacity) could be attributable to a translocation of Ca^{2+} from a CoA-sensitive to a CoA-insensitive pool, without any net Ca^{2+} release into the external medium. The following observations support this possibility. (1) The rate of Ca^{2+} efflux after the inhibition by thapsigargin of Ca^{2+}-ATPase(s) ($t_{\frac{1}{2}} = 94 \pm 10$ s, mean $\pm$ S.D., $n = 3$; e.g. Figure 2a, trace A) was increased by the co-addition of CoA (or palmitoyl-CoA; results not shown) and of thapsigargin to microsomes ($t_{\frac{1}{2}} = 34 \pm 3$ s; e.g. Figure 2a, trace B). On the other hand, in microsomes pre-treated with CoA for a time presumably sufficient to allow Ca^{2+} translocation into CoA-insensitive vesicles, the rate of Ca^{2+} release by thapsigargin was again similar to that in control microsomes ($t_{\frac{1}{2}} = 84 \pm 6$ s; e.g. Figure 2a, trace C). (2) Microsomes loaded with low Ca^{2+} amounts and pre-treated with CoA (or palmitoyl-CoA; results not shown) exhibited a lower Ca^{2+} loading capacity after the addition of Ca^{2+} pulses (Figure 2b, trace B) as compared with control microsomes (Figure 2b, trace A). (3) The rate of Ca^{2+} re-uptake after Ca^{2+} release induced by CoA (and palmitoyl-CoA) became progressively slower as the microsomes were pre-loaded with increasing amounts of Ca^{2+} (see Figure 1).

The dose-dependence of the extent of CoA- and palmitoyl-CoA-induced microsomal Ca^{2+} release is shown in Figure 3. In these experiments, microsomes were loaded with Ca^{2+} concentra-

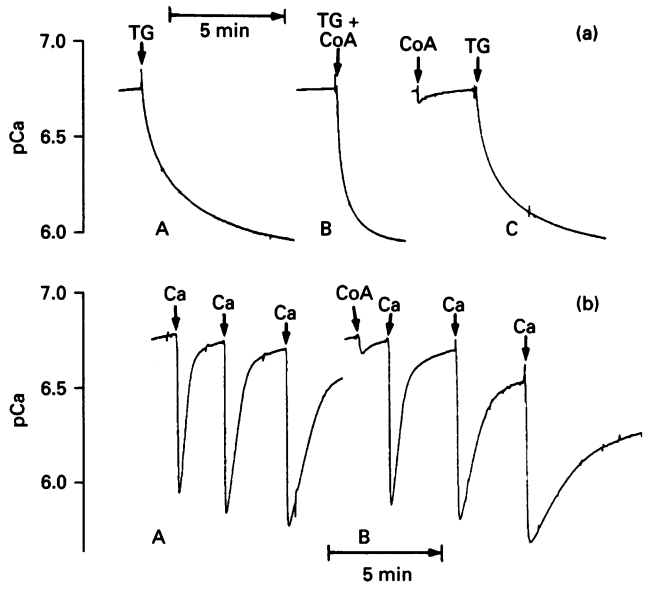

Figure 2 Thapsigargin-induced Ca^{2+} efflux rate (a) and steady-state Ca^{2+} uptake after pulse Ca^{2+} addition (b) in 'low-Ca^{2+}-loaded' microsomes treated with CoA

Experimental conditions were as reported in the legend to Figure 1. Liver microsomes were loaded with approx. 6 nmol of Ca^{2+}/mg of protein. Ca^{2+} fluxes were measured with the Ca^{2+} electrode as detailed in the Experimental section. At steady-state microsomal Ca^{2+} accumulation, CoA, thapsigargin (TG) and Ca^{2+} (Ca) were added (arrows) at final concentrations of 50 μM, 10 μM and 10 μM respectively. Traces are representative of 3–4 experiments undertaken.

