Fatty acyl-CoA–acyl-CoA-binding protein complexes activate the Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum

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We previously reported that fatty acyl-CoA esters activate ryanodine receptor/Ca^{2+} release channels in a terminal cisternae fraction from rabbit skeletal muscle [Fulceri, Nori, Gamberucci, Volpe, Giunti and Benedetti (1994) Cell Calcium **15**, 109–116]. Skeletal muscle cytosol contains a high-affinity fatty acyl-CoA-binding protein (ACBP) [Knudsen, Hojrup, Hansen, H. O., Hansen, H. F. and Roepstorff (1989) Biochem. J. **262**, 513–519]. We show here that palmitoyl-CoA (PCoA) in a complex with a molar excess of bovine ACBP causes a discrete Ca^{2+} efflux or allows Ca^{2+} release from the Ca^{2+}-preloaded terminal cisternae fraction by sub-optimal caffeine concentrations. Both effects were abolished by elevating the free [Mg^{2+}] in the system, which inhibits the Ca^{2+} release channel activity. Sensitization towards caffeine was a function of both the concentration of the complex and the [PCoA]-to-[ACBP] ratio. In all experimental conditions the calculated free [PCoA] was no more than 50 nM, and such concentrations by themselves were inactive on Ca^{2+} release channels. The K_D for PCoA binding was approx. 2 nM for bovine and yeast ACBP, and slightly higher (8 nM) for rat ACBP. The PCoA–rat ACBP complex behaved in the same manner as the PCoA–bovine ACBP complex, whereas the ester complexed with yeast ACBP was more active in activating/sensitizing Ca^{2+} efflux. A non-hydrolysable analogue of PCoA bound to (bovine) ACBP also sensitized the Ca^{2+} release channel towards caffeine. These findings indicate that fatty acyl-CoA–ACBP complexes either interact directly with one or more components in the terminal cisternae membranes or, through interaction with the component(s), donate the fatty acyl-CoA esters to high-affinity binding sites of the membrane, thus affecting (and possibly regulating) Ca^{2+} release channel activity.

INTRODUCTION

Activation of the ryanodine receptor/Ca^{2+} release channels (RyRCs) is a crucial step in muscle contraction. In skeletal muscle, RyRCs are segregated to the membrane of terminal cisternae (TC) of sarcoplasmic reticulum (SR), and are activated upon stimulation of muscle contraction through the voltage-sensitive dihydropyridine receptor on the T-tubule/plasma membrane [1,2]. A variety of exogenous or endogenous compounds (e.g. ryanodine, caffeine, Ca^{2+} ions, calmodulin, cADP-ribose or cell lipid metabolites) have been reported to activate/sensitize RyRCs expressed in skeletal muscle and/or in other cell types (reviewed in [1,2]). The obligatory fatty intermediates in cellular fatty acid metabolism, the fatty acyl-CoA esters (FACoAs) have been shown by us [3] and by other laboratories [4,5] to activate RyRC(s) in skeletal muscle SR preparations. Palmitoyl-CoA (PCoA) has also been reported to potentiate the cADP-ribose-induced activation of RyRC in the endoplasmic reticulum of *Lytechinus pictus* eggs [6], and to remove the Mg^{2+} inhibition of cardiac RyRC [7].

Eukaryotic cells are known to contain a cytosolic high-affinity acyl-CoA-binding protein (ACBP) [8,9] encoded by a highly conserved housekeeping gene [10]. The K_D for FACoA binding is in the low nM range [11–13]. The concentration of ACBPs in rat and bovine skeletal muscle (cytosol) is 1.5 and 3 nmol/g wet weight respectively [9,14], and the total (rat) muscle FACoA concentration has been reported to range from 4.3 to 7.9 nmol/g wet weight [15,16]. However, in muscle more than 95 % of total CoA and 85–90 % of the FACoAs are located in the mitochondria [17]. The level of free FACoA in skeletal muscle cytosol can therefore be expected to be in the low nanomolar range and the cytosolic [FACoAs]-to-[ACBP] ratio to be less than 1. Long-chain acyl-CoAs are amphipathic molecules and bind strongly to phospholipid membranes; the partition coefficient for PCoA binding to liposomes can be calculated as 1.5×10^5 from [18]. ACBP has been shown to be able to extract FACoAs from, and prevent their binding to, biological membranes and liposomes, and to donate FACoAs for mitochondrial β-oxidation and microsomal glycerolipid synthesis [12]. ACBP would therefore serve both to prevent massive non-specific binding of the esters to cell structures and to channel FACoAs to specific metabolic pathways [12].

