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Regulation of CTP:Phosphocholine Cytidylyltransferase by Lipids. 1. Negative Surface Charge Dependence for Activation†

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ABSTRACT: The activity of phosphocholine cytidylyltransferase (CT), the regulatory enzyme in phosphatidylcholine synthesis, is dependent on lipids. The enzyme, obtained from rat liver cytosol, was purified in the presence of Triton X-100 [Weinhold et al. (1986) *J. Biol. Chem. 261*, 5104]. The ability of lipids to activate CT when added as Triton mixed micelles was limited to anionic lipids. The relative effectiveness of the lipids tested suggested a dependence on the negative surface charge density of the micelles. The mole percent lipid in the Triton mixed micelle required for activation decreased as the net charge of the lipid varied from 0 to −2. Evidence for the physical association of CT with micelles and vesicles containing phosphatidylglycerol was obtained by gel filtration. The activation by micelles containing PG was influenced by the ionic strength of the medium, with a higher surface charge density required for activation at higher ionic strength. The micelle surface potential required for full activation of CT was calculated to be −43 mV. A specificity toward the structure of the polar group of the acidic lipids was not apparent. CT was activated by neutral lipids such as diacylglycerol or oleyl alcohol when included in an egg PC membrane, but the activities were reduced by dilution with as little as 10 mol % Triton. Thus Triton mixed micelles are not suitable for studying the activation of CT by these neutral lipid activators. We conclude that one way that lipid composition can control CT–membrane binding and activity is by changing the surface potential of the membrane. Other distinct mechanisms involved in the activation by neutral lipids are discussed.

Cytidylyltransferase (CT)[1] is a key regulatory enzyme in the synthesis of phosphatidylcholine (PC) in higher eucaryotes. The regulation of CT resembles that of a group of proteins (including protein kinase C and 5-lipoxygenase) which have been referred to as *amphitropic* (Burn, 1988). The enzyme is active in its membrane-bound form and inactive in its lipid-free cytosolic form[2] and appears to interconvert between the two forms in response to a variety of effectors of PC biosynthesis (Sleight & Kent, 1980, 1983a,b; Tercé et al., 1988; Weinhold et al., 1981; Pelech et al., 1981, 1983, 1984a,b; Lim et al., 1983; Cornell & Vance, 1987a; Sanghera & Vance, 1989a). There is disagreement as to which cellular membrane CT binds: the endoplasmic reticulum, Golgi, or the nuclear membrane (Tercé et al., 1988; Vance & Vance, 1988; Morand & Kent, 1989).

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[1] Abbreviations: CT, CTP:phosphocholine cytidylyltransferase; PC, phosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; DPPC, dipalmitoylphosphatidylcholine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

[2] A third form is present in some cells—a high molecular weight cytosolic lipid–protein aggregate of undefined physical structure (Weinhold et al., 1989, 1991).

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Three features of the enzyme–membrane interaction have attracted us to the study of the mechanism of the regulation of CT by lipids: the specificity of the lipid component, the reversibility of membrane association, and the involvement of hydrophobic interactions in the binding of CT to membranes. The binding of CT to cellular membranes and to liposomes and its consequent activation shows selectivity for membranes enriched with the anionic phospholipids PG and PI, fatty acids, or diacylglycerol (Weinhold et al., 1984, 1986; Feldman & Weinhold, 1987; Cornell & Vance, 1987a,b; Whitlon et al., 1985). In addition, a depletion in the relative amount of membrane PC by phospholipase C treatment, choline deprivation, or enrichment with mono- or dimethyl-PE has been correlated with increased membrane association of CT (Sleight & Kent, 1983a,b; Jamil et al., 1990). Enzyme–membrane binding can be reversed by various means, such as removal of the fatty acid from the membrane (Weinhold et al., 1984; Cornell & Vance, 1987a,b), enzyme phosphorylation (Sanghera & Vance, 1989b), or withdrawal of phospholipase C from the medium of lipase-treated cultured cells (Wright et al., 1985). Hydrophobic interactions appear to mediate the enzyme–membrane association in that the binding was insensitive to ionic strength or EGTA (Cornell & MacLennan, 1985; Cornell & Vance, 1987a,b).

The development of a method for purifying the enzyme from rat liver cytosol (Weinhold et al., 1986) has enabled the investigation of the mechanism of interaction of the purified transferase with lipids in a chemically defined system. One of our main goals is to decipher the chemical and/or physical basis of the lipid specificity for binding and activation. The choice of the form of presentation of the various lipids is an important consideration in such an undertaking. The enzyme was purified in the presence of the detergent Triton X-100, which was needed as a stabilizer. In the present work we investigated the suitability of a Triton mixed micelle system for testing lipid effectors of CT activity. We examined the relative effectiveness of anionic and neutral lipids as activators of the purified transferase. The micelle systems has an advantage over a membrane system in that *when the detergent is in excess*, the structure of the particle is not dependent on the type of phospholipid used (Robson & Dennis, 1983). We found that only the anionic phospholipids could fully activate CT when presented in micellar form. The dependence on the relative amount of anionic lipid in the micelle showed clearly that the negative surface charge density influences CT activation. Triton seriously interfered with the activation of CT by egg PC or mixtures of egg PC with fatty alcohol or diacylglycerol.

