Lipid Regulation of CTP:Phosphocholine Cytidylyltransferase: Electrostatic, Hydrophobic, and Synergistic Interactions of Anionic Phospholipids and Diacylglycerol†

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ABSTRACT: The contributions of electrostatic and hydrophobic interactions in the activation of cytidylyltransferase (CT) by various negatively charged lipids were analyzed using small unilamellar or multilamellar vesicles (SUVs or MLVs). The activation of CT by SUVs containing increasing mole percentages of anionic phospholipids varied in proportion to the net charge associated with the polar head group, suggesting an electrostatic component to the activation. However, increasing ionic strength to neutralize the surface charge enhanced the potency of SUVs containing PA or PG, suggesting that the hydrophobic effect is a stronger force than electrostatics in driving the interaction of CT with SUVs. On the other hand, electrostatics played a more important role in the activation by MLVs. Increasing ionic strength decreased the potency of MLVs containing PG. CT bound to MLVs in the gel state, but was inactive; the enzyme was only active when the MLVs were in the liquid-crystalline state, suggesting an intercalation event. Lowering the pH from 7.4 to 6.2 resulted in a decrease in the negative surface charge required for activation. The binding of CT to PG vesicles was enhanced at acidic pH. The results suggest that at pH 6.2 one or more amino acids on CT that are involved in lipid binding would be protonated. This could enhance the electrostatic effect by increasing the positive charge on CT, or it could enhance the hydrophobic effect by decreasing the negative charge on CT. In addition, maximal activity of CT was decreased at the lower pH, suggesting that active site residues may also be affected. CT was activated by the synergistic interaction of diacylglycerol and anionic phospholipid in SUVs. The synergy between DG and PA at low concentrations suggests the possibility that these second messenger lipids could concertedly regulate CT and thus PC synthesis in response to agonists that stimulate PC hydrolysis via phospholipases C and/or D.

CTP:phosphocholine cytidylyltransferase (CT)[1] is a key regulatory enzyme in the synthesis of phosphatidylcholine (PC). It is localized in the nucleus (Wang *et al.*, 1993) where its activity is regulated by interconversion between an active membrane-bound and an inactive soluble conformation. The lipid composition of the target membrane is a principle determinant of the distribution of CT between the two forms (Houweling *et al*., 1994; Wang & Kent, 1995). Using pure CT and sonicated vesicles, three classes of lipid modulators of the enzyme's activity have been established: class I activators include anionic phospholipids and fatty acids (Choy & Vance, 1978; Feldman *et al*., 1978, 1981; Feldman & Weinhold, 1987; Cornell, 1991a,b); class II activators include neutral lipids exemplified by diacylglycerol (DG) (Choy *et al*., 1979; Johnson *et al*., 1992; Jamil *et al*., 1993); and class III consists of inhibitory cationic lipids such as sphingosine (Sohal & Cornell, 1990). DG and fatty acids can also activate CT in cultured cells (Pelech *et al*., 1983; Cornell & Vance, 1987; Kolesnick & Hemer, 1990; Slack *et al*., 1991; Utal *et al*., 1991).

The mechanism whereby these diverse lipid structures can modulate enzyme activity is not well understood. The contribution of the surface charge in the case of class I lipid activators has been probed using Triton X-100 mixed micelles (Cornell, 1991a). This study suggested that the surface charge density is more important than head-group structure. Data from different experimental approaches suggest that a portion of CT intercalates into the nonpolar core of the lipids when it binds to activating membranes, implying significant hydrophobic interactions (Cornell, 1991b; Craig *et al*., 1994; Johnson & Cornell, 1994).

There is much evidence implicating a membrane binding function for an amphipathic α-helix located between residues 236 and 293. Deletion of this region, via chymotrypsin proteolysis (Craig *et al*., 1994) or cDNA truncation (Cornell *et al*., 1995; Wang & Kent, 1995; Yang *et al*., 1995), led to loss of binding to or activation by lipid vesicles containing oleic acid or DG. A peptide derived from this domain was transformed from random coil to α-helix by anionic lipid vesicles, and its fluorescence was quenched by anionic vesicles containing brominated PC with bromo groups

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[1] Abbreviations: CL, cardiolipin; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP, phosphatidylinositol monophosphate; PS, phosphatidylserine; DG, diacylglycerol; CT, CTP:phosphocholine cytidylyltransferase; di15:0PC, dipentadecanoyl phosphatidylcholine; [^{3}H]DPPC, [^{3}H]dipalmitoylphosphatidylcholine; di15:0PG, dipentadecanoylphosphatidylglycerol; MLVs, multilamellar vesicles; SUVs, small unilamellar vesicles; SLVs, sucrose-loaded vesicles; PKC, protein kinase C; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; ADA, *N*-(2-acetamido)-2-iminodiacetic acid.

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midway down the acyl chain, implying deep penetration of a tryptophan (Johnson & Cornell, 1994). These studies support a model wherein the amphipathic helix lies with its axis parallel to the plane of the membrane, its nonpolar face penetrating into the bilayer core, and its polar face interacting with lipid head groups, water, and/or other regions of the protein.

Although the specific lipids that promote CT activity and the specific region in the protein that mediates binding have been identified, much work remains to elucidate the chemistry of this interaction. The relative contributions of electrostatic and hydrophobic interactions have never been dissected, nor has the contribution of the charge on the enzyme. Our work examines more closely the contributions of these interactions. In addition, the possibility of cooperative interaction between class I and class II activators has until now not been investigated. Stimulation by activating lipids requires nonphysiological concentrations, in terms of mol percentage of the total lipid, when examined in a lipid vesicle system. We show that in combination, anionic lipids and DG synergistically activate CT at physiological levels of these lipids.

MATERIALS AND METHODS

Materials

The lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (PA), 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol (PG), heart cardiolipin (CL), egg yolk phosphatidylcholine (PC), soybean phosphatidylinositol (PI), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (PS), dipentadecanoylphosphatidylcholine (di15:0PC), and dipentadecanoylphosphatidylglycerol (di15:0PG) were purchased from Avanti Polar Lipids. Diacylglycerol (DG) was prepared from egg PC by phospholipase C digestion as described by Myher and Kuksis (1979). Less than 1% was in the 1,3 isoform as determined by TLC. [methyl-^{3}H]Choline chloride and [^{3}H]dipalmitoylphosphatidylcholine ([^{3}H]DPPC) were obtained from DuPont NEN. [^{3}H]Phosphocholine was prepared from [^{3}H]choline as described by Cornell (1989). Leupeptin, chymostatin, antipain, pepstatin, *p*-aminobenzadine, benzamidine, phenylmethanesulfonyl fluoride (PMSF), cytidine triphosphate, dithiothreitol (DTT), Triton X-100, DEAE-sepharose, and L-α-phosphatidylinositol 4-monophosphate (PIP) were supplied by Sigma. Hydroxyapatite was purchased from Bio-Rad. All other chemicals were reagent grade.

