Characterization of ligand binding to acyl-CoA-binding protein

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Ligand binding to recombinant bovine acyl-CoA-binding protein (rACBP) was examined using a Lipidex 1000 competition assay and an e.p.r. spectroscopy displacement assay. Of all putative ligands tested, rACBP exhibited a high binding affinity only for acyl-CoA esters. No alternative ligands could be found in rat liver fractions purified on an affinity column on which ACBP was coupled to Sepharose 4B. E.p.r. data indicate that both the acyl chain and the CoA head group of acyl-CoA are involved in binding and that the 3'-phosphate group on the ribose moiety of acyl-CoA esters plays a crucial role in the binding of acyl-CoA

to ACBP. E.p.r. competition binding studies show that the binding affinity of acyl-CoA esters for rACBP is strongly dependent on the length of the acyl chain with a clear preference for acyl-CoA esters with 14–22 carbon atoms in the acyl chain. No correlation between the number of double bonds in the acyl chain and the binding affinity was observed. The experimental results strongly indicate that ACBP specifically binds long-chain acyl-CoA esters with a very high affinity, results that indicate that ACBP is likely to be involved in the intracellular transport and pool formation of these compounds.

INTRODUCTION

Acyl-CoA-binding protein (ACBP) is a 10 kDa cytosolic protein which was identified by its ability to induce medium-chain acyl-CoA synthesis by goat mammary-gland fatty acid synthetase *in vitro* (Mogensen et al., 1987). ACBP binds long-chain acyl-CoA esters with high affinity but shows no affinity towards non-esterified fatty acids or free CoA (Mikkelsen et al., 1987; Mikkelsen & Knudsen, 1987; Rasmussen et al., 1990), indicating that both hydrophilic and hydrophobic interactions are involved in binding. The binding stoichiometry is 1 mol of acyl-CoA bound per mol of ACBP (Knudsen et al., 1989; Rasmussen et al., 1990). The apparent dissociation constants (K_d) of native bovine ACBP for *cis*-9-[1-¹⁴C]octadecenoyl-CoA and [1-¹⁴C]hexadecanoyl-CoA have been determined to be 0.22 μ M and 0.14 μ M respectively using the Lipidex 1000 binding assay (Rasmussen et al., 1990).

By amino acid sequence comparison, it was shown that ACBP was identical with diazepam-binding inhibitor (Knudsen et al., 1989). This peptide was isolated on its ability to displace diazepam from the γ -aminobutyric acid (GABA) receptor (Guidotti et al., 1983). These authors suggested that ACBP (or diazepam-binding inhibitor) acted as a neurotransmitter or neuromodulator. ACBP has also been suggested to be involved in the acute regulation of steroid-hormone synthesis (Yanagibashi et al., 1988; Besman et al., 1989). Finally ACBP has been suggested to be involved in regulation of glucose-induced insulin secretion (Chen et al., 1988; Östenson et al., 1990; Borboni et al., 1991).

The suggested role of ACBP in acute regulation of steroid-hormone secretion (Yanagibashi et al., 1988; Besman et al., 1989) has been challenged by the finding that ACBP synthesis in steroid-producing cells is not affected by either adrenocorticotropin or luteinizing hormone (Brown et al., 1992). Furthermore Massotti et al. (1991) have reported that increased plasma concentration of corticosterone precedes the increase in ACBP concentration in rat adrenal gland.

Conclusive evidence that ACBP is a neurotransmitter or neuromodulator is lacking and several facts argue directly against a role for ACBP or ACBP-derived peptides as modulators of the GABA_A receptor. These are: (1) inhibition of diazepam binding

requires micromolar concentrations of ACBP (Guidotti et al., 1983; Costa et al., 1983; Ferrero et al., 1986); (2) direct interaction of ACBP with the GABA_A receptor has never been shown (Knudsen, 1991); (3) displacement of diazepam from the GABA_A receptor complex cannot be repeated with pure rat liver ACBP (Knudsen and Nielsen, 1990); (4) a detailed study of the genomic ACBP gene in rat liver has revealed that ACBP is a typical housekeeping gene (Mandrup et al., 1993a) expressed in all tissues, which is in accordance with the fact that ACBP is found in all tissues tested (Mikkelsen and Knudsen, 1987; Knudsen et al., 1989). Finally, using high-resolution *in situ* hybridization, Tong et al. (1991) have reported that ACBP mRNA is confined to non-neuronal cells in the brain. This finding raises strong doubts about the possible role of ACBP as a neurotransmitter. The possible role of ACBP in regulation of glucose-induced insulin secretion is still an open question.