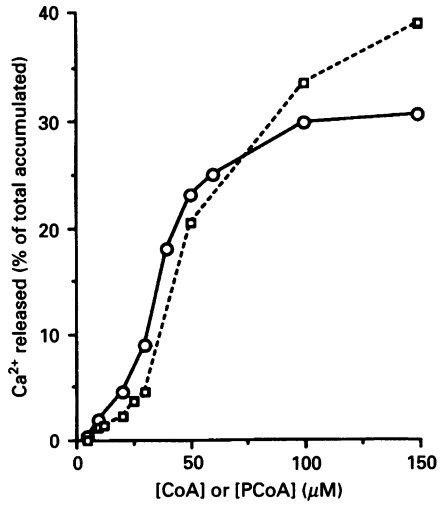

Figure 3 Dose-dependence of Ca^{2+} release induced by CoA and by palmitoyl-CoA

Microsomes were loaded with approx. 23 nmol of Ca^{2+}, and CoA (○) or palmitoyl-CoA (PCoA; □) was added at the indicated concentrations. Ca^{2+} release was quantified as detailed in the Experimental section by measuring extravesicular free [Ca^{2+}] with the Ca^{2+} electrode. Data are means of 3 independent experiments; S.E.M. values were omitted for clarity, but 15% of the mean value was never exceeded.

tions of approx. 23 nmol/mg of protein (e.g. Figure 1, traces B and E). Half-maximal and maximal effects were observed at CoA concentrations of approx. 35 μM and 100 μM respectively. The dose–response curve for palmitoyl-CoA was similar to that for

Table 1 Effect of various concentrations of palmitoyl-CoA and of 50 μM oleoyl-CoA on Ca^{2+}-ATPase activity of liver microsomes

The composition of the reaction mixture was as detailed elsewhere [21] and included 2 mg of microsomal protein/ml. Microsomes were preincubated for 1 min at 37 °C in the presence of the various compounds before adding ATP (1 mM) to start the reaction. Ca^{2+}-ATPase activity was calculated by subtracting ATP hydrolysis measured in the absence of added Ca^{2+} (in the presence of 1 mM EGTA) from ATP hydrolysis measured in the presence of 50 μM added Ca^{2+} (in the absence of EGTA). Data are means of two or means ± S.D. of three independent experiments.

Treatment	Ca^{2+}-dependent ATP hydrolysis (μmol/10 min per mg of protein)
None	120.2 ± 22.1
50 μM palmitoyl-CoA	121.5
50 μM oleoyl-CoA	124.3
100 μM palmitoyl-CoA	118.5 ± 16.8
150 μM palmitoyl-CoA	46.5
200 μM palmitoyl-CoA	13.5 ± 6.0
10 μM thapsigargin	4.5 ± 2.3

CoA up to approx. 50 μM. At concentrations equal to or higher than 100 μM, palmitoyl-CoA appeared to release greater amounts of Ca^{2+} than of CoA. In parallel experiments we verified that [palmitoyl-CoA] higher than 100 μM caused a slow progressive Ca^{2+} release even from microsomes loaded with low Ca^{2+} concentrations (results not shown), and inhibited microsomal Ca^{2+}-dependent ATPase activity (Table 1). Indeed, concentrations of fatty acyl-CoA derivatives higher than 50 μM might result in micelle formation or detergent action [13].

The rapid kinetics of CoA- and palmitoyl-CoA-induced Ca^{2+} release were investigated by measuring variations in free [Ca^{2+}] of the system with the Ca^{2+} indicator Fluo 3 [14,15,23] to avoid the possible delay in the response of the Ca^{2+} electrode to rapid free [Ca^{2+}] variations [15]. At palmitoyl-CoA concentrations between 10 and 50 μM, the rapid phase of Ca^{2+} release was accomplished within approx. 2.0 s (e.g. Figure 4, traces C and D). The rate of Ca^{2+} release induced by CoA was slower (Figure 4, traces A and B); after the addition of 50 μM CoA (Figure 4, trace B), $t_{\frac{1}{2}}$ was 10 ± 3 s (mean ± S.D., $n = 3$). The rate of Ca^{2+} efflux after thapsigargin (at a supramaximal concentration, 10 μM) is also shown in Figure 4, trace F; $t_{\frac{1}{2}}$ was 84 ± 8 s (mean ± S.D., $n = 3$).