Methods

Purification of Cytidylyltransferase. CT was purified from baculovirus-infected insect cells as described by MacDonald and Kent (1993) with modifications (Cornell *et al*., 1995). CT was stored in small aliquots at −80 °C in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 200 mM KH_2PO_4, 2 mM DTT, 0.05% Triton X-100, and protease inhibitors (2.5 μg/mL leupeptin, 2 μg/mL chymostatin, 1 μg/mL antipain, 2 μg/mL pepstatin, 10 μg/mL *p*-aminobenzadine, 10 μg/mL benzamidine, and 2 mM PMSF)

Preparation of Lipids. Lipid stocks were stored as chloroform solutions, and their concentrations were determined by a phospholipid phosphorus assay (Bartlett, 1959). Small unilamellar vesicles (SUVs) were prepared by solvent evaporation under a stream of nitrogen. Residual chloroform was removed by placing the lipids under vacuum. The lipids were rehydrated in buffer containing 20 mM Tris (pH 7.4) or 20 mM ADA (pH 5.3, 6.1, or 7.2) containing 0.5 mM EDTA. Lipids were sonicated using a Branson Sonicator with a fine probe (80% power) for 5−15 min on ice. Negligible sonicated lipid was sedimented by centrifugation at 20000*g* for 5 min. Vesicles were sized using a NICOMP Submicron Particle Sizer Model 370.

Multilamellar vesicles (MLVs) were prepared by drying the appropriate volume of lipid and rehydrating as described above. The lipid samples were vigorously vortexed, incubated for 10 min at 37 °C, vortexed again until all lipid was in solution, and then placed on ice. Lipids were used immediately after preparation. In some cases, the lipid solution was spiked with a small amount of [^{3}H]DPPC to determine if there was any spontaneous formation of unilamellar vesicles. Approximately 10% of the labeled lipid remained in solution after the multilamellar vesicles were pelleted at 20000*g* for 20 min.

CT Activity Assay. A solution containing CT, $MgCl_2$, CTP, DTT, and NaCl was added to lipid vesicles of various composition. The reaction was initiated by the addition of [^{3}H]phosphocholine for a final assay volume of 50 μL containing 50 mM Tris, pH 7.4, 12 mM $MgCl_2$, 3 mM CTP, 10 mM DTT, 200 μM lipid, 0.1 mM EDTA, 88−300 mM NaCl, 0.2−0.3 μg of CT, and 3−7 μM Triton X-100. In some assays ADA (44 mM, final concentration) was used instead of Tris. In experiments where the pH was varied, we tested the pH of the final reaction mixture. The reaction was stopped after 25 min at 37 °C unless otherwise indicated by adding 30 μL of methanol/ammonia (9:1). The rate of the reaction was linear within the time frame of the assay. Analysis of the formation of [^{3}H]CDP-choline was performed as described previously (Sohal & Cornell, 1990).

CT Binding Assay with MLVs. Three micrograms of CT, 10 mM DTT, and 88−300 mM NaCl, with or without 12 mM $MgCl_2$ and 3 mM CTP, were added to 1 mM MLVs containing DOPG/egg PC (1:1) or di150PG/di15:0PC (1:1). The solutions were incubated at room temperature for 5 min, followed by centrifugation at 20000*g* for 20 min, which sedimented 90% of the lipid as assessed by tracking with trace amounts of [^{3}H]DPPC. The quantity of CT in the pellet and supernatant was analyzed by SDS gel electrophoresis (12% polyacrylamide) (Laemmli, 1970) followed by densitometry of Coomassie stained gels (Microtek Scanmaker densitometer). Mg^{2+} had no significant effect on the amount of CT in the pellet.

CT Binding Assay with SLVs. Sucrose-loaded vesicles (SLVs) were prepared by drying chloroform solutions containing trace amounts of [^{3}H]DPPC as described above. Lipids were rehydrated in 20 mM TES, pH 5.8−8.3, containing 170 mM sucrose. Lipid suspensions were subjected to five freeze−thaw cycles followed by extrusion in a Liposofast extruder (Avestin, Inc., Ottawa, ON) through a 0.1 μm polycarbonate filter. The extruded vesicles were diluted 4-fold in a solution containing 50 mM TES, pH 5.8−8.3, and 133 mM NaCl. Following centrifugation at 100000*g*, 20 °C for 30 min, three-fourths of the supernatant was removed, and the lipid pellet was resuspended by vortexing. The concentration of the lipid was monitored by the trace amounts of [^{3}H]DPPC present. These SLVs were then added to the assay mixture for a final concentration of 80 mM TES, pH 5.8−8.3, 3 μg of CT, 10 mM DTT, 110 mM NaCl, 5 mM sucrose, 30 μM TX-100, 9 mM KH_2PO_4, 2 mM Tris, and 0.6 mM lipid (0 and 40 mol % PG). The

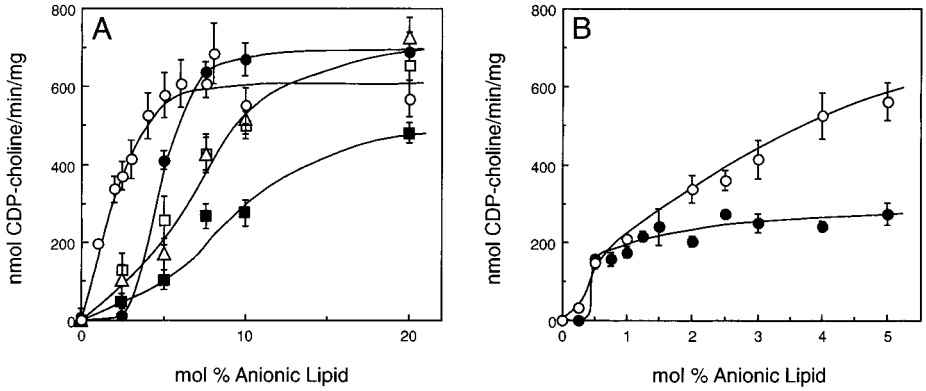

FIGURE 1: Anionic lipid stimulation of CT. (A) CT activity was measured in the presence of egg PC SUVs containing 0−20 mol % of the anionic lipids CL (○), PA (●), PG (△), PI (■), and PS (□) (200 μM total lipid). Error bars represent the standard error of the mean of 6−9 sets of data. B) CT activity was measured in the presence of egg PC SUVs containing 0−5 mol % PIP(●) or CL (○). Error bars represent the standard error of the mean of 3−6 sets of data.

assay mixture was incubated for 10 min at 20 °C and then centrifuged at 100000*g* for 30 min, 20 °C. The pellet contained 90% of the lipid. The quantity of CT in the supernatant and pellet was analyzed by gel electrophoresis followed by densitometric analysis as described above.

