Presence of a Ca^{2+}-Sensitive CDPdiglyceride–Inositol Transferase in Canine Cardiac Sarcoplasmic Reticulum†

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ABSTRACT: Sarcoplasmic reticulum (SR) and plasma membranes from canine left ventricle were used to evaluate the presence of the enzyme CDPdiglyceride–inositol transferase in these membranes. (K^+,-Ca^{2+})-ATPase activity, a marker for SR, was 79.2 ± 5.0 (SE) and 11.2 ± 2.0 $\mu mol \cdot mg^{-1} \cdot h^{-1}$ in SR and plasma membrane preparations, respectively, and (Na^+,K^+)-ATPase activity, a marker for plasma membranes, was 5.6 ± 1.2 and 99.2 ± 8.0 $\mu mol \cdot mg^{-1} \cdot h^{-1}$, respectively. Contamination of SR and plasma membrane preparations by mitochondria was estimated to be 2% and 8%, respectively, and by Golgi membranes, 0.9% and 1.8%, respectively. Transferase activity, measured at pH 6.8, was 1.32 ± 0.04 (SE) and 0.28 ± 0.04 nmol of [^{3}H]phosphatidylinositol ([^{3}H]PtdIns)$\cdot mg^{-1} \cdot min^{-1}$ in three SR and plasma membrane preparations, respectively. The transferase activity detected in the plasma membrane preparation could be accounted for largely, but not entirely, by contaminating SR membranes. The pH optimum for the SR transferase activity was between 8.0 and 9.0; little or no activity was detectable at pH 6.3 and 5.5, the lowest pH tested. Ca^{2+} inhibited the enzyme, half-maximal inhibition occurring at about 10 μM Ca^{2+}; removal of the Ca^{2+} by addition of ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid restored activity. No loss of [^{3}H]PtdIns could be detected when membranes were incubated in the presence or absence of Ca^{2+}. The Ca^{2+} inhibition of the transferase was noncompetitive with respect to CDP–dipalmitin while that with respect to *myo*-inositol was slightly noncompetitive at low [Ca^{2+}] and became uncompetitive at higher [Ca^{2+}]. A portion of the inhibition of the transferase by Ca^{2+} could be accounted for by a competition between Mg^{2+} and Ca^{2+}. It is concluded that CDPdiglyceride–inositol transferase is present on SR membranes and is sensitive to micromolar Ca^{2+}. The data are consistent with a putative role for the inhibition of the SR transferase by Ca^{2+} and acidic pH in the protection of the SR against calcium overload in ischemic myocardium.

Phosphatidylinositol (PtdIns),[1] in addition to its role as a structural component of the phospholipid bilayer of biological membranes, appears to play an important role in the hormonal regulation of cell function (Berridge, 1982). It may be sequentially phosphorylated by two different kinases present on plasma membranes to form PtdIns-4P and PtdIns-4,5P_2. These polyphosphoinositides may then be hydrolyzed by phospholipase C to form Ins-P_2 and Ins-P_3, respectively (Hokin, 1985; Hirasawa & Nishizuka, 1985). The latter compound has been shown to release calcium from endoplasmic reticulum or SR in various cell types. While a function for Ins-P_3 has not yet been established in cardiac muscle (Ochs, 1986), an increase in Ins-P_3, Ins-P_2, and Ins-P in response to α-adrenergic and muscarinic cholinergic agonists has been demonstrated in this tissue (Brown & Jones, 1986). Moreover, a decrease in ^{32}P incorporation into PtdIns was observed in murine atria (Brown & Brown, 1983) and in cardiac myocytes (Mattern et al., 1985) when calcium was present in the incubation medium. This effect might be attributable to an inhibition by Ca^{2+} of the enzyme CDPdiglyceride–inositol transferase. An inhibitory effect of Ca^{2+} on this enzyme was suggested by the work of Moore et al. (1983), who observed a decrease by Ca^{2+} in PtdIns formation from endogenous phosphatidic acid and added CTP and *myo*-inositol in lymphocyte membranes.

Little is known about CDPdiglyceride–inositol transferase in cardiac muscle, and its presumed location on the SR has not been established. A location of this enzyme on the cardiac SR would be of particular consequence in view of the recent report by Suzuki and Wang (1987), which indicates a requirement for a PtdIns microenvironment for protein kinase A catalyzed phosphorylation of phospholamban, a regulatory protein of the calcium pump of cardiac SR (Kirchberger et al., 1974; Tada et al., 1975). Variation in the amount of PtdIns formed by the transferase in the SR could thus affect the amount of myoplasmic Ca^{2+} present as a result of either Ins-P_3-induced calcium release from the SR and/or protein kinase stimulated calcium uptake by these membranes and hence affect the contractile properties of the intact myocardium. Thus, a knowledge of the possible presence of this enzyme on the SR as well as the identification of factors affecting its activity would be of importance. In this report, we provide evidence that CDPdiglyceride–inositol transferase is present on the cardiac SR and that it is inhibited by concentrations of Ca^{2+} that may be of physiological significance.