Materials and Methods

Materials. Phospholipids were from Avanti (Birmingham, AL) except for egg PC, which was from Sigma (St. Louis, MO) or Avanti. The following types of phospholipids were used: beef heart cardiolipin, bovine brain PS, dioleoyl-PG, 1-palmitoyl-2-oleoyl-PA, 2-oleoyl-PA, and bovine liver PI. The phospholipids were >99% pure as assessed by thin-layer chromatography. Oleic acid, oleyl alcohol, and Triton X-100 were from Sigma. Initially, Triton purified by the method of Chang and Bock (1980) and the Sigma-grade Triton were compared for the effects on CT activity by use of a protocol similar to that shown in Figure 1, and no difference was detected. *sn*-1,2-Diacylglycerol was prepared from egg PC by phospholipase C digestion (Myher & Kuksis, 1979). Only minor traces (<5%) of the 1,3 isomer were detectable by TLC. [^{3}H]Phosphocholine was prepared from [^{3}H]choline as previously described (Cornell, 1989). All radioisotopic compounds were from New England Nuclear (Boston, MA): [*phenyl*-^{3}H]Triton X-100, 1.3 mCi/mg; [*2-palmitoyl*-9,10-^{3}H]dipalmitoyl-PC, 50 Ci/mmol; [^{3}H]palmitic acid, 0.5 Ci/mmol. Sephacryl S-400 was from Pharmacia (Uppsala, Sweden). Other chemicals were from Sigma. The anti-CT peptide antibody was the generous gift of H. Jamil and D. Vance.

Purification and Assay of CT. CT was purified according to Weinhold et al. (1986) with some modifications (Cornell, 1989). The purified enzyme was eluted from the final column in buffer A: 50 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 0.2 M K_2HPO_4, 1 mM EDTA, 2 mM DTT, 0.025% NaN_3, and 0.5 mM Triton X-100. The enzyme activity was assayed as described previously (Cornell, 1989). Generally, 2.5 μL of enzyme sample was diluted to a final volume of 50 μL in the assay cocktail [40 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 10 mM $MgCl_2$, 3 mM CTP, 1.5 mM [^{3}H]phosphocholine (15–20 Ci/mol), 10 mM DTT, and variable lipids] and incubated at 37 °C for up to 45 min.

Mixed Micelle Assay and Absorbance Measurements. Liposome stocks were prepared as described in the following paper (Cornell, 1991). Liposomes and an aqueous solution of Triton were mixed at different mole ratios. The assay cocktail was added, and the mixtures were vortexed and equilibrated at room temperature for at least 5 min. Enzyme (2.5 μL) was added, followed by [^{3}H]phosphocholine to initiate the reaction. The assay volume was 50 μL. Alternatively, we mixed the lipids and Triton from chloroform stocks, evaporated the solvent, added buffer, and sonicated for ~3 min as described above. Lipid–Triton mixtures prepared by either of the two methods produced identical mole percent lipid vs activity or absorbance curves. To measure the absorbance, the mixtures were prepared as above but scaled to 0.5 mL. CT, [^{3}H]phosphocholine, and CTP were omitted from these samples. Mole percent lipid = moles of lipid/(moles of Triton + moles of lipid) × 100%. When mixtures of lipids were used, the mole percent lipid refers to the total lipid.

Sephacryl S-400 Gel Filtration. A Sephacryl column (1 × 27.5 cm) was equilibrated and eluted with buffer A containing 0.5 mM Triton X-100. Lipid samples (0.2 mL) containing 10 or 20 mM lipid + Triton were prepared by mixing various amounts of Triton and lipid in $CHCl_3$, evaporating the solvent, and briefly sonicating the suspension in buffer A without Triton. To detect the elution positions, [^{3}H]Triton (1 μCi) or [^{14}C]dipalmitoyl-PC (0.3 μCi) was added to the lipids dissolved in $CHCl_3$ prior to solvent evaporation. Since PC mixes ideally with PG (Findlay & Barton, 1978), it was used as a marker for micelles containing PG as well as those containing PC. The amount of PC added to the PG mixed micelles was only 0.03% of the PG. Fractions of 1 or 0.6 mL were collected, and the radioactivity was measured by liquid scintillation counting. An M_r scale for the Sephacryl column was calibrated with bovine thyroglobulin (6.7×10^5), horse spleen apoferritin (4.4×10^5), yeast isoleucyl tRNA synthetase (1.3×10^5), and bovine serum albumin (6.7×10^4). These proteins were obtained from Sigma, except for the tRNA synthetase, which was the gift of T. Borgford. A plot of K_{av} vs log M_r gave a straight line with a correlation coefficient of 0.95. V_0 and V_t for the column were determined by using Blue Dextran and [^{3}H]glycerol, respectively. Samples containing CT (0.2 mL, ~0.07 nmol) were incubated with 300–600 nmol of lipid + Triton for 5 min at 20 °C prior to chromatography. Calculation of the amount of Triton in the sample included the Triton contributed from the CT stock as well as the lipid–Triton sonicated mixtures.