RESULTS

Activation by Anionic Lipids. The effect of anionic lipids of varying net charge on the activity of CT was tested by the incorporation of varying mole percentage into egg PC SUVs (Figure 1). For all experiments, CT activity in the presence of PC alone was treated as background activity. The difference between CT activity in the absence of lipid and the presence of 200 μM PC was not significant. The mole percent anionic lipid required for full activation was 3% for phosphatidylinositol monophosphate (PIP) (2−3 negative charges at neutral pH), 5% for cardiolipin (CL) (2 negative charges), 7% for PA (1−2 negative charges), and 20% for phosphatidylglycerol (PG), phosphatidylserine (PS), or phosphatidylinositol (PI) (single negative charge. Thus, activation is related to the net charge on the phospholipid head group. The relatively poor magnitude of activation by PI may be a result of the bulky sugar group, inositol, compared to PG (glycerol) or PS (serine). PIP, with a phosphoinositol head group, was also a poor activator (maximal activity was 3-fold lower than that obtained with CL, PA, PG, or PS).

CT activity showed a sigmoidal dependence on the concentration of anionic lipids tested. The sigmoidal behavior may reflect the combined effects of increases in surface charge and curvature. Average vesicle diameters were similar for 100 mol % PC (60 ± 4 nm) and 10 mol % PG (70 ± 8 nm), but decreased for 10 mol % PA (30 ± 4 nm) as measured by the NICOMP Particle Sizer.

Activation by Diacylglycerol. The effect of the neutral lipid DG, incorporated into PC vesicles, on CT activity was examined (Figure 2). CT was half-maximally stimulated by 4 mol % DG with maximal stimulation occurring at 15 mol % DG. The activation was not sigmoidal, in contrast to the activation by anionic phospholipids. Maximal CT activation was approximately 3-fold lower in the presence of DG compared to the anionic lipids CL, PA, PG, PS, and PI.

Effect of Ionic Strength on Lipid Stimulated Activity in SUVs. The role of electrostatics in lipid binding and activation of CT was explored by varying the medium ionic strength. The higher the concentration of the counterion,

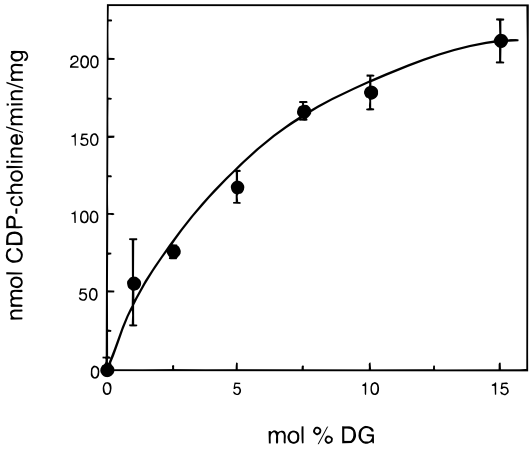

FIGURE 2: Activation of CT by DG. CT activity was measured in the presence of egg PC SUVs containing 0−15 mol % DG (200 μM total lipid). Error bars represent standard error of the mean of triplicate experiments.

sodium, the lower the effective negative charge at the membrane surface. The effect of the medium ionic strength on PA-stimulated CT activity is shown in Figure 3A. Higher ionic strength decreased the maximal activity obtained in the presence of SUVs containing saturating concentrations of PA. A similar effect of medium ionic strength has been observed for the lipid requiring enzyme monoacylglycerol acyltransferase (Bhat et al., 1995). When 300 mM NaCl, instead of 88 mM, was included in the assay mixture, the concentration of PA needed for half-maximal activation of CT shifted from 5 mol % to 3 mol %. The dependence on the PA content of the vesicle was less sigmoidal at the higher NaCl concentration. These results are opposite to what would be expected if the lipid negative charge played an exclusive role in the activation of CT by anionic lipids. Similar results were seen with SUVs containing PG (results not shown). Vesicle size was not affected by change in ionic strength as determined by NICOMP analysis. The average diameter of SUVs containing 10 mol % PA was 30 ± 4 nm in the presence of 88 mM NaCl and 30 ± 10 nm in 300 mM NaCl. Similarly, the average diameter of SUVs containing 10 mol % PG was 70 ± 8 nm in the presence of 88 mM NaCl and 60 ± 10 nm in 300 mM NaCl. Therefore, the observed effect on activity could not be attributed to changes in vesicle size or curvature. The increased NaCl concentration may be screening the negative charge, neutralizing the electrostatic component involved in the activation,

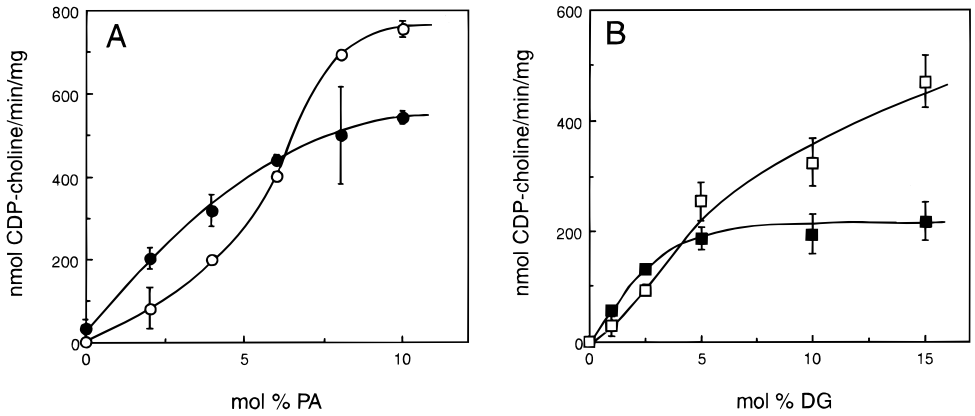

FIGURE 3: Effect of ionic strength on PA-stimulated CT activity in SUVs. (A) CT activity was measured in the presence of egg PC SUVs containing increasing concentrations of PA (0−10 mol %) (200 μM total lipid) and in the presence of 88 (○) or 300 (●) mM NaCl. Data are means of duplicate experiments. (B) CT activity was measured in the presence of egg PC SUVs containing increasing mol % DG (200 μM lipid) in the presence of 88 (□) or 500 (■) mM NaCl. Error bars represent the standard error of the mean of four sets of data.

