Evidence for a single non-arachidonic acid-specific fatty acyl-CoA synthetase in heart which is regulated by Mg^{2+}

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Previous reports indicated that arachidonic acid is incorporated into the isolated perfused rabbit heart in preference to other fatty acids, and that incorporation of arachidonic acid, but not other fatty acids, is inhibited during Mg^{2+} depletion. In this study, we have not been able to demonstrate an arachidonic acid-specific fatty acyl-CoA synthetase in rat or rabbit heart by hydroxyapatite chromatography. Kinetic evidence was consistent with a single enzyme, as the slopes of pseudo-Hill plots were not significantly different from -1 . The single fatty acyl-CoA synthetase present appears to prefer $C_{18:0}$ unsaturated fatty acids to arachidonate, and had about the same affinity for $C_{10:0}$ – $C_{14:0}$ saturated fatty acids as for arachidonate. At $35 \mu M$ arachidonate, enzyme

velocity increased as the total Mg^{2+} was increased from 3 to 80 mM. Calculated $[MgATP]$ indicated that the $MgATP$ complex was not rate-limiting. At low concentrations, Mn^{2+} and Ni^{2+} supported activity, but Cu^{2+} and Zn^{2+} did not. Low Ca^{2+} concentrations activated only oleic acid conversion. Kinetic analysis indicated that the V_{max} of the enzyme was increased with increasing concentrations of ionized Mg^{2+} for both oleic acid and arachidonic acid. The data are consistent with the hypothesis that Mg^{2+} has a direct effect on fatty acyl-CoA synthetase activity, and suggest that preference for oleic acid and arachidonic acid can be influenced by the ionic milieu.

INTRODUCTION

Although stimulus-induced prostaglandin synthesis has been extensively studied, much less is known about the mechanisms that control arachidonic acid re-incorporation into phospholipid. In the isolated perfused rabbit heart, the incorporation of exogenous arachidonic acid is significantly greater than oleic acid, a difference that cannot be attributed to differences in transport or distribution kinetics [1]. Under perfusion conditions that deplete intracellular Mg^{2+} , the incorporation of exogenous arachidonic acid into phospholipid is inhibited, whereas the incorporation of other fatty acids is either unchanged or increased [1,2]. Furthermore isoprenaline-induced prostaglandin synthesis is not changed during Mg^{2+} depletion, whereas basal prostaglandin synthesis rises [1]. These findings suggest that oleate and arachidonate are incorporated via different metabolic pathways.

The initial step in the acylation of both oleate and arachidonate is the formation of the CoA derivative. A long-chain fatty acyl-CoA synthetase specific for arachidonic acid has been described [3,4]. The ubiquitous distribution of arachidonoyl-CoA synthetase has fostered the notion that this enzyme catalyses the first step of arachidonic acid incorporation in virtually all tissues [3]. The preferential incorporation of arachidonate over oleate in the isolated perfused rabbit heart, coupled with the differential inhibition of arachidonate incorporation into phospholipid during Mg^{2+} depletion, led to the hypothesis that cardiac tissue has high levels of arachidonoyl-CoA synthetase, and that arachidonoyl-CoA synthetase activity is reduced during Mg^{2+} depletion. This hypothesis was tested in the experiments reported here.

MATERIALS AND METHODS

Materials

$[^3H]$ Arachidonic acid (76–240 Ci/mmol), $[^3H]$ oleic acid (10 Ci/mmol) and $[^{14}C]$ palmitic acid (57 mCi/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.) and diluted

to the appropriate specific radioactivity with unlabelled fatty acid. Unlabelled arachidonic acid was purchased from NuChek Preps (Elysian, MN, U.S.A.). Other unlabelled fatty acids, CoA (lithium salt) and ATP (disodium salt) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 4'-[3-Carboxy-4-(2,7-dichloro-3-hydroxy-6-oxo-6*H*-xanthen-9-yl)benzamido]-2''-(carboxymethoxy)-*N*-phenyliminodiacetic acid, pentapotassium salt (Magnesium Green) and magnesium standards were purchased from Molecular Probes (Eugene, OR, U.S.A.). All other chemicals were of the highest grade available.