The major evidence that ACBP is able to act as an intracellular acyl-CoA transporter and acyl-CoA pool former is that it binds long-chain acyl-CoA esters with high affinity *in vitro* (Mikkelsen et al., 1987; Knudsen et al., 1989; Rasmussen et al., 1990) and that overexpression of bovine ACBP in yeast dramatically increases the acyl-CoA pool size (Mandrup et al., 1993b). The function of ACBP as an intracellular acyl-CoA transporter and pool former is compatible with the fact that it is a housekeeping protein.

The aim of the present work was to characterize the ligand-binding specificity of ACBP. The results strongly indicate that ACBP, in contrast with other lipid-binding proteins such as liver fatty acid-binding protein, is very specific in binding only acyl-CoA esters. The highest binding affinity was found for long-chain (C₁₄–C₂₂) acyl-CoA esters. The number of double bonds in the acyl chain only slightly affected binding affinity.

MATERIALS AND METHODS

Materials

Non-esterified fatty acids were from Sigma Chemical Co., St. Louis, MO, U.S.A. or Larodan Fine Chemicals, Malmö, Sweden. Spin-labelled fatty acid analogues were from Sigma. CNBr-activated Sepharose 4B was from Pharmacia Biotechnology

International AB, Uppsala, Sweden. Free CoA and 3'-dephospho-CoA were from Pharmacia. Lipidex 1000 was from Packard Instrument Co., Downers Grove, IL, U.S.A. Hexadecyl iodide was from Aldrich Chemie, Steinheim, Germany. H.p.l.c.-grade acetonitrile was from Rathburn Chemicals, Walkersburn, U.K.

Lipidex 1000 competition assay

This assay was set up to see if any ligands, besides acyl-CoA, could be found that were capable of competing with [^{14}C]hexadecanoyl-CoA in binding to ACBP. The putative ligand was mixed with 80 pmol of [^{14}C]hexadecanoyl-CoA (specific radioactivity 10 Ci/mol) in 150 μl of 10 mM potassium phosphate buffer, pH 7.4. Recombinant bovine ACBP (bovine rACBP) (40 pmol) was added in 50 μl of 10 mM potassium phosphate buffer, pH 7.4. The samples were mixed and incubated at 37 °C for 30 min, chilled on ice for 10 min and mixed with 400 μl of an ice-cold 50% slurry of Lipidex 1000 in binding buffer. After 100 min incubation on ice, the samples were centrifuged at 12000 g for 5 min at 0 °C, and the radioactivity in 200 μl of the resulting supernatant was determined by liquid-scintillation counting. The assay was carried out in triplicate, and blanks without added ACBP were run for each concentration of the compounds tested, to ensure that all unbound ligand could be completely removed from the incubation medium by the Lipidex at all concentrations used.

Test of Lipidex 1000-binding assay

cis-9-[^{14}C]Octadecenoyl-CoA (specific radioactivity 7.5 Ci/mol) was dissolved in 150 μl of binding buffer at the indicated concentrations. The samples were chilled on ice and mixed with 400 μl of an ice-cold 50% slurry of Lipidex 1000 in binding buffer. After 30 min incubation on ice, ACBP (40 pmol) was added in 50 μl of 10 mM potassium phosphate buffer, pH 7.4. After mixing, the samples were further incubated for 20 min on ice and then centrifuged at 12000 g for 5 min at 0 °C. The radioactivity in 200 μl of the resulting supernatant was determined by liquid-scintillation counting.

E.I.s.a. of rACBP

The e.i.s.a. of bovine rACBP was performed as described by Mikkelsen and Knudsen (1987).

Preparation of ACBP affinity column

Bovine rACBP (20 mg) was coupled to 1.2 g of CNBr-activated Sepharose 4B as recommended by the manufacturer. The unbound rACBP in the coupling and washing buffers was measured by e.i.s.a. Less than 1% of the originally added rACBP was present in these buffers, indicating that more than 99% had been coupled to the gel. The rACBP-Sepharose (4 ml) was then transferred to a column (1 cm $\times$ 10 cm).