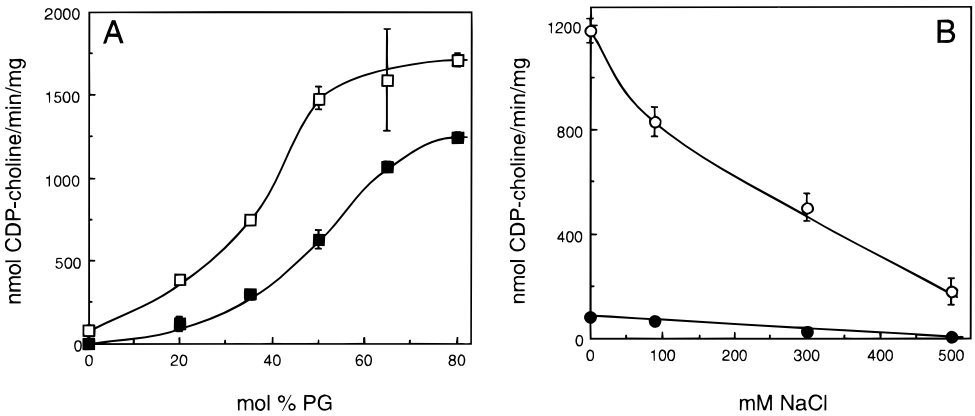

FIGURE 4: Effect of ionic strength on PG-stimulated CT activity in MLVs. (A) CT activity was measured in the presence of egg PC MLVs containing 0−80 mol % DOPG at 88 mM (□) or 300 mM (■) NaCl. Data are means of duplicate experiments. (B) CT activity was measured in the presence of MLVs containing egg PC (●) or DOPG/egg PC (1:1) (○) (500 μM total lipid) at increasing NaCl concentrations. Error bars represent the standard error of the mean of four sets of data.

but at the same time enhancing the hydrophobic component.

The effect of ionic strength on the DG-stimulated CT activity was examined (Figure 3B). Ionic strength should not vary the effective charge at the surface of net neutral membranes. The presence of 500 mM NaCl shifted the DG concentration necessary for maximal activation from greater than 15 mol % to 5 mol %. This result also suggests an enhanced hydrophobic effect as a result of an increase in medium ionic strength.

Effect of Ionic Strength on Lipid-Stimulated Activity in Multilamellar Vesicles. The roles of electrostatics and hydrophobic interactions of CT with planar MLVs were examined. In contrast to the effect of ionic strength on the stimulation by SUVs, higher ionic strength increased the concentration of anionic lipid required for stimulation (Figure 4A). MLVs composed of egg PC or egg PC/DOPG (1:1) were tested as CT activators as a function of medium NaCl concentration (Figure 4B). PG-stimulated activity decreased 30% in the presence of 88 mM NaCl and 85% in the presence of 500 mM NaCl. This indicates a significant ionic strength effect on the stimulation of CT by MLVs containing anionic phospholipid.

If ionic interactions alone suffice to activate CT, the activity would be insensitive to the lipid phase. However, PG/PC MLVs in the gel state were unable to stimulate CT activity (Table 1). Mixtures of di15:0PG/di15:0PC have a phase transition temperature around 36 °C in the presence of 10 mM $MgCl_2$ (Cornell, 1991b). At 20 °C, *i.e.*, in the gel state, these lipids were poor activators compared to their effects in the liquid-crystalline state (37 °C). The activity in the presence of di15:0PG/di15:0PC (1:1) vesicles in the gel state did not increase the activity above that of the lipid minus control samples. CT was in fact bound to the di15:0PG/di15:0PC MLVs at 20 °C, as assessed by sedimentation, and this interaction could be disrupted by 300 mM NaCl, indicating that it was electrostatic in nature (Table 1). These results suggest that electrostatic interactions play a role in the association of CT with MLVs but that activation of the

Table 1: Effect of Lipid Phase on CT Activation[a]

lipid	mM NaCl	sp act., 20 °C	% bound, 20 °C	sp act., 37 °C	% bound, 37 °C
egg PC/DOPG (1:1)	88	250 ± 60	40 ± 8	320 ± 3	60 ± 3
	300	80 ± 40	11 ± 3	130 ± 5	10 ± 2
di15:0PC/di15:0PG (1:1)	88	0	70 ± 6	380 ± 20	70 ± 2
	300	0	0	210 ± 20	30 ± 2

[a] CT activity (nmol of CDP-choline min^{-1} mg^{-1}) was measured in the presence of 1 mM MLVs in the gel state (di15:0PG/PC at 20 °C) and in the liquid-crystalline state (all others) at 88 and 300 mM NaCl. The percent CT bound to MLVs was determined as described in Methods. The lipid-independent activities and the percent CT in the pellet fraction, in the absence of vesicles, were subtracted from the values reported. For the values reported as 0, the average specific activities were less than specific activities obtained in the absence of lipid vesicles. Error is reported as standard error of the mean of 4−5 sets of data at 20 °C and as the average of 2 sets of data at 37 °C.