The ability of fatty acyl-CoA derivatives of varying chain length (50 μM each) to release Ca^{2+} is shown in Table 2. Palmitoyl- and oleoyl-CoA exhibited the highest activity; fatty acyl derivatives whose acyl chain was shorter or longer than 16 carbon atoms appeared to have a relatively lower activity. Other acyl-CoA derivatives tested with acyl carbon number lower than 10 (namely acetyl-CoA, valeryl-CoA, isovaleryl-CoA and octanoyl-CoA) gave little or no Ca^{2+}-releasing activity.

CoA-induced microsomal Ca^{2+} release depends on non-esterified fatty acids

As liver microsomes contain a MgATP-dependent long-chain fatty acyl-CoA ligase activity(ies) [24], the possibility was evaluated that CoA acts via formation of fatty acyl-CoA esters. In an initial set of experiments, we attempted to remove non-esterfied fatty acids embedded in the microsomal membrane by washing the microsomal fraction twice with the usual medium but containing fatty-acid-free BSA (3%, w/v). After this procedure, the extent of Ca^{2+} release induced by CoA (50 μM) was decreased by more than 50% as compared with control microsomes (Figure 5, traces B and A). In microsomes washed with fatty-acid-free

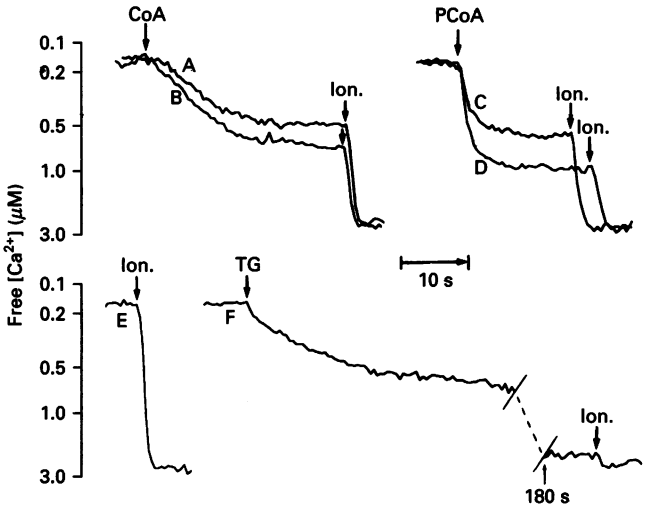

Figure 4 Time course of Ca^{2+} efflux induced by CoA, palmitoyl-CoA and thapsigargin measured with the Ca^{2+} indicator Fluo 3

The volume of the reaction mixture was 2 ml; 7.5 μM Ca^{2+} and 1.0 μM Fluo 3 (free acid) were added, and the incubation was started by adding 2 mg of microsomal protein. Ca^{2+} fluxes were evaluated by monitoring Fluo 3 fluorescence emission at 526 nm (excitation at 506 nm) as detailed in the Experimental section. At steady-state microsomal Ca^{2+} accumulation the various Ca^{2+} dischargers were added (arrows). CoA was at 20 and 50 μM (final concn.) in traces A and B respectively. Palmitoyl-CoA (PCoA) was at 25 and 50 μM in traces C and D respectively. Thapsigargin (TG) and A23187 (Ion.) were 10 μM and 3 μM respectively. Steady-state microsomal Ca^{2+} loading values were approx. 22 nmol of Ca^{2+}/mg of protein. Traces are representative of 3–4 experiments undertaken.

BSA, the addition of palmitic acid (100 μM) fully restored the Ca^{2+}-releasing effect of subsequently added CoA (50 μM; Figure 5, trace C). In additional experiments it was observed that the effect of CoA was prevented by the inclusion of fatty-acid-free BSA (1–0.3%) in the incubation mixture. In the presence of 0.3% BSA, CoA released little Ca^{2+} (Figure 5, trace D; consider the log scale), and the previous addition of palmitic acid (100 μM) resulted in a maximal release of Ca^{2+} by CoA (Figure 5, trace E).