Preparation of rat liver fractions

Male Sprague-Dawley rats (200–250 g) were killed by decapitation. The livers (approx. 10 g) were rapidly excised and homogenized in a Potter-Elvehjem homogenizer in 3 vol. of ice-cold 154 mM KCl, pH 7.0. The homogenate was either centrifuged immediately at 45000 g for 30 min or heat-treated at 80 °C for 30 min before the centrifugation in order to prevent enzymic degradation of putative ACBP ligands. The supernatants were loaded on to the ACBP affinity column and eluted stepwise with

gradients of ethanol in 100 mM ammonium acetate, pH 7.4, at a flow rate of 5.7 ml/h as follows: 5% for 60 min, 10% for 60 min, 20% for 60 min, 50% for 150 min. The eluates from each ethanol concentration were lyophilized and resuspended in 1 ml of potassium phosphate buffer (10 mM). The existence of alternative ligands for ACBP in each fraction was then tested in the e.p.r. displacement assay.

In a different line of experiments, a rat liver was freeze-clamped immediately after excision and ground finely in a mortar under liquid nitrogen. Chloroform/methanol (1:1, v/v; 50 ml) was added to the ground powder. After centrifugation at 45000 g for 30 min, the supernatant was removed and taken to dryness in a vacuum concentrator. The dry residue was resuspended in 100 mM ammonium acetate buffer, pH 7.4, loaded on to the ACBP affinity column and eluted and tested as described above.

Synthesis and purification of acyl-CoA

Medium-, long- and very-long-chain acyl-CoA esters ($\text{C}_8\text{--C}_{24}$) and spin label analogues were synthesized as previously described (Rasmussen et al., 1990). The acyl-CoA esters were purified on an ODS Nucleosil 10 μm particle size and 10 nm pore size column (4.6 mm $\times$ 250 mm) using a linear gradient of the following mobile phases: A, 20% acetonitrile, 80% 25 mM ammonium acetate, pH 5.3, and B, 70% acetonitrile, 30% 25 mM ammonium acetate, pH 5.3. The gradient was: 20% phase B for 15 min, 20–80% phase B for 25 min, 80% phase B for 15 min. The flow rate was 1 ml/min. The acyl-CoA esters were detected by u.v. absorption at 254 nm. After h.p.l.c. purification, the fractions containing the acyl-CoA esters were lyophilized and the acyl-CoA esters were redissolved in 5 mM ammonium acetate, pH 6.0. The acyl-CoA concentration was determined from u.v. absorption at 260 nm using a molar absorption coefficient of 14.7 $\text{mM}^{-1}\cdot\text{cm}^{-1}$. The purified esters were stored at –20 °C until used. The unsaturated acyl-CoA esters were used immediately after purification to prevent oxidation of the double bonds before the assays were performed. The sulphur-substituted acyl-CoA analogues tetradecylthiopropionyl-CoA (MP-CoA) and tetradecylthioacetyl-CoA (ME-CoA) were gifts from Rolf K. Berge, Haukeland Sykehus, Bergen, Norway. The two acyl-CoA esters of 3'-dP-CoA, 3'-dephosphohexadecanoic acid and 3'-dephospho-12-doxyoctadecanoic acid (3'-dP-12-DSC), were synthesized and purified as described above. Butyryl-CoA was synthesized by dissolving free CoA (20 mg) in 1 ml of 0.2 M NaHCO_3 , pH 7.5, and adding butyric acid anhydride until the nitroprusside test (Stadtman, 1957) for free thiol groups was negative. The pH was then reduced to 4.5 and the synthesized butyryl-CoA was purified on a Sephadex G-10 gel-filtration column (60 cm $\times$ 2.5 cm) and eluted with water. The butyryl-CoA-containing fractions were pooled and lyophilized and the butyryl-CoA was redissolved in water. Acetyl-CoA was synthesized and purified as previously described (Hansen et al., 1984).

S-Hexadecyl-CoA, the non-hydrolysable thioether analogue of hexadecanoyl-CoA, was synthesized as described by Ciardelli et al. (1981), with the following modifications. CoA (20 mg) was dissolved in 2 ml of degassed 0.04 M Li_2CO_3 buffer and bubbled through with N_2 for at least 30 min. Hexadecyl iodide (109.2 μmol) was dissolved in 200 μl of benzene and diluted 1:19 (v/v) with ethanol. Both the benzene and the ethanol were previously degassed by sonication for 30 min. The hexadecyl iodide solution was added to the CoA solution under N_2 . The reaction mixture was sealed under an N_2 atmosphere and was stirred overnight to achieve maximum alkylation. S-Hexadecyl-CoA in the reaction mixture was precipitated by adjusting the

pH to 1 with HCl (2 M), and the solvent was removed using a vacuum concentrator. The dry residue was washed three times with 1 ml of diethyl ether, three times with 1 ml of acetone and three times with 1 ml of 0.1 M HCl. Finally the washed precipitate was redissolved and further purified by reversed-phase h.p.l.c. as described above.