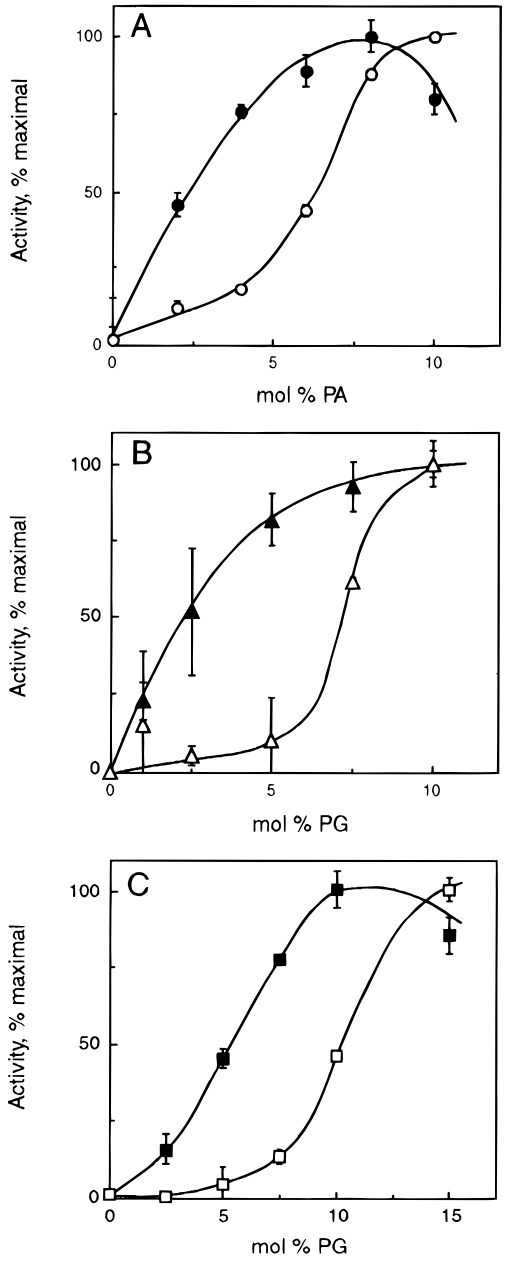

FIGURE 5: Effect of pH on PA- and PG-stimulated CT. CT activity was measured in the presence of egg PC SUVs containing increasing mol % PA or PG (200 μM total lipid). Panels A and B: Tris buffer was used at pH 6.2 (closed symbols) and 7.4 (open symbols). Maximal activity was 300 $\pm$ 20 and 600 $\pm$ 20 nmol of CDP-choline min^{-1} mg^{-1} for PA and 300 $\pm$ 10 and 500 $\pm$ 10 nmol of CDP-choline min^{-1} mg^{-1} for PG at pH 6.2 and 7.4, respectively, and represents the averages of 3 (PA) and 2 (PG) separate experiments. (Panel C) CT activity was measured in ADA buffer at pH 6.1 (closed symbols) and 7.2 (open symbols) Assays were performed at 20 °C . Maximal activity was 280 nmol of CDP-choline min^{-1} mg^{-1} at pH 6.1 and 400 nmol of CDP-choline min^{-1} mg^{-1} at 7.2. Results are the average of duplicate experiments.

enzyme requires additional interactions achieved only by lipids in the liquid-crystalline phase.

Effect of pH on Anionic Lipid-Stimulated Activity. To further explore the electrostatic nature of the interaction between CT and anionic lipids, the effect of pH on PA-stimulated activity was also examined (Figure 5A). The second proton on the phosphate group of PA has of pK_a of 8 (Tocanne & Teissié, 1990). Changing the pH from 7.4 to 6.2 would result in slightly less charge associated with PA. If the lipid negative charge alone dictates the CT−lipid interaction, this shift to lower pH should have weakened the

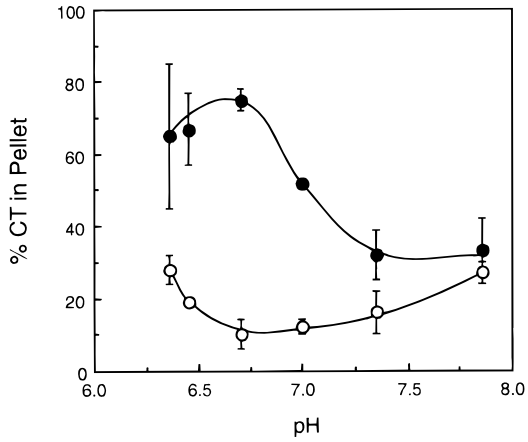

FIGURE 6: Effect of pH on CT binding. CT binding was measured in the presence of egg PC SLVs containing 0 (○) or 40 mol % PG (●) (600 μM total lipid). Greater than 90% of the lipid was in the pellet fraction. Data are (μg of CT in pellet/μg of CT in pellet + supernatant) $\times$ 100. Error bars represent the average of two sets of data.

potency of PA. However, Figure 5A shows that less PA was needed to stimulate CT at pH 6.2 than 7.4. Moreover, the charge on PG would not be affected by a change in pH, yet the same effect of pH was observed with PG as with PA (Figure 5B). Maximal activity of CT in the presence of PA or PG was decreased by approximately 50% at pH 6.2. Maximal activity in the presence of SUVs containing 10 mol % PA was 600 $\pm$ 20 nmol of CDP-choline min^{-1} mg^{-1} at pH 7.4 and 300 $\pm$ 20 nmol of CDP-choline min^{-1} mg^{-1} at pH 6.2. Activity in the presence of SUVs containing 10 mol % PG was 500 $\pm$ 10 nmol of of CDP-choline min^{-1} mg^{-1} at pH 7.4 and 300 $\pm$ 10 nmol of CDP-choline min^{-1} mg^{-1} at pH 6.2.

The shift to a lower lipid charge requirement for activation was also observed at pH 6.1 using ADA buffer (Figure 5C) and pH 5.7 using MES buffer (data not shown). These buffers have pK_as of 6.60 and 6.15, respectively. The ionic strength of the assays was kept constant. Although there were minor differences in the activation curves, depending on the buffer system employed, lower pH consistently reduced the concentration of PA or PG required for the activation. Decreasing the pH lower than 5.7 resulted in an almost complete loss of activity. Maximal activity in the presence of ADA buffer and SUVs containing 15 mol % PG was 400 nmol of CDP-choline min^{-1} mg^{-1} at pH 7.2 and 280 nmol of CDP-choline min^{-1} mg^{-1} at pH 6.1. Thus, it is likely that the pH change was directly affecting CT, *e.g.*, by titrating an acidic group involved in membrane binding or in catalysis, and that the decrease in the charge of PA was compensated by an increase in the positive charge of CT.

A role for an effect of pH on the catalytic domain was investigated using a CT mutant (Δ228) (Cornell *et al*., 1995) lacking the membrane binding domain and the phosphorylation domain, but containing the catalytic domain. Wild type and Δ228 were expressed in COS cells (Cornell *et al.*, 1995), and activity was analyzed in the cytosolic fraction. The activity of this mutant in the presence of 3.5 mol % PA (SUVs) was 360 $\pm$ 7 and 140 $\pm$ 20 nmol of CDP-choline min^{-1} mg^{-1} at pH 7.4 and 6.2, respectively.

If the pH change was directly affecting the membrane binding region of CT, decreasing the pH of the solution should increase the ability of CT to bind to vesicles

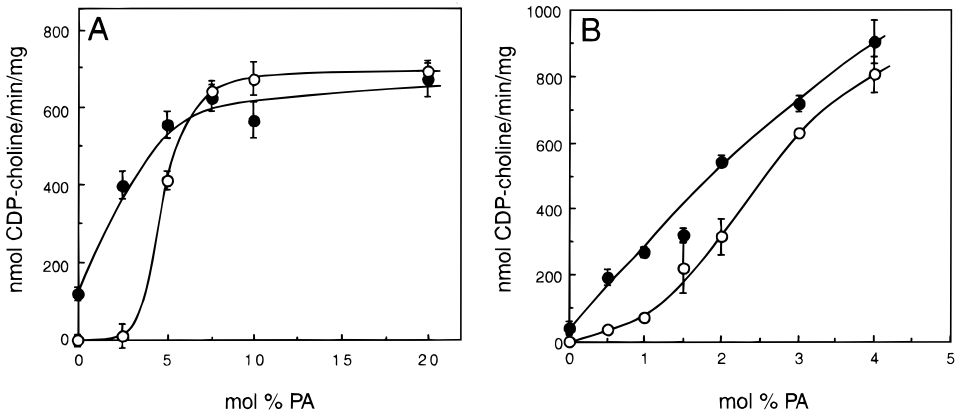

FIGURE 7: Synergistic activation of CT by phosphatidic acid and diacylglycerol. CT activity was measured in presence of egg PC SUVs (200 μM lipid) containing the indicated concentrations of PA without DG (○) or with (A) 2.5 mol % DG (●) or (B) 1 mol % DG (●). Error bars in panel A represent the standard error of the mean of six sets of data. Error bars in panel B represent the standard error of the mean of three sets of data. Notice that the scales of the *x* axes differ in panels A and B.