Identity of CoA- and palmitoyl-CoA-sensitive Ca^{2+} pool

The possibility that CoA and its fatty acyl derivatives mobilize Ca^{2+} from the $InsP_3$-sensitive pool was investigated in permeabilized hepatocytes loaded with Ca^{2+} in the presence of MgATP. Similarly to isolated microsomes, permeabilized cells released Ca^{2+} after addition of μM concentrations of CoA (25 μM; Figure 6) and palmitoyl-CoA (10–50 μM; results not shown), provided that cells were loaded with Ca^{2+} near their maximal loading capacity. Under these experimental conditions, the Ca^{2+}-releasing activity of CoA and of $InsP_3$ appeared not to affect each other. Indeed, similar amounts of Ca^{2+} were mobilized by $InsP_3$ before or after the CoA-induced Ca^{2+} release was completed, and vice versa (Figure 6, traces A and B).

In cells loaded with Ca^{2+} below their maximal loading capacity, CoA did not release appreciable amounts of Ca^{2+}, but enlarged the $InsP_3$-releasable Ca^{2+} pool (Figure 6, traces C and D). As a reasonable explanation, CoA has probably allowed the translocation of Ca^{2+} from a CoA-sensitive (and $InsP_3$-insensitive) Ca^{2+} pool to a CoA-insensitive (but $InsP_3$-sensitive) one, provided that the latter has not been loaded with Ca^{2+} to its maximal capacity. Actually, the extent of $InsP_3$-induced Ca^{2+} release was increased by increasing the cellular Ca^{2+} loading with higher Ca^{2+} concentrations (Figure 6, compare trace C with trace A).

In the above experiments, mitochondrial Ca^{2+} storage was inhibited by including in the media 5 mM NaN_3 (or 20 μM antimycin A plus 10 μg/ml oligomycin in some experiments). Additional experiments in the absence of mitochondrial inhib-

Table 2 Ca^{2+}-releasing effect of various fatty acyl-CoA derivatives in liver microsomes

Experimental conditions were as reported in the legend to Figure 1. Liver microsomes were loaded with approx. 23 nmol of Ca^{2+}/mg of protein, and the various CoA esters were added at a final concentration of 50 μM. Data are means ± S.D. for the numbers of experiments shown in parentheses.

Fatty acyl ester	Ca^{2+} released (% of total accumulated)
Myristoyl-CoA	12.6 + 2.7 (3)
Lauroyl-CoA	14.0 ± 4.2 (3)
Palmitoyl-CoA	19.4 ± 2.3 (5)
Stearoyl-CoA	12.4 ± 2.2 (4)
Oleoyl-CoA	18.8 ± 4.0 (4)
Behenoyl-CoA	5.4 ± 1.7 (3)

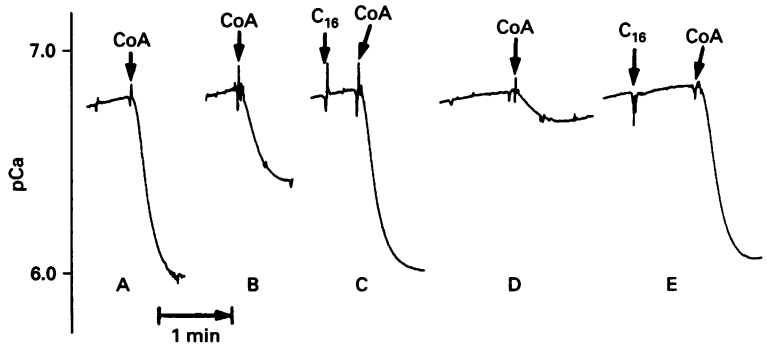

Figure 5 CoA-induced Ca^{2+} release is decreased in microsomes washed with, or incubated in the presence of, fatty-acid-free BSA

Experimental conditions were as reported in the legend to Figure 1 and liver microsomes were loaded with approx. 23 nmol of Ca^{2+}/mg of protein. In traces B and C, microsomes were washed (twice) with the usual medium but including 3% (w/v) fatty-acid-free BSA. In traces D and E, 0.3% fatty-acid-free BSA was included in the incubation mixture. At steady-state microsomal Ca^{2+} accumulation, CoA and palmitic acid (C_{16}) were added (arrows) at final concentrations of 50 μM and 100 μM respectively. Traces are representative of 3–4 experiments undertaken.