Detection of CT by ELISA. Serial dilutions of each column fraction were made in phosphate-buffered saline (PBS). The protein was bound to microtiter plates at 4 °C overnight. The plates were washed with tap water six times, blocked for 1 h at 37 °C with 1% ovalbumin in 0.05% Tween 20 in PBS, and incubated for 2 h at 37 °C with a 1/1000 dilution in 0.5% ovalbumin/0.05% Tween/PBS of an anti-CT antibody generated against a synthetic peptide corresponding to residues 163–176 of rat liver CT (Kalmar et al., 1990). The plates were washed with tap water and incubated for 1–2 h at 37 °C with a 1/1000 dilution of goat anti-rabbit horseradish peroxidase conjugate in 0.5% ovalbumin/0.05% Tween/PBS. The peroxidase was assayed by using 9 mM H_2O_2 and 1 mg/mL of *o*-phenylenediamine dissolved in 0.1 M sodium acetate, pH 6. The reaction was stopped with H_2SO_4 (final concentration 0.4 M). Absorbance ($A_{490nm} - A_{630nm}$) was monitored with a Biotek Model EL309 automated plate reader. Data from the linear portion of the antigen dilution curve were plotted.

Results

Activation of CT by Triton Mixed Micelles Containing Anionic Lipid. In the purification scheme of Weinhold et al. (1986) purified CT was eluted from the final column bound to a Triton X-100 micelle. Initially, we explored the suitability of a Triton–lipid mixed micelle system for investigating the selective activation by lipids. The activity of the transferase bound to pure Triton micelles was very low and was unaltered when the Triton concentration was varied between 1 and 10 mM. In the experiments shown in Figure 1 the Triton concentration was fixed at 1 or 10 mM and the lipid concentration (PC–oleic acid, 1/1) was varied. PC–oleic acid (1/1) sonicated vesicles have been shown previously to stimulate maximally (Weinhold, 1986; Feldman & Weinhold, 1987). The turbidity of the suspensions and the activity of the transferase were measured. The turbidity curves suggested that the transition from micelles to membranes occurred between 30 and 40% lipid. Gel filtration of Triton micelles and Triton–PC–oleic acid mixtures on Sephacryl S-400 indicated micelles and small vesicles of homogeneous size distribution (Figure 2A). 20% PC–oleic acid (1/1) micelles eluted at $K_{av} = 0.52$, corresponding to an apparent M_r of 3×10^5, which describes a micelle containing ~500 monomers. 40% PC–oleic acid mixtures eluted at $K_{av} = 0.43$, corresponding to $M_r = 6 \times 10^5$, which is the mass of a very small vesicle (~20 nm) containing ~1000 monomers. Separation of PC and oleic acid in the eluted fractions by thin-layer chromatography showed coelution of these lipids, suggesting that they were components of the same particle. Pure Triton micelles eluted at $K_{av} = 0.66$, corresponding to an apparent M_r of 1.8×10^5, which is the mass of a micelle containing 286 monomers. The column was calibrated with globular proteins of known molecular masses; thus these micelle molecular weights are only rough approximations. These micelle sizes are larger than those obtained by agarose chromatography (Dennis, 1974) or sedimentation (Yedgar et al., 1974), but are closer to results obtained by Sephacryl S-200 chromatography (Hannun et al., 1985).

The CT activity profile indicated two phases of activation (Figure 1): one associated with the formation of mixed micelles containing >10 mol % PC–oleic acid and another associated with the presence of membranes containing >50% PC–oleic acid. CT activity was 20–25% of maximum when the lipids were in a mixed micelle. Eighty mole percent lipid was required for full activation. The mole ratio of lipid/Triton was important rather than the absolute concentrations, since similar curves were obtained with 1 or 10 mM Triton (Figure 1, panels A and B, respectively). The PC–oleic acid concentration dependency was also tested by using octyl glucoside (22 mM) instead of Triton mixed micelles. Similar results were obtained (data not shown).