Experimental Procedures

Materials

Enzyme-grade sucrose was obtained from Schwarz/Mann Biotech, Cambridge, MA. CDP–Dipalmitin, alamethicin,

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[1] Abbreviations: PtdIns, phosphatidylinositol; PtdIns-4P, phosphatidylinositol 4-phosphate; PtdIns-4,5P_2, phosphatidylinositol 4,5-bisphosphate; Ins-P, inositol phosphate; Ins-P_2, inositol bisphosphate; Ins-P_3, inositol trisphosphate; SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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Na_2ATP, and phosphoinositides, either as a mixture or as separate components, were obtained from Sigma. *myo*-[2-^{3}H]Inositol (16 Ci/mmol) and UDP-[U-^{14}C]galactose (303 mCi/mmol) were obtained from Amersham Corp., Arlington Heights, IL. Formula 963 scintillation fluid was obtained from New England Nuclear, Boston, MA. All organic solvents used in this study were of high-performance liquid chromatography grade and were obtained from Fisher Scientific, Springfield, NJ. Silica gel H plates with organic binder were from Analtech, Newark, DE.

Methods

Preparation of Subcellular Membranes and Assay of Membrane Markers. Microsomes, consisting largely of fragmented SR, were prepared from canine left ventricle as described earlier (Kirchberger & Antonetz, 1982). In order to obtain relatively pure SR vesicles, the microsomes were subjected to calcium loading and sucrose gradient centrifugation as described by Jones et al. (1979) except that 1 mM DTT was included in all buffers. The purified SR vesicles were depleted of loaded calcium as follows. Two milligrams of SR protein was suspended in 1 mL of a solution containing 9 mM Hepes–KOH, pH 6.8, 182 mM sucrose, 0.9 mM DTT, and 0.1% Triton X-100 and maintained on ice. After 3 min, 15 mL of an ice-cold solution containing 1 mM EGTA (brought to pH 6.8 with Tris) and 1 mM DTT was added. The treated vesicles were centrifuged at 170000*g* for 30 min, and the resultant pellet was suspended in a solution containing 10 mM Hepes–KOH, pH 6.8, 200 mM sucrose, and 1 mM DTT for use in subsequent transferase assays. This procedure resulted in a reduction of the calcium content of calcium-loaded vesicles from 3.6 to 0.04 μmol of calcium/mg of SR protein as determined by a filtration assay using ^{45}Ca (Kirchberger et al., 1986). Purified plasma membranes were prepared by the method of Jones and Besch (1984). This membrane preparation will be referred to as plasma membranes rather than sarcolemmal membranes because of the report by Tomlin et al. (1986) which indicates that the sarcolemmal membranes are contaminated by plasma membranes derived from other cell types present in cardiac tissue. The pellet obtained after centrifugation at 17000*g* for 20 min during the plasma membrane isolation was retained and designated the mitochondria-rich fraction. Ouabain-sensitive (Na^+,K^+)-ATPase, (K^+,Ca^{2+})-ATPase, and NaN_3-sensitive ATPase were used as markers for plasma membranes, SR, and mitochondria, respectively, and were assayed by the methods of Jones and Besch (1984). UDPgalactose–*N*-acetylglucosamine galactosyltransferase activity, used as a marker for Golgi membranes, was measured according to Fleischer and Smigel (1978). Membranes were stored in liquid nitrogen. Protein concentrations were determined by the biuret procedure with bovine serum albumin as the standard.

Assay of CDPdiglyceride–Inositol Transferase Activity. CDPdiglyceride–inositol transferase activity of membranes was assayed by determining the incorporation of *myo*-[2-^{3}H]inositol into PtdIns. Unless otherwise indicated in the text, membranes (0.06 mg/mL) were incubated at 25 °C in 125 μL of assay medium containing 40 mM histidine hydrdochloride, pH 6.8, 0.05 mM CDP–dipalmitin, 5 mM $MgCl_2$, 0.1% Triton X-100, 0.25 mM *myo*-[2-^{3}H]inositol at a specific radioactivity of 0.1 μCi/nmol, and varying Ca^{2+} concentrations obtained with $CaCl_2$–EGTA buffers prepared as described previously (Kirchberger et al., 1986). After a 5-min preincubation of membranes in the assay medium without *myo*-inositol, reactions were started by addition of *myo*-[2-^{3}H]inositol. The reactions were stopped after 5 and 10 min by transferring a 50-μL aliquot of reaction mixture to 0.3 mL of ice-cold acidified chloroform–methanol. Phospholipids were extracted, separated, and identified as described below. Under these assay conditions, PtdIns formation was linear with time for at least 20 min at a membrane protein concentration of up to 0.09 mg/mL. *myo*-[2-^{3}H]Inositol incorporation into PtdIns was insignificant in the absence of CDP–dipalmitin; nevertheless, all rates of transferase activity were corrected for the trace counts migrating with the spot on the chromatogram corresponding to PtdIns.

