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BASE-EXCHANGE REACTIONS OF THE PHOSPHOLIPIDS IN CARDIAC MEMBRANES

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Summary

Canine cardiac microsomes were shown to incorporate the nitrogenous bases, serine, ethanolamine, and choline, into their respective phospholipids by the energy-independent, Ca²⁺-stimulated base-exchange reactions. The optimal Ca²⁺ concentration was 2.5 mM. Metal ions other than Ca²⁺ either inhibited or had no effect on the activities. La³⁺ and Mn²⁺ were both potent inhibitors. The pH optimum for the reactions at 2.5 mM Ca²⁺ was approx. 7.8 and depended upon Ca²⁺ concentration. Apparent *K_m* values at 2.5 mM Ca²⁺ were 0.06 mM for L-serine, 0.13 mM for ethanolamine and 0.49 mM for choline. The kinetic and metal ion inhibition studies suggest that the choline-exchange reaction is a separate process from the serine and ethanolamine reactions. The ATP-stimulated Ca²⁺ binding system of the cardiac membranes was not related to the base-exchange reactions; however, the energy-independent Ca²⁺ binding to the membranes appears to be related to the exchange reactions.

Introduction

The incorporation of serine, ethanolamine and choline into phospholipids can occur by a Ca²⁺-dependent, energy-independent exchange with the base of an existing phospholipid [1]. Although these reactions have been demonstrated *in vitro* in several mammalian tissues [2–6], their *in vivo* significance is uncer-

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethyleneglycol bis-(β -aminoethyl ether)-*N*-*N'*-tetraacetic acid.

tain [7–11]. The serine-exchange reaction is, however, the only known pathway for the biosynthesis of phosphatidylserine in mammalian tissues [3,12].

The specialized role of cardiac membranes in the regulation of intracellular Ca^{2+} concentrations [13–16], coupled with the fact that the Ca^{2+} -binding and transport systems have a requirement for acidic phospholipids such as phosphatidylserine [17–22], raise the possibility that the Ca^{2+} -dependent base-exchange reactions in the membranes may function to regulate the Ca^{2+} -binding properties through a modification of the phospholipid composition at specific sites in the membranes. In order to explore this possibility in more detail, we have characterized the Ca^{2+} -dependent base-exchange reactions in cardiac microsomes and compared them to Ca^{2+} binding properties. The results of these studies are presented in this paper.

Experimental

Materials. L-[U- ^{14}C]Serine, [1,2- ^{14}C]ethanolamine hydrochloride, [*Me*- ^{14}C]choline chloride, $^{45}\text{CaCl}_2$ and Aquasol were purchased from New England Nuclear. All other chemicals were of reagent grade and were obtained from commercial supply houses.

Preparation of cardiac microsomes. The cardiac microsomes were prepared by the modification and combination of the methods of Krasnow [23] and Harigaya and Schwartz [24]. Mongrel dogs obtained from Meadowbrook Farms (Detroit, MI) were anesthetized with 60 mg/kg pentobarbital intravenously. The beating heart was excised, chilled in crushed ice, and freed of extraneous tissue. All subsequent steps were performed at 4°C.

The ventricles were cut into small pieces, washed with 0.3 M sucrose, 10 mM Hepes (pH 7.4), ground in a meat grinder, and homogenized in a Waring Blendor for 35 s in 3 vols. 0.3 M sucrose, 10 mM Hepes (pH 7.4). The pH of the homogenate was readjusted to pH 7.4 with 6 N NaOH. The homogenate was centrifuged at $13\,000 \times g$ for 30 min and the pellet discarded. A microsomal preparation was prepared from the supernatant by centrifugation for 45 min at $100\,000 \times g$. The microsomal pellet was suspended in 0.3 M sucrose, 10 mM Hepes (pH 7.4) to obtain a protein concentration of 10 mg/ml. The microsomal preparation was stored at -80°C and was stable for several months.