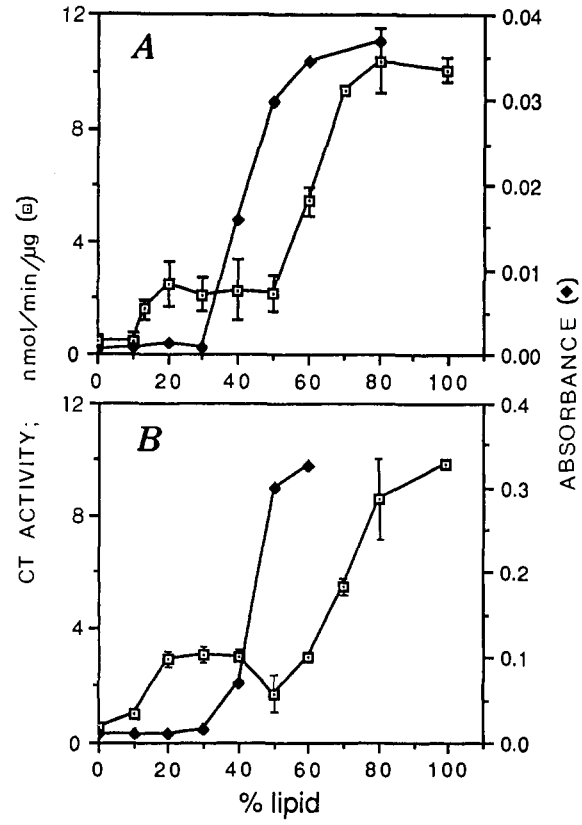

FIGURE 1: Lipid dependence of CT activity and micelle to membrane conversion at constant [Triton]. Triton and sonicated egg PC–oleic acid (1/1) vesicles were combined from separate stocks, purified rat liver CT (Weinhold et al., 1986) was added, and the mixtures were incubated for 10 min at 21 °C prior to enzyme activity assay (□) or measurement of absorbance at 600 nm (◆). The latter samples were identical with those prepared for the enzyme assay except that they lacked CT and CTP. In the samples containing 98% lipid the Triton concentration was 25 μM; otherwise, the Triton concentrations were (A) 1 mM, or (B) 10 mM. The data in panel A are averages of 4 separate experiments, and the data in panel B are averages of 2 separate experiments.

These results suggested that perhaps the active form of CT is stabilized better by a membrane rather than a micellar environment. However, similar studies with anionic phospholipid activators indicated that CT can be fully activated by lipids in micellar form. Absorbance measurements indicated the formation of membranes at ~40 mol % lipid for PG, PI, PA, and PS and 20 mol % lipid for cardiolipin (data not shown). These transitions from micelle to membrane at approximately 2 mol of Triton/mol of lipid are in agreement with previous studies (Dennis, 1974). Since cardiolipin has nearly twice the mass of the other lipids, a transition at a Triton/lipid molar ratio of 4 is reasonable. Vesicles containing 20% PG eluted from the Sephacryl column as a homogeneous peak at the same position as the micelles composed of 20% PC–oleic acid (1/1) (Figure 2A). A mixture of 40% PG eluted at $K_{av} = 0.32$ corresponding to a M_r of $\sim 1 \times 10^6$, the mass of a small vesicle composed of ~1500 monomers.

Sigmoidal activation curves for the anionic lipids cardiolipin, PA, PI, PS, and PG were obtained (Figures 3A and 4). The lipid concentrations required for half-maximal activation of CT were 4–5 mol % for cardiolipin and PA (charge = −2) and 9–13 mol % for PG, PI, and PS (charge = −1). The midpoints of these activation curves corresponded to surface charge densities of only 1 charge per 8–12 molecules. The charge