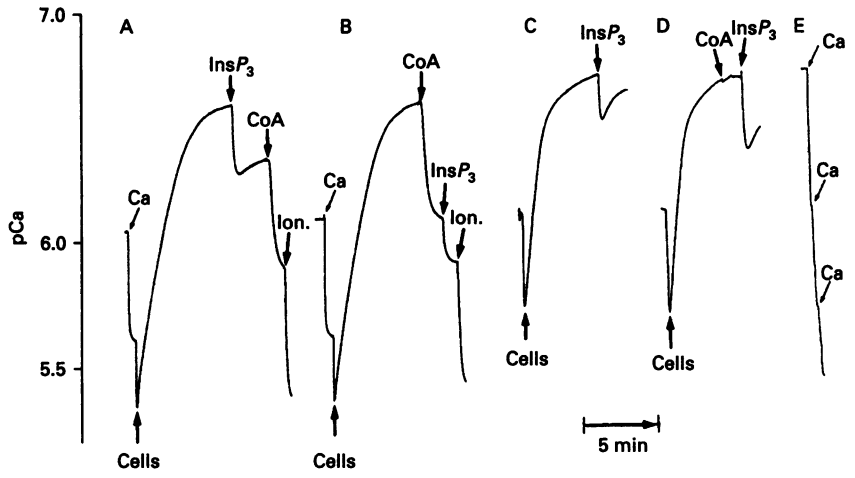

Figure 6 Ca^{2+} release induced by CoA and $InsP_3$ in digitonin-permeabilized isolated hepatocytes

Experimental conditions were as reported in the legend to Figure 1. Cells (6 mg of protein/ml) were loaded with approx. 5.5 nmol of Ca^{2+}/mg of protein in traces A, B and E, and with approx. 3 nmol of Ca^{2+}/mg of protein in traces C and D. The Ca^{2+}-uptake phase was omitted for clarity in trace E. CoA, $InsP_3$, Ca^{2+} (Ca) and A23187 (Ion.) were added (arrows) at final concentrations of 25 μM, 2 μM, 10 μM and 2 μM respectively. Traces are representative of 4 experiments undertaken.

itors revealed that CoA and palmitoyl-CoA were unable to release mitochondrial Ca^{2+}. In fact, both agents at concentrations up to 100 μM did not release any Ca^{2+} from permeabilized cells which had been previously loaded with Ca^{2+} in the presence of MgATP and subsequently allowed to discharge non-mitochondrial (reticular) Ca^{2+} upon addition of thapsigargin (5 μM) until the mitochondrial Ca^{2+}-buffering set point (approx. 0.5 μM) was reached (results not shown).

Finally, the possibility that the ER is the organelle which comprises the CoA-sensitive Ca^{2+} pool(s) was further investigated. In this respect, CoA (and palmitoyl-CoA) released Ca^{2+} from purified rough and smooth reticular preparations (results not shown). Moreover, the increase in the Ca^{2+}-loading capacity of microsomes and permeabilized cells by including glucose 6-phosphate (0.5 mM) in the incubation medium led to an increase in the CoA-releasable Ca^{2+} pool (results not shown). Since the enlargement of the liver microsomal Ca^{2+}-loading capacity by glucose 6-phosphate requires the hydrolysis of the hexose phosphate [25], the presence of the ER marker enzyme glucose-6-phosphatase [26] in the pool(s) responsive to CoA can be envisaged.

DISCUSSION

The major finding of the present work is that CoA and its fatty acyl derivatives are able to mobilize Ca^{2+} from liver microsomes and from the non-mitochondrial (reticular) compartment of permeabilized hepatocytes. Evidence is also presented that CoA acts via the formation of fatty acyl esters.