Enzyme preparation

Male New Zealand White rabbits were killed with an overdose of sodium pentobarbital (60–65 mg/kg; injected intravenously) or by stunning and exsanguination. The hearts were removed and trimmed of atria and large blood vessels. The enzyme was prepared as described by Wilson et al. [4]. Briefly, the tissue was homogenized in 10 vol. of buffer composed of 320 mM sucrose and 10 mM Hepes, pH adjusted to 7.4 with 1 M Tris, and supplemented with 10 mM 2-mercaptoethanol, 20 $\mu g/ml$ PMSF, 1 mM benzamidine and 0.01 % soyabean trypsin inhibitor. The homogenate was centrifuged at 1000 *g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 35000 *g* for 1 h. This pellet was resuspended in 20 mM potassium phosphate buffer, pH 7.4, containing 1 % Nonidet P40, 10 mM 2-mercaptoethanol and 10 mM EDTA, stirred at 4 °C for 2 h, and centrifuged at 35000 *g* for 1 h. The supernatant, which contained the solubilized enzyme, was loaded on to a hydroxyapatite column (void volume 12 ml) equilibrated with 20 mM potassium phosphate buffer, pH 7.4, containing 1 % Nonidet P40 and 10 mM 2-mercaptoethanol. All elution buffers contained 10 mM 2-mercaptoethanol and 1 % Nonidet P40, and were adjusted to pH 7.4. The column was eluted with 100 ml of 80 mM potassium phosphate, followed by a 270 ml linear gradient of potassium phosphate (80–300 mM). The eluate was collected in 6 ml

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fractions. Each fraction was assayed for enzyme activity as described below. Protein content was measured by the method of Lowry et al. [5]. The prepared enzyme was stored at -20°C .

For some studies, the enzyme was prepared as described from normal rabbit liver, normal guinea-pig heart, normal and diabetic rat liver, normal and diabetic rat heart and neonatal rabbit heart. Diabetes was induced by streptozotocin (60–65 mg/kg, 8 weeks before the experiment) and confirmed by blood glucose levels exceeding 400 mg/dl and failure to gain weight. Diabetic rats were killed with an overdose of sodium pentobarbital (50–60 mg/kg; injected intraperitoneally). Normal rats and neonatal rabbits were killed by decapitation.

Enzyme assay

Fatty acyl-CoA synthetase activity was measured as described previously [4]. Briefly, 50 μl of enzyme preparation was added to 100 μl of an assay cocktail containing (final concentration) 100 mM Tris/HCl, pH 8.0, 2 mM Triton X-100, 0.66 mM CoA, 6.6 mM ATP, radiolabelled (^{14}C or ^3H) fatty acid and the appropriate concentrations of unlabelled fatty acid and bivalent cations. The reaction was allowed to proceed for 2–3 min at 37°C , and terminated by the addition of 2.25 ml of stopping solution (propan-2-ol/heptane/2 M H_2SO_4 , 40:10:1, by vol.). Unchanged substrate was extracted by adding 1.5 ml of heptane and 1 ml of water, vortexing vigorously, and removing the upper (organic) layer. The aqueous layer was re-extracted twice with 2 ml of heptane containing 4 mg/ml palmitic acid. The radioactivity in a 1 ml portion of the final aqueous layer was determined by liquid-scintillation spectroscopy. Product formation was calculated as nmol of product formed/min per mg of protein.

Measurement of free Mg^{2+}

Assay cocktail (100 μl) containing 2.83–9.16 mM MgCl_2 , 3–10 mM ATP and 35 μM oleic acid or arachidonic acid was mixed with 50 μl of 80 mM potassium phosphate buffer, containing 1% Nonidet P40, in a 96-well microtitre plate. Magnesium Green, a fluorescent Mg^{2+} indicator, was added to a final concentration of 2 μM , and the plate agitated. The fluorescence was read using a Cytofluor 2300 fluorimeter. Samples were compared with known standards of Mg^{2+} at concentrations ranging from 0 to 35 mM.