containing anionic lipid. As shown in Figure 6, sucrose-loaded vesicles (SLVs) containing 40 mol % PG bound 30% of the CT at pH 7.3 and 75% of the CT at pH 6.7. The change in pH had little effect on the binding of CT to SLVs composed of PC alone. This result directly implicates the membrane binding region of CT in the response to pH change.

Synergistic Interaction between Diacylglycerol and Anionic Lipids. The data in Figures 1 and 2 indicated that the mole percent of anionic phospholipid or DG required for full activation in lipid vesicles exceeds that found in nuclear membranes. We then assessed the effect of combinations of two activators. CT activity was stimulated by egg PC SUVs containing increasing mole percent of PA in the absence or presence of 2.5 mol % DG (Figure 7A). At concentrations <5 mol %, PA had little effect on the CT activity. Similarly, 2.5 mol % DG only slightly activated CT. However, the presence of 2.5 mol % DG with 2.5 mol % PA stimulated CT activity 20-fold over activity in the presence of 2.5 mol % PA alone (from 20 nmol of CDP-choline min^{-1} mg^{-1} to about 430 nmol of CDP-choline min^{-1} mg^{-1}). Thus, the effects of 2.5 mol % PA plus 2.5 mol % DG were synergistic. Lower concentrations of DG were also capable of interacting in a synergistic manner with PA to stimulate CT activity. Figure 7B shows the effect of 1 mol % DG in combination with 0−4 mol % PA. There was no significant stimulation of CT by 1 mol % DG alone. However, the combination of 1 mol % DG and 0.5 mol % PA stimulated CT activity 10-fold over activity in the presence of PA alone. The synergistic activation of CT was not restricted to stimulation by DG and PA. DG also synergized with other anionic phospholipids. CT activation by 2.5 mol % CL, PA, PG, PS, PI, or oleic acid or 0.25 mol % PIP in the absence and presence of 2.5 mol % DG is shown in Table 2. In all cases, the activation of CT by the combination of DG and an anionic lipid was greater than additive. PA had the greatest synergy with DG followed by PIP, PS, oleic acid, PI, PG, and CL. Synergism was observed only when vesicles were employed. No synergism of DG and anionic phospholipids was observed when analyzed as components of Triton X-100 mixed micelles (data not shown). There was no synergistic activation between oleic acid and anionic phospholipids in vesicles (data not shown). Cholesterol had no effect on CT activity, alone or in combination with a negatively charged lipid (data not shown). The effect of two anionic phospholipids in combination on CT activity was also examined and found to be additive (data not shown).

Table 2: Synergistic Effects of Diacylglycerol and Anionic Phospholipids on CT Activity[a]

lipid activator	sp act. without DG	*N*	sp act. with DG	*N*
			70 ± 10	55
CL	370 ± 40	3	700 ± 40	3
PA	20 ± 20	11	430 ± 20	8
PG	130 ± 20	22	370 ± 30	13
PI	50 ± 20	6	290 ± 20	6
PS	90 ± 20	11	380 ± 30	6
oleic acid	20 ± 5	3	190 ± 10	3
PIP	10 ± 10	3	270 ± 101	3

[a] CT activity (nmol of CDP-choline min^{-1} mg^{-1}) was measured in the presence of egg PC SUVs containing 2.5 mol % anionic lipids or oleic acid (except PIP) either without or with 2.5 mol % DG. The total lipid concentration was 200 μM. PIP was present at 0.25 mol % in egg PC SUVs without or with 2.5 mol % DG. *N* is the number of data points averaged, and the error is reported as the standard error of the mean.

DISCUSSION

Role of Electrostatic vs Hydrophobic Interactions. The electrostatic component in the activation of CT by vesicles containing anionic phospholipids was evidenced by the following: First, CT activity was enhanced by an increase in the negative charge associated with the phospholipid in SUVs. The order of potency for stimulation by anionic lipids was PIP > CL > PA > PG ≅ PS > PI ≫ PC. Second, increased ionic strength inhibited the effectiveness of anionic lipids as components of MLVs (Figure 4), large unilamellar vesicles (data not shown), and Triton X-100 mixed micelles (Cornell, 1991a). These results indicate an electrostatic component is involved in the activation of CT.

On the other hand, increasing ionic strength *enhanced* the effectiveness of anionic lipids and DG when tested as components of SUVs. While an increase in ionic strength decreases electrostatic interactions, it enhances hydrophobic interactions. These data indicate that hydrophobic interactions, rather than electrostatic, are more significant in the activation of CT by lipids when presented as SUVs. The differences in lipid packing properties of MLVs versus SUVs could account for these different responses. The enhanced hydrophobic driving force resulting from the increased medium ionic strength may be insufficient to overcome the

penetration barrier of planar MLVs, but sufficient to drive penetration of CT into the more loosely packed SUVs.

Hydrophobic interactions are also important in the activation of CT by MLVs. In the gel state, MLVs containing PG did not activate CT, suggesting that if CT could not insert into the membrane it could not be activated. CT was, however, electrostatically associated with these membranes. An electrostatic interaction with MLVs could be required to concentrate CT at the membrane surface, thereby increasing the probability of overcoming the penetration barrier, resulting in activation.

These data indicate that CT activation follows a two-step process (binding followed by intercalation), with a complex interplay between electrostatic and hydrophobic components. The importance of electrostatics depends on the lipid packing density of the membranes: the tighter the packing, the greater the role of electrostatics in binding. It is clear from all the data that intercalation (hydrophobic interaction) is required for conversion to the enzyme's active form.

Somewhat surprising was the observation that the magnitude of maximal activation was lower for PI or PIP than for the other acidic lipids. The negatively charged head groups of PI and PIP provide electrostatic binding with CT, but their bulkiness might interfere with intercalation into the bilayer core, which provides the hydrophobic stabilization required for activation of CT. In accordance with this proposal, PI was just as effective as PS and PG when tested as components of a Triton X-100 micelle, which is more highly curved and more loosely packed in the interfacial region than bilayer vesicles (Cornell, 1991a). Moreover, the partition coefficient of CT for PI vesicles was 3-fold lower than for PG or PS vesicles, as determined from the equilibrium distribution between membrane and aqueous phases using sucrose-loaded vesicles (Arnold and Cornell, unpublished results).