PtdIns Hydrolysis. PtdIns hydrolysis was measured by using [^{3}H]PtdIns isolated from cardiac microsomes as the substrate. To prepare ^{3}H-labeled PtdIns, cardiac microsomes (0.8 mg/mL) were incubated for 20 min with 25 μCi of *myo*-[2-^{3}H]inositol in 1 mL of medium used for assaying CDPdiglyceride–inositol transferase activity except that histidine–NaOH, pH 8.5, and 0.1 μM Ca^{2+} were used. [^{3}H]-PtdIns was extracted and separated by thin-layer chromatography as described below. The [^{3}H]PtdIns-containing spot was scraped off the plates and transferred to plastic tubes. One milliliter of acidified chloroform–methanol was added to each tube, which was then vortexed for 30 s and centrifuged at 2500*g* for 5 min. The supernatant was filtered through Whatman no. 1 paper. This procedure was repeated 2 more times to maximize recovery of radiolabeled PtdIns. To the filtrate were added 1 mL of chloroform and 2 mL of 2 M KCl, and the tube was vortexed for 10 s. After separation of the solvent layers upon centrifugation as described above, the upper layer was discarded, and the lower layer was dried under N_2.

The dried [^{3}H]PtdIns was dissolved in chloroform, and an aliquot was subjected to thin-layer chromatography in order to obtain the number of counts associated with the PtdIns. An aliquot containing the desired number of counts was dried and suspended in 0.18% Triton X-100 and sonicated 3 times at 0 °C for 30 s with 1-min intervals using a Fisher Sonic Dismembrator, Model 150, with the microtip at 40% control output; unlabeled PtdIns was subjected to the same sonication procedure. The suspension was used in the following assay medium (final concentrations) for measuring [^{3}H]PtdIns hydrolysis: 40 mM histidine hydrochloride, pH 6.8, 5 mM $MgCl_2$, 0.1% Triton X-100, 0.1 or 30 μM Ca^{2+}, 0.06 mg/mL SR membranes, 28 000 cpm of extracted [^{3}H]PtdIns, and 0, 0.1, or 1 mM additional unlabeled PtdIns. Reactions were carried out at 25 °C in a final volume of 60 μL and were stopped after 10 and 20 min by transferring 25-μL aliquots of reaction medium to 150 μL of acidified chloroform–methanol. Control incubates were run using heat-inactivated membrane.

Lipid Extraction of Subcellular Membranes. A further addition of 0.3 volume of chloroform and 0.6 volume of 2 M KCl was made to the reaction samples in the acidified chloroform–methanol obtained in assays of membrane CDPdiglyceride–inositol transferase activity, as described above. The tubes were then vortexed again for 10 s and centrifuged at 2500*g* for 5 min. After the upper layer was aspirated and discarded, the the lower layer was dried under nitrogen. The dried material was dissolved in 25 μL of chloroform of which 20 μL was applied to silica gel H plates along with 5 μL containing 50 μg of a mixture of phosphoinositides. The plates were developed 13–14 cm in a solvent-saturated, paper-lined chamber with chloroform–methanol–ammonium hydoxide–water (90:90:7:22 v/v) (Schacht, 1978) containing 20 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid. Lipids were detected by exposing the plates to iodine vapor. The spots of interest were scraped off the plates and transferred to

Table I: Distribution of CDPdiglyceride–Inositol Transferase and Marker Enzyme Activities in Cardiac Membrane Preparations[a]

enzyme activity	plasma membranes	SR membranes
(K^+,Ca^{2+})-ATPase (A) (μmol of $P_i \cdot mg^{-1} \cdot h^{-1}$)	11.2 ± 2.7	79.2 ± 5.0
(Na^+,K^+)-ATPase (B) (μmol of $P_i \cdot mg^{-1} \cdot h^{-1}$)	99.2 ± 9.8	5.6 ± 1.2
azide-sensitive ATPase (μmol of $P_i \cdot mg^{-1} \cdot h^{-1}$)	8.1	2.0
galactosyltransferase (nmol of UDP-[U-^{14}C]galactose$\cdot mg^{-1} \cdot h^{-1}$)	5.5	2.6
CDPdiglyceride–inositol transferase (C) (nmol of [^{3}H]PtdIns$\cdot mg^{-1} \cdot min^{-1}$)	0.28 ± 0.04	1.32 ± 0.24
C/A	0.025	0.017
C/B	0.003	0.236

