Multiple-site phosphorylation of the 280 kDa isoform of acetyl-CoA carboxylase in rat cardiac myocytes: evidence that cAMP-dependent protein kinase mediates effects of β -adrenergic stimulation

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Two major forms of mammalian acetyl-CoA carboxylase (EC 6.4.1.2), ACC- α and ACC- β , have been described and the sequences of the isoforms deduced. ACC- β is the predominant isoform expressed in heart and skeletal muscles, in which a major role of malonyl-CoA is probably to regulate fatty acid β -oxidation. The regulatory properties of ACC- β are incompletely defined but it is known that some cellular stresses lead to inhibition in parallel with the activation of AMP-activated protein kinase (AMP-PK). Here we examine the phosphorylation state of ACC- β within intact rat cardiac ventricular myocytes. Treatment of myocytes with the β -adrenergic agonist isoprenaline (isoproterenol) led to increased ACC- β phosphorylation that was maximal within 2 min and with 50 nM agonist. Effects of isoprenaline were revealed by the incorporation of ³²P into ACC in cells incubated with [³²P]_i and also by a marked decrease (approx. 80 %) in subsequent phosphorylation *in vitro* with cAMP-dependent protein kinase (PKA). Analysis of tryptic

phosphopeptides revealed that ACC- β was phosphorylated at multiple sites by incubation *in vitro* with PKA or AMP-PK. Treatment of myocytes with isoprenaline affected all the major phosphorylation sites of ACC- β that were recognized *in vitro* by purified PKA, so that subsequent phosphorylation *in vitro* was greatly diminished after cell stimulation. β -Adrenergic stimulation led to decreases in cellular malonyl-CoA concentrations but no changes in kinetic properties of ACC were detected after cell homogenization and partial purification of proteins. The results suggest that: (1) ACC- β is rapidly phosphorylated at multiple sites within intact cardiac ventricular myocytes after β -adrenergic stimulation, (2) ACC- β is phosphorylated *in vitro* by PKA and AMP-PK at multiple sites, including at least one site accessible to each kinase, as well as kinase-selective sites, and (3) PKA is a physiologically significant ACC- β kinase.

Key words: fatty-acid metabolism, lipid metabolism, heart.

INTRODUCTION

Acetyl-CoA carboxylase [acetyl-CoA:carbon-dioxide ligase (ADP-forming), EC 6.4.1.2] (ACC) plays critical roles in lipid metabolism by catalysing the ATP-dependent formation of malonyl-CoA from bicarbonate and acetyl-CoA [1–4]. Malonyl-CoA serves as a substrate for fatty acid synthase and for the fatty acyl chain elongation system; it also acts as a regulator of fatty acid oxidation by inhibiting carnitine palmitoyl transferase I [5–7]. There might be additional functions for ACC, suggested by the apparent failure to compensate for the absence of the enzyme in yeast null mutants by supplying long-chain fatty acids [8,9].

Two major isoforms of mammalian ACC with subunit sizes of 265 and 280 kDa have been recognized [2–4,10,11]; here they are designated ACC- α and ACC- β . Sequences of ACC- α from several animal species, including chicken [12], rat [13], goat [14] and human [15], have been deduced. Two variants of ACC- α have also been described, one of which contains eight additional amino acid residues commencing at Pro-1196 [16]. The second ACC- α variant is highly induced in lactating mammary gland and contains a distinct N-terminus that is 59 residues shorter than the predominant fat and liver isoform [17]. The distinct subunit size and enzymic properties of ACC- β were first noted in rat heart [10,11]; the two ACC isoforms were identified in several cell types with isoform-specific antibodies [11,18]. Substantial differences between the two rat liver ACC isoforms were revealed

by peptide sequencing and profiling by HPLC–MS and led to the prediction that distinct genes might encode the two isoforms [19]. The genes for the human ACC- α and ACC- β isoforms, located on chromosomes 17q and 12q respectively, have now been characterized and demonstrate that the two proteins exhibit approx. 75 % overall identity [20–23]. The human ACC isoforms differ most at the N-terminus, within which human ACC- β has a sequence of approx. 200 amino acids that is absent from ACC- α [20–22].

The relative degrees of expression of the two ACC isoforms vary with the tissue type: ACC- β is undetectable in rat white adipose tissue, the minor component of ACC in liver and lactating mammary tissue (10–25 % of total) but is the major isoform of ACC in both heart and skeletal muscle (90 % of the total). ACC- α is therefore the dominant isoform in tissues capable of high rates of fatty acid biosynthesis *de novo*, whereas ACC- β is the dominant isoform in tissues that perform little or no fatty acid biosynthesis *de novo* and in which malonyl-CoA is required predominantly for chain elongation and for the regulation of fatty acid oxidation (reviewed in [2–4,24]).

Most of our present understanding of the regulation of ACC comes from studies of ACC- α , and much less is known about the properties of ACC- β . ACC- α is subject to complex regulatory mechanisms and exists physiologically in a dynamic equilibrium between inactive dimers and linear polymers composed of 10–20 dimers [25–27]. Co-precipitation with isoform-specific antibodies

Abbreviations used: ACC, acetyl-CoA carboxylase (EC 6.4.1.2); AMP-PK, AMP-activated protein kinase; HRP, horseradish peroxidase; PKA, cAMP-dependent protein kinase.

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suggests that α - and β - isoforms of ACC associate, at least *in vitro*, to form heterodimers and thus heteropolymers [18,19]. The dimer-polymer equilibrium and catalytic activity of ACC are controlled by the interplay between hormone-induced phosphorylation and dephosphorylation and by allosteric mechanisms [2–4,24].

At least eight phosphorylation sites on ACC- α have been identified [4]. Several critical sites on ACC- α are rapidly phosphorylated in intact cells treated with hormones that suppress enzyme activity and rates of fatty acid synthesis. The most important 'inhibitory' phosphorylation sites are probably Ser-79 and Ser-1200, both of which are substrates for AMP-activated protein kinase (AMP-PK) [28,29]. Ser-77 and Ser-1200 might be phosphorylated by cAMP-dependent protein kinase (PKA) *in vitro*, but because Ser-77 seems not to be phosphorylated in intact hepatocytes exposed to glucagon, it has been argued that AMP-PK is the most significant inhibitory ACC- α kinase within intact cells and that Ser-79 is the dominant inhibitory site [4,30]. In contrast, results obtained by site-directed mutation of phosphorylation sites of ACC- α suggest that Ser-1200 contributes most importantly to the inhibition of ACC activity by catabolic hormones [31]. Ser-1215 is also phosphorylated in rat liver *in vivo* [32], although the effect of the phosphorylation of this site on ACC activity is not clear.

So far, the number and identity of the phosphorylation sites within ACC- β remain undefined, and our understanding of the protein kinases that mediate the intracellular phosphorylation of this ACC isoform is still incomplete. *In vitro*, ACC- β is a good substrate for PKA [19,33,34] and for AMP-PK [33,34]. The phosphorylation of ACC- β within intact cells has been inferred from the inhibition of ACC that is observed when AMP-PK is activated in hearts subjected to ischaemia and reperfusion [35,36]. An inverse relationship between ACC and AMP-PK has also been observed in skeletal muscle stimulated electrically [37], by exercise *in vivo* [38] or with an AMP-PK activator [39]. The importance of the phosphorylation of ACC- β in the inhibition of the enzyme after electrical stimulation of skeletal muscle is further supported by the apparent shift in ACC subunit size and by the re-activation of ACC by subsequent treatment with protein phosphatases [38]. Although exposure of tissues to catecholamines leads to the inhibition of ACC- α , effects on ACC- β have not been examined. Treatment of fat or liver cells with insulin leads to a rapid activation of ACC- α but effects of insulin on ACC- β have not yet been clearly resolved. For example, treatment of isolated perfused rat hearts with insulin has been reported to induce the inhibition of AMP-PK and the parallel activation of ACC [40]; however, in studies on mature rabbit heart [41] and on isolated soleus muscle preparations [42], no increase in ACC activity could be detected in tissue isolates after the treatment of intact tissue with insulin.

Here we evaluate the phosphorylation of ACC- β within intact rat cardiac ventricular myocytes. We demonstrate that ACC- β is phosphorylated on multiple sites within intact cardiac myocytes and provide evidence that PKA might be physiologically important in mediating β -adrenergic effects on ACC- β in the heart.

EXPERIMENTAL

Materials

Laboratory chemicals and solvents were obtained from Fisher Scientific (Vancouver, British Columbia) or BDH Chemicals Canada Ltd. (Vancouver, British Columbia). Biochemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), including trypsin, isoprenaline (isoproterenol), the inhibitor of PKA, and Kemptide; alternatively, they were from

Boehringer Mannheim (Laval, Quebec, Canada), including sweet potato acid phosphatase. Avidin was kindly donated by Canadian Inovatech (Abbotsford, British Columbia). Radioisotopes and the streptavidin-horseradish peroxidase (HRP) conjugate were from Amersham International (Oakville, Ontario, Canada). Pepstatin A and leupeptin were from Peptides International (Louisville, KY, U.S.A.), Microcystin-LR was from Calbiochem (La Jolla, CA, U.S.A.) and custom peptides including the 'SAMS' peptide (HMRSAMSGHLVKRR) for the assay of AMP-PK was synthesized by the Nucleic Acid and Protein Service Laboratory (University of British Columbia). Electrophoresis reagents and activated agarose for protein coupling (Affigel-10) were from Bio-Rad Ltd. (Mississauga, Ontario, Canada). Tetrameric avidin-agarose (10 mg of protein/ml of packed resin) was prepared in accordance with the manufacturer's instructions. Where indicated, avidin was subsequently monomerized as described previously [43]. PVDF membranes and concentrators were from Millipore (Bedford, MA, U.S.A.) and Western blotting chemiluminescence reagents were from Pierce (Rockford, IL, U.S.A.). Cellulose plates for TLC were from Kodak (number 13255).

Preparation of cardiac ventricular myocytes and cell extracts

Male Wistar rats were maintained on a 12 h light-dark cycle with continuous access to water and laboratory chow according to the guidelines of the Canadian Council for Animal Care. Cardiac ventricular myocytes from two rats (180–220 g), prepared as described previously [44], were suspended at a final density of 4×10^5 cells/ml in Joklik buffer containing CaCl_2 (1 mM) and exposed to O_2/CO_2 (95:5, v/v). Cell suspensions were divided into aliquots, incubated for the indicated durations in the absence or presence of isoprenaline at 37 °C and then quickly washed twice with 2 vol. of ice-cold Joklik buffer before being snap-frozen. Cells in each treatment group were homogenized on ice in buffer (pH 7.5) containing: Mops (20 mM), sucrose (0.25 M), KCl (150 mM), EDTA (2 mM), benzamidine (2.5 mM), Pepstatin A (3 μM), leupeptin (5 μM), glutathione (2.5 mM), PMSF (0.5 mM) and microcystin-LR (0.1 $\mu\text{g}/\text{ml}$). Other procedures were performed at 4 °C unless stated. The homogenate was centrifuged for 2 min at 1000 *g* and subsequently for 5 min at 15000 *g*; the final supernatant was then incubated with tetrameric avidin-agarose for the purification of immobilized ACC as described below.

To examine the incorporation of ^{32}P into ACC within intact myocytes, cells were prepared as above and incubated to isotopic equilibrium (50 min at 37 °C, with continuous gassing) in Krebs-Henseleit bicarbonate medium, containing glucose (11 mM) and [^{32}P]P_i (0.5 mM, approx. 1000 d.p.m./pmol). After the treatment of cell aliquots (typically 1 ml, containing approx. 1.5×10^6 cells/ml) for a further 2–10 min with the addition of the indicated hormone, cells were collected by brief centrifugation (1 min, 1000 *g*) and rinsed with ice-cold Krebs-Henseleit medium before being snap-frozen. Frozen cell pellets were homogenized and ACC was isolated by binding to tetrameric avidin-agarose as described below, followed by SDS/PAGE, electrophoretic transfer to a PVDF membrane and quantification by phosphorimaging and blotting with streptavidin-HRP.

Purification of ACC

ACC was purified either from myocytes (beginning as described above) or from freshly isolated intact rat tissues. For tissue preparations, two to four male Wistar rats (200–400 g) were killed by brief asphyxiation in CO_2 followed by cervical dislocation. Tissues (heart, liver, quadriceps muscle and epididymal

plus peri-renal white fat) were removed, chilled on ice and (with the exception of liver) were disrupted with a Polytron-PT10 homogenizer in 3 vol. of the homogenization buffer described above, except that phosphatase inhibitors were omitted. Each liver was homogenized, with a Teflon-glass Potter-Elvehjem tissue homogenizer, in 3 vol. of the same homogenization buffer. Homogenates were centrifuged at 1000 *g* for 5 min and then for a further 60 min at 250 000 *g*, yielding high-speed supernatant fractions that were subjected to one of two possible procedures to isolate ACC.

In one procedure, ACC was isolated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ (40 % satn.) followed by immobilization on tetrameric avidin-agarose. The protein fraction obtained after precipitation with $(\text{NH}_4)_2\text{SO}_4$ was resuspended in homogenization buffer to which was added NaCl (150 mM) and Nonidet P40 (0.1 %, v/v). The re-dissolved pellet was incubated with end-over-end rotation (4 h at 4 °C) in the presence of tetrameric avidin-agarose (10–15 μl packed vol./g wet weight of tissue). The agarose beads were then washed five times with buffer (pH 7.2) containing Mops (50 mM), EDTA (5 mM) and NaCl (1 M) and finally twice with the same buffer except that NaCl was replaced with KCl (150 mM), before storage at 4 °C.

In the second purification procedure, soluble ACC was purified by DEAE-cellulose chromatography followed by reversible binding to monomeric avidin-agarose, as described [19]. Fractions containing ACC were concentrated by centrifugation (1500 *g* for 1.5–2.5 h) through membrane filters with a 50 kDa cut-off (Millipore Ultrafree-15 Biomax). Concentrated fractions (0.5–1.5 mg/ml protein) were stored in aliquots at –70 °C.

Purification of protein kinases

AMP-PK was typically purified from the livers of two male Wistar rats through the centrifugation, precipitation with poly(ethylene glycol), and DEAE-cellulose steps described [45]. In some studies, the same procedure was used to purify AMP-PK from rat hearts. Preparations were stored at –20 °C in the presence of glycerol (50 %, w/v) and kinase activity was assessed by filter-paper assays with the SAMS peptide as phosphate acceptor. A unit of kinase activity is defined as the activity that transfers 1 μmol of phosphate/min to the SAMS peptide at 30 °C.

PKA was purified from approx. 500 g of fresh beef heart ventricular muscle by the technique described [46], including the specific elution from DEAE-cellulose with cAMP. The preparations of PKA were stored and assayed as described for AMP-PK, except that the phosphate acceptor was Kemptide.