Fatty acyl-CoA esters have been shown to affect a number of cellular functions [1–13]; however, only a few reports indicate an effect on cellular Ca^{2+} fluxes. A very recent report [14] showed that CoA and its fatty acyl esters suppressed GTP-induced Ca^{2+} release and enlarged the size of the $InsP_3$-mobilizable Ca^{2+} pool, but did not release Ca^{2+} from liver microsomes. The observed lack of Ca^{2+} release was probably due to the use of microsomes loaded with low Ca^{2+} concentrations (approx. 5–7 nmol/mg of protein; data from [14]). At similar values of microsomal Ca^{2+} loading (6–7 nmol/mg of protein), we also observed little or no Ca^{2+} release in the external medium after addition of CoA and palmitoyl-CoA (Figure 1). Moreover, in permeabilized cells (loaded with relatively low Ca^{2+} concentrations) CoA caused no Ca^{2+} release, but enlarged the size of the $InsP_3$-sensitive pool (Figure 6). It is probable that, under circumstances of relatively low reticular Ca^{2+} loading, CoA (and palmitoyl-CoA) favoured Ca^{2+} translocation from an $InsP_3$-insensitive into an $InsP_3$-sensitive pool. This translocation appeared to be due to the simultaneous re-uptake by pool(s) insensitive to CoA (but sensitive to $InsP_3$) of the CoA-released Ca^{2+}, with no or little release of the ion to the ambient medium. Actually, under experimental conditions of suppression of ATP-driven microsomal Ca^{2+} uptake (by thapsigargin), CoA (and palmitoyl-CoA) were able to mobilize Ca^{2+} even from microsomes loaded with low Ca^{2+} concentrations (Figure 2). In microsomes (or cells) loaded with relatively high Ca^{2+} concentrations (up to their maximal capacity) the Ca^{2+}-releasing effect of CoA (and palmitoyl-CoA) was maximal and followed by little or no re-uptake of released Ca^{2+} (Figure 1, trace C). Under these experimental conditions, the CoA-sensitive Ca^{2+} pool was discharged and no re-uptake by, or translocation into, the CoA-insensitive one took place, since the latter was already maximally filled with Ca^{2+}.

Concentrations of (total) Ca^{2+} ranging from 10 to 18 nmol of Ca^{2+}/mg of protein appear to be present in the liver ER compartment *in vivo* (see [27]). At comparable concentrations of Ca^{2+}, CoA (and its esters) released Ca^{2+} from microsomal vesicles to the ambient medium (Figure 1) or favoured Ca^{2+} translocation among ER pools in permeabilized cells (Figure 6).

At variance with the present data, a stimulatory effect by CoA esters on the reticular Ca^{2+} uptake by clonal β-cells has recently been described [13]. However, differences among cell types appear to exist with respect to the effect of CoA esters on Ca^{2+} fluxes. A Ca^{2+}-releasing effect has been observed in sarcoplasmic-reticulum vesicles [28], and a very recent study indicates that palmitoyl-CoA opens the Ca^{2+}-release channel in the terminal cisternae sub-fraction from skeletal-muscle sarcoplasmic reticulum. Moreover, we observed a stimulatory activity by CoA and by its esters on non-mitochondrial Ca^{2+} uptake of digitonin-permeabilized Ehrlich ascites-tumour cells (A. Gamberucci, R. Fulceri, R. Giunti, F. L. Bygrave and A. Benedetti, unpublished work).

CoA appears to be active at concentrations (5–50 μM; Figures 1, 2 and 6) which are considered to be in the physiological range. In fact, the cytosolic concentrations of CoA of isolated hepatocytes has been reported to be approx. 70 μM [29], and similar concentrations (70–30 μM) can be calculated from various reports [30–32], taking into account the water space of the cytosolic compartment [33] and mitochondrial/cytosolic concentration ratio for CoA [29].

As CoA appears to require non-esterified fatty acids to mobilize Ca^{2+}, the availability of non-esterified fatty acids in the hepatocyte and in the ER membrane would be relevant. Under physiological conditions, non-esterified fatty acids do not accumulate and are removed by conversion into the CoA derivatives in the mitochondrial (outer) and ER membrane [24], and subsequently oxidized or incorporated into phospholipids and triacylglycerols. Nonetheless, microsomes and permeabilized hepatocytes used here appeared to contain non-esterified fatty acid concentrations sufficient to allow CoA to form fatty acyl derivatives (ATP was present in the medium) and to promote Ca^{2+} release. In any event, variations in supply of non-esterified fatty acids to the ER membrane and in the subsequent rate of CoA ester formation should result in Ca^{2+} mobilization from, or translocation among, ER pools.