Base-exchange assays. The assay of base incorporation was a modification of the method of Kanfer [4]. The standard incubation media contained 1.2–1.9 mg/ml microsomal protein, 50 mM Hepes (pH 7.8), 2.5 mM CaCl_2 , and base substrate in a total volume of 0.2 ml. The standard concentration of base for serine incorporation was 0.1 mM L-[U- ^{14}C]serine (12 Ci/mol), for ethanolamine incorporation 0.13 mM [1,2- ^{14}C]ethanolamine (2 Ci/mol), and for choline incorporation 0.5 mM [*Me*- ^{14}C]choline (11 Ci/mol). The incorporation was started by the addition of microsomal protein and incubated at 37°C in a water bath. The incubation time for serine incorporation was 2 min, for ethanolamine 8 min, and for choline 6 min. The reaction was stopped, the total lipids extracted, and the extract washed by the method of Folch et al. [25]. The washed solvent phase was transferred to a scintillation vial and evaporated to dryness. Controls containing no microsomal protein or calcium or with zero time of incubation were included with each experiment. The base incorpora-

tion was calculated for the amount of radioactivity incorporated into the total lipid extract and the specific activity of the base substrate. Radioactivity incorporated in the control samples was subtracted from the other samples in each experiment. The rate of incorporation of ethanolamine, choline and serine was constant for 15 min, 5 min and 3 min, respectively. The rate of reaction was proportional to protein up to 2 mg/ml.

Ca²⁺ binding and uptake. Ca²⁺ binding and uptake assays were a modification of the methods used by Sulakhe et al. [26]. For Ca²⁺ binding the standard incubation medium contained 0.02–0.10 mg/ml microsomal protein, 50 mM Tris-maleate (pH 6.7), 5 mM MgCl₂, 5 mM ATP and 0.1 mM ⁴⁵CaCl₂ (5000–10 000 cpm/nmol) in a final volume of 1 or 2 ml. The assay was started by the addition of microsomes, incubated for 2 min at 37°C, and terminated by filtering with suction a 0.2 ml aliquot through a 0.2 mm filter (25 mm type GS Millipore). The filter was washed with 5 ml cold 50 mM Tris-maleate (pH 6.7) and placed in a scintillation vial containing 10 ml Aquasol. The radioactivity retained by the microsomes on the filter was determined and the Ca²⁺ binding was calculated using the specific activity of the added ⁴⁵CaCl₂. Controls containing no ATP and no protein were included. No Ca²⁺ was bound to the filters in the absence of microsomes. The medium for Ca²⁺ uptake was the Ca²⁺ binding medium with 5.0 mM potassium oxalate added. The microsomes were preincubated at 37°C in the media without ⁴⁵CaCl₂ for 4 min. The uptake was started by the addition of 0.1 mM ⁴⁵CaCl₂ (5000–10 000 cpm/nmol), incubated for 2 min at 37°C, and terminated as described for the binding assay. The preincubation led to a 35–40% increase in the Ca²⁺ uptake. The Ca²⁺ uptake was calculated by methods similar to those described for binding. Controls containing no ATP or incubated for zero time gave an insignificant amount of uptake.

Energy-independent Ca²⁺ binding was determined by the Millipore filtration technique except the pH was 7.8 and Mg²⁺ and ATP were omitted from the reaction mixture. Binding was also determined by equilibrium dialysis. In this method, the standard incubation medium contained 30 mM Hepes (pH 7.8), 0.1 mM ⁴⁵CaCl₂ (5000–10 000 cpm/nmol) and 0.8–1.0 mg/ml microsomal protein in a total volume of 0.2 ml. The dialysis was performed in an 8-place equilibrium dialysis cell with 0.5 ml chambers (model 377, Bel-Art Products). The chambers were separated with sheets of cellulose dialysis membranes (pore size 0.28 nm). Opposing chambers contained similar incubation medium without microsomal protein added. The dialysis cell was rotated slowly at 4°C for 20–24 h. A 0.1 ml aliquot was withdrawn from each chamber and placed in a scintillation vial containing 10 ml of Aquasol. The radioactivity was determined, and the Ca²⁺ binding was calculated from the difference in radioactivity in opposing chambers.