[a] (Na^+,K^+)-ATPase, (K^+,Ca^{2+})-ATPase, azide-sensitive ATPase, galactosyltransferase, and CDPdiglyceride–inositol transferase activities were quantitated as described under Experimental Procedures. CDPdiglyceride–inositol transferase activity was measured at 0.1 μM Ca^{2+}, using a $CaCl_2$–EGTA buffer containing 1 mM $CaCl_2$. Values are the means ± SE obtained with three different membrane preparations except for sodium azide sensitive ATPase and galactosyltransferase determinations for which a single determination was made.

counting vials. Radiolabeled phospholipids were identified by comparison of R_f values with those of phospholipid standards. The R_f values for PtdIns, PtdIns-4P, and PtdIns-4,5P_2 were 0.76, 0.54, and 0.32, respectively.

Results

CDPdiglyceride–Inositol Transferase Activity in Cardiac Membranes. The presence of CDPdiglyceride–inositol transferase in cardiac membranes was investigated by measuring the incorporation of *myo*-[2-^{3}H]inositol into the enzyme product. Prior to this assay, the membranes were characterized with respect to marker enzyme activities. In SR, (K^+,Ca^{2+})-ATPase activity was enriched 7-fold compared to plasma membranes while ouabain-sensitive (Na^+,K^+)-ATPase activity was enriched 18-fold in the plasma membrane preparation compared to SR (Table I). Contamination of the SR and plasma membrane preparations by mitochondria was 2% and 8%, respectively, based on the NaN_3-sensitive ATPase activity (100 μmol of $P_i \cdot mg^{-1} \cdot h^{-1}$) observed in the mitochondria-rich fraction (i.e., the fraction sedimenting at 17000*g*, 20 min), which was obtained during the plasma membrane preparation. The Golgi contamination in the SR and plasma membrane preparations was 0.9% and 1.8%, respectively, based on the galactosyl transferase activity (300 nmol$\cdot mg^{-1} \cdot h^{-1}$) observed for rat liver Golgi membranes (Jergil & Sundler, 1983).

myo-[2-^{3}H]Inositol incorporation into PtdIns, a measure of CDPdiglyceride–inositol transferase activity, was observed in both SR and plasma membrane preparations but was approximately 5-fold greater in the SR membranes (Table I). [^{3}H]PtdIns formation was dependent upon exogenously added CDP–dipalmitin, millimolar concentrations of $MgCl_2$, and 0.1% Triton X-100. Approximately 90% of the transferase activity was retained in membranes which had been stored in liquid nitrogen for 3–6 months.

Effects of Ca^{2+} and pH on PtdIns Synthesis in SR. Enzyme present within muscle cells may be subjected to a large fluctuation in intracellular [Ca^{2+}] and may be regulated by this cation if their activity is related to contraction or relaxation. CDPdiglyceride–inositol transferase activity was therefore assayed at Ca^{2+} concentrations ranging from 0.1 to 30 μM (Figure 1). In the presence of 0.1 mM EGTA, the transferase activity was similar to that seen at 0.1 μM Ca^{2+} but was progressively inhibited at increasing Ca^{2+} concentrations above

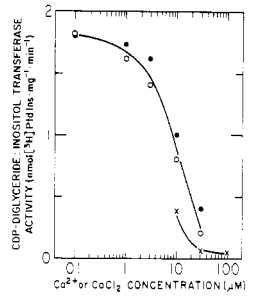

FIGURE 1: Effect of Ca^{2+} concentration on CDPdiglyceride–inositol transferase activity in cardiac SR. SR membranes (0.06 mg/mL) were preincubated for 5 min in assay medium including CDP–dipalmitin as specified under Experimental Procedures. Reactions were started by the addition of 0.25 mM *myo*-[2-^{3}H]inositol and stopped after 5 and 10 min upon addition of 25 μL of the reaction mixture to tubes containing 150 μL of ice-cold acidified chloroform–methanol. $CaCl_2$–EGTA buffers containing either 0.5 mM $CaCl_2$ (○) or 1 mM $CaCl_2$ (●) were used to obtain the indicated Ca^{2+} concentrations, or $CaCl_2$ was added without EGTA (×).

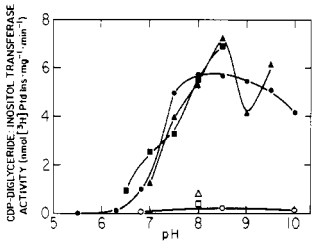

FIGURE 2: pH dependence of CDPdiglyceride–inositol transferase activity in cardiac SR. SR membranes (0.06 mg/mL) were assayed for transferase activity in the presence of either 1 mM EGTA (●, ▲, ■) or 30 μM $CaCl_2$ (○, △, □) using the following buffer systems: 100 mM histidine hydrochloride or histidine–NaOH (●, ○), 100 mM Tris-HCl (▲, △), and 100 mM Hepes–NaOH (■, □).

