Activation of hepatic acetyl-CoA carboxylase by glutamate and Mg^{2+} is mediated by protein phosphatase-2A

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The activation of hepatic acetyl-CoA carboxylase by Na^+-co-transported amino acids such as glutamine has been attributed mainly to the stimulation of its dephosphorylation by accumulating dicarboxylic acids, e.g. glutamate. We report here on a hepatic species of protein phosphatase-2A that activates acetyl-CoA carboxylase in the presence of physiological concentrations of glutamate or Mg^{2+} and, under these conditions, accounts for virtually all the hepatic acetyl-CoA carboxylase phosphatase activity. Glutamate also stimulated the dephosphorylation of a synthetic pentadecapeptide encompassing the Ser-79 phosphorylation site of rat acetyl-CoA carboxylase, but did not affect the dephosphorylation of other substrates such as phosphorylase. Conversely, protamine, which stimulated the dephosphorylation of phosphorylase, inhibited the activation of acetyl-CoA carboxylase. A comparison with various species of muscle protein phosphatase-2A showed that the stimulatory effects of glutamate and Mg^{2+} on the acetyl-CoA carboxylase phosphatase activity are largely mediated by the regulatory A subunit. Glutamate and Mg^{2+} emerge from our study as novel regulators of protein phosphatase-2A when acting on acetyl-CoA carboxylase.

INTRODUCTION

Hepatic lipogenesis is stimulated by Na^+-co-transported amino acids such as glutamine and proline that are converted into glutamate [1–3]. We have previously shown that, in liver extracts, glutamate and other dicarboxylic acids promote the activation of acetyl-CoA carboxylase (ACC), a key enzyme of lipogenesis [1]. Since these effects were blocked by inhibitors of Ser/Thr-protein phosphatases, it was concluded that the activation resulted from the stimulation of an ACC phosphatase. The identity of the phosphatase was not clear, however, since none of the known protein phosphatases have been shown to be stimulated by dicarboxylic acids.

In vitro, ACC can be phosphorylated on as many as eight serine residues by six different protein kinases [4,5]. Most of these sites are also phosphorylated in intact hepatocytes and adipocytes, but the function of these phosphorylation events is not always clear. It has been demonstrated, however, that ACC is inactivated in response to hormones such as glucagon and adrenaline, which increase the level of intracellular cAMP. The effect of cAMP is indirect and appears to be mediated by the rapid phosphorylation of Ser-79 by the AMP-activated protein kinase [4,5]. The much slower phosphorylation of Ser-1200 and Ser-1215 by the AMP-activated protein kinase does not seem to have an additional effect on the activity of ACC.

Initial information on the protein phosphatase(s) that dephosphorylate ACC has been obtained from the screening of subcellular fractions and (partially) purified enzymes for ACC phosphatase activities in the presence of inhibitors and/or activators of specific protein phosphatases [6–11]. However, with one exception [7], the ACC phosphatase activities in these studies were determined solely from the release of ^{32}P-labelled phosphate from ACC that had been phosphorylated *in vitro* by protein kinase A (PKA), casein kinase-1 or casein kinase-2 [8–11]. Since only some phosphorylation sites directly affect the enzyme activity, the results of phosphatase assays with *in vitro* labelled substrates must be interpreted cautiously and are not necessarily informative about the phosphatases that activate the native substrate.

Taking into account the limitations discussed above, assays on subcellular liver fractions suggested that a major part of the ACC phosphatase activity is accounted for by Ser/Thr-protein phosphatases of type 2A (PP-2A) [8,10,11]. These phosphatases have a common core structure consisting of a 36–38 kDa catalytic subunit (PP-$2A_C$) and a 61–65 kDa regulatory subunit (A subunit), and are activated by polyamines and basic polypeptides such as protamine [6,12,13]. In addition to the dimeric species (PP-$2A_D$), several trimeric forms of PP-2A have been identified in cardiac or skeletal muscle. These are named according to the size of their third subunit or B subunit [14], e.g. PP-$2A_{T55}$, PP-$2A_{T54}$, PP-$2A_{T72}$, PP-$2A_{T74}$. In liver, however, only two major species of PP-2A could be identified by fractionation of the cytosol, and these were both shown to dephosphorylate ACC *in vitro* [8,11]. Based upon its subunit structure and the primary structures of some proteolytic peptides, one hepatic species could be identified as PP-$2A_D$ [15,16]. The second species has a trimeric structure [17] and was tentatively identified as the hepatic homologue of PP-$2A_{T55}$, which was previously named PP-$2A_1$ [18]. However, given the growing number of newly discovered trimeric species of PP-2A [12,13], the latter identification remains uncertain.

It has also been noted that the liver contains an ACC phosphatase activity that is stimulated by bivalent cations such as Mn^{2+} or Mg^{2+} [1,7–9]. In one study it was proposed that this enzyme corresponds to the monomeric (42 kDa) Mg^{2+}-dependent phosphatase termed PP-2C [8]. However, given its larger size [7] and its sensitivity to inhibition by microcystin, which does not

Abbreviations used: ACC, acetyl-CoA carboxylase; GAPP, hepatic glutamate-activated protein phosphatase; PKA, protein kinase A; PP-1, protein phosphatase-1; PP-1_C, catalytic subunit of PP-1; PP-2A, protein phosphatase-2A; PP-$2A_C$, catalytic subunit of PP-2A; PP-$2A_D$, dimeric form of PP-2A (previously called PCS_L or $PP2A_2$); PP-$2A_{T55}$, trimeric form of PP-2A (previously called PCS_{H1} or $PP2A_1$); PP-$2A_{T72}$, trimeric form of PP-2A (previously called PCS_M).

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affect PP-2C [1], it seems likely that the liver also contains at least one other bivalent-cation-stimulated ACC phosphatase.

We have now characterized an ACC phosphatase from rat liver cytosol that is stimulated not only by dicarboxylic acids but also by physiological concentrations of Mg^{2+}. We have identified this enzyme as a species of PP-2A and provide data on the substrate and subunit requirements for stimulation by glutamate and Mg^{2+}.