Previously reported effects of FACoAs *in vitro* on skeletal muscle RyRC were obtained by using micromolar concentrations [3–7]. The effect of FACoA *in vivo* on RyRC would therefore be expected to be minor or absent owing to the presence of constitutive cytosolic ACBP, which raises questions about the physiological significance of the previously published data on regulation of the RyRC by FACoA. We have therefore re-investigated the effect of PCoA on RyRC-mediated Ca^{2+} fluxes in a purified TC subfraction of SR in the absence and in the presence of a molar excess of ACBP. PCoA is assumed to be representative of a mixed intracellular FACoA pool because all physiologically relevant FACoAs behave in a similar way with respect to binding to ACBP [11] and activation of Ca^{2+} efflux from TC [3].

Abbreviations used: ACBP, fatty acyl-CoA-binding protein; FACoA, fatty acyl-CoA ester; $[Ca^{2+}]_{ext}$, extravesicular free Ca^{2+} concentration; PCoA, palmitoyl-CoA; RyRC, ryanodine receptor/Ca^{2+} release channel; SR, sarcoplasmic reticulum; TC, terminal cisternae.

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Here we report that PCoA complexed with a molar excess of different ACBPs does activate/sensitize skeletal muscle RyRC(s); the activity of the PCoA–ACBP complexes was, however, far lower than that of the PCoA alone. Our results suggest a dual role for constitutive ACBP: (1) to prevent a massive activation of RyRC by cytosolic muscle FACoAs and (2) to allow the activation to be discrete, thus supporting the hypothesis that these lipid metabolites might be involved in control of Ca^{2+} homoeostasis in skeletal muscle.

EXPERIMENTAL

Preparation of TC fraction

TC vesicles were isolated from the predominantly fast-twitch skeletal muscles of New Zealand white rabbit, as previously reported by Saito et al. [19]. TC fractions were frozen and maintained in liquid N_2 until used.

Preparation of ACBP and of its complex with PCoA

Recombinant bovine ACBP was purified from *Escherichia coli* harbouring the ACBP expression plasmid pKK223ACBPDEI as reported in [20]. Recombinant rat ACBP [10] was produced and purified in the same way as bovine ACBP, except that pH 11 was used during the Q-Sepharose ion-exchange step. Recombinant yeast ACBP was produced as previously described [21]. Stock solutions of ACBP [1.5–3 mM in 100 mM KCl/20 mM NaCl/3.5 mM $MgCl_2$/20 mM Mops (pH 7.2)] and PCoA (3 mM in 5 mM sodium phosphate buffer, pH 6.0) were maintained at −20 °C. For each experiment fresh solutions of PCoA–ACBP complexes were prepared by mixing the ACBP and PCoA stock solutions.

Measurement of Ca^{2+} fluxes

The incubation medium consisted of (in mM): KCl, 100; NaCl, 20; $MgCl_2$, 3.5 (or 5.5 where indicated in the individual experiments); Mops, 20 (pH 7.2); ATP, 3; phosphocreatine, 20; P_i, 10 (as potassium phosphate buffer, pH 7.2). Creatine phosphokinase (10 μM units/ml) was also added. The incubation (37 °C) was started by adding TC fractions (100 μg of protein) to make a final volume of 2 ml in a fluorometer cuvette, and Ca^{2+} uptake and release were measured by using the fluorescent Ca^{2+} indicator Fluo 3 free acid (1 μM), as previously described [3]. The concentrated solution of PCoA–ACBP complexes (2–10 μl) was added to the system to give the final desired concentrations of PCoA and ACBP. The excitation and emission wavelengths were 506 and 526 nm respectively. At the end of each incubation, 1 mM $CaCl_2$ or 10 mM EGTA were added to measure maximal (F_{max}) or minimal (F_{min}) fluorescence values respectively. Fluorescence mV output signals were acquired at 0.5 s intervals with MacLab® hardware equipped with Chart (version 3.2.5) software. The K_D for the Fluo 3/Ca^{2+} complex was determined as 650 nM with a Ca^{2+} electrode [3]. Free Ca^{2+} concentrations in the incubation media ($[Ca^{2+}]_{ext}$) were calculated with CA Cricket Graph III software by using the formula $[Ca^{2+}]_{ext} = K_D[(F-F_{min})/(F_{max}-F)]$.

Determination of K_D for PCoA binding to ACBPs

K_D values for PCoA binding to bovine, rat and yeast ACBPs were measured in the incubation medium used for the Ca^{2+} flux experiments (without TC) by microcalorimetry as described in detail elsewhere [13].