1 μM; half-maximal inhibition was observed at approximately 10 μM Ca^{2+}, and inhibition was greater than 85% at 30 μM Ca^{2+}. A similar Ca^{2+} sensitivity was observed for the transferase activity present in microsome preparations which had not been subjected to the SR isolation procedure. Almost identical results were obtained when $CaCl_2$–EGTA buffers with different buffering capacities were used; i.e., the $CaCl_2$ concentration was fixed at either 0.5 or 1.0 mM, and the EGTA concentration was varied so as to result in the desired Ca^{2+} concentration. Inhibition by Ca^{2+} was also seen with $CaCl_2$ in the absence of EGTA.

The inhibitory effect of Ca^{2+} on the transferase activity in SR was present at each pH tested over a range of pH, extending from 5.3 to 10.0 (Figure 2). The pH optimum of the enzyme was found to be between 8.0 and 9.0; little or no enzyme activity was detected at pH 6.3 and 5.5, the lowest pH tested. A pH of 6.8 was chosen for the experiments in our study because it approximates normal intracellular pH (Garlick et al., 1979) and because a pH around neutrality is most appropriate for use of EGTA as a Ca^{2+} buffer (Smith et al., 1984).

To test whether the apparent inhibition of the SR transferase by Ca^{2+} could be attributable to activation of phospholipase C or other esterases possibly associated with the membranes, the SR membranes were incubated at varying concentrations of [^{3}H]PtdIns together with 0.1 to 30 μM Ca^{2+} (see Methods). No loss of substrate was detectable.

To test whether the Ca^{2+} inhibition of the SR transferase is reversible upon removal of the Ca^{2+}, SR membranes were incubated with either 1 mM EGTA or 30 μM $CaCl_2$ under conditions similar to those used to obtain the data shown in Figure 1 (Table II). Subsequent addition of 1 mM EGTA

Table II: Reversibility of the Inhibition by Ca^{2+} of CDPdiglyceride–Inositol Transferase Activity in SR Membranes[a]

addition subsequent to pretreatment	transferase act. (nmol of [^{3}H]PtdIns·mg^{-1}·min^{-1}) in membranes pretreated with	
	1 mM EGTA	30 μM $CaCl_2$
(1) distilled water	1.60	0.14
(2) $CaCl_2$ (1.03 mM)	0.14	
(3) EGTA (1 mM)		1.44

[a] SR membranes (0.06 mg/mL) were pretreated in the presence of either 1 mM EGTA or 30 μM $CaCl_2$ in a standard medium for assaying CDPdiglyceride–inositol transferase activity except that *myo*-[2-^{3}H]inositol was omitted. After 10 min, distilled water, or $CaCl_2$, or EGTA was added to the final concentration indicated, and the tubes were incubated further for 3 min. The transferase reaction was then started by addition of *myo*-[2-^{3}H]inositol and stopped after 5 and 10 min by transferring 25 μL of assay medium to 150 μL of ice-cold acidified chloroform–methanol. PtdIns was extracted, and *myo*-[2-^{3}H]inositol incorporation into PtdIns were quantitated as described under Methods. Additions 1–3 to the assay medium decreased the concentrations of the reactants present during the pretreatment by 4%.

Table III: Kinetic Analysis of Ca^{2+} Inhibition of CDPdiglyceride–Inositol Transferase of Cardiac SR with *myo*-Inositol as the Varied Substrate[a]

	[Ca^{2+}] (μM)		
	0.1	3.0	10.0
K_m (mM)	1.6 ± 0.2	1.5 ± 0.1	0.6 ± 0.3
V_{max} (nmol of [^{3}H]PtdIns·mg^{-1}·min^{-1})	7.6 ± 0.6	6.0 ± 0.2	2.0 ± 0.4

[a] SR membranes (0.06 mg/mL) were incubated in the presence of 50 μM CDP–dipalmitin and varying concentrations of *myo*-inositol at 0.1, 3, and 10 μM Ca^{2+}. Each reaction tube contained 3 μCi of *myo*-[2-^{3}H]inositol at concentrations ranging from 0.05 to 5 mM. Rates of [^{3}H]PtdIns formation at different *myo*-inositol concentrations were fit to the Michaelis–Menten equation using HYPERBOLIC on the Prophet computer system. SE refers to the standard error of the data from the fitted curve.

to membranes pretreated with 30 μM $CaCl_2$ restored their transferase activity to 90% of that observed as a result of pretreatment with EGTA. Addition to $CaCl_2$ to the membranes pretreated with EGTA, as expected, inhibited the transferase activity.