Analysis of results

Data were analysed by one- or two-way analysis of variance, or by Student's *t* test for paired data. The null hypothesis was rejected at $P < 0.05$.

RESULTS

Protein concentrations were adjusted so that fatty acyl-CoA synthetase activity remained linear for at least 3 min at 35 μM arachidonic acid and 100 mM Mg^{2+} . The minimum detectable concentration of product when arachidonic acid was used as substrate was less than 0.5 fmol. The radioactive product of a scaled-up reaction was subjected to TLC as described by Mishina et al. [6]. More than 98% of the radioactivity co-migrated with authentic arachidonoyl-CoA.

Separation of fatty acyl-CoA synthetases

Hydroxyapatite chromatography of solubilized rabbit heart preparations yielded a single peak of enzyme activity, contained

in fractions 7–12 (Figure 1A). Preparations made from the livers of the same animals yielded two peaks of activity (Figure 1B), the first in fractions 7–12 (during elution with 80 mM potassium phosphate) and the second in fractions 22–35 (during elution with 80–300 mM potassium phosphate gradient). Preparations of normal rat or guinea-pig heart and neonatal rabbit heart each yielded a single peak of activity, eluted in fractions 7–12 (P1H) (results not shown). Preparations of liver obtained from normal or diabetic rats each yielded two peaks of activity eluted in fractions 7–12 (P1L) and 26–40 (P2L) (results not shown).

The lack of a second peak of CoA synthetase activity in hearts might be explained by the presence of an endogenous inhibitor, eluted with the 80–300 mM phosphate gradient. If so, then the activity of the P2L liver fractions should decline in the presence of the corresponding heart fractions (P2H). The mean activity of P2L was 8.30 ± 2.78 pmol/min per 30 μl in the absence of P2H and 10.6 ± 1.99 pmol/min per 30 μl in the presence of 30 μl of P2H (means $\pm$ S.E.M., $n = 3$), which are not significantly different.

Fatty acyl-CoA synthetase substrate preference

The substrate preference of the single peak of fatty acyl-CoA synthetase activity isolated from heart was determined in the presence of 7 mM MgCl_2 . Product formation at 35 μM substrate (^3H)arachidonic acid or ^{14}C palmitic acid) was measured in the presence or absence of increasing concentrations of unlabelled fatty acids. The results were analysed as pseudo-Hill plots, and the concentration of fatty acid required to inhibit the formation of radiolabelled product by 50% (IC_{50}) was calculated. The IC_{50} values for the competing fatty acids were compared with that for unlabelled arachidonic acid and these values were used to construct the preference ratios shown in Table 1. The enzyme preferred each of the unsaturated fatty acids tested to arachidonic acid. Among the saturated fatty acids, the enzyme displayed equal preference for arachidonic acid, $\text{C}_{14:0}$, $\text{C}_{12:0}$ and $\text{C}_{10:0}$ fatty acids. In contrast, there was little preference for $\text{C}_{16:0}$ or $\text{C}_{8:0}$ saturated fatty acids. The substrate preference ratio for the inhibition of ^{14}C palmitoyl-CoA formation was: arachidonic acid = 1.0, palmitic acid = 0.17 ± 0.07 , $\text{C}_{14:0}$ = 0.89 ± 0.26 , $\text{C}_{12:0}$ = 2.44 ± 1.34 , $\text{C}_{10:0}$ = 1.88 ± 0.70 . The average slopes of the pseudo-Hill plots were -0.91 ± 0.052 and -1.1 ± 0.075 for ^3H arachidonic acid and ^{14}C palmitic acid respectively (not significantly different from -1 or from each other; means $\pm$ S.E.M., $n = 3$).

Measurement of Mg^{2+}

Mg^{2+} concentrations measured in the presence of oleic acid are shown in Table 2. Also shown are the calculated Mg^{2+} concentrations, assuming MgATP dissociation constants of 28.7 μM [7] or 50 μM . Assuming a MgATP dissociation constant of 28.7 μM , the mean measured Mg^{2+} values were $157 \pm 12\%$ of the calculated values. Assuming a MgATP dissociation constant of 50 μM , the measured values were $107 \pm 7.6\%$ of the calculated values. For the purpose of further calculations, the MgATP dissociation constant was assumed to be 50 μM .