Other assays

The Ca^{2+}-ATPase activity of TC membranes was measured as described in [5]. TC protein content was by the method of Lowry et al. [22], with BSA as standard.

Materials

ATP, phosphocreatine, creatine phosphokinase (Type III), PCoA and Fluo 3 (free acid) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The non-hydrolysable analogue of PCoA (heptadecan-2-onyldethio-CoA) was a gift from Professor T. Wieland (Heidelberg, Germany). All other chemicals were of analytical grade.

RESULTS

In the present study, as previously [3], we used a purified TC subfraction of SR [19,23] highly enriched in RyRC, derived from rabbit fast-twitch skeletal muscle. TC vesicles actively accumulated Ca^{2+} and were able to buffer ambient $[Ca^{2+}]_{ext}$ down to approx. 100 nM for prolonged incubation times and after Ca^{2+} pulse additions (Figure 1A).

FACoA binding to ACBP has recently been shown to be highly dependent on the ionic strength of the binding buffer [13]. We therefore determined the binding affinity and stoichiometry

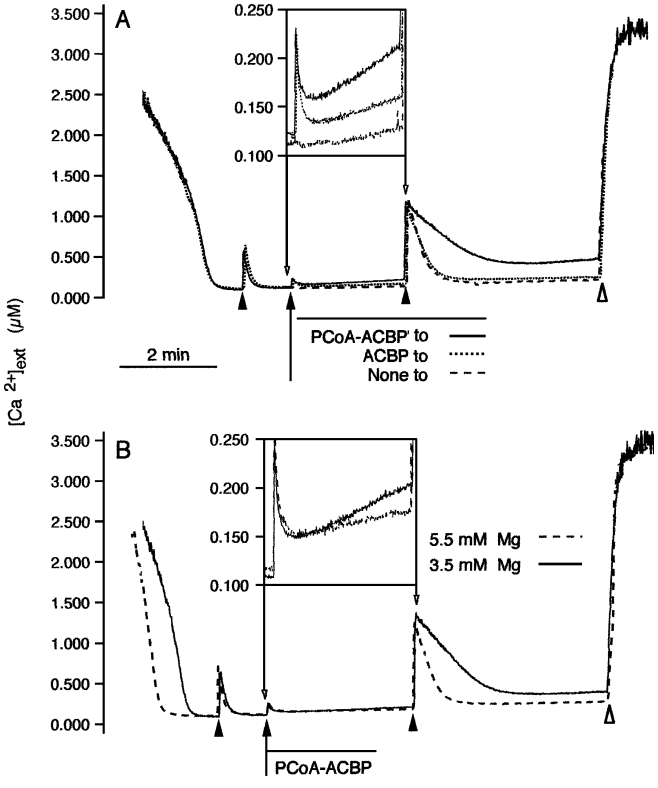

Figure 1 Efflux of Ca^{2+} induced by the PCoA–ACBP complex in Ca^{2+}-preloaded TC vesicles (A) and inhibition of the efflux by elevating $[Mg^{2+}]$ (B)

TC vesicles were allowed to accumulate Ca^{2+} until $[Ca^{2+}]_{ext}$ had decreased to approx. 0.1 μM, in the presence of $MgATP^{2-}$ as detailed in the Experimental section. TC vesicles were successively treated with: 5 μM $CaCl_2$ (filled arrowhead); PCoA (6.0 μM) complexed with bovine ACBP (6.6 μM), bovine ACBP alone (6.6 μM) or 10 μl of vehicle buffer (filled arrow); 10 μM $CaCl_2$ (filled arrowhead). The Ca^{2+} ionophore A23187 (5 μM, open arrowhead) was finally added to release the total Ca^{2+} accumulated by TC. These and the following experiments are representative of 3–12 different replications.

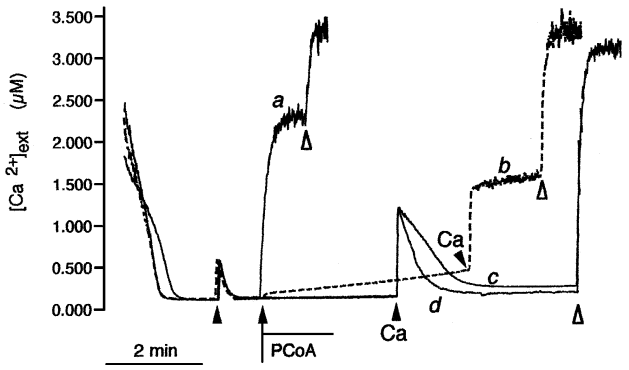

Figure 2 Efflux of Ca^{2+} induced by different concentrations of free PCoA in Ca^{2+}-preloaded TC vesicles

TC vesicles were preloaded with Ca^{2+} as described in the legend to Figure 1. TC vesicles were subsequently treated with: 5 μM $CaCl_2$ (filled arrowheads); PCoA (6, 0.5, 0.1 and 0.05 μM in traces a, b, c and d respectively; filled arrow); 10 μM $CaCl_2$ (Ca^{2+}, filled arrowheads); 5 μM A23187 (open arrowheads).

