Separate myocardial ethanolamine phosphotransferase activities responsible for plasmenylethanolamine and phosphatidylethanolamine synthesis

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Abstract **Ethanolamine phosphotransferase (EPT) is a key enzyme responsible for the synthesis of ethanolamine glycerophospholipids. Plasmenylethanolamine is a predominant molecular subclass of ethanolamine glycerophospholipids in the heart. The present study was designed to identify the selective use of 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycerol as a substrate for EPT as a mechanism responsible for the predominance of plasmenylethanolamine in the rabbit heart. EPT activity in rabbit myocardial membranes using 1,2-diacyl-*sn*-glycerol as substrate is activated by Mn^{2+}, inhibited by dithiobisnitrobenzoic acid (DTNB) and is unaffected by Ca^{2+}. In contrast, ethanolamine phosphotransferase activity using 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycerol as substrate is inhibited by Mn^{2+} and Ca^{2+}, but is activated by DTNB. Additionally, ethanolamine phosphotransferase activity using 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycerol substrate was more sensitive to thermal denaturation compared with that of 1,2-diacyl-*sn*-glycerol. Taken together, these results suggest that separate ethanolamine phosphotransferase activities are present in heart membranes that are responsible for the synthesis of phosphatidylethanolamine and plasmenylethanolamine.**—Ford, D. A. **Separate myocardial ethanolamine phosphotransferase activities responsible for plasmenylethanolamine and phosphatidylethanolamine synthesis.** ***J. Lipid Res.*** **2003.** 44: **554–559.**

Supplementary key words myocardium • ethanolamine glycerophospholipids • choline phosphotransferase

Ethanolamine phosphotransferase (EPT) and choline phosphotransferase (CPT) are critical enzymes involved in the synthesis of ethanolamine and choline glycerophospholipids. CPT and EPT, in concert with diglyceride kinase, coordinate the salvage of diradylglycerols produced as signaling molecules into choline and ethanolamine glycerophospholipids as well as the acidic phospholipids, respectively (1–5). The plasmalogens, plasmenylethanolamine and plasmenycholine, are a molecular subclass of glycerophospholipids that contain a vinyl ether bond linkage of their *sn*-1 aliphatic chain. Furthermore, plasmalogens are the predominant molecular subclass of glycerophospholipids present in the membranes of heart cells as well as many other tissues (6–11). Previously, it has been proposed that phospholipase C and EPT activity are key enzymes for the cycling of 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycerol (AAG) out of and into myocardial plasmenylethanolamine pools while providing an intermediate (i.e., AAG) for the synthesis of plasmenylcholine in the heart (4).

Previous studies have revealed the selectivity of EPT for AAG as substrate (4, 12, 13). In the present study, AAG and DAG utilization by rabbit heart EPT was further characterized. Multiple lines of evidence now suggest that two EPT activities are present in myocardial membranes. One EPT activity is specific for AAG as substrate, is not altered by dithiobisnitrobenzoic acid (DTNB) treatment, and is inhibited by Ca^{2+} and Mn^{2+}. In contrast, a second EPT activity has been identified that utilizes DAG as substrate, is sensitive to DTNB treatment, and is activated by Mn^{2+}. Taken together, these are the first studies to suggest the presence of two EPT activities in heart membranes that are specific for the two major diradyl glycerol substrates produced in the heart, AAG and DAG.

MATERIALS AND METHODS

Preparation of rabbit myocardial membranes

Male New Zealand white rabbits (1 kg) were sacrificed by cervical dislocation, a thoracotomy was performed, and hearts were immediately minced into small pieces (~2 × 4 mm) with scissors

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Abbreviations: AAG, 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycerol; CGP, choline glycerophospholipid; CPT, choline phosphotransferase; DAG, 1,2-diacyl-*sn*-glycerol; EGP, ethanolamine glycerophospholipid; EPT, ethanolamine phosphotransferase; GPC, *sn*-glycero-3-phosphocholine; 16:0-20:4, 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl; a16:0-20:4, 1-*O*-hexadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl.

