Effects of CoA and acyl-CoA on Ca^{2+}-permeability of endoplasmic-reticulum membranes from rat liver

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We have studied the effects of CoA and palmitoyl-CoA on Ca^{2+} movements and GTP-dependent vesicle fusion in rat liver microsomes. (1) Inhibition of membrane fusion by CoA depends on esterification of CoA to long-chain acyl-CoA using endogenous non-esterified fatty acids. (2) Binding of long-chain acyl-CoA to microsomal membranes is inhibited by BSA, which also relieves inhibition of membrane fusion. (3) Under conditions where acyl-CoA binding is inhibited, CoA causes increased Ca^{2+} accumulation, apparently by decreasing the Ca^{2+} leak rate. (4) Conversely, palmitoyl-CoA, in the presence of BSA, causes Ca^{2+} efflux. (5) The decrease in Ca^{2+}-permeability caused by CoA does not depend on the presence of ATP or GTP, and is irreversible in the short term. (6) Using ^{14}C-labelled CoA we show that CoA derivatives can be formed from endogenous components of microsomal membranes in the absence of ATP. (7) The results are interpreted in terms of a Ca^{2+}-permeability which is controlled by CoA and/or long-chain acyl-CoA esters.

INTRODUCTION

Recently there have been several reports of Ca^{2+} movements across endoplasmic-reticulum membranes being affected by CoA or acyl-CoA esters. Deeney et al. [1] found that addition of CoA or long-chain acyl-CoA esters to permeabilized HIT cells (a clonal pancreatic β-cell line) caused enhanced Ca^{2+} uptake into intracellular Ca^{2+} stores, including the InsP_3-sensitive store, apparently by decreasing passive Ca^{2+}-permeability, since the rate of emptying of the stores in the presence of thapsigargin was decreased by acyl-CoA. With isolated endoplasmic-reticulum vesicles from rat liver, we also found [2] that CoA and long-chain acyl-CoA esters increased Ca^{2+} uptake, although in this case there appeared to be at least two mechanisms involved. Firstly, CoA and acyl-CoAs at low concentrations ($< 10\ \mu$M: similar to those used by Deeney et al. [1]) caused a large decrease in the GTP-induced Ca^{2+}-permeability of endoplasmic-reticulum vesicles and an increase in the size of the InsP_3-sensitive pool. Secondly, at higher concentrations of long chain-acyl-CoA [EC_{50} was 20 μM for palmitoyl-CoA (Pm-CoA)] there was inhibition of GTP-dependent vesicle fusion and an increase in vesicle budding, resulting in the formation of small InsP_3-insensitive vesicles.

Using rather higher Ca^{2+} loading, in the absence of GTP and with relatively high concentrations of CoA and long-chain acyl-CoA (approx. 50 μM), Fulceri et al. [3] observed Ca^{2+} release from liver endoplasmic-reticulum vesicles and from permeabilized hepatocytes. At lower concentrations and at lower loading there was an increase in size of the InsP_3-sensitive pool, although without a net increase in Ca^{2+} uptake. Fulceri et al. [4] have also found that much lower concentrations of long-chain acyl-CoA esters (1–15 μM) caused Ca^{2+} release from vesicles derived from skeletal-muscle terminal cisternae, but not from vesicles derived from longitudinal tubules, and suggested that acyl-CoA esters may interact with ryanodine receptors.

In the present paper we describe experiments designed to resolve some of these complex and, in some cases, conflicting observations. We have used BSA to remove endogenous non-esterified fatty acids and to modify binding of long-chain acyl-CoAs, and have measured binding of long-chain acyl-CoA to microsomal membranes. We have also used ^{14}C-labelled CoA to study binding and metabolism of free CoA under the conditions of experiments monitoring Ca^{2+} movements and vesicle fusion. It appears that, as we previously suggested, inhibition of vesicle fusion is due to long-chain acyl-CoA binding to endoplasmic-reticulum membranes, and that inhibition of fusion by CoA requires prior conversion into long-chain acyl-CoA. However, CoA appears to interact with liver endoplasmic-reticulum membranes to cause a decrease in Ca^{2+}-permeability which is, at least partially, reversed by long-chain acyl-CoA. We present evidence that the decrease in Ca^{2+}-permeability caused by CoA does not require ATP, but may involve conversion of CoA into an as-yet unidentified CoA derivative.

MATERIALS AND METHODS

Materials

Dithiothreitol (DTT), ATP, GTP and CoA were supplied by BCL, Lewes, E. Sussex, U.K. Fluorescent probes octadecyl-rhodamine B (R18), 5-(*N*-octadecanoyl)aminofluorescein (F18) and Fluo 3 (free acid) were obtained from Molecular Probes, Eugene, OR, U.S.A. CoA and Pm-CoA were from Sigma Chemical Co., Poole, Dorset, U.K. [*palmitoyl*-1-^{14}C]Pm-CoA (1.5–2.2 GBq/mmol), D-[1-^{14}C]pantothenate (Na salt, 2.0 GBq/mmol) and [U-^{14}C]palmitic acid (29.6 GBq/mmol) were from New England Nuclear–Du Pont, Stevenage, Herts., U.K. Ins(1,4,5)P_3 was generously provided by Dr. R. F. Irvine, Babraham Institute, Cambridge, U.K.

Microsomal preparation

Rat liver microsomes (36000 *g* fraction) were prepared from fed male rats as described previously [5]. Protein concentration was determined by the BCA method [6].

Abbreviations used: Pm-CoA, palmitoyl-CoA; R18, octadecyl-rhodamine B; F18, 5-(*N*-octadecanoyl)aminofluorescein; PEG, poly(ethylene glycol) (average M_r 8000); DTT, dithiothreitol.

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Synthesis of ^{14}C-labelled CoA

^{14}C-labelled CoA was synthesised by the method described by Shimizu et al. [7], using dried cells of *Brevibacterium ammoniagenes* and including 1.5 MBq of [^{14}C]pantothenic acid in the reaction mixture. Labelled CoA was purified by anion-exchange chromatography on DEAE-cellulose using a LiCl gradient. LiCl was removed by cation exchange on Dowex 50W (H^+ form) and the CoA was concentrated by freeze-drying. The radiochemical purity of the final product was checked by f.p.l.c. on a MonoQ column and by reverse-phase h.p.l.c. For f.p.l.c., the mobile phases were (A) 21 mM ammonium acetate, pH 4.6, and (B) 2.5 M ammonium acetate, pH 4.6. At a flow rate of 1 ml/min, the eluting gradient was 0–100% B in A over 20 min, followed by 100% B for 10 min. H.p.l.c. followed the protocol described below for analysing CoA derivatives. Both analytical methods showed the ^{14}C-labelled CoA to be 90% pure.

GTP-dependent vesicle fusion

Vesicle fusion was measured using fluorescence resonance energy transfer from F-18 to R-18 as previously described [2,8]. Briefly, microsomal membranes (about 1.5 mg of protein in each case) were labelled separately with either F-18 or R-18 and then mixed together in a total volume of 1.5 ml in an assay mixture containing (final concentrations): 150 mM sucrose/50 mM KCl/10 mM Hepes/KOH (pH 7.0)/5% (w/v) poly(ethylene glycol) (PEG)/ 1 mM DTT/5 mM ATP/2 mM $MgCl_2$.The microsomes were incubated for 2 min and fusion was started by the addition of 50 μM GTP. Fluorescence was measured in a Shimadzu RF5000 spectrofluorimeter at 30 °C, by using an excitation wavelength of 460 nm and an emission wavelength of 595 nm (5 nm bandwidth in both cases).

Ca^{2+} uptake and release

Ca^{2+} movements were measured as previously described [2]. Briefly, Ca^{2+} uptake was initiated by addition of microsomes (1.5.mg/ml final protein concentration) to 150 mM sucrose/ 50 mM KCl/10 mM Hepes/KOH (pH 7.0)/5% (w/v) PEG/ 1 mM DTT/5 mM ATP/2 mM $MgCl_2$/10 mM phosphocreatine/ creatine kinase (10 μg/ml) and Fluo 3 at 0.63 μM. GTP-dependent Ca^{2+} release was initiated by addition of 50 μM GTP. Additions of CoA, Pm-CoA and Ins(1,4,5)P_3 were as described in the Figure legends. Fluo 3 fluorescence was measured at 30 °C in a Shimadzu RF5000 fluorimeter (excitation 505 nm, emission 530 nm). The digitized output from the fluorimeter was converted into free [Ca^{2+}] concentration, by using a K_d of 275 nM for Fluo 3 [2], via Microsoft Excel software.

Kinetics of conversion of added palmitate into Pm-CoA by microsomal membranes

Microsomes were added to the same buffer as was used for measurement of Ca^{2+} uptake and release without Fluo 3, but containing in addition 2.5 μM CoA and [^{14}C]palmitic acid (5 μM, 3.7 KBq/ml) at either 30 ° or 0 °C. After incubations for 0–5 min, samples were removed, acyl-CoA synthetase activity was stopped, and acyl-CoAs were extracted by the methods of Bar-Tana et al. [9].

Pm-CoA and CoA binding to microsomal membranes

Binding of CoA and Pm-CoA was initially studied under the same conditions as used for Ca^{2+} uptake and release studies, that is in the presence of ATP, the ATP-regenerating system and PEG. Microsomes (0.5–1.0 mg/ml and 1.0–2.0 mg/ml final protein concentration for Pm-CoA and CoA, respectively) were incubated with stirring for 5 min at 30 °C in solutions with the same composition as that used for Ca^{2+} movements before the addition of [^{14}C]Pm-CoA (3–70 μM, 0.06–2.0 kBq/ml) or [^{14}C]CoA (2–60 μM, 0.1–0.9 kBq/ml). After 1.5 min a sample was removed and centrifuged in Eppendorf tubes at 30 °C for 0.5 min at 10000 ***g***. Samples of the supernatant were removed for counting free radioactivity and the rest was removed by aspiration. The sides of the Eppendorf tube were dried with tissue, and residual fluid on the pellet was removed with wicks of filter paper. The surface of the pellet was rapidly washed with the incubation solution, and the pellet was re-dried by the procedure described above. The microsomal pellet was quantitatively transferred to scintillation fluid and dispersed by sonication for counting of the bound radioactivity.

For measurement of binding in the presence of BSA, the same protocol was used, with the inclusion of BSA (final concentration 2.5 mg/ml). BSA was omitted from the solution used to wash the pellet surface.

We also studied binding in the absence of PEG and ATP. Sedimentation of the membranes in the absence of PEG required centrifugation at 14000 ***g*** for 15 min at 30 °C. In the absence of ATP, $MgCl_2$ was omitted from the incubation solutions.

In studying the displacement of bound [^{14}C]CoA by unlabelled CoA or desulpho-CoA, excess of these reagents was added 1 min after [^{14}C]CoA. The incubation was continued for a further 1 min.

Extraction and analysis of CoA derivatives

Long-chain acyl-CoA derivatives were extracted by the method of Woldegiorgis et al. [10]. For microsomes incubated with CoA in the absence of ATP we used an aqueous extraction method. Pellets (3–4 mg of membrane protein) were suspended in 0.1–0.2 ml of 5 mM potassium phosphate buffer, pH 5.8, containing 2 mg/ml BSA. After centrifuging for 10 min at 15000 ***g*** at 4 °C the supernatant was collected. The extraction was repeated two more times. For derivatives formed in the absence of ATP, this procedure extracted 80–85% of the total bound radioactivity. The extracts were stored at −80 °C before analysis. The presence of BSA did not affect the chromatographic analyses.

For t.l.c., the extracts and ^{14}C-labelled standards were spotted on to silica plates and developed in butan-1-ol/acetic acid/water (5:3:2, by vol.). The positions of the CoA derivatives and standards were revealed by autoradiography. The CoA derivatives formed in the absence of ATP were analysed by reverse-phase h.p.l.c. using a Spherisorb S5 ODS2 (250 mm × 4 mm) column maintained at 30 °C. Using 50 mM potassium phosphate, pH 5.3, and acetonitrile, at a flow rate of 0.5 ml/min, we used the following elution sequence: 13.5 min isocratic 5% acetonitrile in potassium phosphate, 28 min isocratic 38% acetonitrile in potassium phosphate, 10 min 70% acetonitrile in water. The column was pre-equilibrated for 20 min under the starting conditions. Analytes were detected by absorbance at 260 nm, and 0.75 min fractions were collected for counting radioactivity.

RESULTS

Since we had previously found that ATP was required to cause inhibition of membrane fusion in the presence of CoA, and that the microsomal fraction which we use is capable of converting CoA into long-chain acyl-CoA [2], we investigated the effects of the addition of BSA to fusion assays, to see if removal of endogenous non-esterified fatty acids blocks the effects of CoA on fusion. Figures 1(a) and 1(b) show the inhibition of vesicle

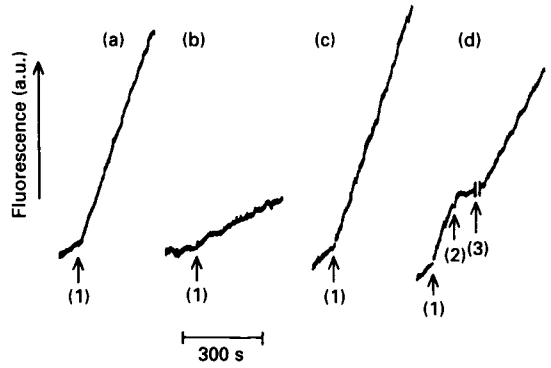

Figure 1 Effects of CoA and BSA on GTP-induced membrane fusion

Microsomal-vesicle fusion was measured as described in the text, with a final protein concentration of 2 mg/ml. In all cases, the arrow marked (1) shows the addition of 50 μM GTP. (**a**), Control, no further addition; (**b**), 10 μM CoA present initially; (**c**), 1 mg/ml BSA and 10 μM CoA present initially; (**d**), 10 μM CoA added at the arrow marked (2), followed by 1 mg/ml BSA at the arrow marked (3). A small addition artefact has been eliminated at the break in the trace at BSA addition in (**d**).