Analysis of phospholipids. Phospholipids were separated by thin-layer chromatography on Silica gel 60 HR plates with CHCl₃/CH₃OH/CH₃COOH/H₂O (25 : 15 : 4 : 2, v/v). Glycerolphosphoryl bases were prepared by mild alkaline methanolysis of the total lipid extract as described by Dawson [27]. The glycerolphosphoryl bases were separated on Q2F cellulose thin-layer plates (Quantum Industries) with phenol/0.12% NH₄ (25 : 35, v/v).

Analytical methods. Protein was determined by the method of Lowry et al.

[28] with bovine serum albumin as standard.

Radioactivity measurements were made in a liquid scintillation spectrometer.

Results

Characteristics of base-exchange reactions

Cardiac microsomes incorporate radioactive choline, ethanolamine and serine into phospholipids when incubated with Ca^{2+} . Ethanolamine was incorporated at the highest rate, followed by serine and choline. The reaction was dependent upon Ca^{2+} and was unaffected by ATP or CTP. The pH optimum was determined to be 7–8 for the three base-exchange reactions at Ca^{2+} concentrations of 2.5 mM. This value is the same as reported for liver [9] and brain [31]. The pH optimums were shifted to slightly higher pH values when the reactions were done at 0.2 mM Ca^{2+} . A similar Ca^{2+} -dependent shift in pH optimum has been reported with liver microsomes [9,29].

Double-reciprocal plots of base concentrations and Ca^{2+} concentrations vs. velocity were linear. The apparent K_m values and maximal velocities are shown in Table I. The apparent K_m for base incorporation was lowest for serine and highest for choline. The V for ethanolamine was approx. five times higher than for serine and choline. The apparent K_m for Ca^{2+} was essentially the same for the three reactions. Each base was a competitive inhibitor for the incorporation of the other bases. Their relative potency as inhibitors was similar to their relative apparent K_m values. Serine was the most potent inhibitor with a K_i value of 0.11 mM for the inhibition of both ethanolamine and choline incorporation. Ethanolamine inhibition was also similar for both serine and choline incorporation (K_i of 0.33 mM and 0.51 mM, respectively). However, the inhibition by choline of serine and ethanolamine incorporation was considerably different (K_i of 0.64 and 6.9 mM, respectively).

Phosphatidylserine decarboxylation

Radioactive choline and ethanolamine were incorporated exclusively into their corresponding phospholipids as judged by both thin-layer chromatography of the lipids and by separation of the glycerylphosphoryl bases after methanolysis. The major portion of [$U\text{-}^{14}\text{C}$]serine was incorporated into phosphatidylserine, however, a small but reproducible amount of radioactivity was incorporated into phosphatidylethanolamine (2% of the total incorporated).

TABLE I

K_m AND V VALUES FOR BASE EXCHANGE

The values are averages \pm S.E. for the number of determinations (shown in parentheses). Each value was from an enzyme preparation from a different animal. Ca^{2+} was maintained at 2.5 mM when base concentrations were varied.