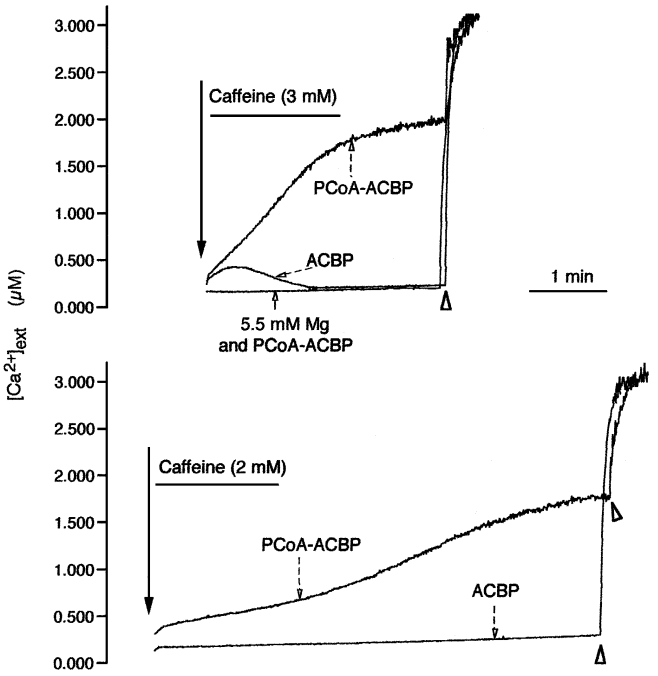

Figure 3 Sensitization of caffeine-induced Ca^{2+} release from Ca^{2+}-preloaded TC vesicles by the PCoA–ACBP complex

TC vesicles were preloaded with Ca^{2+} as described in the legend to Figure 1. The incubation system contained, where indicated, 5.5 mM Mg^{2+}. TC vesicles were pretreated with PCoA (6.0 μM) complexed with bovine ACBP (6.6 μM) or bovine ACBP alone (6.6 μM) 2 min before adding either 2 or 3 mM caffeine (filled arrow). The open arrowhead indicates the addition of 5 μM A23187.

of PCoA binding to the rat, bovine and yeast ACBPs in the medium used for the Ca^{2+} release assays. This information enabled us to calculate the correct free acyl-CoA concentrations under the experimental conditions used. The K_D values obtained for PCoA binding to bovine, rat and yeast ACBP (means $\pm$ S.D., $n = 3$) were 2.0 ± 1.8, 8.9 ± 5.3 and 2.3 ± 3.0 nM respectively, with a binding stoichiometry of 1 mol per mol of ACBP.

The addition of 6 μM PCoA complexed to 6.6 μM (recombinant bovine) ACBP caused a minor release of Ca^{2+} (Figure 1A, inset) and a significant delay in the recovery of $[Ca^{2+}]_{ext}$ after a Ca^{2+} pulse (Figure 1A). Mg^{2+} is known to antagonize RyRC [1,24]; increasing $[Mg^{2+}]$ in the incubation mixture (2.5 mM over [ATP]) completely prevented any effect of the PCoA–ACBP complex on TC (Figure 1B), indicating that PCoA or the PCoA–ACBP complex acted on RyRC. ACBP alone (Figure 1A) and 50 nM PCoA (Figure 2) did not affect Ca^{2+} release. This PCoA concentration is 2.6-fold higher than the calculated free PCoA concentration (19.3 nM) in a mixture of 6.0 μM PCoA and 6.6 μM bovine ACBP on the basis of the above K_D values. The addition of 100 nM free PCoA was necessary to cause a minor activation of RyRC, as revealed by a delay in recovering the steady-state $[Ca^{2+}]_{ext}$ after a Ca^{2+} pulse (Figure 2). As previously observed [3], the addition of 6 μM free PCoA resulted in a massive release of Ca^{2+} from TC (Figure 2).