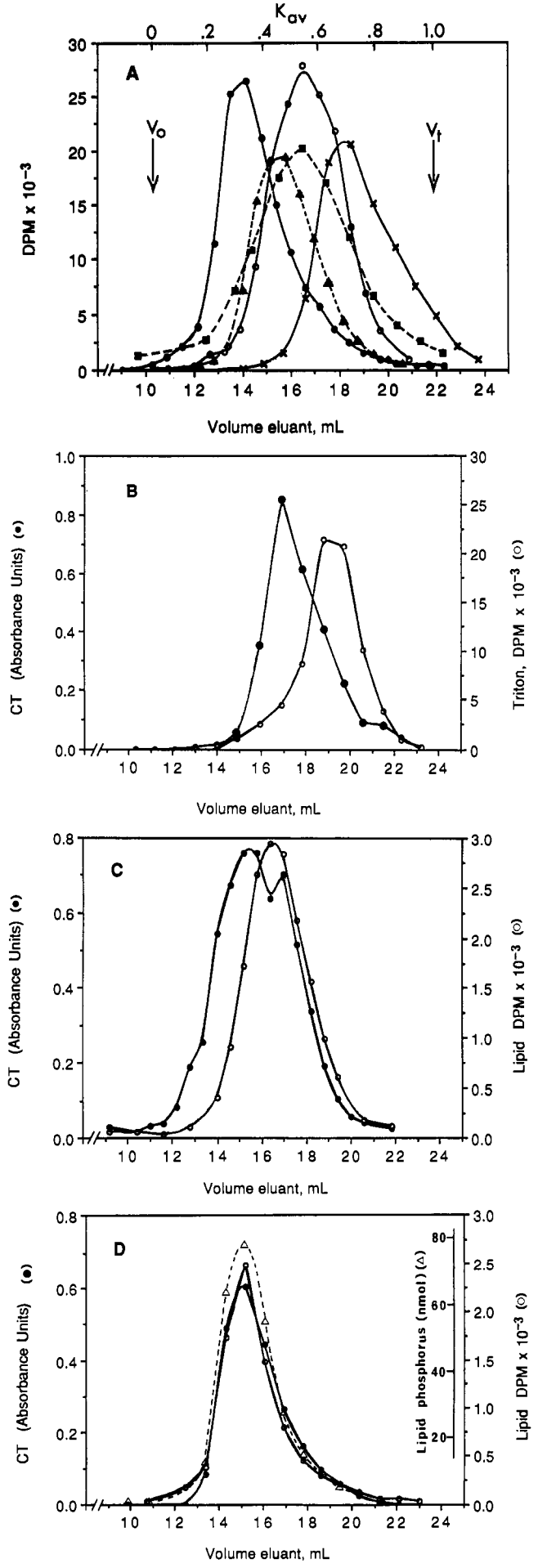

FIGURE 2: (A) Sephacryl S-400 characterization of vesicles and micelles. The samples applied to the column contained 10 or 20 mM Triton plus lipid, prepared and chromatographed as described under Materials and Methods. Triton X-100 (×); 20 mol % DOPG/80 mol % Triton (○); 40 mol % DOPG/60 mol % Triton (●); 10 mol % egg PC/10 mol % oleic acid/80 mol % Triton (■); 20 mol % egg PC/20 mol % oleic acid/60 mol % Triton (▲). The 100% Triton sample was spiked with 1 μCi of [^{3}H]Triton for detection. All other samples were spiked with 0.5 μCi of [^{3}H]DPPC. (B–D) Sephacryl S-400 chromatography of CT, micelles, and vesicles. The samples applied to the column contained ~0.07 nmol of purified rat liver CT (dimer) and 300–600 nmol of Triton–lipid mixtures. Samples were prepared and chromatographed as described under Materials and Methods. The elution buffer was buffer A except for panel C (buffer A with 0.2 M K_2HPO_4). CT was detected by an ELISA. The samples were spiked with [^{3}H]Triton or [^{3}H]DPPC for detection of Triton micelles and PG–Triton mixed micelles. In panel D the position of phospholipid was also monitored by phosphorus assay (Bartlett, 1959). (B) 100% Triton; (C) 20 mol % DOPG/80 mol % Triton; (D) 35 mol % DOPG/65 mol % Triton.

density required for maximum activation by the acidic lipids was −1 per 5 molecules. A higher charge density did not increase the activity. PA did not activate CT to the same extent as the other anionic lipids. Magnesium-induced aggregation was apparent with membranes containing >45 mol % PA, but was not apparent in samples containing high mole ratios of the other anionic phospholipids. This could explain the relative ineffectiveness of PA. The activation curves plateaued at concentrations below the micelle to membrane transition.

Mixtures of Triton and oleic acid were also examined. The charge on the oleic acid would vary between 0 and −1 depending on the concentration of the fatty acid in the micelle (see Discussion). The activation curve was sigmoidal with a midpoint at 18 mol % oleic acid and a plateau at 40 mol % (Figure 3A). Above 70% oleic acid there was a sharp decline in activity from 140 units/mL to 7 units/mL at 80 mol % oleic acid (not shown). Mixtures containing more than 70 mol % oleic acid were turbid; stable micellar suspensions were no longer present. Light scattering measurements using a Nicomp 270 particle sizer indicated particles 100–150 nm in diameter, which readily aggregated to form a microcrystalline phase upon incubation with the CT assay buffer which contained 10 mM $MgCl_2$. Rod-shaped crystals were observed by phase contrast microscopy. Thus a phase change appears to be responsible for lack of activation by oleic acid–Triton mixtures containing >70% fatty acid.

Activation of CT by Triton Mixed Micelles Containing Uncharged or Zwitterionic Lipid. By contrast, egg PC and 1/1 mixtures of egg PC with diacylglycerol or oleyl alcohol did not activate CT below 70 mol % lipid (Figure 3B). Absorbance measurements indicated membrane formation between 30 and 40% lipid for these three mixtures (data not shown). Membranes composed of 99% PC/diacylglycerol (1/1) or PC/oleyl alcohol (1/1) activated CT to the same degree as the anionic lipids, but the addition of only 10 mol % Triton reduced the activity 2.5-fold. Mixtures containing only diacylglycerol or oleyl alcohol and Triton did not activate CT at any concentration between 10 and 99% lipid (data not shown). From these experiments it appeared that a Triton mixed micelle would be an unsuitable system for testing the effects of neutral lipids, since even a few mole percent Triton in a bilayer interfered with the activation by these lipids.

Effect of Medium Ionic Strength on the Activation of CT by PG. The data of Figure 3 suggested that the anionic lipids were more effective than the neutral lipids tested and that the potency of the activator was related to the surface charge

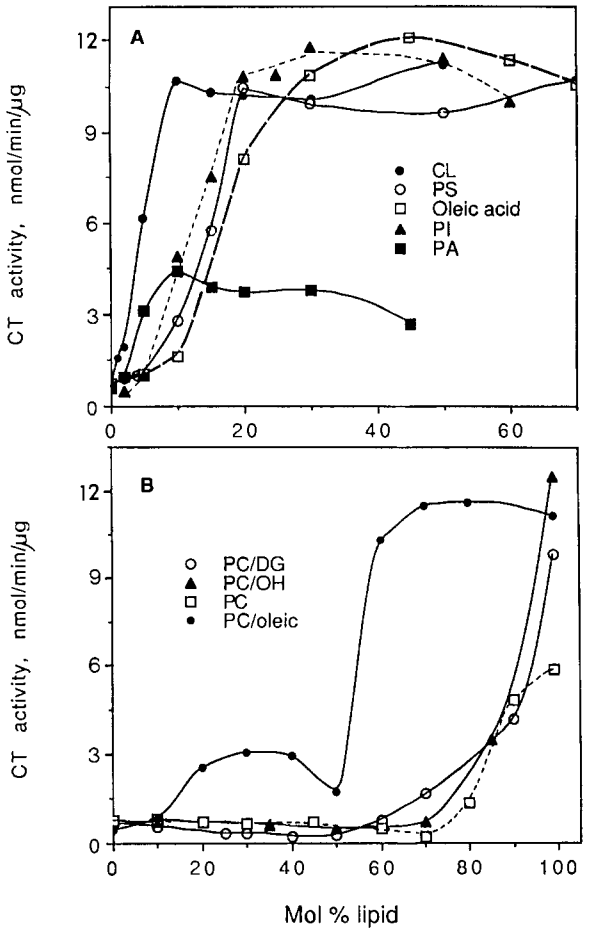

FIGURE 3: Comparison of the mole percent dependencies for CT activation by various lipids. Phospholipid–Triton mixtures were prepared as described in the legend to Figure 1. Other lipid mixtures were prepared from chloroform solutions of the lipids and Triton at various ratios as described under Materials and Methods. Mixtures of egg PC and oleic acid, diacylglycerol (PC/DG), or oleyl alcohol (PC/OH) were 1/1 (M/M). The Triton concentration was 1 mM except when the mole percent lipid was 99. In these samples [Triton] = 20 μM. The same batch of purified CT was used in each experiment. The curves for egg PC (□) and egg PC/oleyl alcohol (▲) were duplicate samples from single experiments which were repeated with similar results. All other curves were averages of 2–4 separate experiments.

density of the micelle. To test the role of the micelle surface potential in the activation of CT by anionic lipids, we examined the effect of raising the ionic strength of the medium on the concentration dependence for activation by PG. The surface potential of a micellar suspension at constant surface charge density should be reduced by increasing the ionic strength according to the Gouy–Chapman theory (McLaughlin, 1982). Figure 4 shows the concentration dependence for CT activation by PG in medium containing 0.125 M salt vs 0.535 M salt. The CT activity maximum was reduced ~20% in the higher ionic strength medium. The surface potential for micelles in the low ionic strength buffer containing 20 mol % PG was calculated to be −43 mV (see Appendix). There was a significant shift in the activation curve to a higher mole percent PG in 0.535 M salt medium. The mole percent lipid required for maximum activation shifted from 20% (at 0.12 M) to 35% (at 0.535 M). This increase in the surface charge density would be sufficient to maintain the surface potential at ~−43 mV (see Appendix). Thus the negative surface potential appears to be a major factor in the activation of CT by anionic lipids.

Binding of CT to Micelles and Membranes. The association of CT with an activating micelle or membrane was examined

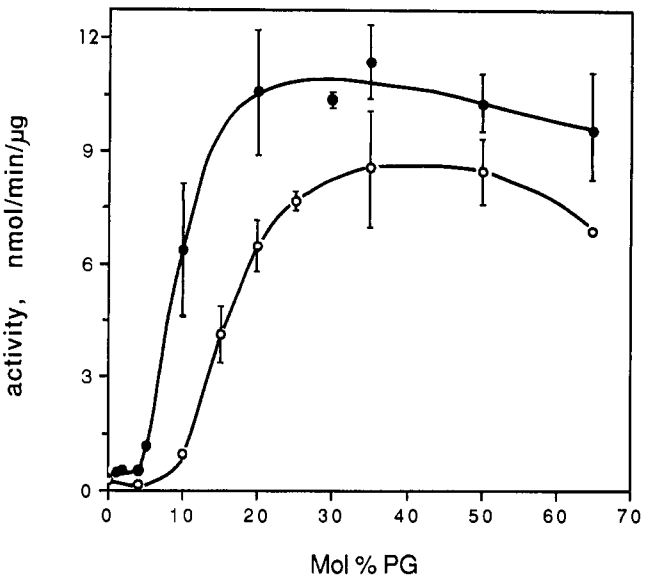

FIGURE 4: Effect of medium ionic strength on the activation by DOPG. (●) Total ion concentration = 0.125 M; (○) total ion concentration = 0.535 M. Data are averages of two separate experiments. The Triton concentration was 1 mM.

by gel filtration on Sephacryl S-400. CT eluted as a progressively larger particle when chromatographed with pure Triton micelles, 20% PG–Triton mixed micelles, and 35% PG vesicles (Figure 2B–D). In the experiments shown in Figure 2B,C the micelles were in at least 20-fold molar excess of CT, and thus the Triton or lipid profiles correspond mainly to micelles unassociated with CT. There are two CT peaks in Figure 2C. The first peak eluted likely corresponds to CT bound to the PG–Triton mixed micelles, and the second peak probably corresponds to CT bound to Triton micelles, since it appears at the exact position of the CT–Triton micelles shown in Figure 2B. This column was eluted with buffer A (see Materials and Methods) containing 0.2 M phosphate; the ionic strength was 0.55 N. At this ionic strength CT was only ~70% activated (Figure 4). The CT elution profile suggests that a similar fraction of the CT was associated with the PG-containing micelles under these conditions.

The difference in molecular weights of the micelles with and without CT, estimated from the elution positions which were calibrated with protein molecular weight standards, was 2×10^5 to 3×10^5. Weinhold et al. (1986) previously determined that purified rat liver CT elutes as a 2.5×10^5 species on Bio-Gel A-1.5m. Chemical cross-linking (Cornell, 1989) and sedimentation analysis (Weinhold et al., 1989) indicate that CT is a dimer of a 42-kDa subunit. The anomalous gel filtration behavior of CT, when referenced to the elution of globular proteins, is due to its elongated dimensions (Weinhold et al., 1989).

In the experiment shown in Figure 2D, the elution of the Triton–35% PG mixture was monitored both by radioactive tracer lipid and by chemical analysis of lipid phosphorus. These peaks coeluted, demonstrating the validity of tracking with labeled DPPC. CT also coeluted with the lipid. If the 35% PG mixture was in the form of small vesicles containing ~1000 molecules, then the molar ratio of CT/vesicles in this experiment would be 1. The peaks would coelute since all the lipid would be a component of a CT-associated vesicle.

Discussion

Anionic lipids such as PG, PI, and fatty acids have been used extensively as activators of CT. On the basis of the effectiveness of anionic lipids compared to zwitterionic lipids such

as PC, PE, and sphingomyelin, Feldman and Weinhold (1987) have proposed that CT–membrane binding and activation may be promoted by increased local concentrations of anionic lipids in the membrane. On the other hand, neutral lipids such as mono- and diacylglycerol and oleyl alcohol were also found to be strong activators of CT in vitro and when added to cell cultures (Cornell & Vance, 1987a,b; Rosenberg et al., 1987). Furthermore, the binding of cytosolic CT to liposomes composed of PC and oleic acid appeared to be insensitive to the ionic strength of the medium (Cornell & Vance, 1987b). These studies suggested that factors other than the lipid negative charge contributed to CT–membrane binding.

In this paper we evaluated the relative effectiveness of lipids ranging in charge from −2 to 0 using Triton as a dilutant of the activating species. The results supported the idea that the negative charge of the lipids does play an important role in the activation of CT. The charge density is a more critical factor than the head group structure, and the effect on CT is probably a response to the surface potential of the Triton–lipid mixed micelle. These conclusions are derived from the following observations.

(1) The greater is the negative charge of the lipid, the lower is the mole percent required for CT activation. Maximal activation by cardiolipin or PA (charge = −2) was achieved at 10 mol % lipid, while maximal activation by PG, PI, or PS (charge = −1) was reached at 20 mol % lipid, and maximal activation by fatty acid (charge = $\sim -^1/_2$) occurred at ~40 mol % lipid. The uncertainty in the charge of the fatty acid is due to incomplete dissociation even at pH 7.4 when concentrated into a membrane or micelle that has a negatively charged surface (Cistola et al., 1988). A pK_a of ~7.4 is a reasonable estimate for oleic acid in a micelle and would account for the mole percent dependence shown in Figure 3. The pK_a of lamellar oleic acid has been determined to be 8.0–8.5 (Cistola et al., 1987). Micellar fatty acid should have a slightly lower pK_a due to a lower surface charge density. (2) PS, PI, and PG have different polar head group structures but identical net charge. The concentration curves for activation of CT by these three lipids were very similar. The same argument holds for PA and cardiolipin. On the other hand, PG and cardiolipin have similar polar head group structures but differ by a net charge of 1. The mole percent PG required for activation of CT was twice that of cardiolipin. (3) The surface potential for mixed micelles containing 20% PG in 0.125 M salt was calculated to be −43.2 mV (see Appendix). Increasing the medium ionic strength 4.4-fold raised the mole percent PG required for full activation from 20 to 35. This charge density increase would be sufficient to maintain an electrostatic surface potential of −42.6 mV in medium of 0.535 M ion concentration. (4) Activation by uncharged lipid activators required much higher ratios in the membrane compared to the anionic lipids. (5) Sphingosine and other positively charged lipids antagonize the effects of anionic lipid activators (Sohal & Cornell, 1990). This finding is consistent with a role for the negative surface potential in promoting the interaction of CT with lipids and consequent activation.

Several other "soluble" proteins preferentially interact with anionic membrane lipids. These include the blood clotting factors Va, VIII, Xa, and prothrombin (Gilbert et al., 1990; Rosing et al., 1988; Mayer et al., 1983), protein kinase C (Rando, 1989), colicin A (Massotte et al., 1989), apocytochrome *c* (Rietveld et al., 1985; Berkhout et al., 1987), and synapsin (Benfenati et al., 1989). Smaller peptides such as mellitin, cardiotoxin, and ACTH also interact preferentially with negatively charged membranes (Verhallen et al., 1984; Battenburg et al., 1987). While the binding of the blood clotting factors and protein kinase C to acidic membranes *may* involve direct coordination of Ca^{2+} with the anionic lipids (Rosing et al., 1988; Ganong et al., 1986), the interactions of synapsin, apocytochrome *c*, and colicin A, like CT, are not Ca^{2+} mediated and involve both hydrophobic and electrostatic interactions. For these proteins two-step models for membrane binding have been proposed in which the anionic phospholipid surface promotes an electrostatic interaction with the proteins (which contain clusters of positive charge) followed by penetration into the hydrophobic core. This latter step has been demonstrated by photolabeling with a diazirine derivative of PC in the case of synapsin (Benfenati et al., 1989) and by tryptophan fluorescence quenching with brominated phospholipids in the case of apocytochrome *c* (Berkhout et al., 1987).

We postulate a similar type of membrane binding and integration for CT. The fact that at high concentrations neutral lipids can activate CT to the same extent as anionic lipids suggests that changes in membrane properties that will stabilize the CT–membrane interaction can be accomplished in more than one way. (1) The correlation between the membrane negative surface potential and CT activity might be due to electrostatic attraction of a cationic membrane-binding domain on the CT molecule. This membrane feature would serve to concentrate CT near the membrane surface. The shifts in the elution positions of CT when chromatographed on Sephacryl with micelles or small vesicles containing PG supported the idea that the activation of CT involves binding of the enzyme to the micelle or vesicle. (2) Enrichment with lipids that have small polar head groups such as diacylglycerol or fatty alcohol might enhance CT binding/activation by the creation of defects in lipid packing. This might facilitate integration of CT into the bilayer. Anionic lipids might have dual roles in promoting CT binding. In addition to the effect on Ψ_0, increases in the proportion of negatively charged lipids would also "loosen" lipid packing due to increased charge repulsions between neighboring molecules.

One other observation contributes to the idea that the interaction of CT with lipid surfaces is affected by the packing density. The activation of CT by mixtures of PC–oleic acid and Triton was biphasic (Figures 1 and 3B). A micelle to membrane transition occurred in the middle of the first phase of the PC–oleic acid activation curve between 30 and 40 mol % lipid. The increase in the negative charge density associated with increased mole percent oleic acid may have been counteracted by an increase in the lipid packing density upon the transition to bilayers above 30 mol % lipid. This could explain the plateau in the curve between 20 and 50 mol % lipid. Biphasic activation curves were not observed for cardiolipin, PG, PA, PI, and PS because CT was fully activated by these lipids at concentrations below the micelle to membrane transition.

The Triton mixed micelle system proved to be an effective means for presentation of the activating lipids only for the anionic lipids. The neutral lipids activated CT only at concentrations above their micelle to membrane transitions. Moreover, CT was sensitive to a low mole percent Triton in bilayers composed of egg PC and diacylglycerol or fatty alcohol. In the following paper (Cornell, 1991) we present a method for removal of 99.9% of the Triton and reconstitution of CT activity with detergent-free lipid bilayers. We have used this preparation of CT to examine the role of lipid packing density, acyl chain length and unsaturation, and the lipid-phase preference.

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APPENDIX

Calculation of the micelle surface potential was based on the Gouy–Chapman theory:

$$\Psi_0 = 2kT/ze \sinh^{-1}(A\sigma/\sqrt{C}) \quad (1)$$

The values used for A and kT/e were from McLaughlin (1977). The values for C, the bulk aqueous ion concentration, took into account all cations in the medium including the Tris buffer. The concentration of $Tris^+$ was determined from its pK_a at 37 °C, which is 7.74. The values for z, the charge of the counterion, took into account the 10 mM Mg^{2+} present as well as the Na^+ contribution and assumed equal attraction of both cations for the micelle surface:

solution 1: [total ions] = 0.125 M; z = 1.08

solution 2: [total ions] = 0.535 M; z = 1.019

The values for σ, the surface charge density in $(Å^2)^{-1}$, were estimated as follows: The area per molecule in a Triton mixed micelle containing 20 or 35 mol % PG was estimated to be 79 or 73 $Å^2$, respectively, on the basis of an equivalent spherical micelle radius of 39 or 43 Å and an aggregation number of 242 or 319 (Yedger et al., 1974). The values for σ were thus $-1/395$ $Å^2$ for 20 mol % PG and $-1/209$ $Å^2$ for 35 mol % PG. Although these radii and aggregation numbers were determined for Triton–sphingomyelin mixed micelles, Robson and Dennis (1978) have shown that the mixed micelle structure is influenced little by the type of phospholipid incorporated.

(a) At 0.125 M ionic concentration and 20 mol % PG:

$$\Psi_0 = (53.6/1.08) \sinh^{-1}\left[\frac{(-137.7/395)}{\sqrt{0.125}}\right] = -43.2 \text{ mV}$$

(b) At 0.535 M ionic concentration and 35 mol % PG:

$$\Psi_0 = (53.6/1.019) \sinh^{-1}\left[\frac{(-137.7/209)}{\sqrt{0.535}}\right] = -42.6 \text{ mV}$$

Similar estimations of σ for the PG compositions at half-saturation of the activation curves in 0.125 vs 0.535 M salt (9 and 15.5 mol % PG, respectively) generated Ψ_0 values of −22 and −20 mV, respectively.

Registry No. CT, 9026-34-0; DOPG, 45322-62-1; Triton X-100, 9002-93-1; oleic acid, 112-80-1; oleyl alcohol, 143-28-2.

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