The Charge on CT Influences Activation by Membranes. There are several examples of pH changes affecting protein−lipid interactions. Tetanus toxin, which interacts with acidic lipids, binds more readily to membranes at an acidic pH. It was suggested that lowering the pH causes protonation of one or more groups on the toxin, leading to higher affinity for membranes (Schiavo *et al*., 1991). The protonation of acidic residues is also required for the insertion of influenza virus hemagglutinin into membranes (Yu *et al*., 1994). Similarly, cytochrome *c* had a higher affinity for CL-containing membranes at a lower pH. Lowering the pH resulted in an increase in the net positive charge on the protein, facilitating binding (Rytömaa *et al*., 1992; Rytömaa & Kinnunen, 1994). In our study, the pH was lowered from 7.4 to ~6 to reduce the negative charge on PA. We anticipated a reduced effectiveness of PA. However, lowering the pH *increased* the potency of PA. It is unlikely the increase was caused by a change in the charge on PA since PG-stimulated activity was affected in a similar manner. Instead, the reduction in pH may have affected the charge on CT by protonating one or more residues at the active site, or in the amphipathic membrane binding domain, or both.

Evidence in support of a change at the active site of CT includes 1) a decrease in maximal activity observed at pH 6.2 vs pH 7.4 and (2) a CT mutant, Δ228 (Cornell *et al*., 1995), lacking the membrane binding domain and the phosphorylation domain, but containing the catalytic domain, was less active at pH 6.2. Other data implicate a pH effect on the membrane binding domain. There was a reduction in the concentration of both PA and PG needed for activity at lower pH (lower membrane charge requirement). In addition, lower pH enhanced CT binding to vesicles containing PG. A model in which pH affects only the catalytic domain cannot account for these results. Rather the results argue favorably for a direct effect of pH on the membrane binding domain of CT. There are 1 histidine and 13 glutamate plus aspartate residues in the membrane binding domain. Protonation of one or more of these amino acids would increase the positive charge associated with this domain. Recently, Leenhouts *et al*., (1995) showed that membrane insertion of a peptide containing glutamic acid was enhanced by anionic phospholipids through protonation of the peptide's glutamyl group. Membrane insertion was related to a lower pH at the membrane interface, a result of the accumulation of protons at the negatively charged membrane surface. Thus, in addition to their role in promoting electrostatic interactions, the mechanism whereby anionic lipids promote CT binding and activation may involve the creation of a more hydrophobic lipid binding surface in CT. This provocative idea can be tested by examining the effects of mutations at specific acidic residues on the lipid specific activation. The recently solved NMR-derived structure for the membrane binding domain of CT bound to SDS reveals three interfacial glutamate residues (257, 268, and 279) which are prime candidates for mutation (S. Dunne, R. B. Cornell, J. Johnson, and A. Tracey, submitted for publication in *Biochemistry*).

Synergism between DG and Anionic Phospholipids. Synergistic interaction between lipids has been demonstrated for other proteins. The synergistic relationship between DG and PS has been well characterized for activation of protein kinase C (PKC) (Bell & Burns, 1991; Newton, 1993). DG and PA act synergistically to stimulate NADPH oxidase (Qualliotine-Mann *et al*., 1993). In addition, previous work by Sleight and Dao (1990) demonstrated a synergistic activation of CT by phosphatidylethanolamine and oleic acid. Our work clearly shows a synergistic interaction between DG and anionic lipids in the stimulation of CT, with the highest degree of synergy between DG and PA. The mechanism of synergism between DG and anionic lipids is unknown. Since a bilayer rather than micellar organization was required for the synergistic effects, a perturbation of bilayer packing is suggested. The activation of CT requires membrane intercalation which is facilitated by defects in the regular bilayer packing (Cornell, 1991b). It is interesting that DG promotes negative curvature, while the anionic lipids promote positive curvature. Both induce packing defects.

CT was activated nearly maximally at concentrations as low as 1 mol % DG and 2 mol % PA in PC SUVs. These concentrations approach those found in membranes of stimulated cells (Getz *et al*., 1968; Preiss *et al*., 1986; Farese *et al*., 1987; Leach *et al*., 1992). The synergism between DG and anionic lipids may have physiological relevance to the control of PC metabolism in cells. Stimulation of cells with growth factors, or other agonists which promote PC degradation, leads to increases in levels of DG, PA, and arachidonic acid. These same agonists also stimulate PC synthesis (Billah & Anthes, 1990). PC synthesis may be stimulated by the synergistic effects of DG, PA, and/or arachidonic acid on the activity of CT, the rate-limiting enzyme.

Mechanism of CT−Lipid Interactions. How does CT compare to other amphitropic proteins in its interaction with

lipids? A mechanism emerging from studies on the membrane interaction of the synapsins (Benfenati *et al*., 1989a,b; Valtorta *et al*., 1992) and SecA (Breukink *et al*., 1992; Hendrick & Wickner, 1991; Ulbrandt *et al*., 1992) involves an initial electrostatic interaction, which locates the protein at the surface, followed by a hydrophobic insertion of the protein into the membrane core. CT also appears to follow this mechanism. These proteins interact electrostatically and hydrophobically with anionic phospholipids with no apparent specificity toward phospholipid head group.

The mechanism of CT interaction with membranes differs from that employed by PKC or prothrombin, which show specific requirements for PS (Bell & Burns, 1991; Newton, 1993; Gerads *et al*., 1990; Lentz *et al*., 1991; Orr & Newton, 1992). Like PKC, CT activity is affected by the synergistic interaction of DG with anionic lipids. The apparent cooperativity is probably not a result of specific binding sites on CT since there is a lack of specificity in anionic lipid stimulation. Rather, the sigmoidal curve may result from the simultaneous increase in surface charge and vesicle curvature with increase in mole percent anionic phospholipid, which perturbs phospholipid packing. The interaction of DG with the two proteins also appears to be different. Protein kinase C has a specific binding site for DG, a cysteine-rich motif (Zhang *et al*., 1995), which is not present in CT. PKC is activated by Triton X-100 mixed micelles containing PS and 1 mol % DG whereas CT is not activated by DG in Triton X-100 micelles. Thus, the activation of CT by DG is more likely due to DG-induced perturbation of membrane lipid packing, similar to the interaction of apolipophorin III and DG (Soulages *et al*., 1995).