PCoA-induced Ca^{2+} release in the presence of ACBP cannot be explained by an inhibition of active Ca^{2+} uptake because the Ca^{2+}-dependent ATPase activity of TC was unaffected by the addition of PCoA plus ACBP. The measured ATPase activities were 2.11 ± 0.45 and 2.44 ± 0.39 μmol of ATP consumed/min per mg of protein (means $\pm$ S.D., $n = 3$) respectively [5].

The PCoA–ACBP complex strongly sensitized RyRC to its classic agonist caffeine [1,2]. As shown in Figure 3, caffeine at submaximal concentrations (2 or 3 mM) did not promote Ca^{2+} efflux from TC (2 mM), or only promoted a minor transient efflux (3 mM). However, both caffeine concentrations induced Ca^{2+} efflux from TC when added in the presence of 6 μM PCoA complexed with 6.6 μM ACBP. This release was also blocked by increasing $[Mg^{2+}]$ in the incubation mixture. Again, ACBP alone (Figure 2) or PCoA concentrations ranging from 10 to 50 nM (results not shown) were totally ineffective in potentiating caffeine-stimulated Ca^{2+} release.

These results indicate that the PCoA–ACBP complex can interact directly with TC components, or that ACBP, by preventing non-specific membrane binding of the ester, is able to form a soluble PCoA pool, which can donate PCoA to the TC membranes. To investigate the effect of changing the concentration of free PCoA or of the PCoA–ACBP complex, TC membranes were exposed to increasing concentrations of the complex at two different molar ratios of PCoA to ACBP: 0.9 as used in the above experiments, and 0.45. Both the rate and extent of caffeine-induced Ca^{2+} releases and the calculated concentration of free PCoA decreased markedly when the molar ratio of PCoA to ACBP was decreased from 0.9 to 0.45 (Figures 4A and 4B). In contrast, the concentrations of the PCoA–ACBP complexes were practically unchanged during this manoeuvre (owing to the high binding affinity of the ACBP for PCoA). This observation could indicate that it is free PCoA, and not the PCoA–ACBP complex, that acts on one or more components in the TC membrane, thereby sensitizing Ca^{2+} release. However, this cannot be the correct interpretation because at both [PCoA]-to-[ACBP] ratios, 0.9 and 0.45, the calculated free [PCoA] remained constant or changed only very little (approx. 19 and 1.6 nM; Figures 4A and 4B respectively), but increasing concentrations of the PCoA–ACBP complex increased the sensitivity of TC to caffeine-induced Ca^{2+} release.

To investigate the role of increasing the concentration of unbound ACBP on the sensitization of caffeine-induced Ca^{2+} release by the PCoA–ACBP complex, TC membranes were incubated with constant amounts of PCoA (6 μM) in the presence of increasing amounts of ACBP (6.6–16.5 μM). The results (Figure 4C) show that increasing the concentration of unbound

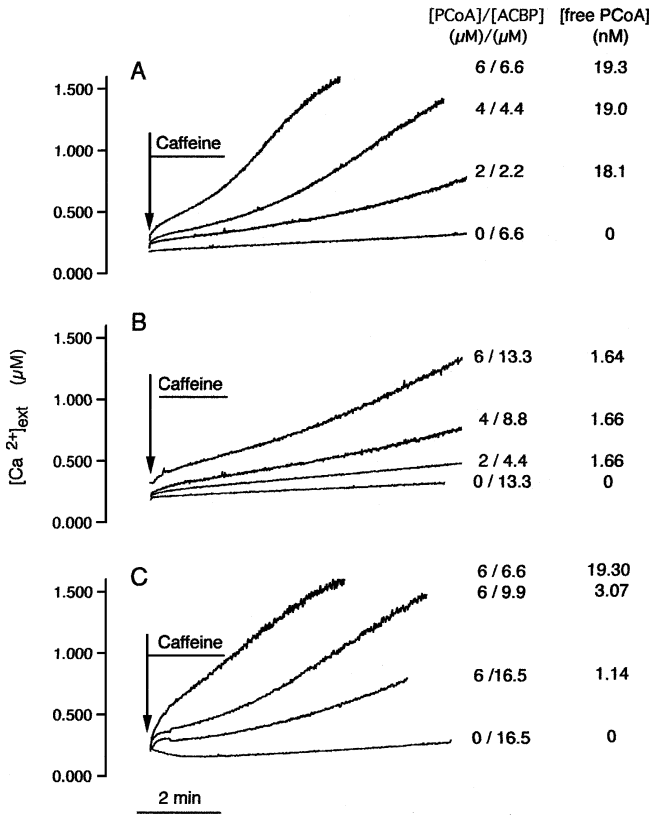

Figure 4 Sensitization of caffeine-induced Ca^{2+} release from TC vesicles by PCoA–ACBP complexes at different [PCoA]-to-[ACBP] ratios

