Effects of CoA and acyl-CoAs on GTP-dependent Ca^{2+} release and vesicle fusion in rat liver microsomal vesicles

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(1) CoA (IC_{50} 23 μM) and acyl-CoAs (IC_{50} values 15–18 μM) inhibit GTP-dependent vesicle fusion in rat liver microsomal vesicles. Acyl-CoAs of carbon chain length C_8 and C_{20} are much less effective than acyl-CoAs of carbon chain length C_{14} – C_{18} . The effect of CoA is mimicked by dephospho-CoA, but not by desulpho-CoA. High acyl-CoA concentrations (50 μM) appear to favour formation of small vesicles (budding), while 50 μM CoA does not. (2) Low concentrations of CoA (EC_{50} 2 μM) and palmitoyl-CoA (10 μM) cause re-accumulation of Ca^{2+} released in response to GTP. This re-accumulation is into an $\text{Ins}(1,4,5)\text{P}_3$ -

sensitive compartment. By investigation of the effects of CoA and palmitoyl-CoA on the thapsigargin-induced passive leak rate of Ca^{2+} , and on the latency of the mannose-6-phosphatase of the vesicles, we conclude that CoA and palmitoyl-CoA cause decreased vesicle permeability rather than stimulation of Ca^{2+} pumping activity. (3) It is suggested that GTP-induced membrane fusion in rat liver microsomes involves an as yet uncharacterized acylation–deacylation reaction which is required to produce complete vesicle sealing.

INTRODUCTION

Previously we have reported that, in rat liver microsomal fractions (microsomes), $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release is greatly increased by low concentrations of GTP, in the presence or absence of poly(ethylene glycol) (PEG) (Dawson, 1985; Dawson et al., 1986; Comerford and Dawson, 1991a,b). Under these conditions GTP mediates slow but extensive Ca^{2+} mobilization (Dawson et al., 1987). We have shown that micromolar concentrations of GTP can also induce fusion of rat liver microsomes, using the techniques of electron microscopy, light scattering analysis (Dawson et al., 1987) and fluorescence-resonance energy transfer (Comerford and Dawson, 1988, 1989). Similarities between GTP-dependent Ca^{2+} release and vesicle fusion in relation to requirements for PEG, Mg^{2+} and GTP, and sensitivity to guanosine 5'-[γ -thio]triphosphate (GTP[S]) and to limited proteolysis, have led us to suggest that GTP-dependent Ca^{2+} mobilization in rat liver microsomes may be due to a change in vesicle permeability to Ca^{2+} accompanying vesicle fusion (Dawson and Comerford, 1989).

We have suggested that one or more of the small- M_r GTP-binding proteins (G_n proteins; Bhullar and Haslam, 1987) of rat liver microsomes may be involved in the mechanism of vesicle fusion (Comerford and Dawson, 1989), as at least two of these G_n proteins (M_r values of 21900 and 24100) are sensitive to proteolytic digestion, and this sensitivity to proteolysis correlates well with the inhibitory effects of proteolysis on GTP-dependent Ca^{2+} release and fusion (Comerford and Dawson, 1989). Further, under appropriate experimental conditions, pretreatment of microsomal vesicles with fluoroaluminate results in inhibition of GTP-dependent vesicle fusion and high-affinity PEG-stimulated GTPase activity (Comerford and Dawson, 1991a). Similar pretreatment of nitrocellulose strips containing SDS-separated polypeptides of G_n proteins from rat liver microsomes results in inhibition of [^{35}S]GTP[S] binding by at least one of the G_n proteins (Comerford and Dawson, 1991a), again implicating G_n proteins as being involved in these processes.

As well as the involvement of the G_n proteins in microsomal fusion, we have found that fusion is inhibited by GTP[S] (Dawson et al., 1987), *N*-ethylmaleimide (NEM) (Comerford and Dawson, 1991b) and AlF_4 (Comerford and Dawson, 1991a). These characteristics of microsomal vesicle fusion are very similar to the reported properties of the mechanism of intracellular membrane trafficking (reviewed by Rothman and Orci, 1992), in relation to the involvement of G_n proteins (see Bourne et al., 1990; Rothman and Orci, 1992), and inhibition by NEM (Glick and Rothman, 1987), GTP[S] and AlF_4 (Melançon et al., 1987). Further, it has been suggested that the fusion events in intracellular membrane trafficking are stimulated by long-chain acyl-CoAs (palmitoyl-CoA) via an acylation reaction (Glick and Rothman, 1987; Pfanner et al., 1989, 1990). In the present paper we have investigated the effects of long-chain acyl-CoAs, CoA and CoA derivatives on GTP-dependent vesicle fusion and Ca^{2+} release, and GTP-dependent potentiation of $\text{Ins}(1,4,5)\text{P}_3$ -dependent Ca^{2+} release. We have found that both CoA and long-chain acyl-CoAs cause inhibition of GTP-dependent vesicle fusion, and that CoA and acyl-CoAs have substantial, complex and differing effects on Ca^{2+} movements in rat liver microsomal vesicles.

EXPERIMENTAL

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 the fluorescent calcium indicator Fluo 3 (free acid) were obtained from Molecular Probes, Eugene, OR, U.S.A. Dephospho-CoA, desulpho-CoA, palmitoyl-CoA, stearoyl-CoA, myristoyl-CoA, acetyl-CoA, octanoyl-CoA, arachidoyl-CoA, glutathione (reduced), ADP-ribose, palmitic acid, palmitoyl-carnitine, pantothenic acid, pantethine and CHAPS were obtained from Sigma Chemical Co., Poole, Dorset, U.K. [*palmitoyl*-1- ^{14}C]Palmitoyl-CoA (40–60 mCi/mmol), [^{14}C]-

palmitic acid (500 mCi/mmol) and [γ - 32 P]GTP (30 Ci/mmol) were obtained from New England Nuclear–Du Pont, Stevenage, Herts., U.K. Ins(1,4,5) P_3 was generously provided by Dr. R. F. Irvine, AFRC Institute of Animal Physiology, Babraham, Cambridge, U.K. All other chemicals used were of the highest grade available.

Microsomal preparation

Rat liver microsomes (36000 g fraction) were prepared from fed male rats as described by Dawson and Irvine (1984), with the addition of the following proteinase inhibitors to the final homogenization step (all to the final concentration indicated): pepstatin A (10 μ g/ml), aprotinin (10 μ g/ml), benzamidine hydrochloride (10 μ M) and phenanthroline (1 μ g/ml). The addition of this proteinase mixture produces microsomes which are stable in relation to Ca^{2+} movements and fusion for a longer period of time than untreated microsomes (J. G. Comerford, unpublished work). Protein concentrations were determined by the method of Lowry et al. (1951).

Effect of acyl-CoAs, CoA and CoA derivatives on GTP-dependent vesicle fusion

The basic procedures used for probe incorporation and fluorescence measurement were essentially as detailed by Comerford and Dawson (1988). Briefly, these were as follows. Microsomal membranes (1–2 mg of protein) were suspended in a total volume of 0.75 ml of 150 mM sucrose/50 mM KCl/10 mM Hepes/KOH (pH 7.0)/2 mM dithiothreitol. The suspension was then split into two equal halves. R18 (1.2 μ l of 3.4 mM stock solution) was added to one half, and F18 (1.2 μ l of 2 mM stock solution) was added to the other half. In both cases the probes were added while mixing the suspension vigorously on a vortex mixer. The suspensions were incubated at 30 °C for 5 min to allow incorporation of probes into the membranes of the microsomal vesicles, after which time the two halves were recombined with thorough mixing and added to the fluorimeter cell, which already contained 0.75 ml of 150 mM sucrose, 50 mM KCl, 10 mM Hepes/KOH (pH 7.0), 10% (w/v) PEG, 5 mM ATP and 2 mM $MgCl_2$. The microsomes were then incubated at 30 °C for 1.5 min, after which acyl-CoAs, CoA or CoA derivatives (as indicated in individual Figure legends) were added. After 30 s, vesicle fusion was initiated by the addition of GTP to a final concentration of 50 μ M. In all experiments energy transfer from F18 to R18 was measured, using a Shimadzu RF 5000 spectrofluorimeter system, by monitoring the fluorescence increase at 595 nm with an excitation wavelength of 460 nm.

Determination of vesicle fusion by this method does not provide any indication of the rate of budding of vesicles, owing to the randomization of the two probes in the fused vesicle membranes. Therefore, in experiments to investigate the possibility that the effect of CoA derivatives and acyl-CoAs is to bring about budding of vesicles, fusion and budding were determined by light scattering as described by Dawson et al. (1987). Incubation conditions were as described above, with the exception that light scattering was determined in a final volume of 4 ml.

Effects of acyl-CoAs, CoA and CoA derivatives on GTP-dependent and Ins(1,4,5) P_3 -dependent Ca^{2+} efflux from rat liver microsomes

Ca^{2+} uptake and release were determined using the fluorescent Ca^{2+} indicator Fluo 3 (Minta et al., 1989). The incubation conditions were as follows: Ca^{2+} uptake was initiated by addition of microsomes (1.5 mg/ml final 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/10 μ g/ml creatine kinase containing Fluo 3 at a final concentration of 0.67 μ M. GTP-dependent Ca^{2+} release was initiated by addition of GTP (50 μ M). Ins(1,4,5) P_3 -dependent Ca^{2+} release was initiated by the addition of Ins(1,4,5) P_3 to a final concentration of 2 μ M. The effect of acyl-CoAs, CoA and CoA derivatives was determined by the addition of these compounds as indicated in individual Figure legends. Fluo 3 fluorescence was measured at 30 °C using a Shimadzu RF 5000 spectrofluorimeter with an excitation wavelength of 505 nm and emission detection at 530 nm. Under our experimental conditions a K_d value of 275 nM was determined for Fluo 3 at 30 °C, using the Ca^{2+} electrode to determine pCa values which were then correlated to fluorescence values in the spectrofluorimeter. pCa values in the experiments were determined using this K_d value.

Effect of CoA and acyl-CoA on GTPase activity

The basic procedure for determination of PEG-stimulated GTPase activity has been detailed previously (Comerford and Dawson, 1991a). CoA, dephospho-CoA and palmitoyl-CoA (50 μ M final concentration) were added 2 min prior to initiation of GTPase activity by the addition of GTP (1 μ M GTP containing 0.5–1 μ Ci of [γ - 32 P]GTP).

Effects of CoA and palmitoyl-CoA on the latency of mannose-6-phosphatase

To investigate further the effects of CoA and palmitoyl-CoA on microsomal vesicle permeability, we examined the effects of both of these compounds on the GTP-dependent increase in mannose 6-phosphate permeability (Nicchitta et al., 1987). Mannose 6- $[^{32}P]$ phosphate, which was used as substrate, was prepared as described by Nicchitta et al. (1987). Mannose-6-phosphatase activity was assayed by measuring the formation of $[^{32}P]P_i$, which was determined by extraction of $[^{32}P]$ phosphomolybdate complex into butan-2-ol essentially as described by Arion et al. (1972).

RESULTS

Figure 1 shows typical traces of the change in fluorescence of microsomal vesicles prelabelled with R18 and F18 which demonstrate the inhibition of GTP-dependent microsomal vesicle fusion by CoA and acyl-CoAs (as represented by palmitoyl-CoA). Addition of GTP (50 μ M) induced microsomal vesicle fusion in the absence of pretreatment with CoA or palmitoyl-CoA (Figure 1a), as shown by an increase in fluorescence. However, addition of CoA (Figure 1b) or palmitoyl-CoA (Figure 1c) 1.5 min prior to GTP addition resulted in inhibition of microsomal vesicle fusion (55% inhibition of fusion observed using 35 μ M CoA, compared with 75% inhibition observed with 35 μ M palmitoyl-CoA). At similar concentrations myristoyl-CoA, stearoyl-CoA and dephospho-CoA also caused inhibition of GTP-dependent vesicle fusion (results not shown). Addition of acetyl-CoA (33 μ M), octanoyl-CoA (33 μ M), desulpho-CoA (50 μ M), glutathione (reduced) (30 μ M), ADP-ribose (50 μ M), pantothenic acid (30 μ M), pantethine (50 μ M), cysteine (30 μ M), palmitoyl carnitine (50 μ M) or palmitic acid (50 μ M) had little or no effect on GTP-dependent vesicle fusion (results not shown).

Having shown that CoA, dephospho-CoA and various acyl-CoAs (chain length C_{14-18}) cause inhibition of GTP-dependent vesicle fusion, we examined the effect of CoA/acyl-CoA concentrations on inhibition of initial rates of vesicle fusion. Initial rates of fusion after GTP addition (with and without prior addition of various concentrations of CoA/acyl-CoAs) were

Figure 1 Inhibition of rat liver microsomal vesicle fusion by CoA and palmitoyl-CoA

Microsomes (2 mg/ml) were labelled and fluorescence energy transfer was determined as described in the text. Labelled microsomes were combined, with the following additions: (a) GTP (50 μM) at G; (b) CoA (35 μM) at C followed by GTP (50 μM) 1.5 min later at G; (c) palmitoyl-CoA (35 μM) at P followed by GTP (50 μM) 1.5 min later at G.

Figure 3 Effect of ATP on inhibition of fusion by CoA and palmitoyl-CoA in rat liver microsomal vesicles

Microsomes (2 mg/ml) were labelled and incubated under conditions similar to those described for Figure 1, with the exceptions that initially ATP was absent and the MgCl_2 concentration was 270 μM . (a) Control trace in which GTP (135 μM) was added at G and MgCl_2/ATP (400 $\mu\text{M}/1.68 \text{ mM}$) were added at M/A. (b) CoA (65 μM) was added at C, 1.5 min prior to GTP addition (G) followed by MgCl_2/ATP addition (M/A), as in (a). (c) Palmitoyl-CoA (65 μM) was added at P, 1.5 min prior to GTP addition at G followed by MgCl_2/ATP addition at M/A, as in (a).

Figure 2 Effect of CoA and palmitoyl-CoA concentrations on inhibition of initial rates of GTP-dependent rat liver microsomal vesicle fusion

Incubation conditions for fluorescence energy transfer were as previously described for Figure 1, except that both CoA and palmitoyl-CoA concentrations were varied as indicated. (a) Initial rates of GTP-induced fusion under the experimental conditions of Figure 1(b) (CoA concentration varied). (b) Initial rates of GTP-induced fusion under the experimental conditions of Fig. 1(c) (palmitoyl-CoA concentration varied). Results shown are typical of three independent experiments.

determined from traces similar to those in Figure 1, these being corrected for the basal levels of fluorescence increase observed prior to GTP addition. Figure 2 shows typical results obtained from these types of experiments. Figure 2(a) shows the effect of CoA concentration on the initial rate of GTP-dependent vesicle fusion, whereas Figure 2(b) shows similar data for the effect of palmitoyl-CoA concentration. From these data the IC_{50} for CoA is 23 μM and that for palmitoyl-CoA is 18 μM . Further, it is observed that although palmitoyl-CoA (80 μM) can cause 100% inhibition of GTP-dependent vesicle fusion, the maximum inhibition of fusion obtained with CoA was only 80% (at 150 μM).

Figure 4 Effects of GTP, CoA and palmitoyl-CoA on light scattering of rat liver microsomal vesicles

Microsomes (2 mg/ml) were incubated under the experimental conditions of Figure 1, with the exception that the total volume was 4 ml. After 5 min of incubation, GTP (50 μM) was added at G. (a) CoA (50 μM) was added (C), 5 min after GTP. (b) Palmitoyl-CoA (50 μM) was added (P), 5 min after GTP. In both experiments the broken line shows the time course without addition of CoA or palmitoyl-CoA. These traces are typical of at least three independent experiments.

A further increase in CoA concentration caused no further inhibition of vesicle fusion. From experiments of this type, IC_{50} values were determined for octanoyl-CoA (65 μM), myristoyl-CoA (17 μM), stearoyl-CoA (15 μM), arachidoyl-CoA (60 μM) and dephospho-CoA (18 μM). For all acyl-CoAs investigated, 100% inhibition was observed at the highest concentrations. However, dephospho-CoA, like CoA, appeared to produce a maximum of about 80% inhibition.

The ineffectiveness of desulpho-CoA compared with CoA suggests the possibility that CoA may be metabolized prior to or during the inhibition of fusion. This possibility is supported by the finding (Figure 3) that ATP is required for inhibition of vesicle fusion by CoA. Figure 3 shows typical results from

Figure 5 Effects of CoA and palmitoyl-CoA on Ca^{2+} movements in rat liver microsomal vesicles

This Figure shows Ca^{2+} uptake and release traces, and the effects of treatment with CoA and palmitoyl-CoA before (**b** and **c** respectively), and after (**e** and **d** respectively), GTP treatment. Ca^{2+} movements were determined by measurement of changes in the fluorescence of Fluo 3 as described in the Experimental section. In (**a**)–(**e**) Ca^{2+} uptake was initiated by the addition of microsomes (2 mg/ml final concentration). Arrows: M, microsomes; G, GTP (50 μM); I, $\text{Ins}(1,4,5)\text{P}_3$ (2 μM); Ca, Ca^{2+} (10 nmol); C, CoA (50 μM); P, palmitoyl-CoA (50 μM). The apparent transient Ca^{2+} efflux observed on palmitoyl-CoA addition is an addition artifact caused by the relatively high volume of palmitoyl-CoA added. pCa values were determined and traces generated using Shimadzu RF5000 software feeding into Microsoft Excel software. These traces are typical of at least four independent experiments.

experiments to investigate this possibility by examining the effects of CoA and palmitoyl-CoA in the absence and presence of ATP. These data show that, in the absence of ATP, GTP-dependent vesicle fusion was not inhibited to any great extent by CoA (65 μM) (Figure 3b), but under similar conditions inhibition was observed with palmitoyl-CoA (65 μM) (Figure 3c). In the absence of ATP and at low added Mg^{2+} concentrations, GTP causes fusion which rapidly reaches a steady state but at a lower extent of fusion than observed in our standard assay conditions (Comerford and Dawson, 1991b). The effect of GTP in the absence of ATP reflects the utilization of GTP by both the fusion process and other endogenous nucleoside triphosphatase activities (Nicchitta et al., 1986). Addition of ATP and Mg^{2+} to the final concentrations present in our standard assay, in the absence of CoA or acyl-CoA, results in a re-initiation of vesicle fusion (Figure 3a), due to the regeneration of GTP by endogenous nucleoside diphosphokinase activity (Dawson et al., 1986). In experiments in which the microsomes were treated with CoA (Figure 3b) or palmitoyl-CoA (Figure 3c), dramatic inhibition of the second phase of vesicle fusion was observed. These results show clearly that inhibition of vesicle fusion by CoA is ATP-dependent, whereas inhibition by acyl-CoA is not, suggesting that CoA may have to be metabolized to cause inhibition of vesicle fusion.

While fluorescence energy transfer gives data on fusion rate, it does not provide information about the final size of the vesicles produced. Once fusion has taken place the probes are randomized within the vesicle membranes and further membrane fusion or budding will cause no change in fluorescence. To investigate the possibility of changes in vesicle size (i.e. possible effects on budding), we have examined the effects of GTP, CoA and palmitoyl-CoA on light scattering of microsomal vesicles, as described by Dawson et al. (1987). Figure 4 shows typical results from experiments investigating the effects of GTP, CoA and palmitoyl-CoA on microsomal vesicle fusion determined by examination of light scattering of microsomal vesicles. Figures 4(a) and 4(b) show that on addition of GTP (50 μM) to microsomes under standard incubation conditions, an increase in light scattering was observed which in control experiments reached a plateau after about 5 min and subsequently remained at steady state (broken line on Figure). The addition of CoA (50 μM) 5 min after GTP caused a very small decrease in light scattering (Figure 4a). In contrast, the addition of palmitoyl-CoA (50 μM) 5 min after GTP caused a very large decrease in light scattering (Figure 4b). Further, addition of palmitoyl-CoA (50 μM) prior to GTP prevented the increase in light scattering caused by GTP (results not shown). The results from these experiments suggest that palmitoyl-CoA, but not CoA, brings

Figure 6 Effects of CoA and palmitoyl-CoA concentrations on Ca^{2+} re-accumulation by rat liver microsomal vesicles

This Figure shows Ca^{2+} uptake and release traces obtained under experimental conditions similar to those of Figure 5(a), after GTP-dependent Ca^{2+} efflux has reached a steady state. The microsomal protein concentration was 2 mg/ml. (a) Effect of CoA concentration. In traces A–C, arrows are: 1, addition of 1 μM CoA (trace A), 10 μM CoA (trace B) and 50 μM CoA (trace C); 2, addition of $\text{Ins}(1,4,5)\text{P}_3$ (2 μM) and 3, addition of Ca^{2+} (10 nmol). (b) Effect of palmitoyl-CoA concentration. For trace A, arrows are: 1, addition of $\text{Ins}(1,4,5)\text{P}_3$ (2 μM); 2, addition of Ca^{2+} (10 nmol). For traces B–D arrows are: 1, addition of 1 μM (trace B), 10 μM (trace C) or 50 μM (trace D) palmitoyl-CoA; 3, addition of $\text{Ins}(1,4,5)\text{P}_3$ (2 μM). (c) Effect of CoA concentration on both the rate (○) and extent (●) of Ca^{2+} re-accumulation, determined from experiments similar to those in (a). These data are typical of three independent experiments.

about a change in the light-scattering properties of rat liver microsomal vesicles which is consistent with the idea that palmitoyl-CoA induces budding of microsomal vesicles. This effect is not mimicked by similar concentrations of palmitoyl-carnitine or palmitate (results not shown). Nicchitta et al. (1987) have described a PEG-stimulated high-affinity GTPase activity which correlates well with effects on Ca^{2+} movements, and also on the fusion process (Comerford and Dawson, 1991a). However, neither CoA (50 μM) nor palmitoyl-CoA (50 μM) inhibits this GTPase activity (results not shown).

Figure 5 shows the effects of CoA and palmitoyl-CoA on Ca^{2+} movements in rat liver microsomal vesicles. Figure 5(a) shows typical Ca^{2+} uptake and release in microsomal vesicles, as determined by change in the fluorescence of Fluo 3. Ca^{2+} uptake

was initiated by the addition of microsomes, with Ca^{2+} release occurring on the addition of GTP (50 μM) (after 4 min) and $\text{Ins}(1,4,5)\text{P}_3$ (2 μM) (after 9 min). Figures 5(b) and 5(c) show the effects of adding CoA (50 μM) and palmitoyl-CoA (50 μM) respectively after the efflux caused by GTP addition had reached a steady state. Both CoA and palmitoyl-CoA are observed to cause re-uptake of Ca^{2+} , this being more rapid and extensive in the case of CoA. The effects on $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release are, however, very different. $\text{Ins}(1,4,5)\text{P}_3$ addition after CoA treatment caused a rapid and extensive efflux of Ca^{2+} , corresponding to most of the Ca^{2+} that had been accumulated by the vesicles. Addition of $\text{Ins}(1,4,5)\text{P}_3$ after palmitoyl-CoA treatment resulted in a very small release of Ca^{2+} , similar in size to the $\text{Ins}(1,4,5)\text{P}_3$ -releasable Ca^{2+} pool observed in the absence of GTP pretreatment (results not shown). Figures 5(d) and 5(e) show the effects on Ca^{2+} movements in microsomes of adding palmitoyl-CoA and CoA respectively prior to GTP. As would be predicted from the results in Figures 2(b) and 2(c), both palmitoyl-CoA and CoA greatly decreased GTP-dependent Ca^{2+} release. Also, palmitoyl-CoA addition caused inhibition of $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release, while CoA caused a considerable potentiation of this release. The latter observation is in agreement with the findings that inhibition of fusion by CoA is not complete and that addition of CoA after GTP-dependent Ca^{2+} release causes rapid Ca^{2+} re-uptake into an $\text{Ins}(1,4,5)\text{P}_3$ -releasable pool (Figure 5e).

Figure 6 shows the effects of CoA and palmitoyl-CoA concentrations on the re-accumulation of Ca^{2+} after GTP treatment. Figures 6(a) and 6(b) show the relevant parts of experimental traces obtained using the protocol of Figure 5, and illustrate the effects of adding CoA and palmitoyl-CoA (1, 10 and 50 μM) after GTP-dependent Ca^{2+} efflux had reached a steady state. Figure 6(a) (traces B and C) shows that addition of 10 and 50 μM CoA caused extensive Ca^{2+} re-uptake and enhanced $\text{Ins}(1,4,5)\text{P}_3$ -dependent Ca^{2+} release, whereas 1 μM CoA (trace A) resulted in greatly reduced re-accumulation of Ca^{2+} and had little effect on $\text{Ins}(1,4,5)\text{P}_3$ -dependent Ca^{2+} release. Figure 6(b) shows similar data for the effect of palmitoyl-CoA at these concentrations. As indicated previously (Figure 5c), 50 μM palmitoyl-CoA (Figure 6b, trace D) caused substantial re-accumulation of Ca^{2+} , but with greatly reduced $\text{Ins}(1,4,5)\text{P}_3$ -dependent Ca^{2+} release. However, at lower concentrations (1 and 10 μM) (traces B and C), the effect of palmitoyl-CoA on Ca^{2+} re-uptake and $\text{Ins}(1,4,5)\text{P}_3$ -dependent Ca^{2+} release was very similar to that of CoA, albeit that the re-uptake rates are lower than those observed with CoA. Figure 6(c) shows the effect of CoA concentration on both the rate and the extent of Ca^{2+} re-uptake. The EC_{50} for CoA in relation to the extent of uptake was 2.2 μM, and for the rate of re-uptake the EC_{50} was 4 μM. Because of the complex effects of palmitoyl-CoA on the size of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pool, no similar analysis was attempted for palmitoyl-CoA.

From the data described above it seems that, as well as influencing fusion rates, CoA and palmitoyl-CoA are also having major effects on Ca^{2+} movements, possibly by decreasing vesicle permeability. There is a striking distinction between the data shown in Figures 5(a) and 5(b), where addition of CoA caused Ca^{2+} re-uptake, and, for example, the effects of GTP[S] under similar experimental conditions (Dawson et al., 1987) where, although fusion is inhibited, GTP-induced Ca^{2+} efflux is not reversed. While the effects of CoA and palmitoyl-CoA could be due either to stimulation of ATP-dependent Ca^{2+} uptake or to inhibition of the outward Ca^{2+} leakage rate, the latter seems the most likely possibility, for two reasons. Firstly, as shown in Figure 7, CoA addition (50 μM) inhibited by 35% the passive

Figure 7 Effect of CoA on the thapsigargin-induced passive efflux of Ca^{2+} from rat liver microsomal vesicles

This Figure shows Ca^{2+} uptake and release traces obtained under experimental conditions similar to those of Figure 5(a), after GTP-dependent Ca^{2+} efflux had reached a steady state. Trace 1 (broken line) shows the effect of addition of thapsigargin (final concentration 330 nM) at T. Trace 2 (solid line) shows the effect of addition of CoA (50 μM) at C prior to thapsigargin addition (330 nM) at T. These traces are typical of three independent experiments.

Ca^{2+} outflow rate observed after addition of the Ca^{2+} -ATPase inhibitor thapsigargin (Thastrup et al., 1990). Similar results (not shown) were obtained with palmitoyl-CoA (50 μM). Secondly, both CoA and palmitoyl-CoA (50 μM) also reversed the decrease in the latency of mannose-6-phosphatase observed after GTP addition, indicating a general decrease in membrane permeability. Using mannose 6- ^{32}P phosphate as substrate, GTP-treated microsomes showed $28 \pm 3.5\%$ (mean $\pm$ S.E.M., $n = 3$) of the mannose-6-phosphatase activity observed in the presence of 0.2% CHAPS, and this was decreased to $16.5 \pm 1.2\%$ ($n = 4$) and $17.4 \pm 1.9\%$ ($n = 3$) in the presence of CoA (50 μM) and palmitoyl-CoA (50 μM) respectively.

DISCUSSION

In the present paper we have shown that CoA, derivatives of CoA and acyl-CoAs can cause inhibition of GTP-dependent vesicle fusion and GTP-dependent Ca^{2+} release in rat liver microsomal vesicles. Inhibition of GTP-dependent vesicle fusion and Ca^{2+} release exhibits a high structural specificity for CoA and the length of carbon chain attached to acyl-CoAs. Of all the CoA analogues tested, only 3'-dephospho-CoA could cause a degree of inhibition of these processes comparable to that of CoA. Further, we have demonstrated that acyl-CoAs with chain lengths of C_{14} , C_{16} and C_{18} are equally effective in causing maximal inhibition of GTP-dependent vesicle fusion and Ca^{2+} release, whereas acyl-CoAs with chain lengths of C_8 and C_{20} and acetyl-CoA are much less effective. These findings differ from the observations of Pfanner et al. (1990), who demonstrated that palmitoyl-CoA promoted fusion of transport vesicles with Golgi cisternae and that CoA had no effect on fusion. Inhibition of GTP-dependent vesicle fusion by CoA and palmitoyl-CoA differs. The IC_{50} for CoA is about 23 μM , with maximal inhibition (only 80%) obtained at about 150 μM . The IC_{50} for palmitoyl-CoA is about 18 μM , and maximal (100%) inhibition of vesicle fusion is obtained at about 80 μM . The effects of CoA on Ca^{2+} movements do not appear to be very closely related to its effects on fusion rates. Firstly, as pointed out above, inhibition of fusion (e.g. by GTP[S]) does not in itself result in changes in Ca^{2+} movements. Secondly, the concentration-dependences of the effects are very different. GTP-dependent Ca^{2+} release is fully reversed by the addition of 10 μM CoA, while fusion requires a