E.p.r. spectroscopy

E.p.r. spectra of free acyl-CoA esters of spin-labelled acids (5-, 12-, 16-DSC and 3'-dP-12-DSC) in solution were obtained in the following way: 1.5 nmol of spin-labelled acyl-CoA in 50 μ l of 5 mM ammonium acetate, pH 6.0, was added to 150 μ l of 10 mM potassium phosphate, pH 7.0. To obtain spectra of ligands complexed to ACBP, 1.5 nmol of rACBP or 1.5 nmol of native bovine ACBP was added in 50 μ l of 10 mM potassium phosphate, pH 7.0, replacing 50 μ l of phosphate buffer in the above assay. The samples were allowed to equilibrate for 30 min at room temperature before the spectra (X-band) were recorded with a Varian E line spectrometer using 100 kHz field modulation. Measurements were carried out in plain micro-haematocrit tubes.

E.p.r. displacement assays were carried out as follows: bovine rACBP (0.75 nmol) was incubated with 0.75 nmol of 12-DSC in 100 μ l of 10 mM potassium phosphate buffer, pH 7.0. The putative displacers/ligands were added and the final volume was adjusted to 150 μ l with 5 mM ammonium acetate, pH 6.0. The samples were allowed to equilibrate for 30 min at room temperature before the e.p.r. spectra were recorded as described above.

RESULTS AND DISCUSSION

In the present study, bovine rACBP was used instead of native ACBP because it is easy to obtain in large amounts. A synthetic gene encoding ACBP was constructed and rACBP was expressed in *Escherichia coli* (Mandrup et al., 1991). The rACBP is identical with the native form except that it lacks the N-terminal acetyl group. No discrepancies in the ability to bind acyl-CoA have been observed between the two forms of ACBP (Mandrup et al., 1991), and the three-dimensional structures of ACBP and rACBP in solution are very similar (Andersen et al., 1991). On this background we found it safe to assume that data obtained with bovine rACBP are representative of bovine native ACBP.

Figure 1 Displacement of [14 C]hexadecanoyl-CoA in the Lipidex 1000 displacement assay

Results are means $\pm$ S.D. ($n=1$) of triplicates. The concentration of [14 C]hexadecanoyl-CoA was 0.4 μ M. For further details, see the Materials and methods section.

Binding studies with Lipidex 1000

In the Lipidex 1000 competition binding assay, an alternative ligand to ACBP is expected to be able to displace [14 C]hexadecanoyl-CoA from the binding site as illustrated in Figure 1, using unlabelled hexadecanoyl-CoA.

The following compounds were tested: ATP, NADH, NADPH, palmitoylcarnitine, cholesterol and GABA. They were selected on the basis of having structural similarities to CoA (ATP, NADH and NADPH) or having amphiphilic characteristics like long-chain acyl-CoA (palmitoylcarnitine and cholesterol). GABA was selected to test the possibility that the postulated effect of ACBP on diazepam binding to GABA_A could be indirect through binding of GABA to ACBP.

None of the compounds tested could compete with [14 C]hexadecanoyl-CoA (0.4 μ M) in binding to ACBP, in the concentration range 0.4–40 μ M, indicating that ACBP specifically binds acyl-CoA esters (results not shown).

The Lipidex 1000-binding assay was originally developed to determine binding stoichiometry and dissociation constants for ligands binding to fatty-acid-binding protein (Glatz and Veerkamp, 1983). The method is based on the assumption that the Lipidex 1000 effectively removes all unbound ligand without interfering with ligand binding to the protein when added at 0 $^{\circ}$ C and vice versa. As we originally used this binding assay for the determination of binding constants for acyl-CoA binding to ACBP (Rasmussen et al., 1990), we tested the validity of this assumption with regard to the binding of acyl-CoA esters to ACBP. The assumption that there is no ligand exchange between Lipidex 1000 and binding protein when incubated at 0 $^{\circ}$ C does not hold in the case of acyl-CoA esters binding to ACBP. ACBP is clearly able to extract acyl-CoA esters bound to Lipidex (Figure 2). Therefore the Lipidex-binding assay cannot be used to determine true binding constants and relative binding affinity of different ligands as the results will express competition between Lipidex 1000 and ACBP for the ligand in question. For this reason we turned to e.p.r. and developed a binding assay based on ligand displacement of spin-labelled acyl-CoA bound to ACBP. The above result showing that ACBP can extract acyl-CoA bound to Lipidex 1000 at 0 $^{\circ}$ C shows that previously obtained results for acyl-CoA binding to protein using this assay (Bass, 1985; Burrier et al., 1987; Paulssen et al., 1988) should be treated with caution.