EXPERIMENTAL

Materials

PP-1_C (i.e. the catalytic subunit of PP-1) [19], PP-$2A_C$ [20], PP-$2A_D$ [21], PP-$2A_{T55}$ [21], inhibitor-2 [22] and phosphorylase *b* [23] were purified from rabbit skeletal muscle. PKA from beef heart was purchased from Sigma. AMP-activated protein kinase was partially purified from fed rat liver extracts by precipitation with poly(ethylene glycol) and fractionation on Mono Q [24]. ACC was purified to homogeneity from the livers of rats that had been starved for 2 days and then refed with a low-fat, high-carbohydrate, diet for 3 days [1]. The purified active ACC was used as a substrate for the AMP-activated protein kinase. Inactive ACC, which was used for the ACC phosphatase assay, was obtained by injection of the rats with glucagon before they were killed [1].

ACC, avidin, polylysine and glutamate were coupled to CNBr-activated Sepharose-4B according to the recommendations of Pharmacia. Iminodiacetic acid Sepharose-6B for Mn^{2+}-affinity chromatography was obtained from Sigma. A kit for the development of Western blots by enhanced chemiluminescence was purchased from Amersham. Microcystin-LR and okadaic acid were obtained from Calbiochem. Microcystin–Sepharose was prepared as previously described [25]. Poly(vinylidene difluoride) membranes (Immobilon) were purchased from Millipore. Rabbit polyclonal antibodies against a synthetic peptide (GEPHVTRRTPDYFL) comprising the C-terminal domain of human $PP2A_C$ were obtained from Upstate Biotechnology Inc. Polyclonal antisera against synthetic peptides encompassing the C-terminal domains of the α-isoform (RPITPPRNSAKAKK) and the δ-isoform (PRTANPPKKR) of rat PP-1_C were raised in rabbits.

The 'T_3-peptide' (RRPTVA) is based on the sequence surrounding the PKA phosphorylation site (Thr-35) of inhibitor-1. The 'SAMS-peptide' (HMRSAMSGLHLVKRR) comprises the sequence surrounding the Ser-79 phosphorylation site for AMP-activated protein kinase, except that Ser-77 was replaced by an alanine residue. This change was introduced to prevent *in vitro* phosphorylation of residue 77 by PKA, which has been shown to block phosphorylation of Ser-79 by AMP-activated protein kinase [26].

Phosphorylase *b* was phosphorylated in the presence of [γ-^{32}P]ATP by phosphorylase kinase [27]. The T_3-peptide was similarly phosphorylated by the catalytic subunit of PKA [27], and the SAMS-peptide by AMP-activated protein kinase. Both peptides were phosphorylated to a stoichiometry of about 0.1 mol of phosphate/mol of peptide. The peptides were separated from [^{32}P]ATP by anion-exchange chromatography on Dowex 1-X8 in the presence of 30 % acetic acid [28].

Partial purification of glutamate-activated protein phosphatase (GAPP)

The livers of 10 female Wistar rats of about 250 g body weight were homogenized in 3 vol. of an ice-cold buffer containing 50 mM glycylglycine, pH 7.4, 1 mM dithiothreitol, 0.1 mM PMSF, 0.1 mM benzamidine, 0.1 mM 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK'), 0.1 mM 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK') and 5 μM leupeptin (buffer A), supplemented with 0.5 mM EDTA, 0.5 mM EGTA and 0.25 M sucrose. Successive centrifugations (8000 ***g*** for 10 min and 140000 ***g*** for 40 min) yielded a cytosolic fraction, which was stirred for 60 min at 4 °C with 100 ml of DEAE-Sephadex-A50 equilibrated in a buffer containing 50 mM glycylglycine, pH 7.4, and 1 mM dithiothreitol (buffer B). After washing with 10 vol. of buffer B on a Büchner funnel, the gel was poured into a column. The bound proteins were eluted with a linear 1000 ml gradient of 0–0.8 M NaCl in buffer A. Fractions containing the glutamate- and magnesium-stimulated ACC phosphatase activity were pooled, diluted with cold water to a NaCl concentration of about 150 mM, and applied to a 15 ml column of polylysine–Sepharose-4B equilibrated in buffer B. The column was washed with 5 vol. of buffer B and developed with a 150 ml linear gradient of 0–0.6 M NaCl in buffer A. The pooled fractions containing glutamate- and magnesium-stimulated ACC phosphatase activity were concentrated to about 0.5 ml by ultrafiltration (Amicon Centriprep 30), supplemented with 0.8 M $(NH_4)_2SO_4$ and applied on to a phenyl-Superose column (5 cm × 0.5 cm) equilibrated in buffer B plus 0.8 M $(NH_4)_2SO_4$. The proteins were eluted with a 15 ml linear gradient of $(NH_4)_2SO_4$ (0.8–0 M) in buffer B and the fractions were dialysed overnight against buffer B. The fractions containing glutamate- and magnesium-stimulated ACC phosphatase activity were pooled, concentrated to about 0.2 ml by ultrafiltration (Amicon Centriprep 30 and Centricon 30) and applied to a Superdex-200 gel-filtration column (30 cm × 1 cm) equilibrated in buffer B plus 0.1 M NaCl. The peak fractions containing glutamate- and magnesium-stimulated ACC phosphatase activity were pooled, dialysed against buffer B plus 50 % glycerol and stored at −20 °C.

Assays

Protein phosphatase activities were assayed either as such ('spontaneous' activity) or in the presence of 30 μM protamine plus 15 mM ammonium sulphate [29], or still in the presence of 100 mM glutamate and/or 10 mM magnesium acetate. The ACC phosphatase activity was measured at 37 °C in a final volume of 30 μl containing 50 mM glycylglycine, pH 7.4, 1 mM dithiothreitol, 5 mg/ml BSA and 0.15–0.50 μM purified ACC. At various times, samples were taken for the assay of ACC. The linear, time-dependent, increase in ACC activity was taken as a measure of ACC phosphatase activity. One unit of ACC phosphatase was defined as the amount that generates 1 unit of ACC/min under the specified conditions. ACC was determined at 37 °C from the incorporation of [^{14}C]HCO_3^- into acid-soluble compounds (method 1 in [1]). The ACC assay was performed in the presence of 5 mM NaF plus 0.1 μM microcystin to block further dephosphorylation, and in the presence of a subsaturing concentration of magnesium citrate (0.5 mM), an allosteric activator of ACC [30,31]. One unit of ACC converts 1 μmol of substrate/min.