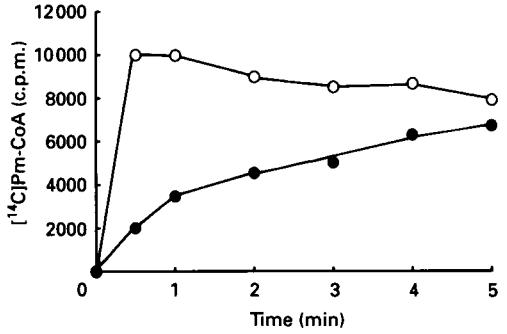

Figure 2 Kinetics of conversion of [^{14}C]palmitate into Pm-CoA

Conditions were as described in the text, with a microsomal protein concentration of 1.5 mg/ml. ○, 30 °C; ●, 0 °C.

fusion caused by the addition of 10 μM CoA to the standard fusion assay. Figure 1(c) shows that inclusion of BSA (1 mg/ml) in the assay mixture blocks the effect of CoA, consistent with removal of endogenous non-esterified fatty acids. Figure 1(d) shows that, when CoA is added while the assay is in progress, there is a very rapid onset of inhibition, but that also this inhibition is immediately and almost completely reversible by addition of BSA. Assuming that the inhibition is due to conversion of CoA into long-chain acyl-CoA derivatives by using endogenous fatty acids, this would suggest that the inhibition by long-chain acyl-CoA is also reversed by the presence of BSA. Binding of Pm-CoA by BSA has been reported by Richards et al. [11]. In accordance with this, under the conditions of Figure 1 there is no inhibition by 20 μM Pm-CoA in the presence of 1 mg/ml BSA. T.l.c. of the radioactive material extracted from membranes incubated with [^{14}C]CoA and ATP under these experimental conditions showed the presence of a radioactive spot running at a similar R_F value to Pm-CoA. Figure 2 shows that incubation of membranes under these experimental conditions with 5 μM [^{14}C]palmitate plus 2.5 μM CoA leads to an approximately steady-state level of labelling of long-chain acyl-CoA, which is reached within 1 min at 30 °C.

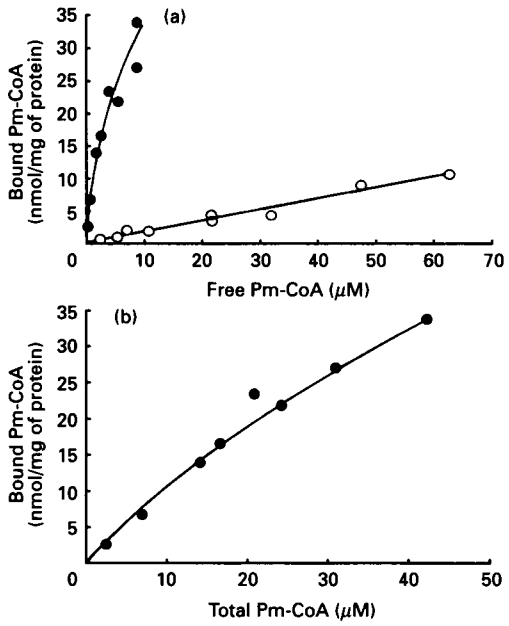

Figure 3 Binding of Pm-CoA to microsomal membranes

Binding measurements were carried out as described in the Materials and methods section. Binding was measured either in the absence (●) or in the presence (○) of 2.5 mg/ml BSA. The data in the absence of BSA in (**a**) are replotted in (**b**) as bound concentration as a function of total Pm-CoA concentration to illustrate the fact that over this concentration range the great majority of the Pm-CoA is membrane-bound. For both sets of data, microsomal protein concentration was 0.8 mg/ml.

Binding of Pm-CoA to microsomal membranes is shown in Figure 3. The binding is complete within 1 min and is not affected by the presence or absence of ATP or GTP (results not shown). Under conditions similar to those used in the fusion assay of Figure 1, it is clear that over the concentration range of interest most of the Pm-CoA added is actually bound to the membranes. This is made clear by replotting the experimental data that were used for Figure 3(a) in the absence of BSA in Figure 3(b), where we have plotted the concentration of bound Pm-CoA as a function of the total amount of Pm-CoA added. (In these experiments the membrane protein concentration was 0.8 ± 0.05 mg/ml.) As is predicted by the results of Figure 1, the binding of Pm-CoA is largely prevented by the inclusion of BSA in the assay medium. Analysis of the binding curve in the absence of BSA suggests the presence of two types of binding site, one high-affinity (K_d about 1.5 μM, $B_{max.}$ about 20 nmol/mg) and one low-affinity (K_d about 60 μM, $B_{max.}$ about 200 nmol/mg). The K_d for the high-affinity site agrees with the value reported by Deeney et al. [1] for binding of Pm-CoA to permeabilized HIT cells, and the $B_{max.}$ value for this site is also of a similar order of magnitude, allowing for the fact that their value of 0.5 nmol/mg was for cell protein rather than microsomal membrane protein. Figure 4 shows equivalent data for CoA binding. In the presence of ATP, there is considerable binding of CoA to the membranes, which is complete within 1 min and is largely prevented by the inclusion of BSA. Binding of labelled CoA is reversed by the addition of an excess of unlabelled CoA, but not by desulpho-CoA. In the absence of ATP (Figure 4b), there is still some binding of CoA to the membranes, but it appears to be of rather low affinity, and is unaffected by the presence or absence of BSA, PEG or GTP. The $B_{max.}$ value of around 4 nmol/mg observed in Figure 4(a) in the presence of ATP is likely to reflect the level of

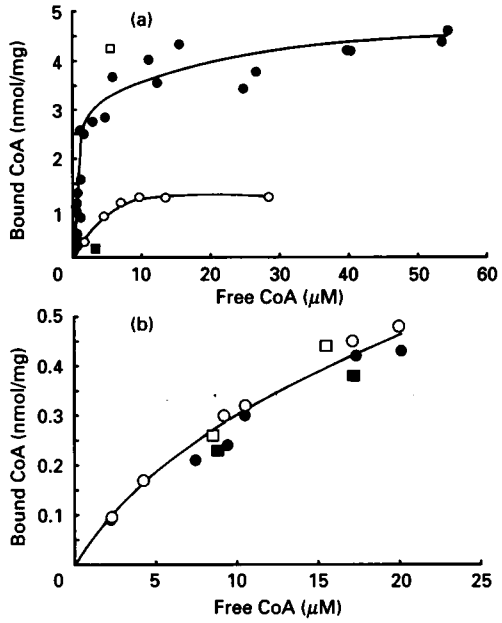

Figure 4 Binding of CoA to microsomal membranes

Binding measurements were carried out as described in the text. (**a**) Binding in the presence of 5 mM ATP/2mM $MgCl_2$/5% PEG: ●, no further additions; ○, plus 2.5 mg/ml BSA; □, plus 100 μM desulpho-CoA; ■, plus 100 μM unlabelled CoA. (**b**) Binding in the absence of ATP: ●, no further additions; ○, plus 2.5 mg/ml BSA; ■, PEG omitted, no BSA; □, PEG omitted, plus 2.5 mg/ml BSA.

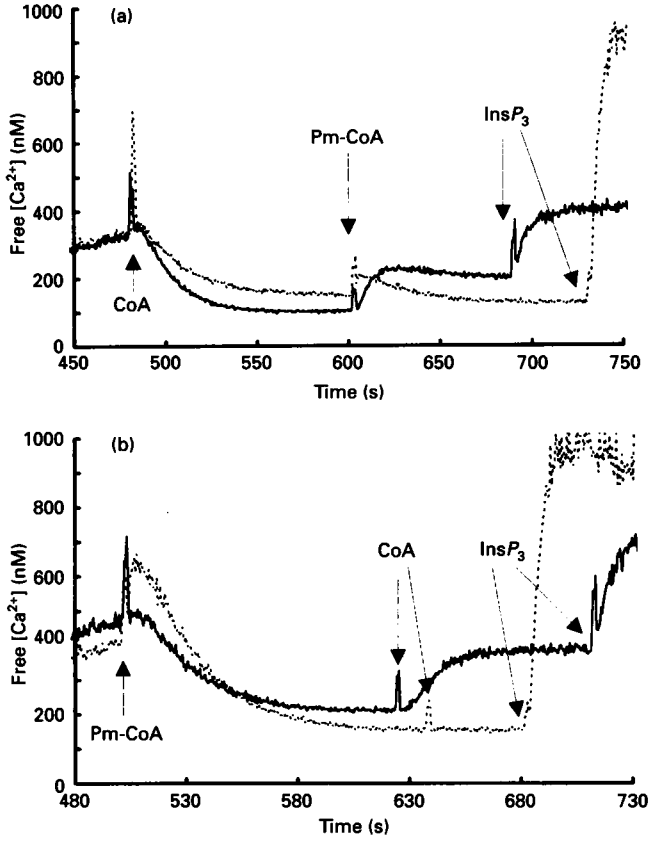

Figure 5 Effects of CoA and Pm-CoA on Ca^{2+} movements across microsomal membranes

Ca^{2+} movements were measured by using Fluo 3 fluorescence as described in the Materials and methods section. Protein concentration was 2 mg/ml, and $t = 0$ corresponds to time of addition of microsomes to an assay medium complete except for GTP and CoA or Pm-CoA. Then 50 μM GTP was added after 4 min, and the section of the trace shown starts when GTP-dependent Ca^{2+} efflux was essentially complete. The arrows marked 'CoA' show the addition of 10 μM CoA; 'Pm-CoA' marks the addition of 20 μM Pm-CoA, and '$InsP_3$' the addition of 2 μM Ins(1,4,5)P_3. In both (**a**) and (**b**) the dotted trace is that obtained in the presence of 1 mg/ml BSA, present from $t = 0$. The apparent large increase in signal noise after $InsP_3$ addition in the presence of BSA is because the extensive Ca^{2+} release brings the fluorescence value close to $F_{max.}$ for Fluo 3.

endogenous non-esterified fatty acids present and the conversion of CoA into acyl-CoA (see above). These observations are thus in accord with the data shown in Figure 1: in the presence of ATP, CoA is converted into long-chain acyl-CoA, the binding of which is prevented or reversed by BSA. Furthermore, the K_d reported for Pm-CoA binding to BSA (0.65 μM [11]) is consistent with BSA being able to remove long-chain acyl-CoA esters from the membrane.