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REFERENCES

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466−468.
Bell, R. M., & Burns, D. J. (1991) *J. Biol. Chem. 266*, 4661−4664.
Benfenati, F., Greengard, P., Brunner, J., & Bähler, M. (1989a) *J. Cell Biol. 108*, 1851−1862.
Benfenati, F., Bähler, M., Jahn, R., & Greengard, P. (1989b) *J. Cell Biol. 108*, 1863−1872.
Bhat, B. G., Wang, P., & Coleman, R. A. (1995) *Biochemistry 34*, 11237−11244.
Billah, M. M., & Anthes, J. C. (1990) *Biochem. J. 269*, 281−291.
Breukink, E., Demel, R. A., de Korte-Kool, G., & de Kruijff, B. (1992) *Biochemistry 31*, 1119−1124.
Choy, P. C., & Vance, D. E. (1978) *J. Biol. Chem. 253*, 5163−5167.
Choy, P. C., Farren, S. B., & Vance, D. E. (1979) *Can. J. Biochem. 57*, 605−612.
Cornell, R. B. (1989) *J. Biol. Chem. 264*, 9077−9082.
Cornell, R. B. (1991a) *Biochemistry 30*, 5873−5880.
Cornell, R. B. (1991b) *Biochemistry 30*, 5881−5888.
Cornell, R. B., & Vance, D. E. (1987) *Biochim. Biophys. Acta 919*, 26−36.
Cornell, R. B., Kalmar, G. B., Kay, R. J., Johnson, M. A., Sanghera, J. S., & Pelech, S. L. (1995) *Biochem. J. 310*, 699−708.
Craig, L., Johnson, J. E., & Cornell, R. B. (1994) *J. Biol. Chem. 269*, 3311−3317.
Farese, R. V., Konda, T. S., Davis, J. S., Standaert, M. L., Pollet, R. J., & Cooper, D. R. (1987) *Science 236*, 586−589.
Feldman, D. A., & Weinhold, P. A. (1987) *J. Biol. Chem. 262*, 9075−9081.
Feldman, D. A., Kovac, C. R., Dranginis, P. L., & Weinhold, P. A. (1978) *J. Biol. Chem. 253*, 4980−4986.
Feldman, D. A., Brubaker, P. G., & Weinhold, P. A. (1981) *Biochim. Biophys. Acta 665*, 53−59.
Gerads, I., Govers-Riemslag, J. W. P., Tans, G., Zwaal, R. F. A., & Rosing, J. (1990) *Biochemistry 29*, 7967−7974.
Getz, G. S., Bartley, W., Lurie, D., & Notton, B. M. (1968) *Biochim. Biophys. Acta 152*, 325−339.
Hendrick, J. P., & Wickner, W. (1991) *J. Biol. Chem. 266*, 24596−24600.
Houweling, M., Jamil, H., Hatch, G. M., & Vance, D. E. (1994) *J. Biol. Chem. 269*, 7544−7551.
Jamil, H., Hatch, G. M., & Vance, D. E. (1993) *Biochem. J. 291*, 419−427.
Johnson, J. E., & Cornell, R. B. (1994) *Biochemistry 33*, 4327−4335.
Johnson, J. E., Kalmar, G. B., Sohal, P. S., Walkey, C. J., Yamashita, S., & Cornell, R. B. (1992) *Biochem. J. 285*, 815−820.
Kolesnick, R. N., & Hemer, M. R. (1990) *J. Biol. Chem. 265*, 10900−10904.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Leach, K. L., Ruff, V. A., Jarpe, M. B., Adams, L. D., Fabbro, D., & Raben, D. M. (1992) *J. Biol. Chem. 267*, 21816−21822.
Leenhouts, J. M., van den Wijngaard, P. W. J., de Kroon, A. I. P. M., & de Kruijff, B. (1995) *FEBS Lett. 370*, 189−192.
Lentz, B. R., Wu, J. R., Sorrentino, A. M., & Carleton, J. N. (1991) *Biophys. J. 60*, 942−951.
MacDonald, J. I. S., & Kent, C. (1993) *Protein Expression Purif. 4*, 1−7.
Myher, J. J., & Kuksis, A. (1979) *Can. J. Biochem. 57*, 117- 124.
Newton, A. C. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 1−25.
Orr, J. W., & Newton, A. C. (1992) *Biochemistry 31*, 4667−4673.
Pelech, S. L., Pritchard, P. H., Brindley, D. N., & Vance, D. E. (1983) *J. Biol. Chem. 258*, 6782−6788.
Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E., & Bell, R. M. (1986) *J. Biol. Chem. 261*, 8597−8600.
Qualliotine-Mann, D., Agwu, D. E., Ellenburg, M. D., McCall, C. E., & McPhail, L. C. (1993) *J. Biol. Chem. 268*, 23843−23849.
Rytömaa, M., & Kinnunen, P. K. J. (1994) *J. Biol. Chem. 269*, 1770−1774.
Rytömaa, M., Mustonen, P., & Kinnunen, P. K. J. (1992) *J. Biol. Chem. 267*, 22243−22248.
Schiavo, G., Demel, R., & Montecucco, C. (1991) *Eur. J. Biochem. 199*, 705−711.
Slack, B. E., Breu, J., & Wurtman, R. J. (1991) *J. Biol. Chem. 266*, 24503−24508.
Sleight, R. G., & Dao, H. N. T. (1990) *Lipids 25*, 100−107.
Sohal, P. S., & Cornell, R. B. (1990) *J. Biol. Chem. 265*, 11746−11750.
Soulages, J. L., Salamon, Z., Wells, M. A., & Tollin, G. (1995) *Proc. Natl. Acad. Sci. USA 92*, 5650−5654.
Tocanne, J.-F., & Teissié, J. (1990) *Biochim. Biophys. Acta 1031*, 111−142.
Ulbrandt, N. D., London, E., & Oliver, D. B. (1992) *J. Biol. Chem. 267*, 15184−15192.
Utal, A. K., Jamil, H., & Vance, D. E. (1991) *J. Biol. Chem. 266*, 24084−24091.
Valtorta, F., Benfenati, F., & Greengard, P. (1992) *J. Biol. Chem. 267*, 7195−7198.
Wang, Y., & Kent, C. (1995) *J. Biol. Chem. 270*, 18948−18952.
Wang, Y., Sweitzer, T. D., Weinhold, P. A., & Kent, C (1993) *J. Biol. Chem 268*, 5899−5904.
Yang, W., Boggs, K. P., & Jackowski, S. (1995) *J. Biol. Chem 270*, 23951−23957.
Yu, Y. G., King, D. S., & Shin, Y.-K. (1994) *Science 266*, 274−276.
Zhang, G., Kazanietz, M., Blumberg, P., & Hurley, J. (1995) *Cell 81*, 917−924.

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