Search for alternative ligands

To test the ligand-binding ability of the ACBP affinity column coupled with 0.55 μ mol of ACBP, excess [14 C]hexadecanoyl-CoA (1 μ mol) was applied to the column; unbound [14 C]hexadecanoyl-CoA was eluted with 10 mM ammonium acetate buffer, pH 7.4. The ammonium acetate eluted 0.45 μ mol (45%) of the loaded [14 C]hexadecanoyl-CoA. The remaining [14 C]hexadecanoyl-CoA, presumably specifically bound to ACBP, could only be eluted with 50% ethanol (Figure 3). This is in good agreement with the reported binding stoichiometry of 1 mol of acyl-CoA/mol of ACBP (Knudsen et al., 1989; Rasmussen et al., 1990). Furthermore the results indicated that the acyl-CoA-binding site of the coupled rACBP was intact and accessible.

Alternative ligands to ACBP in rat liver

In an attempt to identify alternative endogenous ligands to ACBP besides acyl-CoA esters, rat liver fractions were prepared and fractionated on the ACBP affinity column as described in the Materials and methods section. No alternative ligands were found in any of the tested ethanol eluates from the affinity

Figure 2 Test of Lipidex 1000-binding assay

ACBP (0.4 μ g) was added after incubation of *cis*-9-[1- 14 C]octadecenyl-CoA with Lipidex 1000 at 0 $^{\circ}$ C. The extraction of *cis*-9-[1- 14 C]octadecenyl-CoA from Lipidex by ACBP is shown as a function of the concentration of *cis*-9-[1- 14 C]octadecenyl-CoA. Results are means $\pm$ S.D. ($n=1$) of triplicates. ●, ACBP added; ○, ACBP not added. For further details, see the Materials and methods section.

Figure 3 Elution profile of [1- 14 C]hexadecanoyl-CoA from the ACBP affinity column

[1- 14 C]Hexadecanoyl-CoA (1 μ mol; specific radioactivity 0.11 Ci/mol) was applied to the ACBP affinity column (●) and the control column without ACBP bound (○). The arrows indicate where the elution buffer was changed as follows: 1, pH lowered to 3.0; 2, made 1 M with NaCl; 3, made 25% with ethanol; 4, made 50% with ethanol.

column (results not shown). However, we cannot completely exclude the possibility that ACBP does bind other ligands, since labile compounds may have decomposed during the procedure.

Binding of 12-DSC to ACBP

In free solution, the nitroxide moiety of 12-DSC yields a simple three-line e.p.r. spectrum. When an equimolar amount of ACBP is added, the resonance peaks are substantially broadened (Figure 4b), thus indicating a strong immobilization of the spin-labelled acyl chain. The high-field (right-hand) resonance peak is particularly broadened and as this peak contributes only negligibly to the total spectrum of the bound ligand, it can be used as a direct measure of the concentration of unbound 12-DSC (Fournier et al., 1983). No free ligand is detectable at a ligand/protein ratio of 1:1; it was therefore not possible to obtain a dissociation constant by a traditional Scatchard plot (Scatchard, 1949). Even at increased concentrations of ligand-rACBP complex (0.4 mM),

Figure 4 Binding of spin-labelled octadecenyl-CoA analogues to rACBP

E.p.r. signals of free (---) spin-labelled octadecenyl-CoA analogues (DSC) with the doxyl group in different positions on the acyl-chain bound to bovine rACBP (—) as shown. (a) 5-DSC; (b) 12-DSC; (c) 16-DSC. The bar represents 10^{-3} T. The measurements were carried out as described in the Materials and methods section.

no free ligand could be detected, indicating that the binding affinity is extremely high. Measurements by n.m.r. spectroscopy have further revealed that the ligand exchanges slowly and that the binding is too strong for a dissociation constant to be measured on the n.m.r. time scale (B. B. Kragelund, personal communication). We did not observe any differences between native bovine ACBP and rACBP in the binding of 12-DSC (results not shown), which is in accordance with earlier findings (Mandrup et al., 1991). The e.p.r. spectrum of 3'-dP-12-DSC did not show any peak-broadening on addition of rACBP (result not shown), which strongly indicated that rACBP was not able to bind the spin-labelled 3'-dephosphoacyl-CoA. This finding led us to the conclusion that the 3'-phosphate group on the ribose moiety is involved in crucial electrostatic interactions with positively charged amino acid residues in the binding site of ACBP. To test the mobility of the acyl chain, binding studies were carried out using DSC with the spin-label in three different positions (Figure 4). Extensive peak-broadening was observed with the spin label in the 5, 12 or 16 position on the acyl chain of octadecenyl-CoA. These results strongly suggest that the

Figure 5 Displacement of 12-DSC from rACBP by acyl-CoA

Displacement assay using heptadecanoyl-CoA to displace 12-DSC from equimolar concentrations ($5 \mu\text{M}$) of rACBP. The assay was carried out as described in the Materials and methods section. The results presented here are means $\pm$ range of two determinations. The displacement isotherms were constructed by using non-linear regression. For experimental details see the Materials and methods section.

Table 1 RBAs of different ligands to ACBP

IC_{50} values and $\text{RBA}_{12\text{-DSC}}$ for acyl-CoA esters measured by the e.p.r. displacement assay are shown. The $\text{RBA}_{12\text{-DSC}}$ for $\text{C}_{17:0}$ is calculated as follows: $5 \mu\text{M}$ divided by the measured IC_{50} for $\text{C}_{17:0}$ ($5 \mu\text{M}/3.20 \mu\text{M} = 1.56$). Values are means $\pm$ S.D. ($n-1$). For experimental details see the Materials and methods section.

Displacer	IC_{50} (μM)	$\text{RBA}_{12\text{-DSC}}$
Decanoyl-CoA ($\text{C}_{10:0}$)	31.80 ± 2.00 (5)	0.16 ± 0.01
Dodecanoyl-CoA ($\text{C}_{12:0}$)	9.51 ± 0.31 (5)	0.53 ± 0.02
Tetradecanoyl-CoA ($\text{C}_{14:0}$)	5.18 ± 0.34 (4)	0.97 ± 0.06
Hexadecanoyl-CoA ($\text{C}_{16:0}$)	3.80 ± 0.08 (4)	1.32 ± 0.03
Heptadecanoyl-CoA ($\text{C}_{17:0}$)	3.20 ± 0.07 (3)	1.56 ± 0.04
Octadecanoyl-CoA ($\text{C}_{18:0}$)	3.20 ± 0.17 (3)	1.56 ± 0.08
Icosanoyl-CoA ($\text{C}_{20:0}$)	2.96 ± 0.05 (3)	1.69 ± 0.03
Docosanoyl-CoA ($\text{C}_{22:0}$)	4.46 ± 0.15 (3)	1.12 ± 0.01
Tetracosanoyl-CoA ($\text{C}_{24:0}$)	25.54 ± 1.57 (4)	0.20 ± 0.01
S-Hexadecyl-CoA	3.17 ± 0.05 (3)	1.58 ± 0.03
<i>cis</i> -9-Octadecenoyl-CoA ($\text{C}_{18:1}$)	4.17 ± 0.26 (4)	1.20 ± 0.08
<i>cis,cis</i> -9,12-Octadecadienoyl-CoA ($\text{C}_{18:2}$)	6.80 ± 0.25 (3)	0.74 ± 0.03
All- <i>cis</i> -9,12,15-octadecatrienoyl-CoA ($\text{C}_{18:3}$)	5.55 ± 0.40 (3)	0.90 ± 0.07
All- <i>cis</i> -5,8,11,14-icosatetraenoyl-CoA ($\text{C}_{20:4}$)	8.65 ± 0.60 (4)	0.58 ± 0.05
MP-CoA	3.03 ± 0.13 (4)	1.65 ± 0.07
ME-CoA	3.17 ± 0.13 (4)	1.58 ± 0.07
Dephosphohexadecanoyl-CoA	> 100	< 0.05

acyl chain is highly immobilized in its entire length, thus contributing to the strong binding by hydrophobic interaction.

Displacement of 12-DSC by acyl-CoA esters

Displacement assays were performed using a number of acyl-CoA esters. The amount of 12-DSC displaced was calculated from the height of the high-field resonance peak by comparing it with a standard curve of free ligand. The height of the high-field resonance peak was linear in the tested concentration range of 0–20 μM (result not shown). The data from displacement assays were plotted as free 12-DSC versus the concentration of added displacer. Results in Figure 5 show an example of such a displacement isotherm using heptadecanoyl-CoA as a displacer. To compare the binding affinity of the displacers, we calculated

Figure 6 $\text{RBA}_{12\text{-DSC}}$ as a function of acyl chain length

Graphic representation of the $\text{RBA}_{12\text{-DSC}}$ for the saturated acyl-CoA esters listed in Table 1.

relative binding affinities ($\text{RBA}_{12\text{-DSC}}$), defined as the ratio between the putative IC_{50} for 12-DSC displacing itself and the IC_{50} for a given displacer. The IC_{50} and $\text{RBA}_{12\text{-DSC}}$ values for the tested acyl-CoA esters are shown in Table 1. For direct comparison, the $\text{RBA}_{12\text{-DSC}}$ values for the saturated fatty acyl-CoA esters from C_{10} to C_{24} are shown in Figure 6. The inability of 3'-dephosphohexadecanoyl-CoA to displace 12-DSC (Table 1), even when added in large excess, confirmed the data from the direct binding studies with 3'-dP-12-DSC which indicated that the 3'-phosphate on the ribose moiety is indeed essential for binding. Acetyl-CoA, butyryl-CoA and octanoyl-CoA were also tested in this assay but they were all unable to displace 50% of the 12-DSC even when added 20 times in excess of 12-DSC. All the compounds tested in the Lipidex competition binding assay and lysophosphatidylcholine as well as diazepam, which ACBP is reported to displace from the binding site on the GABA_A receptor, were tested in the e.p.r. displacement assay. None of the compounds tested were able to displace any 12-DSC from rACBP even when added 20 times in excess.

From the displacement data presented here (Table 1 and Figure 6), it is evident that the binding affinity of rACBP towards acyl-CoA esters is strongly dependent on the length of the acyl chain with a clear preference for acyl-CoA esters with between 14 and 22 carbon atoms in the acyl chain. Although short- and medium-chain length ($\text{C}_2\text{--C}_6$) acyl-CoA esters are unable to displace 12-DSC, they can bind to ACBP with low affinity (Mikkelsen et al., 1987; Knudsen et al., 1989). The findings suggest that the physiological ligands *in vivo* are long-chain acyl-CoA esters ($\text{C}_{14}\text{--C}_{22}$) only. This conclusion is further strengthened by the fact that ACBP only increases the content of C_{16} and C_{18} acyl-CoA esters when overexpressed in yeast (Mandrup et al., 1993b). There is no clear correlation between the number of double bonds in the acyl chain and the binding affinity, and it is striking that rACBP is able to bind acyl-CoA esters with little or no conformational mobility in the acyl chain, such as all-*cis*-9,12,15-octadecatrienoyl-CoA (18:3) and all-*cis*-5,8,11,14-icosatetraenoyl-CoA (20:4), with a relative high affinity. This indicates that the acyl moiety of the acyl-CoA is not wrapped around ACBP but is bent back on itself in the binding site. The exact conformation of the acyl-CoA when bound to rACBP will be a subject of future investigations. The strong influence of the 3'-phosphate on ligand binding cannot be explained by a conformational difference between CoA and dephospho-CoA, since there is only a marginal difference between

the conformation of the two species of CoA (Lee and Sharma, 1974).

The results presented here suggest an interaction between the ligand and amino acid residues that are able to accommodate the negative charge of the phosphate, most likely an interaction between the 3'-phosphate and the one or more positively charged amino acid residues in the binding site. Neither the substitutions of a methylene group for sulphur in the acyl chain of ME-CoA and MP-CoA nor the substitution of the carbonyl group for a methylene group (*S*-hexadecyl-CoA) has any significant effect on the ability of ACBP to bind these compounds. It thus seems that the CoA head group conveys the specificity of binding acyl-CoA esters to ACBP, whereas the only demand on the acyl chain is that it expresses a certain degree of hydrophobicity.

Finally, ACBP has been found not to possess phospholipid-transfer activity (J. Østergaard, personal communication). Also a test for cholesterol-transfer activity has proved negative (J. T. Billheimer, personal communication).

In conclusion, the results presented here strongly indicate that ACBP specifically binds long-chain acyl-CoA esters and is therefore likely to be involved in the intracellular transport and pool formation of these compounds. It is established that both hydrophilic and hydrophobic forces are involved in binding, and that binding is very specific with regard to chain length but not to degree of desaturation of the acyl chain.

These results do not exclude the possibility that ACBP may have one or more alternative functions *in vivo*. However, no conclusive evidence to prove or disprove such functions is available at present.