Figure 5 shows the effects of CoA and Pm-CoA on Ca^{2+} movements in the presence and absence of BSA. In all cases, the microsomal vesicles have been allowed to reach a steady state of Ca^{2+} loading in the presence of GTP, ATP and an ATP-regenerating system. Figure 5(a) shows that, unlike the effects on membrane fusion, the addition of BSA has rather little effect on the Ca^{2+} uptake caused by CoA addition. Also, in both the presence and the absence of BSA, Pm-CoA added after CoA causes Ca^{2+} release. However, in the presence of BSA this release is transient, and full $InsP_3$ sensitivity is maintained, whereas in the absence of BSA the Ca^{2+} release is maintained and the size of the $InsP_3$-sensitive pool is substantially decreased. Figure 5(b) shows the effect of reversing this order of addition. As we found previously, Pm-CoA in the absence of BSA causes Ca^{2+} uptake, although surprisingly the subsequent addition of CoA causes Ca^{2+} efflux. This appears to be due to conversion into long-chain acyl-CoA, since it is completely prevented by BSA [Figure 5(b), dotted trace]. Pm-CoA addition in the presence of BSA causes a large Ca^{2+} efflux, followed by a re-uptake phase, and the size of the $InsP_3$-sensitive pool is maintained. With permeabilized hepatocytes under the conditions of Figure 5 (but in the absence of BSA) Pm-CoA caused Ca efflux, as found by Fulceri et al. [3]. CoA reversed the Ca^{2+} efflux caused by GTP, but only after washing of the permeabilized hepatocytes, suggesting that endogenous CoA is sufficient to decrease Ca^{2+}-permeability (results not shown).

Although it is clear that the effect of CoA on fusion requires large-scale conversion of CoA into long-chain acyl-CoA esters, and that ATP is required for this, it is not clear whether or not ATP is required to cause the effects of CoA on Ca^{2+}-permeability. Figure 6 shows an experiment designed to address this question. We have previously shown [2] that when Ca^{2+} efflux from loaded microsomal vesicles is triggered by inhibition of the Ca-ATPase by thapsigargin, the passive rate of Ca^{2+} outflow is inhibited by the presence of CoA (see also Deeney et al. [1]). In the experiment shown in Figure 6, passive efflux is initiated by the addition of glucose and hexokinase, to decrease the ATP concentration rapidly to very low levels. CoA addition after glucose/hexokinase causes a decrease in Ca^{2+}-efflux rate to an extent similar to that seen after thapsigargin addition. Although not being absolutely definitive, since we cannot eliminate the possibility that there are very low levels of ATP remaining at the time of CoA addition,

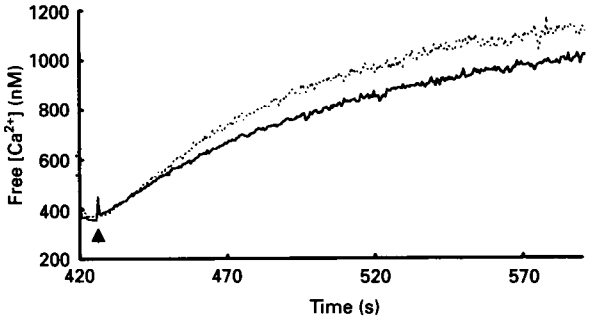

Figure 6 Passive Ca^{2+} efflux from Ca^{2+}-loaded microsomal vesicles

Microsomes were loaded with Ca^{2+} as described in the Materials and methods section and in the legend to Figure 5, except that GTP (50 μM) was present initially and the ATP-regenerating system (phosphocreatine and creatine kinase) was omitted. After steady-state Ca^{2+} loading had been reached, 4 mM $MgCl_2$ was added at $t = 300$ s to complex excess ATP (this eliminates the otherwise large artefact when ATP is converted into ADP). When a new steady level of $[Ca]_{free}$ was established, passive Ca^{2+} outflow was started by the addition of 11.2 units/ml hexokinase and 5 mM glucose simultaneously at $t = 420$ s. The arrow shows the addition of 10 μM CoA to the experiment that gave the continuous trace.