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prior to homogenization in ice-cold buffer [100 mM Tris-HC1 buffer (pH 7.4) containing 0.25 M sucrose, 2 mM EGTA, and 2 mM EDTA] utilizing a Potter-Elvehjem apparatus. Myocardial membranes were prepared by sequential centrifugation of homogenates at 10,000 g_{max} for 20 min followed by centrifugation of the resultant supernatant at 100,000 g_{max} for 60 min. The pellets from the 100,000 g_{max} spin (membranes) were resuspended in ice-cold homogenization buffer at a final concentration of ~6.5 mg of protein/ ml.

Preparation of diradylglycerols

Commercially-available phosphatidylcholine (16:0-20:4-GPC) (Avanti) and synthetic plasmenylcholine (a16:0-20:4-GPC) were treated with 10 units of *Bacillus cereus* phospholipase C, and the resultant diradylglycerols were purified by HPLC utilizing a silica stationary phase and a mobile phase comprised of hexane-isopropanol (100:1, v/v) (R_t = 5.8 min for AAG and R_t = 8.9 min for DAG) (14). These synthetic diradylglycerols were quantitated by capillary gas chromatography of their acid-methanolysis derivative products utilizing arachidic acid as internal standard. HPLC analyses, as well as capillary gas chromatographic analyses, demonstrated that each of the semi-synthetic diradylglycerol molecular species was greater than 98% pure. Diradylglycerols were stored in $CHCl_3$, under N_2 at −20°C and were typically utilized within 1 week. Under these conditions, acyl migration of *sn*-2 aliphatic groups was minimized as determined by straight phase HPLC using the system described above that resolved 1,2-DAG from 1,3-DAG with retention times of 8.9 and 5.7 min, respectively.

EPT and CPT assay

Myocardial EPT activities were determined as previously described (4). Briefly, 100 μg of microsomal protein was incubated with 100 μM [ethanolamine-l,2-^{14}C]CDP-ethanolamine (specific activity, 10 μCi/μmol) in 100 μl of assay buffer [50 mM Tris-HCl, 10 mM $MgCl_2$ (pH 8.0)] containing 0.005% Tween 20 (final concentration) for 15 min at 37°C. Selected concentrations of exogenous diradylglycerols in $CHCl_3$ were evaporated to dryness and subsequently solubilized by vigorous sonication in 25 μl of assay buffer containing 0.02% Tween 20 (v/v). Next, 15 μl of membranes (~100 μg of protein) were added and reactions were initiated by addition of 60 μl of assay buffer containing [ethanolamine-l,2-^{14}C]CDP-ethanolamine. Following 15 min at 37°C, incubations were terminated by the addition of 250 μl of butanol and subsequent vortexing. Reaction products in the butanol phase were resolved by TLC utilizing Silica Gel G plates and a mobile phase comprised of chloroform-methanol-ammonium hydroxide (65:35:5, v/v/v) (the R_f value for ethanolamine glycerophospholipids in this system was 0.34). After visualization of ethanolamine glycerophospholipids by brief exposure to iodine vapor, appropriate regions of the TLC plate were scraped into scintillation vials, and radioactivity in ethanolamine glycerophospholipids was quantified by scintillation spectrometry. Reactions were linear with respect to time and protein content under the condition employed. CPT activity was measured under similar conditions to that for EPT using Tween to solubilize diradylglycerols, an identical assay buffer, [methyl-^{14}C]CDP-choline (specific activity, 10 μCi/μmol) as substrate, and an identical TLC system to resolve the reaction product, CPG. EPT activities were also quantified utilizing the Triton X-100 mixed micellar assay system described by Hjelmstad and Bell (15). In these experiments, selected mole fractions of diradylglycerols incorporated as substitutional impurities (1–10 mol%) into Triton X-100 mixed micelles which were incubated with 100–200 μg of microsomal protein in 100 μl of assay buffer containing 100 μM [ethanolamine-1,2-^{14}C]CDP-ethanolamine (specific activity, 40 μCi/μmol) for 15 min at 37°C. The resultant radiolabeled ethanolamine glycerophospholipids were quantified by scintillation spectrometry after TLC separation as described above.