Influence of Mg^{2+} on enzyme activity

Fatty acyl-CoA synthetase activity was measured at 35 μM ^3H arachidonic acid or 35 μM ^3H oleic acid in the presence of 3–80 mM MgCl_2 (Figure 2). Assuming that the MgATP dissociation constant is 50 μM , the free Mg^{2+} concentration ranged from 40.6 μM to 73.4 mM and MgATP complex varied from about 2.96 to 6.6 mM, with maximal MgATP achieved at about

Figure 1 Elution profiles of fatty acyl-CoA synthetase activity in rabbit heart (A) and rabbit liver (B)

Tissues were obtained from the same animal. Fractions 1–4 represent the void volume; fractions 5–17 represent elution with 80 mM phosphate buffer; fractions 18–55 represent elution with an 80–300 mM linear gradient of phosphate buffer. Fractions were screened for enzyme activity with either 5.6 nM [^3H]arachidonic acid or 5.6 nM [^3H]oleic acid. These elution profiles are representative of six animals.

Table 1 IC_{50} for inhibition of [^3H]arachidonoyl-CoA formation by fatty acids

The [^3H]arachidonic acid concentration was about 35 μM and calculated free Mg^{2+} was 792 μM (7 mM MgCl_2). *Significantly different from the IC_{50} for unlabelled arachidonic acid ($P < 0.05$). The IC_{50} for octanoic acid was arbitrarily set at 1000, and this value was used to compute the relative potency.

Fatty acid	IC_{50} (μM)	Relative to arachidonic acid	Number of enzyme preparations
20:4(5,8,11,14)	52.6 $\pm$ 7.43	1.00	7
18:1(9)	25.9 $\pm$ 4.8*	2.38 $\pm$ 0.77	4
18:2(9,12)	17.3 $\pm$ 2.5*	3.08 $\pm$ 0.53	4
18:3(6,9,12)	16.8 $\pm$ 2.1*	3.38 $\pm$ 0.84	4
20:3(8,11,14)	37.0 $\pm$ 11.9*	2.10 $\pm$ 0.55	4
16:0	354 $\pm$ 82.7*	0.17 $\pm$ 0.06	3
14:0	73.6 $\pm$ 16.2	0.83 $\pm$ 0.28	3
12:0	39.7 $\pm$ 9.65*	1.51 $\pm$ 0.63	3
10:0	44.6 $\pm$ 13.1	1.55 $\pm$ 0.82	3
8:0	> 1000	< 0.05	3

Table 2 Measured Mg^{2+} concentrations at various concentrations of MgCl_2 and ATP, compared with calculated values

Numbers in parentheses indicate the measured Mg^{2+} expressed as a percentage of the calculated Mg^{2+} . The best correspondence between measured and calculated values was obtained by using the MgATP dissociation constant of 50 μM .

MgCl_2 (mM)	ATP (mM)	Measured Mg^{2+} (μM)	Calculated Mg^{2+} ($K_{\text{dMgATP}} = 50 \mu\text{M}$) (μM)	Calculated Mg^{2+} ($K_{\text{dMgATP}} = 28.7 \mu\text{M}$) (μM)
2.83	3	329	283 (116)	204 (161)
4.67	5	309	328 (94.2)	227 (136)
6.5	7	338	358 (94.4)	242 (140)
9.17	10	455	367 (124)	239 (190)

Figure 2 Fatty acyl-CoA synthetase activity in the presence of increasing concentrations of Mg^{2+}

The substrate concentration was 35 μM arachidonic acid ($\circ$) or 35 μM oleic acid ($\blacksquare$); data are expressed as a fraction of the observed activity for each substrate at 80 mM total Mg^{2+} . At all concentrations of Mg^{2+} , the absolute velocity of the reaction was significantly greater with oleic acid than with arachidonic acid. Rates at 80 mM MgCl_2 were 132 $\pm$ 46.3 and 36.4 $\pm$ 16.3 nmol/min per mg of protein for oleic acid and arachidonic acid respectively (mean $\pm$ S.E.M.; $P < 0.05$; $n = 4$).

8.0 mM MgCl_2 . At all concentrations of MgCl_2 , the absolute velocity of the reaction was significantly greater with oleic acid than with arachidonic acid. The ratio of the activity in the presence of arachidonate was compared with that in the presence of oleate for each of the Mg^{2+} concentrations. At 3 mM MgCl_2 (calculated Mg^{2+} 40.6 μM), the activity was 4.91 $\pm$ 0.9 times greater with oleic acid than with arachidonic acid, a value that was significantly greater than 3.03 $\pm$ 0.26, the average ratio at all other MgCl_2 concentrations.

Since the CoA synthetase reaction requires ATP as a co-substrate, it is highly likely that Mg^{2+} participates in the reaction by forming a MgATP complex (which is probably the true co-substrate). This raises the possibility that the apparent effect of

Figure 3 Fatty acyl-CoA synthetase activity in the presence of increasing concentrations of MgATP, at 334 μM ($\triangle$) and 10 mM ($\blacktriangle$) free Mg^{2+} and with 35 μM oleic acid as substrate

Enzyme activity is expressed relative to the activity observed at 10 mM free Mg^{2+} and 10 mM MgATP. The total ATP concentration varied from about 3 to 10 mM. Activity was significantly greater at 10 mM free Mg^{2+} than at 334 μM free Mg^{2+} ; at both concentrations of free Mg^{2+} , enzyme activity did not increase significantly with increasing MgATP concentrations. Results are means $\pm$ S.E.M. ($n = 4$).

Table 3 Apparent K_m and $V_{\max}$ of fatty acyl-CoA synthetase at 3, 7 and 80 mM total MgCl_2 , for arachidonate and oleate

Fatty acyl-CoA synthetase activity was measured at substrate concentrations ranging from 2.5 to 300 μM . The apparent K_m was estimated from double-reciprocal plots. The calculated free Mg^{2+} concentrations (assuming a MgATP dissociation constant of 50 μM) were 40.6 μM (3 mM MgCl_2), 792 μM (7 mM MgCl_2) and 73.4 mM (80 mM MgCl_2). Results are means $\pm$ S.E.M. ($n = 7$). *Significantly different when compared with 3 mM MgCl_2 for a single substrate ($P < 0.05$). †Significantly different between substrates for a single MgCl_2 concentration ($P < 0.05$). Units of $K_{m,\text{app}}$ = μM ; units of $V_{\max,\text{app}}$ = nmol/min per mg of protein.

	Arachidonic acid		Oleic acid	
	$K_{m,\text{app}}$	$V_{\max,\text{app}}$	$K_{m,\text{app}}$	$V_{\max,\text{app}}$
3 mM MgCl_2	22.2 $\pm$ 3.4†	17.6 $\pm$ 6.7†	13.1 $\pm$ 1.3	35.5 $\pm$ 11.7
7 mM MgCl_2	32.1 $\pm$ 6.3*†	21.2 $\pm$ 6.2†	21.7 $\pm$ 4.3*	59.1 $\pm$ 19.9*
80 mM MgCl_2	37.9 $\pm$ 3.7*†	34.9 $\pm$ 11.5*†	21.1 $\pm$ 3.5*	95.2 $\pm$ 33.3*

MgCl_2 in increasing the activity of fatty acyl-CoA synthetase might simply be the result of increased formation of MgATP complex. This possibility was addressed by measuring fatty acyl-CoA synthetase activity at approx. 334 μM and 10 mM free Mg^{2+} (calculated values, using 50 μM as the MgATP dissociation constant), 35 μM oleic acid and ATP concentrations varying from about 3 to 10 mM. The results of these experiments are shown in Figure 3. At each calculated MgATP concentration between 3 and 10 mM, enzyme activity was significantly greater in the presence of 10 mM Mg^{2+} than in the presence of 334 μM Mg^{2+} . At either concentration of Mg^{2+} , enzyme activity did not significantly increase with increasing MgATP concentrations.

The effect of MgCl_2 on the apparent K_m and $V_{\max}$ was determined for both arachidonic acid and oleic acid as substrates (2.5–300 μM). Seven different enzyme preparations were assayed for fatty acyl-CoA synthetase activity at 3, 7 and 80 mM MgCl_2 . The K_m and $V_{\max}$ were estimated from double-reciprocal plots. The results are shown in Table 3. At all concentrations of MgCl_2 , each of the enzyme preparations showed a significantly lower K_m for oleate than for arachidonate ($P < 0.05$). The apparent K_m for both fatty acids was significantly lower at 3 mM MgCl_2 than at

7 or 80 mM MgCl_2 ($P < 0.05$). The affinity at 7 mM MgCl_2 was not different from that at 80 mM MgCl_2 for either substrate. At all concentrations of MgCl_2 , each of the enzyme preparations showed an apparent $V_{\max}$, which was significantly greater for oleate than for arachidonate ($P < 0.05$). For both substrates, maximal velocity increased significantly as the MgCl_2 concentration increased.

Effect of other bivalent cations on fatty acyl-CoA synthetase activity

Fatty acyl-CoA synthetase activity was measured in the presence of increasing concentrations of MnCl_2 , NiCl_2 , CaCl_2 , CuCl_2 or ZnCl_2 , using 35 μM arachidonic or oleic acid as substrate. Enzyme activity for either substrate at 80 mM MgCl_2 was taken as 100% (control value). CuCl_2 and ZnCl_2 did not support activity for either substrate. At low concentrations, both NiCl_2 and MnCl_2 were able to support enzyme activity about equally well for both substrates (Figure 4). In contrast, low concentrations of CaCl_2 supported enzyme activity when oleic acid was the substrate, but did not support activity at any concentration when arachidonic acid was the substrate. At high concentrations, NiCl_2 , MnCl_2 and CaCl_2 all failed to support activity, regardless of substrate.

DISCUSSION

If one or more of the enzymes required to incorporate non-esterified fatty acids into tissue lipids were highly selective for arachidonate, then the preferential incorporation [1,2] and differential inhibition of arachidonate incorporation [1] into cardiac lipids could be explained. Arachidonic acid-specific CoA synthetase appears to be widely distributed [3,4], suggesting that there is high arachidonoyl-CoA synthetase activity in the rabbit heart, which is inhibited during Mg^{2+} depletion. However, the elution pattern of solubilized preparations of rabbit heart from a hydroxyapatite column was consistent with a single non-selective fatty acyl-CoA synthetase.

The evidence that there is only a single fatty-acyl CoA synthetase present in heart is extensive. The presence of a single peak of fatty acyl-CoA synthetase in heart and two peaks of activity in liver indicates that the animals are competent to produce multiple fatty acyl-CoA synthetases. The slopes of the pseudo-Hill plots were not significantly different from -1 , regardless of the substrate, which is consistent with the presence of a single enzyme. There does not seem to be an endogenous inhibitor of arachidonoyl-CoA synthetase in the heart preparation. Finally, only a single peak of enzyme activity is present for rat, guinea-pig and rabbit heart, demonstrating that heart as a tissue does not produce arachidonoyl-CoA synthetase.

Tissues that derive energy primarily from fatty acids (such as normal rabbit heart [8] or livers from diabetic animals) might have relatively low levels of arachidonic acid-specific compared with non-specific fatty acyl-CoA synthetase. However, the hydroxyapatite elution profiles of enzyme isolated from livers of diabetic rats were not different from those of normal rat or rabbit liver. Conversely, preparations of enzyme from neonatal rabbit heart, a tissue that does not rely on β -oxidation for the generation of metabolic energy [9], yielded only a single peak of activity. Taken together, these data suggest that the presence or absence of two peaks of CoA synthetase activity is not related to energy metabolism.