Kinetic Analysis of Ca^{2+} Inhibition of CDPdiglyceride–Inositol Transferase Activity. To determine the type of inhibition of CDPdiglyceride–inositol transferase activity by Ca^{2+}, *myo*-[2-^{3}H]inositol incorporation into PtdIns was measured at 0.1, 3, and 10 μM Ca^{2+} at increasing *myo*-inositol concentrations. CDP–dipalmitin and $MgCl_2$ were fixed at 50 μM and 5 mM, respectively. Under these conditions, double-reciprocal plots of PtdIns formation at different Ca^{2+} concentrations as a function of *myo*-inositol concentration resulted in the data given in Table III. The K_m of the enzyme for *myo*-inositol was the same at 0.1 and 3.0 μM Ca^{2+} but decreased at 10 μM Ca^{2+} while the V_{max} values were decreased with increasing [Ca^{2+}]. This kinetic pattern suggests that the inhibition is noncompetitive with respect to *myo*-inositol at lower Ca^{2+} concentrations and uncompetitive at higher Ca^{2+} concentrations.

The inhibition by Ca^{2+} of *myo*-inositol incorporation into PtdIns was then analyzed with respect to CDP–dipalmitin. Enzyme activity was measured at 0.1 and 10 μM Ca^{2+} with increasing CDP–dipalmitin concentrations (Figure 3). *myo*-Inositol and $MgCl_2$ concentrations were fixed at 0.25 and 5 mM, respectively. Double-reciprocal plots of enzyme velocity measured in the presence of 0.1 and 10 μM Ca^{2+} versus CDP–dipalmitin concentrations resulted in a pattern of inhibition where V_{max} was changed and K_m remained the same

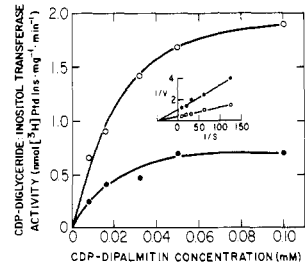

FIGURE 3: Effect of Ca^{2+} on CDP–dipalmitin concentration dependency of CDPdiglyceride–inositol transferase activity in cardiac SR. SR membranes were preincubated for 5 min at increasing concentrations of CDP–dipalmitin and at 0.1 μM (○) and 10 μM (●) Ca^{2+}. Reactions were started by addition of 0.25 mM *myo*-[2-^{3}H]inositol and stopped with acidified chloroform–methanol. The $CaCl_2$ concentration in the $CaCl_2$–EGTA buffers was 1 mM. Insert: Lineweaver–Burk plot of the data.

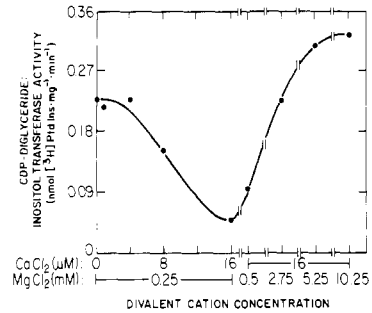

FIGURE 4: Partial reversal of the inhibition of CDPdiglyceride–inositol transferase activity by Ca^{2+} upon addition of $MgCl_2$. Transferase activity was assayed in SR membranes (0.06 mg/mL) in the presence of 0.02 mM EGTA, 50 μM CDP-dipalmitin, 0.25 mM *myo*-[2-^{3}H]inositol, 0.25 mM $MgCl_2$, and increasing concentrations of $CaCl_2$. At 16 μM Ca^{2+}, when approximately 80% inhibition had been attained, the $MgCl_2$ concentration was increased in increments as indicated.

(Figure 3, insert). This kinetic pattern indicates that the inhibition is noncompetitive with respect to CDP–dipalmitin. Rates of [^{3}H]PtdIns formation at different CDP–dipalmitin concentrations were fit to the Michaelis–Menten equation using the nonlinear program HYPERBOLIC on the PROPHET computer system (Figure 3). At 0.1 and 10 μM Ca^{2+}, the K_m values of the enzyme for CDP–dipalmitin were (mean ± SE) 19.50 ± 3.76 and 19.29 ± 0.67 μM, respectively. The corresponding V_{max} values were 2.22 ± 0.14 and 0.86 ± 0.10 nmol of [^{3}H]PtdIns·mg^{-1}·min^{-1}. The inhibition with respect to CDP-dipalmitin is noncompetitive at 10 μM Ca^{2+}. It would appear that the action of Ca^{2+} is likely to involve an interaction with the enzyme at a site other than the active site.