HPLC analysis of EPT products

Radiolabeled ethanolamine glycerophospholipids were first resolved utilizing straight phase HPLC employing an Ultrasphere Si column (4.6 × 250 mm; 5-μm particles) and gradient elution with hexane-isopropanol-water (48.5:48.5:3, v/v/v) as the initial mobile phase and hexane-isopropanol-water (46.5:46.5:7, v/v/v) as the final mobile phase as described previously (6). Column eluates were dried under nitrogen, resuspended in 50 μl of hexane-isopropanol (l:l, v/v), and individual molecular species of ethanolamine glycerophospholipids were resolved by reversed phase HPLC utilizing an octadecyl silica stationary phase (4.6 × 250 mm; 5-μm particles) with isocratic elution at 2 ml/min and a mobile phase comprised of methanol-acetonitrile-water (90.5:2.5:7, v/v/v) containing 20 mM choline chloride (16). Column eluates were monitored by UV detection at 203 nm and radioactivity in column fractions was determined by liquid scintillation spectrometry.

Materials

Phosphatidylcholine, 16:0-20:4-GPC, was purchased from Avanti Polar Lipids. Plasmenylcholine, a16:0-20:4-GPC, was synthesized utilizing semisynthetically prepared 1-*O*-hexadec-1'-enyl-GPC, and arachidonoyl chloride (NuChek Prep) was purified by HPLC and was quantitated by gas chromatography as previously described (17). *B. cereus* phospholipase C and Tween 20 were purchased from Boehringer Mannheim and Pierce Chemical Co., respectively. [ethanolamine-1,2-^{14}C]CDP-ethanolamine and [methyl-^{14}C]CDP-choline were purchased from ICN Radiochemicals and DuPont NEN, respectively. HPLC-grade solvents were purchased from Fisher Scientific Co.

RESULTS

Ethanolamine phosphotransferase activity in Triton X-100 mixed micelles containing diradyl glycerol molecular subclasses

EPT activity was measured in Triton X-100 mixed micelles containing selected concentrations of diradylglycerols and rabbit myocardial membranes as the enzyme source. **Figure 1** demonstrates that AAG is preferentially converted to plasmenylethanolamine by EPT in comparison to DAG conversion to phosphatidylethanolamine. Additional experiments were performed with binary mixtures of AAG and DAG under conditions in which AAG was constant at 5 mol% and DAG was increased from 1–10 mol% (Fig. 1). From these experiments, EPT activity did not change significantly as DAG increased. Thus, DAG does not inhibit AAG incorporation into plasmenylethanolamine. In fact, reversed phase HPLC analysis of the products of the EPT assay in the presence of 5 mol% AAG and 5 mol% DAG confirmed that greater than 90% of the ethanolamine glycerophospholipid formed was plasmalogen (data not shown). The inability of DAG to inhibit AAG utilization suggested that the use of these two substrates by EPT is through two distinct enzymic activities.

DTNB-sensitivity of EPT activity using DAG substrate

As a first step to delineate differences in the utilization of DAG and AAG substrates by myocardial EPT, the sensi-

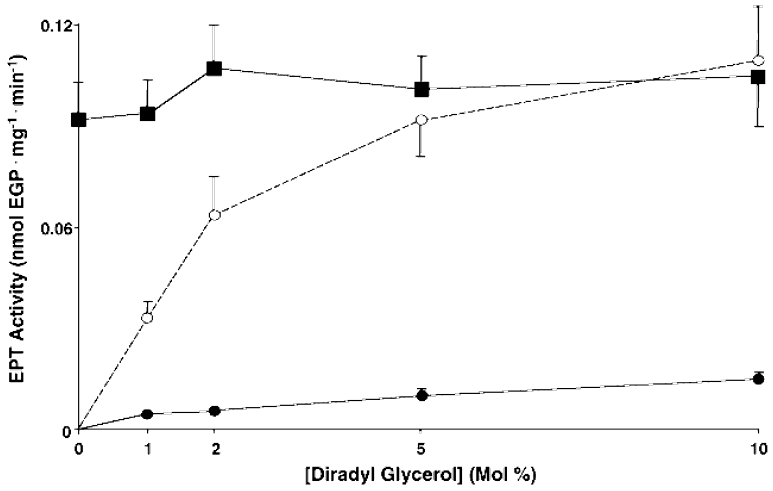

Fig. 1. Rabbit heart microsomal ethanolamine phosphotransferase (EPT) activity using diradylglycerols dispersed in Triton X-100 mixed micelles. Mixed micelles comprised of Triton X-100 (1.8%) and either indicated concentrations of 16:0–20:4 1,2-diacyl-*sn*-glycerol (DAG) (circle), indicated concentrations of a16:0–20:4 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycerol (AAG) (open circle), or indicated concentrations of 16:0–20:4 DAG in the presence of 5 mol% of a16:0–20:4 AAG (square) were incubated with membranes (100 μg) and 100 μM [^{14}C]CDP ethanolamine in assay buffer for 15 min at 37°C. Assays were terminated by the addition of butanol, and EPT activity was determined as described in Materials and Methods. Each data point represents the mean ± SE of six or more independent measurements.

tivity of EPT to chemical modification by DTNB was determined. AAG utilization by myocardial EPT was relatively unaffected by DTNB treatment of the membranes (**Fig. 2**). In contrast, DAG utilization by myocardial EPT decreased over 2-fold by DTNB treatment of the membranes (Fig. 2). The disparate sensitivity of EPT activity using DAG and AAG as substrates provides further support that two EPT enzymic activities are present in rabbit heart membranes. One activity uses AAG as substrate and is DTNB insensitive and the other activity is DTNB sensitive and uses DAG as a substrate. It should be appreciated that the residual DTNB-insensitive component of DAG utilization by EPT might be mediated by the AAG-utilizing EPT catalyzing the conversion of DAG to phosphatidylethanolamine. In contrast, CPT utilization of both DAG and AAG is sensitive to DTNB treatment (**Fig. 3**). Furthermore, Fig. 3 also shows another important difference between CPT and EPT since DAG is slightly preferred as a substrate of CPT compared with AAG substrate. Thus, it seems unlikely that the plasmalogen selective EPT in heart membranes is mediated by CPT.

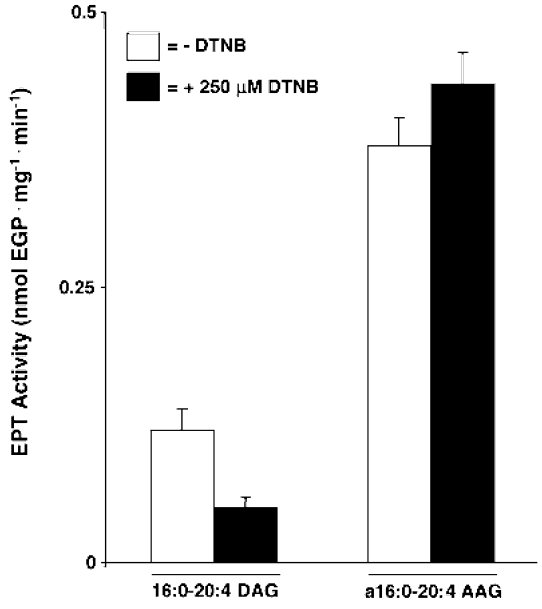

Fig. 2. Dithiobisnitrobenzoic acid (DTNB) sensitivity of rabbit heart microsomal ethanolamine phosphotransferase (EPT) activity. EPT activity in rabbit heart membranes (100 μg) was determined in EPT assays using Tween 20 to disperse individual diradyl glycerols (1 mM) as substrate as indicated, and 100 μM [^{14}C]CDP ethanolamine as cosubstrate. Reactions were incubated for 15 min at 37°C in the presence and absence of 250 μM DTNB as indicated. EPT activity was determined as described in Materials and Methods. Each data point represents the mean ± SE of six or more independent measurements.

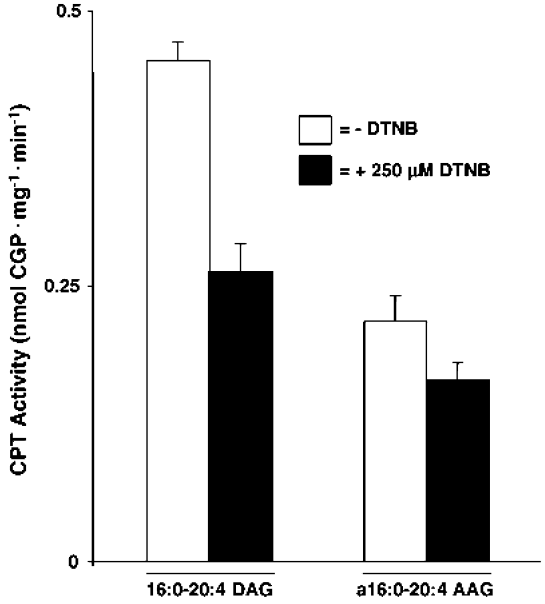

Fig. 3. DTNB sensitivity of rabbit heart microsomal CPT activity. CPT activity in rabbit heart membranes (100 μg) was determined in CPT assays using Tween 20 to disperse individual diradyl glycerols (1 mM) as substrate as indicated and 100 μM [^{14}C]CDP choline as cosubstrate. Reactions were incubated for 15 min at 37°C in the presence and absence of 250 μM DTNB as indicated. CPT activity was determined as described in Materials and Methods. Each data point represents the mean ± SE of five independent measurements.

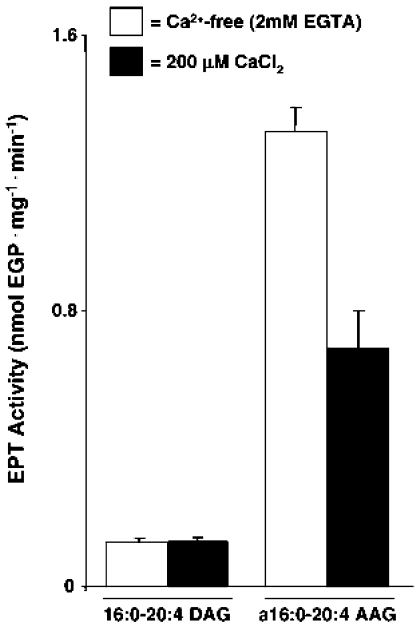

Fig. 4. Ca^{2+} sensitivity of rabbit heart microsomal EPT activity. EPT activity in rabbit heart membranes (100 μg) was determined in EPT assays using Tween 20 to disperse individual diradyl glycerols (1 mM) as substrate as indicated and 100 μM [^{14}C]CDP ethanolamine as cosubstrate with 10 mM $MgCl_2$ and 2 mM EGTA present. Reactions were incubated for 15 min at 37°C in the absence (Ca^{2+}-free) and presence of 2.2 mM $CaCl_2$ (final Ca^{2+} concentration, 200 μM) as indicated. EPT activity was determined as described in Materials and Methods. Each data point represents the mean ± SE of six or more independent measurements.