TC vesicles were treated with different concentrations of the PCoA–bovine ACBP complex at molar ratios of PCoA to ACBP of 0.9 (**A**) or 0.45 (**B**), or with a constant concentration of the PCoA–bovine ACBP complex (6 μM) in the presence of increasing concentrations of unbound ACBP (**C**). TC vesicles were preloaded with Ca^{2+} as described in the legend to Figure 1 and treated with the PCoA–ACBP complex (at the concentrations of both PCoA and ACBP indicated at the right of each trace) 2 min before adding caffeine (2 mM, arrow). The free PCoA concentration in each incubation medium (but in the absence of TC) was calculated (see the Experimental and Results sections) and is indicated at the right of each trace.

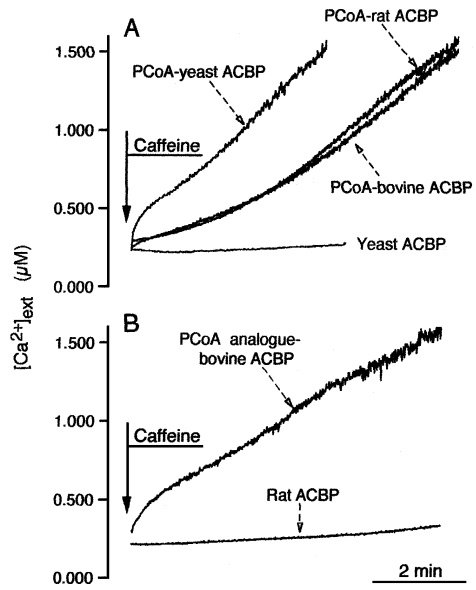

Figure 5 Sensitization of caffeine-induced Ca^{2+} release from TC vesicles by PCoA complexed with yeast, rat or bovine ACBP (A) and by a non-hydrolysable analogue of PCoA complexed with (bovine) ACBP (B)

TC vesicles were preloaded with Ca^{2+} as described in the legend to Figure 1. Where indicated, TC vesicles were treated with PCoA (6.0 μM) complexed with yeast, rat or bovine ACBP (6.6 μM), and with the non-hydrolysable analogue of PCoA (6.0 μM) complexed with bovine ACBP (6.6 μM), 2 min before the addition of 2 mM caffeine (filled arrow).

ACBP decreased the sensitization of RyRC to caffeine by PCoA–ACBP (6 μM).

It should be observed that caffeine-induced Ca^{2+} releases were eventually biphasic: an initial linear phase was followed by a second, more rapid, phase (Figures 3–5). This behaviour possibly reflected the further activation of RyRCs by the increased $[Ca^{2+}]_{ext}$ and/or a delayed transfer of PCoA from ACBP to TC. The non-linear kinetics of Ca^{2+} release prevented us from quantifying the sensitizing effect of PCoA–ACBP to caffeine. However, the differences in rate and extent of Ca^{2+} efflux under the experimental circumstances of Figure 4 were sufficiently evident to demonstrate the dose dependence of the phenomenon.

We used recombinant bovine ACBP for the above experiments because rabbit ACBP was not available. The primary sequence of ACBP is highly conserved between mammalian species, and rat and bovine ACBP have identical sequences in 83% of their residues [9]. The K_D values for PCoA binding are similar for bovine and rat ACBP (see above). An ACBP from a different mammalian species would therefore be expected to have a similar effect on PCoA-mediated Ca^{2+} release from TC. However, to evaluate species-specific differences, we also performed experiments with (recombinant) rat ACBP. The sensitization of caffeine-induced Ca^{2+} release by PCoA (6.0 μM) complexed with bovine or rat ACBP (6.6 μM) was almost identical (Figure 5A). Yeast ACBP binds PCoA with the same affinity as bovine ACBP in the buffer used in the present assay (see above). Despite this, the observed sensitization of RyRC to caffeine by the PCoA–yeast ACBP complex was much stronger than that by PCoA complexed with bovine or rat ACBP [see Figure 5(A), with 6 μM PCoA complexed to 7.5 μM yeast ACBP]. Consistently, the direct activation of Ca^{2+} efflux from (and the delay in Ca^{2+} reuptake by) TC were also more pronounced by using PCoA complexed with yeast ACBP (results not shown) than by using a complex with bovine ACBP (see, for example, Figure 1A).