The third component of the transferase reaction is Mg^{2+}. To test the possibility that Mg^{2+} may be involved in the inhibition by Ca^{2+}, the experiment shown in Figure 4 was carried out. The enzyme activity was first assayed at increasing EGTA concentrations (0.01–0.05 mM) and at 0.25 mM $MgCl_2$ (concentrations above 2 mM were saturating). The activity of the enzyme was progressively increased and reached a maximum at 0.05 mM EGTA. An EGTA concentration of 0.02 mM was chosen, and the enzyme activity was measured at increasing $CaCl_2$ concentrations. Enzyme activity was inhibited, maximum inhibition being attained at 16 μM $CaCl_2$. Furthermore, fixing the $CaCl_2$ concentration at 16 μM and increasing the total $MgCl_2$ concentration from 0.25 to 10.25 mM gradually abolished the Ca^{2+} inhibition of transferase activity with essentially a maximum reversal of inhibition being observed at 5.25 mM $MgCl_2$. An increase in $MgCl_2$ concentration to 10.25 mM did not further increase the transferase activity to its uninhibited state, i.e., 1.32 ± 0.24 nmol of [^{3}H]PtdIns·mg^{-1}·min^{-1} (Table I).

Discussion

Evidence has been presented to indicate the presence of a Ca^{2+}-sensitive CDPdiglyceride–inositol transferase in cardiac SR (Table I). A much lower transferase activity was found in association with cardiac plasma membrane preparations. The ratios of the transferase activity to the (K^+,Ca^{2+})-ATPase and (Na^+,K^+)-ATPase activities were quite similar with respect to (K^+,Ca^{2+})-ATPase (0.025 versus 0.017) whereas the ratios with respect to (Na^+,K^+)-ATPase were very dissimilar (0.003 versus 0.236). This indicates that the transferase is associated with the marker for SR membranes. The slightly higher ratio obtained for the plasma membrane preparation with respect to (K^+,Ca^{2+})-ATPase (0.025 vesus 0.017) could be due to the presence of some transferase activity on the plasma membranes. The association of transferase activity with plasma membranes obtained from GH_3 cells was recently reported by Imai and Gershengorn (1987).

The CDPdiglyceride–inositol transferase present in the cardiac SR is similr in several respects to the enzyme described for the endoplasmic reticulum in a number of tissues (Takenawa & Egawa, 1977; Parries & Hokin-Neaverson, 1984; Egawa et al., 1981; Bleasdale et al., 1979). Like the transferase present in rabbit lung (Bleasdale et al., 1979) and rat liver (Takenawa & Egawa, 1977), the SR transferase has a pH optimum between 8.0 and 9.0. The reported K_m of the enzyme for inositol ranged from 0.1 mM in rabbit lung (Bleasdale et al., 1979) to 2.5 mM in rat liver (Takenawa & Egawa, 1977); the corresponding value for the cardiac SR enzyme is 1.6 mM (Table III). The K_m of the cardiac enzyme for CDPdiglyceride is 19 μM (Figure 3), which is almost identical with that reported for the canine pancreatic transferase (Parries & Hokin-Neaverson, 1984) but lower than the values reported for rat liver (Takenawa & Egawa, 1977) and lung (Bleasdale et al., 1979), which were 0.17 and 0.18 mM, respectively. Incorporation of *myo*-[2-^{3}H]inositol into PtdIns in cardiac membranes cannot be attributed to an exchange of the inositol moiety of endogenous PtdIns with the exogenous *myo*-[2-^{3}H]inositol because [^{3}H]PtdIns formation was dependent upon exogenous CDPdiglyceride (see Methods).

The apparent inhibition of the SR transferase by Ca^{2+} can be attributed neither to endogenous Ca^{2+}-activated protease activity (DeMartino & Croall, 1987), because the inhibition is reversible upon addition of EGTA, nor to Ca^{2+}-activated esterase activity associated with the membranes because no esterase activity could be detected. Both substrates and the metal cofactor of the transferase appear to participate in the inhibition by Ca^{2+}. The inhibition with respect to the CDP–dipalmitin is noncompetitive while that with respect to *myo*-inositol is slightly noncompetitive at low Ca^{2+} concentrations and becomes uncompetitive at higher Ca^{2+} concentrations. Under the conditions of the experiments, i.e., at 5 mM $MgCl_2$, Mg^{2+} does not appear to participate in the inhibition of the transferase by Ca^{2+}. At a subsaturating concentration of $MgCl_2$, i.e., at 0.25 mM, there is an inhibition by 16 μM $CaCl_2$ which is reversed by increasing concentrations of $MgCl_2$ (Figure 4). This reversal, however, is maximal at 5 mM $MgCl_2$. The level of maximal reversal by $MgCl_2$ of the observed inhibition by $CaCl_2$ seen in Figure 4 corresponds to the maximal inhibition by $CaCl_2$ seen in Figure 1. Since an increase in $MgCl_2$ concentration from 5 to 10 mM does not alter the stimulation by Mg^{2+}, a simple competition between Ca^{2+} and Mg^{2+} does not appear to be a sufficient explanation for the inhibition. The vast difference in the concentrations of Mg^{2+} (millimolar) and Ca^{2+} (micromolar) would strengthen this conclusion. The fact that the transferase activity is sensitive to micromolar concentrations of Ca^{2+} would suggest that it is susceptible to cytoplasmic Ca^{2+} and not to intraluminal SR calcium, which has been estimated to be in the millimolar concentration range (Chiu & Haynes, 1980) and would thus result in a constant inactivation of the transferase. Inhibition of transferase by millimolar concentrations of ambient Ca^{2+} has previously been reported for rabbit lung (Bleasdale et al., 1979) and GH_3 cells (Imai & Gershengorn, 1987). In vas deferens, the transferase was inhibited to 80% by 10 μM added $CaCl_2$ (Egawa et al., 1981).