It is difficult to compare directly the substrate specificity of the single enzyme isolated from heart with the non-specific enzyme described by other investigators [3,4]. In studies of crude platelet or liver extracts, the formation of [^3H]arachidonoyl-CoA was

Figure 4 Fatty acyl-CoA synthetase activity in the presence of increasing concentrations of MnCl_2 (A), NiCl_2 (B) or CaCl_2 (C) using $35 \mu\text{M}$ oleic acid (■) or $35 \mu\text{M}$ arachidonic acid (○) as substrate

Data are expressed as a fraction of activities at 80 mM MgCl_2 (control) which were 35.7 ± 12.6 (arachidonic acid) and 96.7 ± 37.8 (oleic acid) nmol/min per mg of protein (Means $\pm$ S.E.M., $n = 4$).

inhibited by unlabelled arachidonic acid and 8,11,14-eicosatrienoic acid, but not by stearic acid, oleic acid, or linoleic acid. On the other hand, the formation of [^3H]oleoyl-CoA was inhibited by oleic acid = linoleic acid > 8,11,14-eicosatrienoic acid > arachidonic acid [3]. In later studies [4], two peaks of enzyme activity were separated, both of which catalysed the formation of arachidonoyl-CoA. The second peak of activity appeared to contain the arachidonic acid-specific enzyme as well as some non-specific enzyme activity. The designation 'specific' was based on the observation that, even in the presence of high concentrations of oleic acid, the formation of arachidonoyl-CoA was catalysed [4]. As the single peak of activity isolated from the heart has a fatty acid preference similar to the non-specific enzyme of [3] and is eluted with 80 mM phosphate, it seems likely that this is the enzyme that others have designated as 'non-specific'.

Enzymes that require ATP as a co-substrate usually also require Mg^{2+} for activity, as MgATP complex is probably the true co-substrate. However, recent examination of several ATP-requiring enzymes has forced a re-examination of this assumption. A Mg^{2+} -binding pocket has been described for protein kinase C [10–12]. When this site is occupied by Mg^{2+} , the conformation and catalytic activity of the protein change [12]. In a similar fashion, calmodulin-dependent protein kinase [13] and insulin-dependent tyrosine kinase [14] also have a requirement for Mg^{2+} , which appears to be separate from the requirement for MgATP . Fatty acyl-CoA synthetase appears to represent still another ATP-requiring enzyme that is regulated by the concentration of Mg^{2+} . The data are consistent with a direct influence of Mg^{2+} on the activity of fatty acyl-CoA synthetase isolated from rabbit heart. Catalytic activity continues to increase even after virtually 100 % of the ATP is complexed to Mg^{2+} . In further support, when the MgATP concentration is held constant and the free Mg^{2+} is allowed to increase, the rate of formation of fatty acyl-CoA increases, regardless of which fatty acid is the substrate. Increasing the MgCl_2 concentration from 3 to 80 mM decreases the affinity of the enzyme for both oleic acid and arachidonic acid by about 40 %. However, over this range of MgCl_2 , the maximal velocity is increased by 98 % for arachidonic acid and 168 % for oleic acid. Taken together, these data indicate that enzyme activity is Mg^{2+} -dependent, apart from the requirement for MgATP .

Activity is only partially supported by low concentrations of MnCl_2 and NiCl_2 . At high concentrations, these cations failed to support activity, whereas ZnCl_2 and CuCl_2 failed to support activity at any concentration tested. These data suggest that the effect of MgCl_2 cannot be attributed to increased ionic strength of the reaction mixture. Other data support the notion that the substrate preference of fatty acyl-CoA synthetase can be changed by the ionic milieu. Low concentrations of CaCl_2 support enzyme activity when oleate, but not arachidonate, is the substrate.

It seems clear that fatty acyl-CoA synthetase activity is influenced by the ionic milieu and, more particularly, the Mg^{2+} concentration. However, as the changes in activity seem to be about equal for both oleic acid and arachidonic acid, it does not seem likely that the direct influence of Mg^{2+} on fatty acyl-CoA synthetase activity can be invoked to explain the reduced incorporation of arachidonic (but not oleic) acid into cardiac lipids that is observed during experimental Mg^{2+} depletion.

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