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REFERENCES

- Andersen, K. V., Ludvigsen, S., Mandrup, S., Knudsen, J. and Poulsen, F. M. (1991) *Biochemistry* **30**, 10654–10662
- Bass, N. M. (1985) *Chem. Phys. Lipids* **38**, 95–114
- Besman, M. J., Yanagibashi, K., Lee, T. D., Kawamura, M., Hall, P. E. and Shiveky, J. E. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4897–4901
- Borboni, P., Condorelli, L., De Stefanis, P., Sesti, G. and Lauro, R. (1991) *Neuropharmacology* **30**, 1399–1403
- Brown, A. S., Hall, P. F., Shoyab, M. and Papadopoulos, V. (1992) *Mol. Cell. Endocrinol.* **83**, 1–9
- Burrier, R. E., Manson, C. R. and Brecher, P. (1987) *Biochim. Biophys. Acta* **919**, 221–230
- Chen, Z. W., Agerberth, B., Gell, K., Andersson, M., Mutt, V., Ostenson, G. G., Efendi, S., Barros-Soderling, J., Persson, B. and Jornvall, H. (1988) *Eur. J. Biochem.* **174**, 239–245
- Ciardelli, Y., Stewart, C. J., Seliger, A. and Wieland, T. (1981) *Liebigs. Ann. Chem.* **81**, 828–841
- Costa, E., Corda, M. G. and Guidotti, A. (1983) *Neuropharmacology* **22**, 1481–1492
- Ferrero, P., Santi, M. R., Conti-Tronconi, B., Costa, E. and Guidotti, A. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 827–831
- Fournier, N. C., Zucker, M., Williams, R. E. and Smith, C. P. (1983) *Biochemistry* **22**, 1863–1872
- Glatz, J. F. and Veerkamp, J. H. (1983) *Anal. Biochem.* **132**, 89–95
- Guidotti, A., Forchetti, P. L., Corda, M. G., Konkel, D., Bennet, C. D. and Costa, E. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3531–3535
- Hansen, H. O., Grunnet, I. and Knudsen, J. (1984) *Biochem. J.* **209**, 521–527
- Knudsen, J. (1991) *Neuropharmacology* **30**, 1405–1410
- Knudsen, J. and Nielsen, M. (1990) *Biochem. J.* **265**, 927–929
- Knudsen, J., Højrup, P., Hansen, H. and Roepstorff, P. (1989) *Biochem. J.* **262**, 513–519
- Lee, C. and Sharma, R. H. (1974) *J. Am. Chem. Soc.* **97**, 1225–1236
- Mandrup, S., Højrup, P., Kristiansen, K. and Knudsen, J. (1991) *Biochem. J.* **276**, 817–823
- Mandrup, S., Hummel, R., Ravn, S., Jensen, G., Andreasen, P. H., Gregersen, N., Knudsen, J. and Kristiansen, K. (1993a) *J. Mol. Biol.* **228**, in the press
- Mandrup, S., Jepsen, R., Skøtt, H., Rosendal, J., Højrup, P., Kristiansen, K. and Knudsen, J. (1993b) *Biochem. J.*, in the press
- Massotti, M., Slobodyansky, E., Konkel, D., Costa, E. and Guidotti, A. (1991) *Endocrinology* **129**, 591–596
- Mikkelsen, J. and Knudsen, J. (1987) *Biochem. J.* **248**, 709–714
- Mikkelsen, J., Højrup, P., Nielsen, P. F., Roepstorff, P. and Knudsen, J. (1987) *Biochem. J.* **248**, 857–861
- Mogensen, I. B., Schulenberg, H., Hansen, H. O., Spener, F. and Knudsen, J. (1987) *Biochem. J.* **241**, 189–192
- Östenson, C.-G., Åhrén, B., Karlsson, S., Sandberg, E. and Efendic, S. (1990) *Regul. Pept.* **29**, 143–151
- Paulssen, R. J. A., van der Logt, P. E. and Veerkamp, J. H. (1988) *Arch. Biochem. Biophys.* **264**, 533–545
- Rasmussen, J. T., Borchers, T. and Knudsen, J. (1990) *Biochem. J.* **265**, 849–855
- Scatchard, G. (1949) *Ann. NY Acad. Sci.* **51**, 660–672
- Stadtman, E. R. (1957) *Methods Enzymol.* **3**, 938–940
- Tong, Y., Toranzo, D. and Pelletier, G. (1991) *Neuropeptides* **20**, 33–40
- Yanagibashi, K., Okno, Y., Kawamura, M. and Hall, P. F. (1988) *Endocrinology (Baltimore)* **123**, 2075–2082