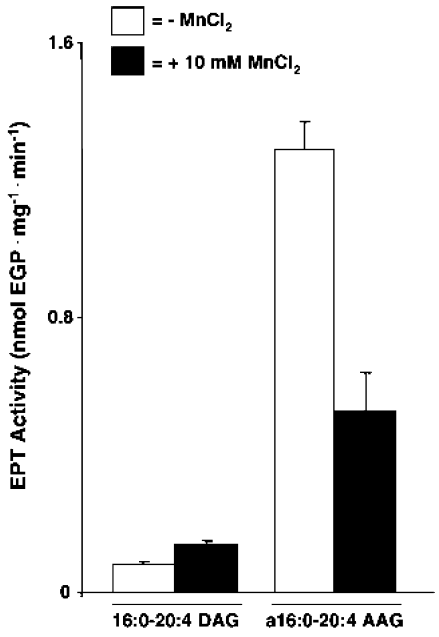

Fig. 5. Mn^{2+} sensitivity of rabbit heart microsomal EPT activity. EPT activity in rabbit heart membranes (100 μg) was determined in EPT assays using Tween 20 to disperse individual diradyl glycerols (1 mM) as substrate as indicated and 100 μM [^{14}C]CDP ethanolamine as cosubstrate with 10 mM $MgCl_2$ and 2 mM EGTA present. Reactions were incubated for 15 min at 37°C in the absence and presence of 10 mM $MnCl_2$ present as indicated. EPT activity was determined as described in Materials and Methods. Each data point represents the mean ± SE of six or more independent measurements.

Disparate divalent cation sensitivity of EPT activity using AAG and DAG substrates

Further analysis was performed to delineate differences in EPT activity with the two molecular subclasses of diradylglycerols (i.e., DAG and AAG) as substrates in the presence of divalent cations since EPT in general is considered to be dependent on Mg^{2+}. EPT activity was dependent on the presence of Mg^{2+} for both DAG and AAG as substrates, and accordingly 10 mM $MgCl_2$ was present in all assays. With the addition of only 200 μM Ca^{2+}, the activity of EPT using AAG as substrate was decreased about 2-fold (**Fig. 4**). In contrast, 200 μM Ca^{2+} did not alter EPT activity with DAG as substrate (Fig. 4). The disparity of the effect of Ca^{2+} on EPT activity was evident at concentrations of Ca^{2+} at and below 200 μM, but at 300 μM Ca^{2+}, EPT activity was inhibited for both AAG and DAG substrates.

Similar to Ca^{2+}, Mn^{2+} also inhibited EPT activity using AAG as substrate to levels that were less than half that observed in the absence of Mn^{2+} (**Fig. 5**). Again, EPT activity using DAG as substrate in the presence of Mn^{2+} was not inhibited but was significantly activated about 80% in comparison to that observed in the absence of Mn^{2+} (Fig. 5).

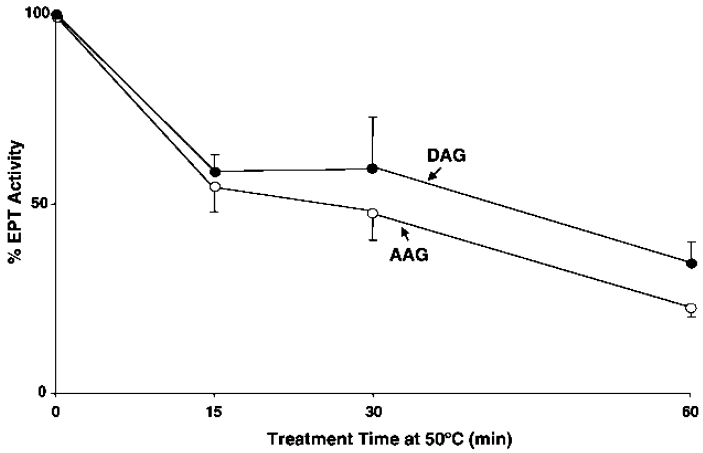

Fig. 6. Thermal lability of rabbit heart microsomal EPT activity. Rabbit heart membranes at a concentration of 6.5 mg/ml homogenization buffer were treated for indicated time intervals at 50°C. Immediately following heat treatments, EPT activity in heat-treated rabbit heart membranes (100 μg) was determined by EPT assays using Tween 20 to disperse either 1 mM 16:0–20:4 DAG or 1 mM a16:0–20:4 AAG substrates as indicated in the presence of 100 μM [^{14}C]CDP ethanolamine, 10 mM $MgCl_2$, and 2 mM EGTA. Reactions were incubated for 15 min at 37°C. EPT activity was quantitated as described in Materials and Methods. Each data point represents the mean ± SE of six or more independent measurements. Data is expressed as a percent of activity at each time point compared with that with no heat treatment.