We have previously observed [3] that the activation of RyRC by (free) PCoA alone was mimicked by a non-hydrolysable thioether analogue of PCoA [25], indicating that the PCoA effect was not mediated by a protein-acylation process. The non-hydrolysable analogue complexed with (bovine) ACBP also sensitized RyRC to caffeine-induced Ca^{2+} release (Figure 5B). The analogue seemed to be even more potent than the parent compound, and this effect is most probably due to the higher effective acyl-CoA concentration in the presence of the non-hydrolysable analogue. The TC fraction, in fact, had hydrolytic activity towards PCoA, even when it was complexed with excess (bovine) ACBP (R. Fulceri, J. Knudsen, P. Volpe and A. Benedetti, unpublished work). In any event, the effect of the PCoA–ACBP complex on RyRC-mediated Ca^{2+} release also seemed not to involve protein-acylation processes.

DISCUSSION

The results demonstrate that PCoA in the presence of molar excess of its high-affinity binding protein, ACBP, still induces a discrete Ca^{2+} efflux from isolated skeletal muscle TC; this was, however, far lower than those previously observed by treating

SR vesicles with comparable concentrations of FACoAs alone. Assuming that our experimental circumstances mirror the conditions *in vivo* (ACBPs are cytosolic widespread proteins; see the Introduction section), this observation is consistent with a role *in vivo* for ACBP in preventing uncontrolled massive effects on SR Ca^{2+} fluxes. The PCoA–ACBP complex also allowed suboptimal concentrations of caffeine to release a significant portion of Ca^{2+} stored in the TC. Caffeine was instrumental in highlighting discrete effects of the complex on the Ca^{2+} efflux pathway in the TC. The observed discrete activation/sensitization by the PCoA–ACBP complex is compatible with a regulatory role on SR Ca^{2+} fluxes *in vivo* for both FACoAs and ACBP.

The activity of the PCoA–ACBP complex is primarily due to sensitization/activation of the TC RyRCs because: (1) the complex synergizes with the RyRC agonist caffeine, (2) it is suppressed by the RyRC inhibitor Mg^{2+}, and (3) the activity of Ca^{2+}-dependent ATPases in the TC is not affected by the PCoA–ACBP complex. The RyRC inhibitors Mg^{2+} and Ruthenium Red have been previously shown to counteract Ca^{2+} release induced by the addition of free PCoA to cardiac microsomes [7] and to skeletal muscle TC [3] respectively.

The effect of PCoA on Ca^{2+} release is decreased by lowering the free [PCoA] from approx. 19 nM to 1.6 nM by changing the [PCoA]-to-[ACBP] ratio from 0.9 to 0.45. These observations imply that it is the free PCoA that is recognized by the TC membranes and suggest that binding of PCoA to the membranes occurs in competition with ACBP from the free pool of PCoA. However, additions of free PCoA at concentrations similar to those calculated for the incubation systems, including the various PCoA–ACBP complexes, do not cause any RyRC activation/sensitization. Concentrations of free [PCoA] at least 5-fold higher (e.g. 100 nM; Figure 2) are necessary to enhance RyRC activity. These observations are not compatible with the direct binding of free PCoA to the membrane. If the TC membrane component(s) involved in RyRC activation were able to compete with ACBP in binding PCoA, it or they should also be able to compete with other potential non-specific membrane binding sites in the TC, to which ACBP can prevent binding.

The activation of RyRC could therefore be due to a direct regulation through interaction with the PCoA–ACBP complex. Alternatively, the donation of PCoA to one or more components in the TC membrane from the complex in a direct interaction-dependent manner, or by the mediation of complex formation, can also take place. The increased caffeine-induced Ca^{2+} release by increased concentrations of PCoA–ACBP complexes, resulting in approximately constant free [PCoA] (Figures 4A and 4B), strongly supports a direct interaction model. The fact that the PCoA–yeast ACBP complex is more potent than the PCoA–bovine ACBP or PCoA–rat ACBP complex in affecting RyRC activity, even though the binding affinities are similar, indicates that the yeast ACBP complex interacts differently from the bovine (or rat) ACBP complex with the TC membrane. This observation further supports the suggestion that it is the complex (and not the free ligand) that interacts with the TC membrane. This interpretation would only be compatible with the observations in Figures 4(A) and 4(B) if it were assumed that the interaction with the complex is specific, and that the ligand-free ACBP can compete with the ACBP complexed to PCoA. A similar mechanism has been hypothesized for the interaction of the retinol/cytosolic retinol-binding proteins complex with the lecithin–retinol acyltransferase from rat liver [26,27]. If this interpretation is correct it can be predicted that increasing the concentration of unbound ACBP at a constant concentration of PCoA–ACBP complex will lead to decreased sensitization (to caffeine) of the RyRC. The experiment shown in Figure 4C demonstrates that this is indeed so, indicating that the TC membrane component(s) recognize both complexed and ligand-free ACBP. The TC membrane component(s) involved could be domains of the RyRC itself, or protein(s) functionally associated with the RyRC. However, a thorough understanding of the molecular mechanisms of action of FACoAs on RyRCs awaits further investigation.