Kinetic constant	Substrate		
	Serine	Ethanolamine	Choline
Substrate K_m (mM)	0.06 \pm 0.01 (5)	0.13 \pm 0.04 (5)	0.49 \pm 0.07 (3)
V (nmol/min per mg)	0.44 \pm 0.08 (3)	2.18 \pm 0.09 (4)	0.45 \pm 0.04 (3)
Ca^{2+} K_m (mM)	0.22 \pm 0.09 (11)	0.36 \pm 0.22 (4)	0.39 \pm 0.23 (3)

Furthermore, the use of [$1\text{-}^{14}\text{C}$]serine resulted in a 3-fold decrease in the amount of incorporation of radioactivity into phosphatidylethanolamine. Thus, the microsomal preparation apparently contains some phosphatidylserine decarboxylase activity. The Ca^{2+} -dependent incorporation of serine into phospholipid was considerably less in mitochondria than in microsomes (2.1 nmol/mg protein for microsomes vs. 0.09 nmol/mg protein for mitochondria after 35 min of incubation). However, 32% of the radioactivity incorporated by mitochondria was in phosphatidylethanolamine. These data suggest that mitochondria have a relatively low base-exchange activity but contain relatively active phosphatidylserine decarboxylase activity. This result is consistent with previous studies with liver and brain [30,31]. The small amount of decarboxylase activity in the microsomes may result from mitochondrial contamination. However, since no independent assessment of mitochondrial contamination was made, the occurrence of phosphatidylserine decarboxylase in microsomes cannot be completely discounted.

Relationship between base-exchange reactions and Ca^{2+} binding and transport systems

The observation that ATP did not stimulate the base-exchange reaction even at low concentrations of Ca^{2+} suggests that binding of Ca^{2+} by the ATP-stimulated process in cardiac microsomes was not related to the base-exchange reaction. Furthermore, the optimum pH for ATP-stimulated Ca^{2+} binding is approx. 6.7 [28], whereas the pH optimum for the base-exchange reaction is 7.8. EGTA has been shown to extract a number of Ca^{2+} binding proteins from sarcoplasmic reticulum, many of which are associated with the ATP-stimulated Ca^{2+} binding and uptake [32–35]. Cardiac microsomes were isolated as described in Experimental except that half of the $100\,000 \times g$ pellet was resuspended in 0.3 M sucrose, 2.5 mM EGTA, 10 mM Hepes (pH 7.8). This was allowed to stand with stirring at 4°C for 30 min. The other half of the pellet was prepared according to the standard procedure. The EGTA-extracted microsomes were centrifuged at $100\,000 \times g$ for 45 min and from that point the preparation was processed according to the standard procedure. The EGTA extraction had very little effect on the apparent Ca^{2+} K_m values for base-exchange; however, the Ca^{2+} K_d for the ATP-stimulated Ca^{2+} binding was more than tripled (Table III).

The K_d for energy-independent Ca^{2+} binding was 0.20 mM and 0.37 mM as measured by Millipore and dialysis, respectively. The K_d for Ca^{2+} is very similar

TABLE II
EFFECT OF EGTA ON CALCIUM K_m FOR THE BASE-EXCHANGE REACTIONS
All determinations were performed at pH 7.8.

Microsomes	Base-exchange Ca^{2+} K_m (mM)			ATP calcium-binding K_d (mM)
	Serine	Ethanolamine	Choline	
Control	0.11	0.13	0.15	0.06
EGTA-extracted	0.09	0.13	0.20	0.20

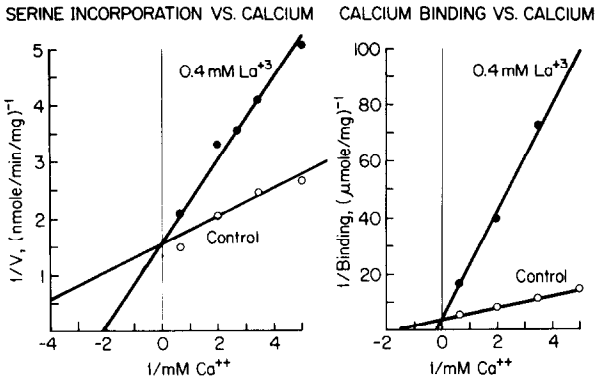


Fig. 1. Effect of La^{3+} on serine incorporation on energy-independent calcium binding. The standard incubation medium for serine incorporation contained 0.2 mM serine, 30 mM Hepes (pH 7.3) and La^{3+} as indicated. Calcium binding was determined by equilibrium dialysis. Each point is an average of three assays.

to the apparent K_m for Ca^{2+} in the serine-exchange reaction (Table I), suggesting that a major portion of the energy-independent binding of Ca^{2+} may be related to the base-exchange reaction. All three exchange reactions were strongly inhibited by Mn^{2+} and La^{3+} . The effects of La^{3+} on both the Ca^{2+} binding and the serine-exchange reactions are shown in Fig. 1. La^{3+} competitively inhibited Ca^{2+} binding, a result in agreement with other reports [36–38]. La^{3+} also appears to competitively inhibit the serine-exchange reaction, thus providing additional evidence that the energy-independent Ca^{2+} binding sites are closely associated with the base-exchange reactions. Mn^{2+} , however, was an uncompetitive inhibitor of Ca^{2+} binding (Fig. 2) and showed mixed inhibition towards serine-base exchange. Although the kinetics of Mn^{2+} inhibition of serine-exchange reaction were not strictly competitive, a strong tendency towards competitive inhibition was apparent.

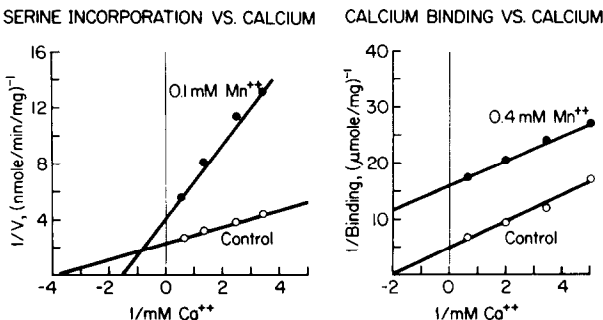


Fig. 2. Effect of Mn^{3+} on serine incorporation and energy-independent calcium binding. The standard incubation medium for serine incorporation contained 0.2 mM serine, 30 mM Hepes (pH 7.8) and Mn^{2+} as indicated. Calcium binding was determined by equilibrium dialysis. Each point is an average of three assays.

Discussion

The base-exchange reactions have been reported to occur in a variety of tissues. Although Kiss [39] has reported that L-serine is incorporated into phosphatidylserine by rat heart homogenates by a Ca^{2+} -dependent process, a complete characterization of the base-exchange reactions in heart has not previously been made. In the present work, canine cardiac microsomes which contain the ATP-stimulated calcium binding and transport were also shown to incorporate the nitrogenous bases, serine, ethanolamine, and choline, into their respective phospholipids by the energy-independent, calcium-stimulated base-exchange reactions. The characteristics of these reactions in heart microsomes were for the most part similar to those reported for other tissues.

The Ca^{2+} binding sites on the membranes that are associated with the active transport of Ca^{2+} apparently are not related to the Ca^{2+} -dependent base exchange reactions. However, the energy-independent binding of Ca^{2+} to the membranes is related to the Ca^{2+} -dependent base-exchange reactions. The exact nature of these sites is unknown. The competitive inhibition by La^{3+} suggests that the sites may be related to a divalent cation carrier system since La^{3+} has been shown to block the cation process in other tissues [40].

Although the synthesis of phospholipids by muscle tissue has not been extensively studied, sarcoplasmic reticulum membranes from skeletal muscle have been shown by Sarzala and Pilanska [41] to synthesize phospholipids by the same pathways as endoplasmic reticulum of other tissues. Similar studies with cardiac sarcoplasmic reticulum membranes have not been reported. Our results demonstrate that cardiac membranes synthesize phosphatidylserine by the same pathway that occurs in other mammalian tissues, i.e. the Ca^{2+} -dependent exchange. The significance of the other base-exchange reactions is less clear but may be important in altering or maintaining the microenvironment at specific positions in membranes.

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