Thermal sensitivity of EPT activity using AAG and DAG substrates

To further support that two different EPT activities mediate the synthesis of phosphatidylethanolamine and plasmenylethanolamine using DAG and AAG substrates, respectively, the thermal labilities of these activities were determined. For these studies, membranes were treated at 50°C for selected time intervals and then EPT activity was immediately assayed using the two different substrates. At each time point of thermal treatment, EPT activity using AAG as substrate was more sensitive to the themal treatment in comparison to the EPT activity using DAG as substrate (**Fig. 6**). Furthermore, following 60 min of treatment at 50°C, EPT activity using DAG as substrate was statistically greater than that using AAG as substrate (Fig. 6). It should be appreciated that for these studies 50°C was chosen since at higher temperatures membranes rapidly denatured and this temperature was found experimentally to delineate differences in thermal sensitivity of EPT using AAG and DAG substrates.

DISCUSSION

Plasmalogens are the predominant glycerophospholipid molecular subclass in the heart and are enriched in many other tissues (6–9). Previous studies have determined that in rabbit myocardium, the synthesis of the *sn*-1 vinyl ether aliphatic group of plasmalogens is ~100-fold slower than the turnover of the *sn*-2 fatty acid and the *sn*-3 polar head group of plasmalogens (3). Furthermore, rabbit myocardial EPT selectively uses AAG substrate and it is likely that the selectivity of myocardial EPT plays a role in maintaining plasmenylethanolamine levels (4). Others have also shown that EPT selectively uses AAG substrate in brain tissue (4, 12, 13). One study has shown that AAG was preferentially used as the substrate while the other suggested that DAG was the preferred substrate (12, 13). In the present study, under every condition employed for the EPT activity measurements, AAG was the preferred substrate compared with DAG. Even under conditions that EPT is relatively inhibited such as DTNB treatment, AAG is preferentially used by myocardial EPT.

Other support that two enzyme activities mediate the separate synthesis of phosphatidylethanolamine and plasmenylethanolamine in rabbit heart membranes is provided by the finding that increasing amounts of DAG in the presence of a fixed concentration of AAG does not inhibit EGP production by EPT. Additionally, reversed phase analysis has shown that both phosphatidylethanolamine and plasmenylethanolamine are produced at approximately a 1 to 10 ratio in the presence of equal mol percents (5 mol%) of DAG and AAG as substrate. These results contrast with previous studies on heart and brain EPT, which demonstrated that AAG inhibits DAG incorporation into EGP pools (12, 18). These differences may be due to differences in assay conditions, including detergents and divalent cations.

It should also be noted that others have suggested that Mn^{2+} is a better activator of EPT than Mg^{2+} (19, 20). Using DAG as substrate for EPT, the present studies are in agreement with these previous studies that Mn^{2+} is an activator of EPT (19, 20). However, the present results clearly demonstrate that AAG is the preferred substrate of the EPT present in myocardial membranes, and this activity is substantially inhibited by Mn^{2+}. Furthermore, myocardial EPT activity using AAG substrate was optimal with 10 mM $MgCl_2$. Similar to the sensitivity of AAG utilizing EPT activity to Mn^{2+}, this activity was also very sensitive to micromolar concentrations of Ca^{2+}.

Taken together, the present results suggest that separate enzymes are responsible for the conversion of DAG and AAG to their respective ethanolamine glycerophospholipids catalyzed by EPT activities. This conclusion is drawn from disparate sensitivities to DTNB treatment, heat treatment, and divalent cations for EPT activity using AAG and DAG substrates. The present studies employing chemical treatments, analysis of divalent cation requirements as well as inhibition, and assessment of thermal lability of activities have provided important information suggesting that at least two EPT activities are present in the heart that separately mediate the production of phosphatidylethanolamine and plasmenylethanolamine.

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