Regardless of the mechanism, the sensitization/activation of skeletal muscle RyRC occurs at physiologically relevant concentrations of both ACBP and FACoAs, and can therefore be expected to occur *in vivo*. Changes in the cell level of the obligatory metabolites FACoAs, and of the FACoA–ACBP complex in turn, could occur under a variety of physiological and pathological conditions. Variations in lipid supply to muscle (for example during muscle exercise, adrenergic stimulation, fasting or diabetic status), in the rate of mitochondrial fatty acid oxidation, or in the production of fatty acids by muscle lipases [28,29] could all increase the muscle levels of FACoAs.

Under circumstances that increase the energetic utilization of lipids by muscle the sensitivity of RyRC might be positively up-regulated. For instance, during prolonged moderate to hard exercise, muscle is fuelled mainly by long-chain fatty acids supplied by higher fatty acid concentrations in the plasma. This could, under extreme conditions, lead to a large increase in muscle cytosolic [FACoAs], induce alterations in muscle Ca^{2+} fluxes and thus be involved in the generation of fatigue. Under extreme conditions, moreover, the rate of utilization of FACoAs by mitochondria might decrease owing to the oxygen debt and contribute to elevating muscle cytosolic [FACoAs]. Both transient and irreversible muscle damage by strenuous exercise have been correlated with a marked increase in intracellular Ca^{2+} levels [30,31]. Prolonged exercise has been reported to induce both decreased Ca^{2+} storage in muscle SR [32] and an adaptive increase in the proportion of functional SR Ca^{2+}-ATPase protein [33].

An increase in futile Ca^{2+} cycling in the SR, through RyRC activation/sensitization owing to increased lipid metabolism, has been hypothesized to underline non-shivering muscle thermogenesis [5,28]. Muscle cytosolic free fatty acids, fatty acyl carnitines, or 'free' FACoAs have been regarded as putative 'thermogenic factors' being all capable of increasing myoplasmic [Ca^{2+}], because they all activate RyRCs *in vitro* (reviewed in [28]). However, fatty acids are expected, similarly to their CoA ester, not to be free in the cytosolic compartment but bound to specific fatty acid-binding proteins [34]. So far, specific binding proteins for fatty acyl carnitines have not been identified; however, these esters have been reported to bind with relatively high affinity to cytosolic fatty acid-binding proteins *in vitro* [35]. The finding that FACoAs complexed with ACBP still activate muscle RyRCs strengthens their role as putative 'thermogenic factors'. The 'malignant hyperthermia' syndrome is characterized by mutations in the gene of skeletal muscle RyRC [29,36] that render the channel more susceptible to activation by a variety of agents [29]. Increased muscle levels of free fatty acids, and of their esters with CoASH or carnitine, might be involved in the generation of the hyperthermic attack [28,29]. Interestingly, a small subset of individuals affected by the 'malignant hyperthermia' syndrome have been reported to have a deficiency in (mitochondrial) carnitine palmitoyltransferase activity [37], a condition known to cause an abnormal cell accumulation of FACoAs [38]. Several other inherited diseases characterized by muscle dysfunction are also accompanied by abnormally high tissue levels of FACoAs, caused by deficiencies in various enzymes involved in mitochondrial long-chain fatty acid oxidation [38]. In these diseases, as well as in the 'malignant hyperthermia' syndrome, the level of

muscle FACoAs might overcome that of ACBP, thus causing abnormal activation of RyRCs. ACBP expression can be induced by insulin-differentiation in 3T3-L1 cells [39]. Nothing is currently known about the (level of) expression of ACBPs in muscle under these pathological conditions. It can be speculated that a different degree of ACBP expression is related to the severity of the diseases, because lowering the [FACoAs]-to-[ACBP] ratio decreases the activation of RyRCs. A deeper knowledge of these aspects might form the theoretical basis for a genetic therapy of these diseases.

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