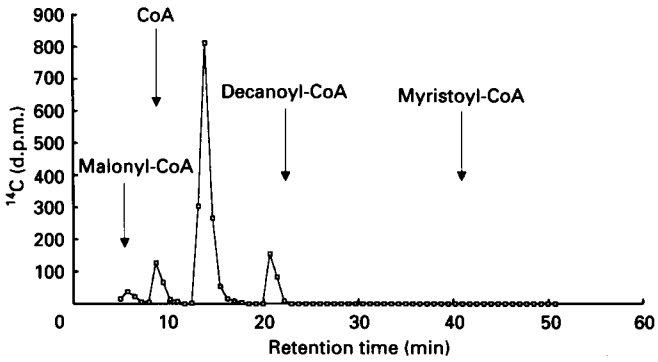

Figure 7 H.p.l.c analysis of extracts of [^{14}C]CoA-treated microsomal membranes

Microsomal membranes were treated with [^{14}C]CoA as described in the Materials and methods section, and the extracts were analysed on a Spherisorb S5 ODS2 reversed-phase column as described in the text. The labelled arrows show the elution times of unlabelled standards.

the experiment indicates that there is probably not an ATP requirement for the effect of CoA on Ca^{2+}-permeability.

We found previously that desulpho-CoA was completely ineffective in causing decreased Ca^{2+}-permeability [2] and, furthermore, does not inhibit the effect of CoA even when added in 10-fold excess (results not shown). It seems that the thiol group of CoA is absolutely required, and that it is thus quite likely that it becomes chemically modified during the process of decreasing Ca^{2+}-permeability. This possibility is supported by the results of experiments where ^{14}C-labelled CoA was incubated with microsomal membranes in the absence of ATP. H.p.l.c. analysis of the products (Figure 7) shows that at least two radioactive peaks are present which do not coincide with the parent compound. Although we cannot as yet identify the peaks, the experiment demonstrates that there are systems/compounds present in the microsomal membranes which can modify CoA in the absence of ATP.

Since preincubation of microsomal membranes with CoA led to the generation of one or more CoA derivatives, it was possible

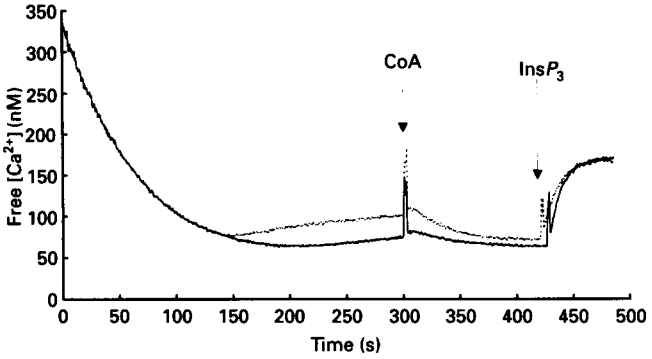

Figure 8 Effect of CoA pretreatment on Ca^{2+}-permeability of microsomal membranes

Microsomes (0.1 ml; 2 mg/ml final protein concentration) were added to 1.4 ml of 150 mM sucrose/50 mM KCl/10 mM Hepes/KOH (pH 7.0)/5% (w/v) PEG/1 mM DTT, and 1 mg/ml BSA plus 10 μM CoA (continuous trace) or with no further addition (dotted trace). The mixture was incubated for 2 min at 30 °C, then centrifuged (12000 *g*, 1 min) at 2 °C. The supernatant was removed, and the membranes were resuspended in 1.4 ml of the same buffer but with no CoA and centrifuged as above. Finally the pellet was resuspended in 1.4 ml of complete reaction medium [150 mM sucrose/50 mM KCl/10 mM Hepes/KOH (pH 7.0)/5% (w/v) PEG/1 mM DTT/5 mM ATP/2 mM $MgCl_2$/10 mM phosphocreatine/creatine kinase (10μg/ml)/50 μM GTP and Fluo 3 at 0.63 μM] and rapidly transferred to the fluorimeter at 30 °C. The arrows show the addition of 10 μM CoA and 2 μM Ins(1,4,5)P_3.

that the effect of CoA on Ca^{2+}-permeability was irreversible, at least over a short time scale. Accordingly, we carried out the experiment shown in Figure 8, where microsomes were pretreated briefly with CoA, the CoA was removed by washing in a BSA-containing medium, and then, after Ca^{2+} loading, the microsomes were re-challenged with CoA. For microsomes which went through a similar preincubation and washing procedure, but in the absence of CoA (dotted trace in Figure 8), the initial phase of Ca^{2+} uptake is followed by a slow Ca^{2+} release, due to the presence of GTP, which then stabilizes into a steady-state level of Ca^{2+} loading. CoA addition then causes Ca^{2+} uptake, as in Figure 5. However, for microsomes pretreated with CoA, the slow release of Ca^{2+} following the uptake phase is decreased and delayed, the steady-state loading level is higher, and the addition of CoA has only a very slight effect on Ca^{2+} uptake. This strongly suggests that indeed the effect of CoA is not readily reversible and persists after removal of CoA. Also, since ATP was not present during the preincubation, it lends further support to the data described above suggesting that ATP is not required for the effect of CoA on Ca^{2+}-permeability. Similarly, since GTP was not present during the preincubation, it is clear that the effects do not depend on the presence of GTP. As discussed below, this has considerable implications when considering the possible mechanisms involved in CoA action.