Phosphorylation of ACC with purified protein kinases

Phosphorylation of ACC was performed either with soluble, affinity-purified ACC or with ACC immobilized on tetrameric avidin-agarose. ACC preparations were incubated in the absence or presence of indicated protein kinase at 30 °C in buffer (pH 7.4), containing Mops (20 mM), MgCl_2 (10 mM), EDTA (5 mM) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (100 μM , 500–2000 d.p.m./pmol). Typically, 20 μg -units of PKA or AMP-PK (plus 200 μM AMP) were employed in each assay. Reactions were terminated by holding mixtures at 95 °C for 5 min in SDS sample buffer before SDS/5 %-(w/v)-PAGE [47]. Proteins were detected by staining with Coomassie Blue R-250; ^{32}P was detected either by autoradiography coupled with densitometric scanning (with a Bio-Rad Model 620 video densitometer) or with a PhosphorImager (Molecular Dynamics). Absolute ^{32}P incorporation in the protein

Figure 1 Phosphorylation of soluble and avidin-immobilized preparations of ACC

ACC was partially purified from rat livers by precipitation with $(\text{NH}_4)_2\text{SO}_4$; the preparation was then divided equally for further purification with (1) monomeric avidin-agarose (for soluble ACC, □) or (2) by irreversible binding to tetrameric avidin-agarose (immobilized ACC, ■). Equal amounts of the two ACC preparations were incubated with PKA in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$; the incorporation of label into ACC- β was assessed by phosphorimaging after SDS/PAGE and electrophoretic transfer. The ^{32}P content of ACC- β is expressed as phosphorimaging units (upper panel) or as a percentage of the maximum (lower panel). Phosphorimaging values were corrected for ACC subunit protein (determined by streptavidin-HRP blotting) and then log-transformed to accommodate the variation in absolute values between experiments. Results are presented as means ($\times 10^{-3}$) for four independent experiments with separate ACC preparations. The error bars in the upper panel were no larger than the symbols.

bands was quantified by scintillation counting after the digestion of gel slices with H_2O_2 [48].

The advantages and potential pitfalls of using tetrameric avidin-agarose to isolate ACC should be noted. One disadvantage is that immobilized ACC might be so constrained that exogenous protein kinases are unable to gain access to cognate phosphorylation sites. We evaluated this potential problem by comparing the properties of ACC- β immobilized on tetrameric avidin-agarose with the soluble enzyme purified by reversible monomeric avidin-agarose affinity chromatography. The ACC- β in both soluble and immobilized preparations reached maximal phosphorylation within 30 min incubation with PKA but soluble ACC exhibited a somewhat higher maximum ^{32}P incorporation

than an equivalent protein mass of immobilized enzyme (Figure 1). In four independent experiments, the maximum incorporation of ^{32}P into soluble ACC- β exceeded that into immobilized enzyme by an average of 60 % (the absolute values in PhosphorImager units corrected for protein loading were 140 800 for soluble ACC and 87 100 for immobilized enzyme). Despite the lower maximal phosphorylation, two-dimensional TLC peptide analysis revealed essentially identical patterns of phosphopeptides, suggesting that added PKA could indeed access all the possible target sites on immobilized ACC. In accordance with the designation of peptides described in Figure 3(C), peptides 1–3 each accounted for 20–35 % of total ^{32}P and therefore together accounted for more than 80 % of ACC labelling whether the enzyme was soluble or immobilized. Of the remaining peptides (nos. 4–9), none accounted for more than 7 % of the total ACC labelling. The identical pattern of phosphopeptides suggests that one of the two subunits in each dimer must be fully accessible to PKA and that avidin-immobilized ACC acts as a suitable 'reporter' for the phosphorylation achieved within the soluble enzyme. Because it is not feasible to purify small (sub-microgram amounts) of homogeneous ACC from small samples of cardiac myocytes by conventional approaches, the use of tetrameric avidin–agarose provided an essential, rapid and quantitative recovery of ACC that would not have been possible with other available techniques.

Quantification of ACC recovered after SDS/PAGE and electroblotting

The recovery of ACC isoforms was estimated in each gel/membrane lane by blotting with streptavidin–HRP, coupled with chemiluminescence detection. ACC subunits were separated by SDS/PAGE and transferred to a PVDF membrane with the use of a semi-dry transfer system (50 min at 3 mA/cm² of gel) by using a transfer buffer (pH 9.0) containing Tris (48 mM) and glycine (39 mM). The membrane was blocked for 1 h with Tris-buffered saline/Tween (TBS-T), pH 7.6, containing Tris/HCl (20 mM), NaCl (137 mM) and Tween 20 (0.1 %, w/v). The membrane was next incubated for 2 h with streptavidin–HRP diluted 1:5000 with TBS-T that also contained BSA (2 %, w/v). ACC subunits were detected and quantified by incubation with chemiluminescence reagents and subsequent densitometry of photographic films. ^{32}P incorporation was then determined by phosphorimaging and values were corrected for subunit protein recovery in each individual gel lane.

Analysis of phosphopeptides

Preparations of ACC from liver, heart and adipose tissue were immobilized on tetrameric avidin–agarose and incubated with PKA or AMP-PK in the presence of [γ - ^{32}P]ATP (2 h, 30 °C). The individual ACC isoforms were isolated by SDS/PAGE and transferred to PVDF as described above. After the location and quantification of ACC subunits by phosphorimaging, membrane slices were blocked (30 min with TBS-T) and then incubated (4 h at 37 °C) in 0.5 ml of ammonium bicarbonate buffer (50 mM, pH 8.2) containing trypsin (10 μg initially, with a further 10 μg added after 2 h). Trypsin digests were subjected to three cycles of drying under vacuum and rehydration. Dried residues were resuspended and applied to TLC plates with 2,4-dinitrophenyllysine and Xylene Cyanol FF as internal standards. Two-dimensional peptide mapping, involving high-voltage electrophoresis followed by ascending chromatography, was performed as described [48,49].

Determination of the malonyl-CoA content of cell preparations

Cardiac myocytes (approx. $5 \times 10^5/\text{ml}$) were incubated with appropriate hormone treatments, pelleted by brief centrifugation (1 min, 1000 g), snap-frozen and then extracted by sonication in 2 vol. of ice-cold HClO_4 (6 %, w/v). After centrifugation (15 min, 10000 g), protein-free acid supernatants were stored at -20 °C before analysis by HPLC as described [50]. In brief, samples of acid extracts (20 μl) were applied to a C_{18} reverse-phase column (Waters Nova-Pak; 3.9 mm $\times$ 300 mm) via a precolumn cartridge (Waters Nova-Pak; C_{18}) at a flow rate of 0.5 ml/min. A gradient was applied with buffers A and B, both pH 5.0, containing: A, 0.2 M NaH_2PO_4 ; B, 0.25 M NaH_2PO_4 and acetonitrile (20 %, v/v). The composition of the buffer was 2 % B at the time of sample application. The gradient rose linearly to 57 % B at 16.7 min, remained at 57 % B until 18.0 min and finally rose to 90 % B at 22.0 min before returning to the starting composition at 30 min. The elution of CoA and its esters was detected with a flow-through monitor set at 254 nm. The column was calibrated with a range of CoA ester standards, with molar absorption coefficients of 14.9 and 14.4 mM⁻¹·cm⁻¹ for acetyl-CoA and malonyl-CoA respectively.

RESULTS AND DISCUSSION

Phosphorylation of ACC- β within intact rat cardiac myocytes

The phosphorylation of ACC- α , established in studies with intact adipose cells and hepatocytes, was facilitated by the abundance of the enzyme, which is in the range of 50–250 μg of ACC/g of tissue [24]. ACC- β is undetectable in rat white adipose tissue and represents a small fraction (10–25 %) of total ACC in a number of tissues, including liver and mammary gland. For studies of intact cells we therefore employed freshly isolated cardiac ventricular myocytes, in which ACC- β is the dominant expressed isoform of ACC and because these cells are non-immortalized and exhibit hormone-sensitive metabolic responses.

On the basis of earlier experiments [51], we examined the phosphorylation of ACC- β by incubating myocytes to isotopic equilibrium in medium containing [^{32}P]P_i. Myocytes from each heart were divided into two samples that were labelled in parallel and then incubated in the absence or presence of isoprenaline (500 nM, 2 min). ACC was then isolated by binding to tetrameric avidin–agarose and by SDS/PAGE; the ^{32}P content was determined by phosphorimaging as described in the Experimental section. In a set of three experiments, the incorporation of ^{32}P into ACC- β was increased by a factor of 1.8 ± 0.1 (mean $\pm$ S.E.M., $P < 0.02$) after exposure of myocytes to isoprenaline (500 nM, 2 min). More than 95 % of the ^{32}P detected on gel lanes was incorporated into ACC- β ; the remaining incorporation was into ACC- α . No other phosphoproteins were consistently detected.

Studies of ^{32}P incorporation into ACC subunits by metabolic labelling of myocytes are limited by two important factors. First, the expression of ACC is maximally approx. 2 μg of ACC protein/g wet weight of heart tissue, corresponding to only ~ 8 pmol ACC subunit/g wet weight. Secondly, the specific radioactivity readily achieved in the γ -phosphate of ATP is limited because of the high intramyocardial ATP concentration (approaching 10 mM). Extensive studies, especially those requiring phosphopeptide analysis, were therefore not feasible by metabolic labelling. As an alternative strategy we used a 'reverse phosphorylation' procedure in which myocytes were treated, ACC was isolated by binding to avidin–agarose and residual unoccupied sites were then phosphorylated by incubation *in vitro* with purified protein kinases and [γ - ^{32}P]ATP. This approach was based on the assumption that increases in the phosphorylation of

Figure 2 Phosphorylation of ACC- β within intact cardiac ventricular myocytes

ACC was isolated after incubations of cells by binding to tetrameric avidin-agarose and was then treated with purified PKA in the presence of [γ -³²P]ATP. The incorporation of ³²P into ACC subunits was determined by phosphorimaging after SDS/PAGE and electroblotting. A typical phosphorimage (top panel) illustrates the incorporation of ³²P into ACC- β after the incubation of myocytes (2 min) in the presence of isoprenaline ('isoproterenol') at 0.5, 5.0, 50, 500 and 0 nM (lanes 1–5 respectively). Lane 6 contained the ³²P-labelled 280 kDa subunit of a sample of affinity-purified cardiac ACC (arrowed) that was applied as a standard to verify the subunit migration. The dose dependence and time dependence (middle and bottom panels respectively) of the effects of isoprenaline are summarized as means and S.E.M. for three independent experiments with separate batches of myocytes. The effects of isoprenaline were significantly different from the control ($P < 0.01$) at 50 and 500 nM (all time points) on the basis of Student's *t*-test and analysis of variance.

ACC within intact cells (e.g. after hormone treatment) should lead to diminished subsequent phosphorylation *in vitro*. We employed tetrameric avidin-agarose to achieve a rapid and

quantitative recovery of ACC from heart extracts; the validation of this approach is discussed in the Experimental section. Cell recovery and extraction were performed rapidly to minimize changes in adenine nucleotides that might lead to the activation of stress-activated protein kinases, and in the presence of metal ion chelators and microcystin-LR to prevent the dephosphorylation of ACC.

Previous studies had demonstrated that rat liver ACC- β is rapidly phosphorylated by PKA [19], so we first employed PKA to phosphorylate residual sites on cardiac ACC isolated after treatments of myocytes. Results obtained with this indirect strategy confirmed the results of direct metabolic labelling and demonstrated that exposure of cells to isoprenaline led to a marked phosphorylation of ACC- β and therefore a corresponding decline in subsequent phosphorylation *in vitro* with PKA (Figure 2). The phosphorylation of ACC- β *in vitro* was decreased by more than 80 % after a brief exposure of cells to isoprenaline, indicating that extensive phosphorylation of PKA sites was achieved within the intact cells (Figure 2). When ACC was isolated from non-stimulated cells, treatment with potato acid phosphatase caused little increase (less than 10 %) in subsequent phosphorylation with PKA. In contrast, the phosphorylation *in vitro* of ACC isolated from cells treated with isoprenaline was restored by first treating it with the acid phosphatase (the average increase in two experiments was 2.2-fold). These results indicate that sites on ACC- β that might be phosphorylated by PKA are not substantially occupied in non-stimulated cells.

Examination of the dose-dependence of ACC- β phosphorylation in intact cardiac myocytes demonstrated that maximal phosphorylation was achieved with isoprenaline concentrations of 50 nM; half-maximal phosphorylation occurred with agonist concentrations in the range of 1–10 nM (Figure 2, middle panel), which is consistent with the dose-dependence of cardiac mechanical responses and the stimulation of cAMP formation. The phosphorylation of ACC- β also occurred rapidly in response to isoprenaline, reaching a maximum within 2 min and remaining near maximal for at least 10 min with sustained stimulation, indicating that there was little or no significant dephosphorylation of ACC during this period (Figure 2, bottom panel).

ACC- β exhibits multiple phosphorylation sites

Because the two ACC isoforms are closely related structures and ACC- α contains at least eight phosphorylation sites, it is important to define the complexity of phosphorylation of ACC- β . After the incubation of myocytes in the absence or presence of isoprenaline (500 nM, 2 min), ACC was recovered by binding to avidin-agarose and phosphorylated with PKA in the presence of [γ -³²P]ATP; the specific ACC subunits were then isolated and tryptic phosphopeptides were analysed. Five significant phosphopeptides (nos. 1–5) consistently accounted for more than 90 % of the ³²P detected on the two-dimensional maps (Figure 3a). Peptide 1 was the dominant tryptic phosphopeptide derived from ACC- β and represented 38 ± 1 % of the total ³²P detected on two-dimensional TLC plates (mean $\pm$ S.E.M. for three independent experiments). The incorporation of ³²P into the other peptides designated in Figure 3(e) were as follows (as a percentage of total isotope incorporation): peptide 2, 20 ± 2 %; peptide 3, 11 ± 4 %; peptide 4, 10 ± 1 %; peptide 5, 12 ± 1 %. Incorporation into other phosphopeptides was no greater than 6 % of total ³²P. The treatment of myocytes with isoprenaline led to greatly diminished radiolabelling of all five major ACC- β phosphopeptides, suggesting that all the peptides contain sites phosphorylated during β -adrenergic stimulation (compare Figures 3b and 3a). It is therefore concluded that ACC- β is

Figure 3 Multiple-site phosphorylation of ACC- β in intact cardiac myocytes in response to isoprenaline

Myocytes were incubated in the absence (a) or presence (b) of isoprenaline (500 nM, 2 min). ACC was then isolated by binding to tetrameric avidin-agarose and phosphorylated by incubation with PKA and [γ - ^{32} P]ATP. In addition, purified dephosphorylated ACC from rat liver was phosphorylated in the presence of PKA (c) or AMP-PK (d). In each case, ACC- β was isolated by SDS/PAGE and electroblotting, located by phosphorimaging and digested with trypsin. Phosphopeptides were separated by peptide mapping with the use of two-dimensional TLC with high-voltage electrophoresis (horizontal dimension) and chromatography (vertical dimension) from the origin [O; indicated by a star in (e)]. ^{32} P-peptides were detected by phosphorimaging and designated as in the key (e). Abbreviation: DNP, dinitrophenyl-lysine.

subjected to rapid, multiple-site phosphorylation in intact cardiac myocytes in response to isoprenaline.

Effects of phosphorylation on the activity of ACC- β

It has been demonstrated previously that total cellular malonyl-CoA concentrations are decreased after treatment of rat cardiac myocytes with adrenaline [52]. In a set of eight independent experiments, we observed a similar decrease in total cell malonyl-CoA after treatment of myocytes with isoprenaline (500 nM for 20 min). Values for control and isoprenaline-treated cells respectively were 1.33 ± 0.29 and 0.81 ± 0.15 nmol of malonyl-CoA/g wet weight of cells (means $\pm$ S.E.M.), a decline of $42 \pm 5.8\%$ in response to isoprenaline ($P < 0.01$ by paired *t*-test). We cannot conclude unambiguously from these values that ACC activity itself is decreased in response to isoprenaline, but rather that the balance between the synthesis and removal of malonyl-CoA (presumably via cytoplasmic malonyl-CoA decarboxylase) is altered. Cardiac cytoplasmic malonyl-CoA decarboxylase has not been characterized and there is no evidence for a rapid

regulation of its activity. The evidence so far suggests that a decline in ACC activity is the most likely explanation for the effects of isoprenaline on cardiomyocyte malonyl-CoA concentrations.

ACC activity is too low in initial myocyte homogenates or supernatant fractions to permit detection by conventional or HPLC-based assay. We therefore examined the activity of ACC after partial purification and concentration by precipitation with $(\text{NH}_4)_2\text{SO}_4$. So far we have been unable to detect changes in ACC activity or sensitivity to citrate that persist through the partial purification. Further, in agreement with a previous report [34], we have not been able to detect any change in the maximum activity of myocardial ACC or any changes in sensitivity to citrate after incubation with purified PKA. If PKA-mediated phosphorylation of ACC- β is physiologically important, as suggested by the speed, apparent stoichiometry and sensitivity of the response within intact cells, then the effect of covalent modification on enzyme function must be reflected in a more complex aspect of ACC regulation, perhaps involving other allosteric and/or protein modulators as reported previously [24,53].

Evidence that PKA might have a significant role in phosphorylation of myocardial ACC- β in response to isoprenaline

The balance of evidence suggests that PKA has a minor role in the regulation of ACC- α in fat and liver cells and that AMP-PK is the dominant inhibitory ACC kinase (see the Introduction section). However, an analysis of rat liver ACC isoforms suggested that ACC- β is a substantially better substrate than ACC- α for PKA [19] and we confirmed that ACC- β prepared from rat heart or skeletal muscle was also an excellent substrate *in vitro* for PKA (results reviewed but not shown). Three lines of evidence suggest that PKA might have a significant role in ACC- β phosphorylation in the heart during β -adrenergic stimulation.

Phosphorylation of ACC- β by PKA and AMP-PK after treatment of intact myocytes with isoprenaline

In a set of eight experiments with separate batches of myocytes, treatment of cells with isoprenaline (500 nM, 2 min) led to a decrease in the subsequent PKA-mediated phosphorylation of ACC- β by $78 \pm 6.0\%$ compared with controls (mean $\pm$ S.E.M., $P < 0.001$). In contrast, treatment of myocytes with isoprenaline led to only a modest apparent decrease in sites of ACC- β available for subsequent phosphorylation by AMP-PK ($24 \pm 11.9\%$, no significant difference). For most of these studies we employed AMP-PK purified from rat liver, as this is an excellent source of the enzyme. Because it is possible that a distinct cardiac AMP-PK might exhibit different properties, we also purified this kinase from rat heart and compared the phosphorylation of heart and liver ACC subunits in parallel assays with the two sources of AMP-PK. The maximum extents of phosphorylation of ACC subunits were very similar to the liver and heart preparations of AMP-PK, when activities were normalized with respect to phosphorylation of SAMS peptide (results not shown).

PKA-specific sites are phosphorylated in response to isoprenaline

As noted above (Figure 3a), five phosphopeptides accounted for most of the phosphorylation of cardiac ACC- β by PKA: peptides 1–3 together accounted for 65–70% of ^{32}P , whereas peptides 4 and 5 each accounted for 10–12% of ^{32}P incorporated. Essentially identical phosphopeptide patterns were observed in studies with ACC- β from rat liver (Figure 3c). In contrast, treatment of ACC- β with AMP-PK followed by two-dimensional peptide analysis revealed a pattern of phosphopeptides (Figure 3d) differing significantly from that seen in response to PKA. AMP-PK phosphorylated sites within two dominant phosphopeptides. Peptide 1 accounted for $37 \pm 1\%$ of total ^{32}P on phosphopeptide maps (mean $\pm$ S.E.M. for three independent experiments). Peptide 6 (a very minor peptide after treatment with PKA) represented $29 \pm 8\%$ of the ^{32}P after treatment with AMP-PK. Peptides 2–5, prominent after treatment with PKA, were phosphorylated very weakly with AMP-PK (less than 3% of total ^{32}P) and seem to represent 'PKA-selective' phosphorylation sites. In contrast, a phosphopeptide (no. 9) was significantly phosphorylated by AMP-PK, accounting for $12 \pm 4\%$ of total ^{32}P but was absent from maps after phosphorylation with PKA. Importantly, the PKA-selective phosphopeptides 2–5 contain sites that are phosphorylated within intact cardiac myocytes treated with isoprenaline (Figure 3b), strongly suggesting that PKA is an ACC- β kinase activated by β -adrenergic stimulation.

Phosphopeptide 1 was a significant product whether PKA or AMP-PK was used to phosphorylate ACC- β . When samples of

peptides from PKA and AMP-PK treatments were co-chromatographed by two-dimensional TLC, the apparently identical phosphopeptides (designated 1) co-migrated exactly (results not shown). It is possible that AMP-PK and PKA phosphorylate the same or two distinct serine or threonine residues within this common phosphopeptide 1. The presence of a common phosphopeptide is reminiscent of the situation in ACC- α , in which Ser-77 and Ser-79 occur within the same tryptic phosphopeptide and are mutually exclusive substrates for PKA and AMP-PK respectively [54].

Low abundance has so far prevented the sequencing of the major phosphopeptides derived from ACC- β but some inferences can be drawn from the sequence of human ACC- β [20–22] and of rat ACC- β [55]. For example, the motif containing Ser-77 and Ser-79 of ACC- α [HMRSSMSGLH] is modified in human and rat ACC- β [TMRPSMSGLH]. The presence of proline might well disrupt the structure around this putative ACC- β phosphorylation site (Ser-216 of rat ACC- β), so it is not certain that it will remain a substrate for PKA. The other major PKA phosphorylation site of rat ACC- α occurs at Ser-1200 and in rat ACC- β the equivalent residue (Ser-1338) occurs in a context that is unfavourable for PKA [PINVSFAS]. Intriguingly, Ser-1215 of rat ACC- α is a poor substrate for PKA [THVASVSDVL] but the sequence is modified in rat ACC- β [LLRHSKELF], so it might in fact be a potential target for PKA. Phosphoamino acid analysis indicated that ACC- β is phosphorylated exclusively on serine and not threonine residues (results not shown); however, there are still a substantial number of potential seryl residues that might provide sites for phosphorylation within this large protein. Peptide sequencing is therefore essential to resolve the exact nature of the dominant phosphorylation sites of ACC- β .

Rapid phosphorylation of cardiac ACC- β by PKA *in vitro* suggests that this isoform might be a relevant substrate in intact cells

Previous studies with purified proteins had demonstrated that rat liver ACC- β was phosphorylated more rapidly than ACC- α in the presence of PKA [19]. Similarly, PKA was found to catalyse the rapid phosphorylation of ACC- β purified from rat heart or skeletal muscle: ^{32}P incorporation into ACC- β was appreciable within 1–3 min and maximal within 30 min. In contrast, phosphorylation of ACC- α in these same preparations was barely detectable at times earlier than 30 min, even though ACC- α subunit protein represented approx. 20% of the total ACC protein, on the basis of staining with Coomassie Blue. Similarly, ACC- α purified from rat white adipose tissue was also phosphorylated slowly by PKA; ^{32}P incorporation did not reach a plateau until at least 2 h. To address the possibility that ACC- α might have been phosphorylated more extensively than ACC- β in the purified preparations, treatment with phosphatases was performed. Incubation of purified ACC with acid phosphatase for up to 60 min led to only modest increases (less than 10%; three independent experiments) in the subsequent incorporation of ^{32}P into ACC subunits. Commercial preparations of protein phosphatases 1 and 2A also had little or no effect on the phosphorylation of ACC by PKA or AMP-PK. The same concentrations of phosphatases catalysed substantial (more than 80%) removal, within 30–60 min, of ^{32}P from ACC that had been prelabelled in the presence of PKA or AMP-PK. It should be stressed that, to purify ACC for use as a protein kinase substrate, we specifically chose to avoid the use of protein phosphatase inhibitors, thus allowing sufficient opportunity for action by endogenous protein phosphatases during purification.

Conclusions

1. ACC- β is phosphorylated within intact rat cardiac myocytes and the extent of phosphorylation increases rapidly on the exposure of cells to isoprenaline.

2. ACC- β is phosphorylated on sites within five or more phosphopeptides that are released by digestion with trypsin.

3. Although β -adrenergic stimulation leads to a decline in cellular malonyl-CoA concentrations, no change in kinetic properties of ACC were found to persist through homogenization and partial purification.

4. Several lines of evidence suggest that PKA might have a significant role in the isoprenaline-stimulated phosphorylation of ACC- β : first, sites on ACC- β that are substrates for PKA become substantially phosphorylated within intact cells during treatment with isoprenaline, whereas sites for AMP-PK are much less affected; secondly, the sites affected by isoprenaline stimulation include those that are substrates for PKA but not for AMP-PK; thirdly, although PKA seems to have a minor role in phosphorylation of ACC- α , ACC- β is a substantially better substrate for PKA *in vitro*.

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