Data on the cytosolic levels of fatty acyl-CoA derivatives would be essential also, before one could be confident about a physiological role for these metabolites in the regulation of hepatocellular Ca^{2+} handling. The total concentrations of fatty acyl-CoA esters have been reported to be 94 and 219 nmol/g dry wt. in the liver of fed and fasted rats respectively [32]. However, direct measurements of these metabolites in the subcellular compartments are few. Indirect calculations attribute 78 % of the total tissue fatty acyl-CoA esters to the cytosolic compartment [13]. On this basis, and taking into account the water space of the cytosolic compartment [33], the calculated concentrations of total fatty acyl-CoA derivatives should be 44 and 103 μM in the liver cytosolic compartment of fed and fasted rats respectively. Therefore, the concentrations of fatty acyl-CoA derivatives active in promoting Ca^{2+} release (Figure 2) appear to be similar to, or even lower than, those envisaged for total cytosolic fatty acyl-CoA esters. However, as fatty acyl-CoA esters are amphiphilic and tend to accumulate in the (ER) membrane, concentrations attained in the membrane rather than total concentrations should also be relevant to the effect described here. In the intact hepatocyte, steady-state concentrations of fatty acyl-CoA esters in the ER membrane might be affected by soluble high-affinity fatty acyl-CoA binding proteins, which have been isolated from various tissues including the liver [34].

In any event, the total cellular concentrations of fatty acyl-CoA esters can vary depending on the nutritional and/or the metabolic status. For instance, glucagon [29], starvation [32] or oleate feeding [30] have been reported to increase the concentration of fatty acyl-CoA esters in perfused livers and/or isolated hepatocytes. A marked elevation of these metabolites, associated with a stimulation of insulin release, has been described for the clonal β-cell line (HIT) supplemented with various fatty acids [35]. Activation of neutrophils with *N*-formylmethionyl-leucyl-phenylalanine caused a prompt 100 % increase in the absolute level of long-chain fatty acyl-CoA, which could be consistent with a signalling role for these compounds [5].

With respect to the mechanisms involved in the Ca^{2+}-releasing effect induced by long-chain CoA esters, it seems reasonable that a Ca^{2+}-efflux pathway of the reticular membrane is activated. An unspecific activity (i.e. ionophore-like) of the (amphiphilic) compounds appears to be unlikely, as they release Ca^{2+} from a discrete hepatocellular ER pool (or from a discrete portion of the microsomal vesicles). The possibility that these compounds act via inhibition of the reticular ATP-driven transport is also unlikely, since, at least at concentrations of a possible physiological relevance (below 100 μM), they were unable to inhibit Ca^{2+}-dependent ATP hydrolysis (Table 1) and did not promote Ca^{2+} efflux from microsomes loaded with low Ca^{2+} concentrations (Figure 1, trace D). Instead, CoA and palmitoyl-CoA markedly increased the Ca^{2+}-efflux rate after Ca^{2+}-ATPase inhibition by a supra-maximal dose of thapsigargin (Figure 2), and palmitoyl-CoA-induced Ca^{2+} efflux was completed within times (> 2 s) far shorter than those required to discharge microsomal Ca^{2+} after Ca^{2+}-transport inhibition by thapsigargin ($\geqslant 160$ s; see Figures 2 and 4) or by the removal of ATP (> 180 s; results from [27]). For CoA-induced Ca^{2+} release, the relatively longer efflux times ($t_{\frac{1}{2}}$ approx. 10 s; Figure 4) might be accounted for by the time necessary to form sufficient concentrations of CoA-derived fatty esters. The mechanism(s), however, for the activation by fatty acyl esters of Ca^{2+} efflux in liver reticular membranes are not the subject of the present study, and need further clarification.

In conclusion, the data shown suggest that CoA and/or its fatty acyl derivatives could play a role in regulating Ca^{2+} fluxes in liver cells. Studies are in progress in our laboratory to assess the possible role of this phenomenon in liver cells subjected to a range of stimuli.

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