The phosphorylase phosphatase and peptide phosphatase activities were determined at 30 °C from the linear rate of dephosphorylation of the substrates, which were present at a final concentration of 5–10 μM [27]. The released ^{32}P-labelled phosphate was measured by liquid scintillation counting after precipitation of phosphorylase with 10 % trichloroacetic acid plus 3 mg/ml BSA, or after extraction of the liberated ^{32}P as a phosphomolybdic complex [27]. One unit of phosphorylase phosphatase or peptide phosphatase produces 1 μmol of phosphate/min under the specified conditions.

Western blotting

Antisera against PP-1_C were used at a final dilution of 1:1000. Antibodies against human PP-$2A_C$ were used at a final dilution of 1 μg/ml. Incubation with the primary antibodies was for 2 h at room temperature. Peroxidase-labelled secondary antibodies were detected by enhanced chemiluminescence.

RESULTS

Partial purification of GAPP

In accordance with our previous findings [1], 'spontaneous' ACC phosphatase activity in liver extracts, as determined from the rate of activation of exogenous ACC, was barely detectable, but was stimulated at least 20-fold by 100 mM glutamate and 10 mM Mg^{2+} (Table 1). Upon subcellular fractionation nearly all of the glutamate- and Mg^{2+}-stimulated ACC phosphatase activity was recovered in the cytosol (Table 1). Unexpectedly, when assayed at the same dilution, the cytosolic fraction contained a 2-fold higher ACC phosphatase activity than the extract. Combination experiments showed that the lower activity in the extract was due to inhibition of the phosphatase exerted by an unidentified component of the glycogen/microsomal fraction.

After chromatography of the cytosolic fraction on DEAE-Sephadex-A50, only a single peak of glutamate- and Mg^{2+}-stimulated ACC phosphatase activity was detected (Figure 1B). This activity was not affected by the addition of up to 10 μM inhibitor-2 (results not shown), ruling out a contribution of PP-1 enzymes [6,32]. The column fractions were also assayed for their ability to dephosphorylate phosphorylase *a*, a model substrate of PP-1 and PP-2A [6,32]. The spontaneous phosphorylase phosphatase activity was relatively low, and was eluted as a single broad peak. In the presence of protamine, a well known stimulator of PP-2A [6], two activity peaks were obtained, one slightly in front of and the other immediately after the single peak of ACC phosphatase (Figure 1B). That the protamine-stimulated phosphorylase phosphatase activity stemmed from PP-2A was confirmed by a similar elution of PP-$2A_C$, as detected by Western blot analysis (Figure 1A).

Further isolation of GAPP on polylysine–Sepharose, phenyl-Superose and Superdex-200 yielded an overall purification of only about 50-fold (Table 2). In spite of extensive efforts, the lability of GAPP and its low recovery during chromatography prevented further purification of the phosphatase. Besides a number of classical chromatographic procedures, we have unsuccessfully tried to purify GAPP by affinity chromatography on immobilized substrate (ACC), activators (glutamate, manganese) or inhibitor (microcystin). In view of a report on the co-purification of ACC and an ACC phosphatase from adipose tissue [33], we have also investigated whether this applies to GAPP. However, we did not find any evidence for the co-purification of an ACC phosphatase with ACC during affinity chromatography on avidin–Sepharose (results not shown).

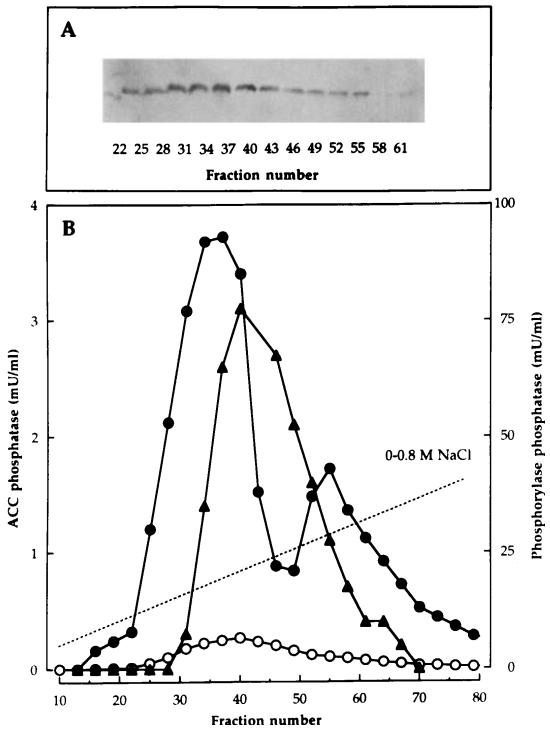

Figure 1 Elution profile of ACC phosphatase and phosphorylase phosphatase activities from DEAE-Sephadex-A50

The cytosolic fraction prepared from 10 livers was chromatographed on a DEAE-Sephadex-A50 column, as detailed in the Experimental section. The concentration of PP-$2A_C$ in the eluted fractions (10 ml) was determined by Western analysis (**A**). The fractions were also assayed for spontaneous (○) and protamine-stimulated (●) phosphorylase phosphatase activities, and for glutamate- and Mg^{2+}-stimulated (▲) ACC phosphatase activity (**B**). Activities are given in m-units/ml.

Table 1 Subcellular localization of ACC phosphatase in rat liver

The glutamate- and Mg^{2+}-stimulated ACC phosphatase activity was assayed in a liver extract (8000 ***g*** for 10 min), and in the supernatant (cytosol) and resuspended pellet (glycogen/microsomes) obtained by high-speed centrifugation (140 000 ***g*** for 40 min) of the same extract. The extracts and cytosolic fractions were filtered through a Sephadex-G25 column prior to the assays. The results are represented as means ± S.E.M. ($n = 4$). The ACC phosphatase activity measured in the extract in the absence of glutamate and Mg^{2+} amounted to less than 0.05 m-unit/g of liver.

Fraction	ACC phosphatase (m-units/g of liver)
Extract	0.95 ± 0.09
Cytosol	1.82 ± 0.07
Glycogen/microsomes	0.13 ± 0.04
Cytosol + glycogen/microsomes	1.02 ± 0.14

Several lines of evidence suggested that GAPP belongs to the PP-2A family. First, GAPP was inhibited by the same concentrations of okadaic acid (Figure 2) or microcystin (not shown) that also inhibited PP-$2A_C$. Thus half-maximal inhibition of GAPP and PP-$2A_C$ was obtained at 3 ± 2 nM ($n = 3$) and 4 ± 1 nM ($n = 3$) okadaic acid respectively, while 367 ± 67 nM ($n = 3$) toxin was required for half-maximal inhibition of PP-1_C (Figure 2). Secondly, from the polylysine–Sepharose purification step onwards the ACC phosphatase co-purified with a protamine-stimulated phosphorylase phosphatase (results not shown). During gel filtration both activities migrated as a protein of 285 kDa (Figure 3B) and the peak fractions also contained the catalytic subunit of PP-2A, as visualized by Western analysis (Figure 3A). On the other hand, no signal was obtained with antibodies directed against the C-termini of the α- and δ-isoforms of PP-1_C (results not shown). Thirdly, antibodies against PP-$2A_C$ inhibited the ACC activity of GAPP by 75 ± 2 % ($n = 3$). At the same concentration, these antibodies also decreased the phosphorylase phosphatase activity of PP-$2A_C$ by 80 %, while barely affecting PP-1_C activity (8 % decrease). That the phosphorylase phos-

Table 2 Partial purification of ACC phosphatase from rat liver

ACC phosphatase was purified from the cytosolic fraction of 10 rat livers, as detailed in the Experimental section.

Step	Total protein (mg)	ACC phosphatase (m-units/mg of protein)	Recovery (%)	Purification (fold)
Cytosol	5800	0.09	100	1
DEAE-Sephadex	432	0.65	54	7
Polylysine–Sepharose	68	1.60	21	18
Phenyl-Sepharose	15	2.90	8	32
Superdex-200	9	4.30	7	48

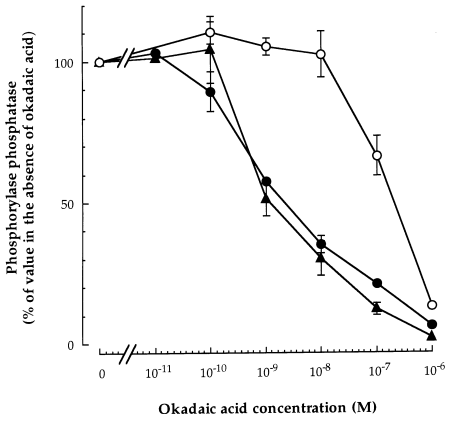

Figure 2 Sensitivity of GAPP to inhibition by okadaic acid

The spontaneous phosphorylase phosphatase activities of PP-1_C (○), PP-$2A_C$ (●) and GAPP (▲) were measured in the presence of the indicated concentrations of okadaic acid. The results are expressed as a percentage of the control value and represent means ± S.E.M. ($n = 3$).

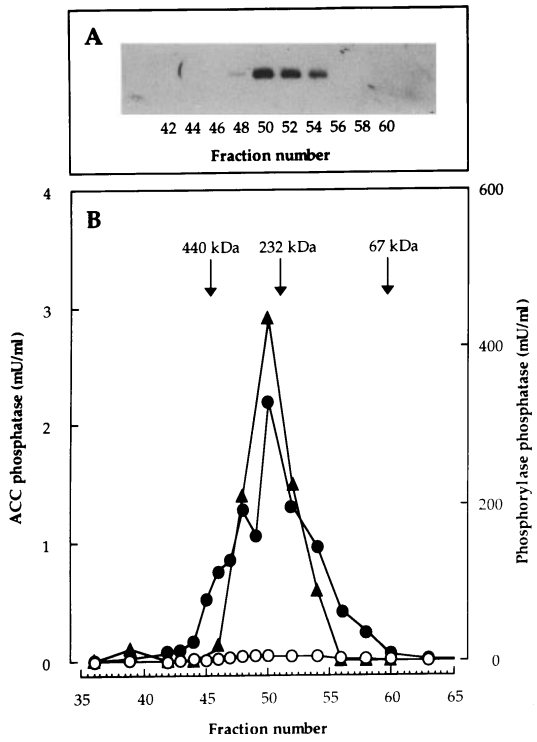

Figure 3 Filtration of GAPP on Superdex-200

Following the partial purification of GAPP on DEAE-Sephadex, polysine–Sepharose and phenyl-Sepharose, 250 μl of the concentrated enzyme was applied to a Superdex-200 gel-filtration column. The fractions (0.25 ml) were analysed for the presence of PP-$2A_C$ by Western blotting (**A**), and for spontaneous (○) and protamine-stimulated (●) phosphorylase phosphatase activities and glutamate- and Mg^{2+}-stimulated ACC phosphatase activity (▲) (**B**). The arrows indicate the elution positions of the marker proteins ferritin (440 kDa), catalase (232 kDa) and BSA (67 kDa). The apparent molecular mass of GAPP was calculated by linear regression analysis.

phatase and ACC phosphatase activities of GAPP were derived from the same phosphatase is also suggested by substrate competition experiments showing that the ACC phosphatase activity was nearly completely blocked by the addition of 5 μM phosphorylase *a* to the assay mixture. In contrast, the addition of the same concentration of phosphorylase *b* was without effect.

Effects of dicarboxylic acids and metals

Figure 4(A) shows the concentration-dependence of the stimulation of GAPP by glutamate. At 100 mM glutamate, the concentration routinely used in this study, phosphatase activity was increased about 9-fold. However, a 3-fold stimulation was obtained at 20 mM glutamate, which is the concentration that is found in hepatocytes incubated with 10 mM glutamine [34]. Remarkably, the dephosphorylation of the synthetic SAMS-pentadecapeptide, which is modelled on the sequence surrounding the Ser-79 phosphorylation site of ACC, was also stimulated severalfold by glutamate (Figure 4A). The stimulation of GAPP was not specific for glutamate and was also obtained with other dicarboxylic acids, including aspartate, malonate and succinate (Table 3). The greatest stimulation was obtained with succinate, which at 50 mM was a 3-fold better stimulator than glutamate. On the other hand, oxalate was inhibitory.

Glutamate not only promoted the dephosphorylation of ACC by GAPP, but also acted as a stimulator of the basal activity of ACC, i.e. the activity measured in the presence of phosphatase inhibitors and without prior incubation with protein phosphatases. The activity of ACC purified from glucagon-treated rats was increased between 3- and 14-fold ($n = 6$ different preparations) by 100 mM glutamate. In spite of this severalfold activation, the glutamate-stimulated activity of ACC remained relatively low: it was always less than 5 % of the activity measured after prior activation by PP-2A.

In the absence of dicarboxylic acids, the activity of GAPP was also stimulated up to 9-fold by magnesium acetate (Figure 4B). The stimulation displayed saturation kinetics and was half-maximal at about 1 mM magnesium acetate. In contrast to dicarboxylic acids, magnesium acetate only slightly stimulated the dephosphorylation of the SAMS-peptide. The spontaneous and magnesium-stimulated activities of GAPP were completely blocked by 1 μM microcystin, ruling out a contribution by the Mg^{2+}-stimulated phosphatase PP-2C. Table 3 shows that GAPP was stimulated even more extensively by $MgCl_2$ than by magnesium acetate, indicating that the positive effect was due to Mg^{2+} and not to the monocarboxylate. Stimulation of GAPP was also observed with Mn^{2+}, and to a limited extent with Ca^{2+}. Mg^{2+} increased the V_{max} of the ACC phosphatase reaction about 6-fold, without affecting the K_m for ACC (Table 4). In contrast, glutamate only doubled the V_{max}, but increased the affinity for the substrate ACC about 7-fold.

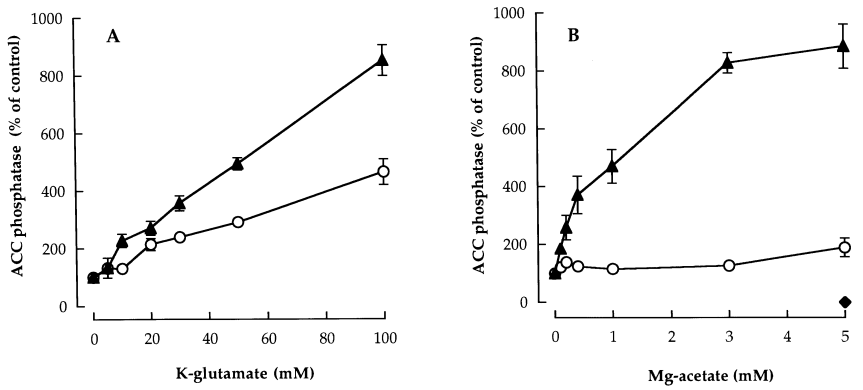

Figure 4 Stimulation of GAPP by glutamate or Mg^{2+}

The effects of the indicated concentrations of potassium glutamate (K-glutamate) (**A**) or magnesium acetate (Mg-acetate) (**B**) on the phosphatase activity of GAPP were studied using either exogenous ACC (▲) or the SAMS-peptide (○) as substrate. Panel (**B**) also shows the effect of 1 μM microcystin (◆) on the Mg^{2+}-stimulated ACC phosphatase activity. ACC phosphatase and SAMS-peptide phosphatase activities of 100% correspond to 1.7 ± 0.2 and 22 ± 1 m-units/mg of protein respectively. The results represent means ± S.E.M. ($n = 3$).

Table 3 Stimulation of GAPP by dicarboxylic acids and bivalent cations

The effects of the indicated dicarboxylic acids (50 mM) or bivalent cations (10 mM) were studied on the ACC phosphatase activity of GAPP. The results represent means ± S.E.M. for three experiments.

Effectors	ACC phosphatase (m-units/mg of protein)
Control	1.7 ± 0.2
Potassium oxalate	0.4 ± 0.2
Potassium malonate	11.8 ± 2.3
Potassium succinate	27.1 ± 3.2
Potassium glutamate	8.4 ± 0.4
Potassium aspartate	10.7 ± 0.8
$MgCl_2$	26.5 ± 2.6
$CaCl_2$	4.7 ± 0.8
$MnCl_2$	17.9 ± 2.3
Magnesium acetate	15.4 ± 2.4

Table 4 Kinetic analysis of the effects of glutamate and Mg^{2+} on GAPP

The V_{max} and K_m values for the ACC phosphatase reaction were determined from Lineweaver–Burk plots and are represented as means ± S.E.M. ($n = 3$). *$P < 0.05$ compared with control value (unpaired t test).

Condition	V_{max} (m-units/mg of protein)	K_m (μM)
Control	5.1 ± 0.4	1.03 ± 0.29
Glutamate (100 mM)	10.0 ± 1.0*	0.14 ± 0.01*
Magnesium acetate (10 mM)	31.9 ± 3.9*	0.86 ± 0.17

Comparison with PP-2A from skeletal muscle

Since it was not possible to determine the subunit composition of GAPP, we have tried to gain further insight into the mechanism of the stimulatory actions of glutamate and Mg^{2+} by comparing their effects on several well characterized species of PP-2A from rabbit skeletal muscle. During fractionation of skeletal muscle cytosol on DEAE-Sephadex-A50, three peaks of glutamate- and Mg^{2+}-stimulated ACC phosphatase activity were observed (results not shown). These activities coincided with the protamine-stimulated phosphorylase phosphatase activities that have previously been identified as PP-$2A_{T55}$, PP$2A_{T72}$ and PP-$2A_D$ respectively [21].

Figure 5 shows the effects of 100 mM glutamate and of protamine (30 μg/ml) on the phosphatase activities of the catalytic subunits of PP-1 and PP-2A, as well as of PP-$2A_D$, PP-$2A_{T55}$ and GAPP. For reasons of comparison, the phosphatases were diluted to give the same spontaneous phosphorylase phosphatase activity (Figure 5A). They were then tested with other substrates (Figures 5B–5D) and the results were expressed for the same concentration of the phosphatase used in Figure 5(A). Glutamate did not affect the activity of any of the phosphatases investigated when assayed with phosphorylase *a* (Figure 5A). As expected, the basic polypeptide protamine markedly stimulated the phosphorylase phosphatase activities of PP-$2A_D$, PP$2A_{T55}$ and GAPP. The spontaneous phosphatase activity assayed using purified ACC was barely detectable, except with PP-$2A_{T55}$ and GAPP (Figure 5B). Glutamate promoted the activation of ACC by all multimeric species of PP-2A. Remarkably, while protamine stimulated the ACC phosphatase activity of PP-$2A_{T55}$ manifold, it did not affect the ACC phosphatase activity of PP-$2A_D$ to any great extent, and even blocked the ACC phosphatase activity of GAPP. The phosphatase activity measured with the SAMS-peptide was very low for all phosphatases except for GAPP. Interestingly, glutamate stimulated the dephosphorylation of the SAMS-peptide by all PP-2A holoenzymes, and in particular by GAPP. Protamine caused a partial inhibition of all the SAMS-peptide phosphatase activities. The T_3-phosphopeptide, as anticipated [27], was rapidly dephosphorylated by all species of PP-2A. It was a particularly good substrate for GAPP, similar to the SAMS-peptide in the presence of glutamate. On the other hand, glutamate had no effect on the dephosphorylation of the T_3-peptide by any of the phosphatases, while protamine was inhibitory.

In order to delineate further the phosphatase subunit requirements for the stimulatory effects of glutamate and Mg^{2+}, we compared their effects on the same molar concentration of various forms of PP-2A (Table 5). A first striking feature was that the basal ACC phosphatase activity of GAPP was much higher than that of the muscle enzymes. This confirms the data of Figure 5. On the other hand, both glutamate and Mg^{2+}

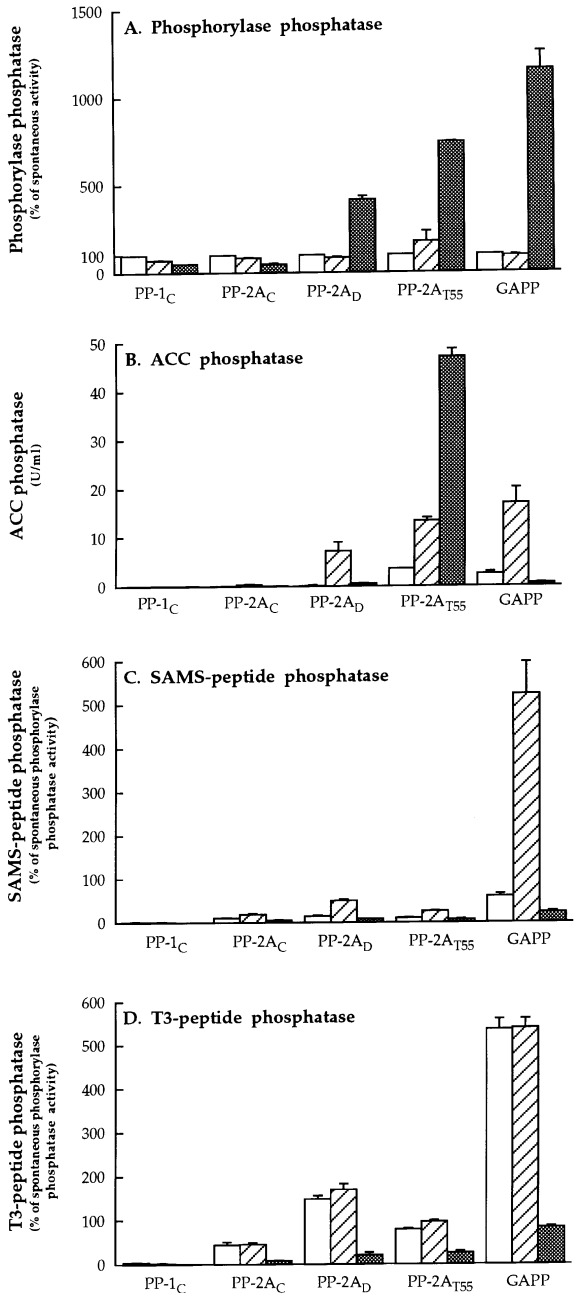

Figure 5 Substrate requirements for the stimulatory actions of glutamate and protamine on various species of PP-1 and PP-2A

Phosphorylase (**A**), ACC (**B**), SAMS-peptide (**C**) and T_3-peptide (**D**) were used as substrates for GAPP as well as for various phosphatases from rabbit skeletal muscle ($PP\text{-}1_C$, $PP\text{-}2A_C$, $PP\text{-}2A_D$ and $PP\text{-}2A_{T55}$). The assays were performed as such (empty bars), in the presence of 100 mM potassium glutamate (hatched bars) or in the presence of 30 μg/ml protamine (shaded bars). Spontaneous phosphorylase phosphatase activity of 100% corresponded to 38 ± 3 units (U)/ml. The values represent means $\pm$ S.E.M. for three experiments.

stimulated the ACC phosphatase activity of all enzymes, although the extent of stimulation was quite varied (Table 5). Indeed, while all multimeric enzymes were stimulated to a similar maximal activity by glutamate and by Mg^{2+}, these compounds increased the ACC phosphatase activity of the catalytic subunit only to 5–15% of the activity obtained with the holoenzymes. Since maximal stimulation was obtained with $PP\text{-}2A_D$, these data suggest that the stimulatory effects of glutamate and Mg^{2+} are largely mediated by the regulatory A subunit.

Table 5 Subunit requirements for the stimulation of PP-2A by glutamate and Mg^{2+}

All data have been calculated for the same concentration of catalytic subunit, which was determined by scanning of Western blots. Results represent means $\pm$ S.E.M. for three observations.

	ACC phosphatase activity (m-units/ml)		
Phosphatase	Control	+ Glutamate	+ Mg^{2+}
GAPP	134 ± 47	678 ± 96	918 ± 40
$PP\text{-}2A_{T55}$	14 ± 8	619 ± 48	905 ± 48
$PP\text{-}2A_D$	15 ± 14	1286 ± 27	2095 ± 47
$PP\text{-}2A_C$	20 ± 6	90 ± 15	91 ± 6

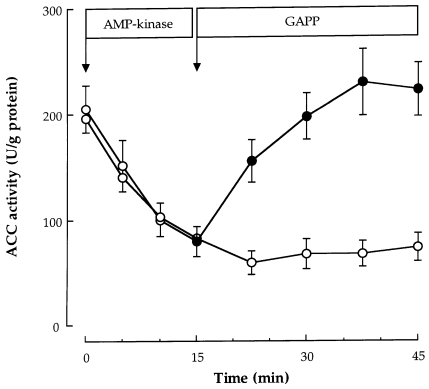

Figure 6 Antagonistic regulation of ACC by GAPP and AMP-activated protein kinase

Active ACC (40 μg) was incubated at 30 °C with 0.5 m-unit of AMP-activated protein kinase (AMP-kinase) in 0.2 ml of a solution containing 25 mM glycylglycine, pH 7.4, 0.2 mM ATP, 0.2 mM AMP, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol and 5 mg/ml BSA. After 15 min the AMP-activated protein kinase was blocked by the addition of 4.5 mM EDTA and the mixture was supplemented with partially purified GAPP (0.4 mg/ml) in the absence (○) or presence (●) of 100 mM potassium glutamate. At the indicated times aliquots were withdrawn for assay of ACC activity (units/g of protein). The results are means $\pm$ S.E.M. for three experiments.

GAPP antagonizes the inactivation of ACC by the AMP-activated protein kinase

Although ACC is controlled through phosphorylation by various protein kinases, its activity seems to be predominantly regulated by the AMP-activated protein kinase (see the Introduction section). We have investigated whether the activation of ACC by GAPP could result from the dephosphorylation of the site(s) that are phosphorylated by the AMP-activated protein kinase. For this purpose, ACC was purified in its active, dephosphorylated, form and inactivated *in vitro* by incubation with the AMP-activated kinase. As shown in Figure 6, the subsequent re-activation of ACC by GAPP required the addition of glutamate, indicating that GAPP and the AMP-activated protein kinase indeed act antagonistically.

DISCUSSION

Identification of GAPP as a novel hepatic species of PP-2A

When the ACC phosphatase activity in liver extracts is assessed from the rate of activation of exogenous ACC, it is hardly detectable unless glutamate and/or Mg^{2+} are added to the assay mixture ([1]; the present study). We have now obtained firm evidence that this ACC phosphatase activity stems exclusively from PP-2A. First, the ACC phosphatase was entirely soluble (Table 1), like all well characterized species of PP-2A [6]. Secondly, the ACC phosphatase co-purified with the catalytic subunit of PP-2A (Figure 3). Thirdly, GAPP was inhibited by antibodies against PP-$2A_C$ and by the same concentrations of okadaic acid that also inhibited PP-2A (Figure 2). Fourthly, substrate competition experiments showed that the ACC phosphatase also dephosphorylated the PP-2A model substrate phosphorylase and that this dephosphorylation was stimulated manifold by the basic polypeptide protamine (Figure 5). Finally, the observed stimulatory effects of glutamate and Mg^{2+} were also obtained with homogeneously purified species of PP-2A from skeletal muscle (Figure 5; Table 5).

The lability of GAPP and its low recovery during chromatography have not allowed us to purify the enzyme to homogeneity or to determine its subunit structure. It should be noted, however, that the use of a similar procedure as illustrated in Table 2 enabled 500–2000-fold purification of PP-$2A_{T55}$ and PP-$2A_D$ from rat liver ([15–17]; see also the Introduction section). The latter two species most likely correspond to the first and second peaks respectively of the protamine-revealed phosphorylase phosphatase activity on DEAE-Sephadex, which did not correspond to GAPP (Figure 1). The molecular mass of GAPP, as determined by gel filtration (285 kDa), was higher than the 150 kDa of hepatic $PP2A_D$ [15,18] and suggests a trimeric structure. GAPP can also be differentiated from $PP2A_{T55}$ by the opposite effects of protamine on the ACC phosphatase activities of these enzymes (Figure 5B). Finally, using phosphorylase as a reference substrate, GAPP displayed a much higher phosphatase activity towards the SAMS-peptide and the T_3-peptide than any other tested species of PP-2A (Figure 5). Taken together, our data suggest that GAPP, which is quantitatively by far the most important ACC-activating phosphatase (Figure 1), represents a novel hepatic species of PP-2A.

Physiological regulation

The activation of PP-2A holoenzymes by basic polypeptides and polyamines has been recognized for a long time, but its physiological relevance has been questioned [6]. Recent evidence points to regulation of the catalytic subunit by reversible phosphorylation and methylation [35–37]. It has also been demonstrated that trimeric PP-2A is activated by the phospholipid precursor ceramide [38,39].

Our present data indicate that the activity of PP-2A may also be controlled by dicarboxylic acids. We propose that the activation of GAPP by dicarboxylic acids represents an essential step in the well known lipogenic action of Na^+-co-transported amino acids (Figure 7). The first step is the cellular uptake and accumulation of the amino acids and their conversion into dicarboxylic acids such as glutamate and aspartate [1–3]. The associated cell swelling [40] may further enhance the uptake of these amino acids [41]. We found that the intracellular concentration of dicarboxylic acids measured during incubation of hepatocytes with lipogenic amino acids [34] is sufficient to cause a severalfold stimulation of the ACC phosphatase activity of GAPP (Figure 4A). The relevance of these observations is further

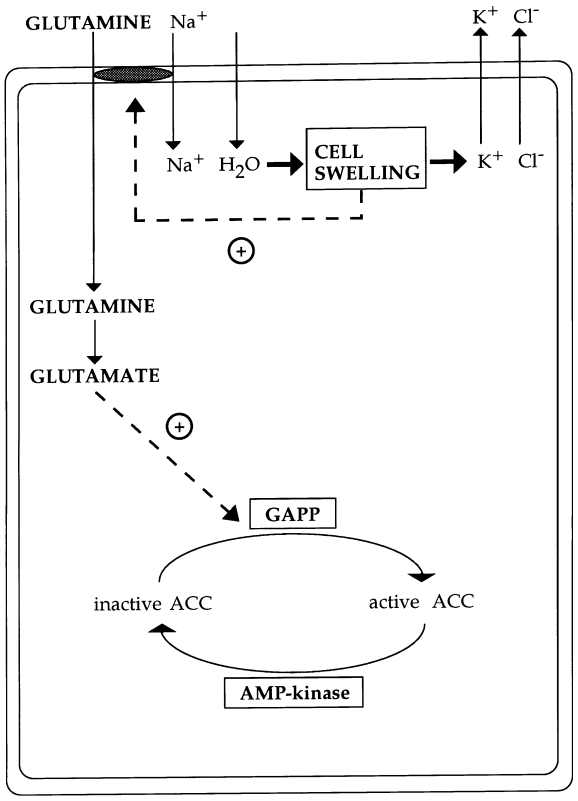

Figure 7 Mechanism of stimulation of liver lipogenesis by glutamine

Glutamine is taken up by a Na^+-co-transport system and is converted intracellularly into glutamate. The accumulation of glutamine and glutamate causes cell swelling, which further promotes the uptake of glutamine. Glutamate promotes the dephosphorylation of Ser-79 of ACC by GAPP, resulting in activation of the enzyme and stimulation of lipogenesis. It is proposed that the effect of GAPP is antagonized by the AMP-activated protein kinase.

strengthened by the fact that the measured ACC phosphatase activities were derived from the activation rate of native ACC, rather than from the dephosphorylation of an *in vitro* ^{32}P-labelled substrate. Moreover, the stimulatory effect of glutamate was also observed with the SAMS-peptide substrate (Figure 4A) and ACC (Figure 6) that were solely phosphorylated by the AMP-activated protein kinase, which is the main activity-controlling ACC kinase *in vivo* (see the Introduction section).

A similar mechanism to that proposed for the lipogenic action of Na^+-co-transported amino acids (Figure 7) may also apply to other pathways that are controlled by these amino acids. For example, it has been demonstrated that the same amino acids that cause lipogenesis also stimulate hepatic glycogen synthesis [33,42]. Initial evidence points to the stimulation of a glycogen synthase phosphatase by glutamate [1,42]. Interestingly, it has recently been found that glutamine also provokes cell swelling and initiates glycogen synthesis in skeletal muscle [43]. Combined with our observations that muscle PP-2A holoenzymes are also stimulated by dicarboxylic acids (Figure 5), this suggests that glycogen synthesis in skeletal muscle may be controlled by regulation of synthase phosphatase(s) by dicarboxylic acids.

Surprisingly, we found that the ACC phosphatase activity of various species of PP-2A was also stimulated by bivalent cations such as Mg^{2+}. At 0.5 mM, which is the mean basal cytosolic concentration of free Mg^{2+} in hepatocytes [44–46], the ACC phosphatase activity amounted to $387 \pm 62\%$ ($n = 3$) of the

control activity. For unknown reasons, partially purified GAPP was reproducibly about 10 times more sensitive to stimulation by Mg^{2+} (Figure 4B) than the non-purified enzyme [1].

Mechanism of stimulation of ACC phosphatase by glutamate and Mg^{2+}

Although the stimulation of PP-2A by polyamines and basic polypeptides has been known for many years, their mechanism of action remains elusive. A substrate-directed mechanism is suggested by observations that basic polypeptides bind to the substrates phosphorylase [32] and phospho-opsin [47], and that the stimulatory action is lost with peptide substrates (Figure 5). On the other hand, optimal stimulatory effects of basic polypeptides have been obtained at concentrations that are more than 30 times lower than the substrate concentration [10], which argues for a phosphatase-directed mechanism.

The same uncertainties arise concerning the mechanism by which dicarboxylic acids promote the activation of ACC by PP-2A. Indeed, on the one hand we have found that glutamate stimulated the activity of ACC by a mechanism that does not involve dephosphorylation. One could therefore envisage that binding of glutamate to ACC induces conformational changes that make the enzyme more active and turn it into a better substrate for dephosphorylation by PP-2A. Such a mechanism could also account for the substrate specificity of the effects of glutamate (Figure 5). On the other hand, the stimulatory action of glutamate was also observed with the small SAMS-pentadecapeptide. This suggests either that glutamate acts by binding to a small domain surrounding the Ser-79 phosphorylation site or that the effects of glutamate are phosphatase-directed. As to the phosphatase subunit requirements, we found that the stimulatory action of glutamate clearly depends upon the presence of the regulatory A subunit (Figure 5; Table 5). This finding also rules out the possibility that glutamate acts by the dissociation of the B subunit, which has previously been shown to occur in the presence of polyanions such as heparin [48]. The low glutamate-revealed ACC phosphatase activity of the catalytic subunit was apparent when phosphorylase was used as a reference substrate (Figure 5) as well as when the phosphatases were compared at the same molar concentration (Table 5).

The Mg^{2+}-dependent stimulation of the ACC phosphatase activity of PP-2A was also largely dependent upon the presence of the A subunit (Table 5). However, in contrast to the stimulatory action of glutamate, the effect of Mg^{2+} was largely lost when the SAMS-peptide was used as substrate. Moreover, the stimulation by both compounds could also be differentiated kinetically (Table 4). These data may indicate that glutamate and Mg^{2+} stimulate the ACC phosphatase activity of PP-2A by different mechanisms. However, these are only indirect arguments, and other interpretations are not ruled out.

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