DISCUSSION

In our previous work on this topic [2], rather than invoke a separate mechanism for the effects of CoA on Ca^{2+} movements and vesicle fusion, we suggested that they might be in some way related. The possibility which we considered was that CoA was involved in producing tight sealing of membranes during fusion, hence decreasing the GTP-induced Ca^{2+} leak, but that the fusion mechanism became inhibited at higher CoA concentrations. However, the experiments described above do not support this view. It is now clear that inhibition of membrane fusion by CoA requires the conversion of CoA into long-chain acyl-CoA, by

using ATP, and that the inhibitory effect depends on extensive binding of acyl-CoA to the microsomal membrane. In contrast, the effects of CoA on Ca^{2+}-permeability do not require ATP, do not involve extensive binding of amphipathic CoA derivatives to the membrane (because BSA does not inhibit the effect), are, at least in the short term, irreversible, and do not depend on the presence of GTP. A more plausible explanation of the results may be as follows. If we suppose that a population of microsomal vesicles contains an open Ca^{2+}-leak pathway, then as these vesicles fuse, by the GTP-dependent mechanism, to Ca^{2+}-pumping non-leaky vesicles, Ca^{2+} will be released. Anything that inhibits this leak pathway (i.e. CoA in this case) would therefore block Ca^{2+} release associated with GTP-promoted vesicle fusion and cause Ca^{2+} re-uptake if added after GTP. Such a mechanism would be in accord with the observation that compounds such as guanosine 5-[γ-thio]triphosphate, which inhibit fusion, do not by themselves cause Ca^{2+} re-uptake [12]. It would also be in agreement with the observations by Deeney et al. [1], showing a rather similar decrease in Ca^{2+}-permeability in HIT-cell endoplasmic reticulum in the absence of GTP. However, it raises questions as to the nature of the effects of long-chain acyl-CoA esters on Ca^{2+} movements, and also about the target for CoA action.

Considering first the effects of acyl-CoAs, previous observations are rather confusing. Acyl-CoAs were found to cause Ca^{2+} uptake in HIT cells [1] or Ca^{2+} release from hepatocytes [3] and terminal cisternae from sarcoplasmic reticulum [4]. We found that they caused Ca^{2+} uptake into liver microsomes at low concentrations [2], but at higher concentrations interpretation of the data was complicated, due to effects on vesicle fusion and budding. In the experiments described above, the latter complications were eliminated by the presence of BSA. Under these circumstances, Pm-CoA causes a Ca^{2+} release, followed by a phase of Ca^{2+} re-uptake. This re-uptake is much more marked if CoA is not present as well [Figure 5(a), cf. Figure 5(b)] and could be due to generation of low (but persistent) concentrations of CoA due to acyl-CoA hydrolysis. Since the effect of CoA is irreversible, the presence of a low free CoA concentration for an extended period would be expected to produce similar effects to CoA addition. Coupled with the observations by Fulceri et al. [3] and our own very similar observations on permeabilized hepatocytes, it therefore appears that, in contrast with HIT cells [1], the primary effect of long-chain acyl-CoAs on liver cell endoplasmic reticulum is to cause Ca^{2+} efflux.

Since treatment of ^{14}C-labelled CoA with microsomal membranes leads to the production of various CoA derivatives, there is evidently a reservoir of groups which can be transferred to CoA in the absence of ATP. There seems no thermodynamic reason why, for example, palmitoyl groups on cysteine residues on proteins should not be reversibly transferred back to CoA (although there is no detectable formation of Pm-CoA under the conditions of our experiment). A possibility is, then, that there is a component of endoplasmic-reticulum membranes which can alter the membrane permeability to Ca^{2+}, depending on whether it is acylated (high permeability) or deacylated (low permeability). The ryanodine receptor has been suggested as a target for Pm-CoA [4], and liver endoplasmic reticulum has been shown to contain ryanodine-binding sites [13–15]. Furthermore, ryanodine has been shown to have effects on the shape of Ca^{2+} spikes in hepatocytes [16], so that we cannot exclude modifications of a form of ryanodine receptor being responsible for the observed actions of CoA and acyl-CoAs on Ca^{2+} movements. However, we have not so far found conditions where we can see any major effects of ryanodine on Ca^{2+} movements, using the sorts of experimental protocols described above. Irrespective of whether or not the ryanodine receptor is the target, the data presented here, together with previous work, suggest that there is a system which modulates permeability of the endoplasmic-reticulum membrane by means of interaction with CoA and long-chain acyl-CoA esters.

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