concentration of 23 μM for 50% inhibition. Because of the requirement for ATP in the study of Ca^{2+} movements, we have not as yet obtained any data on whether or not ATP is required for CoA to have effects on Ca^{2+} movements, as it is for effects on fusion (Figure 3). As a preliminary hypothesis, however, we would suggest that ATP and CoA are involved in the fusion process (possibly via acyl group transfer), in such a way that vesicle fusion produces much more tightly sealed vesicles than in the absence of CoA. Rather than invoke a separate mechanism for the inhibition of fusion by CoA, it seems more plausible to suggest that, in some related fashion, the mechanism becomes inhibited at higher CoA concentrations.

Clearly a real possibility is that an acyl derivative is formed during the process of fusion. If this is the case, such a compound must be formed very rapidly and be effective at very low concentrations to explain the data of, for example, Figure 5. Such a hypothesis does, however, bring us to consider the effects of the acyl-CoA derivatives. If acyl group transfer is indeed involved in the fusion mechanism, effects of acyl-CoAs would certainly be expected. Low concentrations of palmitoyl-CoA (e.g. 10 μM) have effects on Ca^{2+} movements which are rather similar to those of 10 μM CoA, i.e. a decrease in the GTP-dependent Ca^{2+} permeability and Ca^{2+} re-uptake into an $\text{Ins}(1,4,5)\text{P}_3$ -accessible pool. Higher concentrations of palmitoyl-CoA, however, have rather different effects, in that it appears (from light scattering data) that the formation of small vesicles is favoured and, coincidentally, $\text{Ins}(1,4,5)\text{P}_3$ sensitivity is lost. We have also shown that both CoA and palmitoyl-CoA, at all concentrations tested, have no effect on $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release in rat cerebral microsomes (results not shown), which suggests that CoA and palmitoyl-CoA are not having direct effects on the $\text{Ins}(1,4,5)\text{P}_3$ receptor.

Preliminary experiments have shown that the rat liver microsomal system is capable of generating [^{14}C]palmitoyl-CoA from [^{14}C]palmitate and added CoA, so that an endogenous acyl group transfer mechanism seems to be present. Metabolism of [^{14}C]palmitoyl-CoA by rat liver microsomes seems, in initial experiments, to be very limited (J. G. Comerford, unpublished work).

We cannot as yet offer a detailed mechanistic explanation for these observations. The findings that CoA and palmitoyl-CoA do not inhibit PEG-stimulated GTPase activity may indicate effects on a process other than the GTP-utilizing step. Lavoie et al. (1991) have shown that GTP-induced fusion of rat liver microsomal vesicles is correlated with an increase in free polyunsaturated fatty acids. It is possible that the effects we observe are in some way related to these changes in lipid metabolism. It should be noted, also, that Mundy and Warren (1992) have suggested a relationship between membrane trafficking and reversible palmitoylation of a protein of M_r 62000. However, it appears that the effects of CoA and acyl-CoA derivatives on vesicle permeability may, at least, provide a resolution to the long-standing problem of GTP-dependent Ca^{2+} release. We have always regarded the latter as being due to the formation of large leaky vesicles (Dawson and Comerford, 1989), but have been faced with the dilemma of why GTP in the intact cell does not cause large-scale Ca^{2+} leakage from the stores. The results in the present paper would suggest that such GTP-induced Ca^{2+} leakage is unlikely to occur in the intact cell, where sufficient CoA will be present to ensure complete membrane sealing after any GTP-dependent membrane fusion process.

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