The physiological significance of the regulation of cardiac SR transferase activity by Ca^{2+} and of its low activity at acidic intracellular pH values which have been estimated to occur under certain conditions (Garlick et al., 1979) is unknown. It is conceivable that inhibition of PtdIns formation by these factors may be involved in the regulation of SR calcium transport by phospholamban. Suzuki and Wang (1987) have provided evidence that phospholamban is embedded in a PtdIns microenvironment and that this environment is necessary for phospholamban phosphorylation. The inhibition of PtdIns formation by Ca^{2+} and acidic pH and hence an impairment of the catecholamine-induced stimulation of calcium transport by phospholamban phosphorylation may be speculated to play a role in delaying calcium overload of the SR (Fabiato & Fabiato, 1975) in the ischemic myocardium in which intracellular pH is reduced (Garlick et al., 1979) and which is believed to have increased intracellular Ca^{2+} (Fleckenstein, 1981; Bristow et al., 1985) and is characterized by an increased release of catecholamines (Hirche et al., 1980). Calcium overload of the SR has been associated with spontaneous contractions (Fabiato, 1985) and the genesis of cardiac arrhythmias (Capogrossi et al., 1987). As less Ca^{2+} would be taken up by the SR when phospholamban phosphorylation is inhibited, more would remain available for uptake by the mitochondrdia, which exhibit a lower affinity for Ca^{2+} (K_m = 10 μM) and are now generally believed to store calcium only when cytoplasmic Ca^{2+} concentration is abnormally high (Fiskum & Lehninger, 1982). As a result of the postulated shunting of Ca^{2+} to the mitochondria when protein kinase A catalyzed phosphorylation of phospholamban is impaired, there would be less or delayed danger of calcium overload of the SR and the induction of focal ventricular arrhythmias. Decreased phosphorylation of phospholamban by this putative mechanism could thus play a protective role in the ischemically compromised myocardium.

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Composition of Octyl Glucoside–Phosphatidylcholine Mixed Micelles

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ABSTRACT: The compositon of mixed micelles of egg phosphatidylcholine (PC) and octyl glucoside was studied by a novel technique based on measuring resonance energy-transfer efficiency between two fluorescent lipid probes present in trace amounts. Equations were derived for calculating the stoichiometry of the composition of mixed micelles from the energy-transfer measurements. These were applied to determining the average number of lipid molecules in the octyl glucoside–egg PC mixed micelle as a function of detergent concentration. The average number of detergent molecules in these mixed micelles also was determined by varying the lipid concentration. The stoichiometry of egg PC–octyl glucoside mixed micelles was independent of lipid concentration in the range studied (0–500 μM). The dependence of mixed micelle stoichiometry on the concentration of aqueous (monomeric) octyl glucoside is consistent with the assumptions of ideal mixing of the two amphiphiles in the mixed micelles and that mixed micelles can be treated as a distinct phase.

Solubilization of biological membranes by detergents and formation of a bilayer membrane from a mixture of lipids and membrane proteins dissolved in detergent are two important steps in achieving functional reconstitution of membrane proteins. Knowledge of the behavior of the lipid–detergent–protein system, and its dependence on various conditions (concentrations of detergent, temperature, ionic strength, etc.), is of importance to optimize solubilization and functional insertion of the membrane proteins into the lipid bilayer. For these reasons, we have been studying the physicochemical details of a lipid–detergent mixture, egg phosphatidylcholine (PC)[1]–octyl glucoside. Characterization of this system, in the

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[1] Abbreviations: RET, resonance energy transfer; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PC, phosphatidylcholine; Rho-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; SUV, small unilamellar vesicle(s); OG, *n*-octyl β-D-glucopyranoside; EDTA, ethylenediaminetetraacetic acid; cmc, critical micelle concentration; $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether.