# Selective Phosphorylation of Cationic Polypeptide Aggregated with Phosphatidylserine/Diacylglycerol/$Ca^{2+}$/Detergent Mixed Micelles by $Ca^{2+}$-Independent but Not $Ca^{2+}$-Dependent Protein Kinase C Isozymes

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ABSTRACT: Mixed micelles containing Nonidet P40 (NP-40) (829 $\mu$M or 4.8 mM), phosphatidylserine (PS) (14.5 or 8 mol %), and 1,2-diacylglycerol (DG) (0.5 or 1 mol %) when preincubated with protein kinase C (PKC) assay mixture containing cationic substrate and $CaCl_2$ (400 $\mu$M) formed aggregates in a time-, temperature-, and substrate concentration-dependent manner with a $t_{1/2}$ ~3−12 min (22 °C). Concomitant with the formation of these aggregates there was a substantial loss of substrate phosphorylation catalyzed by the $Ca^{2+}$-dependent PKC $\alpha$, $\beta$, and $\gamma$ but not the $Ca^{2+}$-independent PKC $\delta$ and $\epsilon$. All cationic PKC substrates tested, neurogranin peptide analog$_{29-47}$, neurogranin, and histone III-S, formed aggregates with PS/DG/NP-40/$Ca^{2+}$ mixed micelles in a time-dependent fashion. The poly(cationic−anionic) PKC substrate protamine sulfate also forms aggregates with the mixed micelles in the presence of $Ca^{2+}$, but without affecting the substrate phosphorylation by the kinase. Under similar conditions, but at 4 °C, neither aggregation nor loss of cationic substrate phosphorylation was observed. Another nonionic detergent, octyl glucoside, behaved similarly to NP-40. Phosphatidylinositol (PI) and phosphatidylglycerol like PS, were effective in forming aggregates with NP-40/cationic polypeptide/DG/$Ca^{2+}$ as monitored by light scattering, yet without affecting substrate phosphorylation. Phosphorylation of cationic substrates by M-kinase, derived from trypsinized PKC $\beta$, was also greatly diminished by the aggregation. In contrast, [$^3$H]phorbol 12,13-dibutyrate binding to PKC $\beta$ was unaffected. Formation of the aggregates that were selectively utilized by the $Ca^{2+}$-independent PKCs was dependent on the ratio of cationic substrate to the number of mixed micelles. Lipid vesicles containing PS and DG or a phospholipid composition mimicking that of the cell membrane, phosphatidylcholine/phosphatidylethanolamine/sphingomyelin/PI/PS (247, 127, 120, 47, and 40 $\mu$g/mL), 80 $\mu$g/ml DG, and $Ca^{2+}$, did not form such aggregates in the absence of nonionic detergent. These results indicate that the aggregation of cationic polypeptide with nonionic detergent/PS/DG/$Ca^{2+}$ renders the substrate phosphorylation sites inaccessible to the $Ca^{2+}$-dependent subgroup of PKCs. The current findings suggest caution in the use of cationic polypeptides that form aggregates with phospholipid/nonionic detergent/$Ca^{2+}$ mixed micelles in the assay of $Ca^{2+}$-dependent PKCs.

The protein kinase C (PKC)[1] family of serine/threonine-specific protein kinases consists of at least 11 members, $\alpha$, $\beta1$, $\beta2$, $\gamma$, $\delta$, $\epsilon$, $\eta$, $\theta$, $\zeta$, $\iota$, and $\mu$, and can be divided into four groups: group A (conventional), $\alpha$, $\beta1$, $\beta2$, and $\gamma$, which are activated by $Ca^{2+}$, phosphatidylserine (PS), and diacylglycerol (DG); group B (novel), $\delta$, $\epsilon$, $\eta$, and $\theta$, which are $Ca^{2+}$-independent, but are activated by PS and DG; group C (atypical), $\zeta$ and $\iota$, which are $Ca^{2+}$- and DG-independent but are activated by PS; and group D, $\mu$, which is $Ca^{2+}$-independent but is PS- and DG-dependent and has an extended N-terminus containing a signal peptide and transmembrane domain [Selbie et al., 1993; Johannes et al., 1994; for review, cf. Nishizuka (1992) and Mahoney and Huang (1994)]. Mouse PKC $\lambda$, which belongs to the group C PKCs, is 98% homologous to human PKC $\iota$ and hence appears to be the mouse homologue of human PKC $\iota$ (Akimoto et al., 1994). Recently, chimeric PKC isoforms which contain a homologous PKC catalytic domain and leucine zipper domains have been reported (Dorow et al., 1993; Mukai & Ono, 1994).

Group A PKCs, which have been the most studied PKCs, and their cationic substrates undergo complex interactions with the PKC activators PS, DG, and $Ca^{2+}$. In the presence of $Ca^{2+}$, PS and DG bind to the N-terminal regulatory domain of PKC, dissociating the pseudosubstrate inhibitory region from the C-terminal catalytic domain, thereby allowing for catalytic activity (House & Kemp, 1987; Pears et al., 1990; Orr et al., 1992). Protamine sulfate, an activator-independent substrate of PKC, and short-chain PCs can activate PKC by a similar mechanism (Orr & Newton, 1994). Polyanionic phospholipid vesicles interact with polycationic substrates such as histone (Bazzi & Nelsestuen, 1987a), and aggregation has been correlated with PKC activation [Bazzi & Nelsestuen, 1987b, 1992; for review, cf. Nelsestuen and Bazzi (1991)]. In contrast to PS being an activator of PKC, in the absence of $Ca^{2+}$, PS interacts with both the regulatory and

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[1] Abbreviations: PKC, protein kinase C; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; SM, sphingomyelin; DG, diacylglycerol; NP-40, Nonidet P-40; PKA, cAMP-dependent protein kinase; PDBu, phorbol 12,13-dibutyrate; MBP, myelin basic protein; MBP$_{4-14}$, myelin basic protein peptide$_{4-14}$; NG(F-W)$_{29-47}$, neurogranin peptide analog$_{29-47}$; SDS, sodium dodecyl sulfate.

catalytic domains of PKC and irreversibly inactivates the kinase (Huang & Huang, 1990). PS-containing lipid vesicles can interact with an activate PKC in the presence of $Ca^{2+}$, yet since small unilamellar vesicles contain ~4000 phospholipid molecules, it is difficult to impossible to examine systematically the stoichiometry and interactions of lipid with PKC under these conditions [for review, cf. Bell and Burns (1991)]. The mixed micelle system, developed by Bell and co-workers (Hannun et al., 1985), overcomes these limitations by providing a neutral scaffold of nonionic detergent, into which small numbers of lipid activator molecules can be inserted. By using the mixed micelle system it was demonstrated that the interaction with and regulation of PKC by PS is a highly cooperative process (Hannun et al., 1985). Approximately 6−12 molecules of PS in the mixed micelle can cooperatively and maximally activate a single PKC molecule on a single mixed micelle with a Hill coefficient of 8−11, whereas only a single molecule of DG is necessary to activate a single PKC in the presence of PS and $CaCl_2$ (Hannun et al., 1985; Newton & Koshland, 1989). The interaction of $Ca^{2+}$ with PKC in the presence of PS is also highly cooperative with a maximum of ~8 $Ca^{2+}$ molecules bound per PKC molecule (Bazzi & Nelsestuen, 1990). PKC can also bind to other acidic phospholipids and depending on the conditions can be poorly or partially activated (Kaibuchi et al., 1981; Asaoka et al., 1988; Hannun & Bell, 1986; Hannun et al., 1986; Huang et al., 1988; Orr et al., 1992; Orr & Newton, 1992a,b; Bazzi & Nelsestuen, 1987b, 1992). PA, PE, oleic acid, and lyso-PS in combination with PS can supplement the requirement for PS by lowering the amount of PS required for activation in the mixed micellar system (Lee & Bell, 1992). There is a high degree of specificity, including stereoselectivity, for the phosphate, carboxyl, and amino groups of PS in the activation but not for the binding of PKC to lipid in the mixed micellar system (Lee & Bell, 1989). The binding of PKC to PS is a strong interaction with a $K_d$ = 5 nM (Nelsestuen & Bazzi, 1991).

Here we examine additional details in the complex interactions between PS, $Ca^{2+}$, cationic substrates, and PKC. We demonstrate that cationic polypeptide can form aggregates with mixed micelles containing nonionic detergent, $Ca^{2+}$, PS, and DG in a time-, temperature-, and polypeptide concentration-dependent fashion. Concomitant with the formation of these aggregates there was a progressive loss of substrate phosphorylation catalyzed by the $Ca^{2+}$-dependent but not the $Ca^{2+}$-independent PKC isozymes. Although the phosphorylation of cationic substrates associated with these aggregates by the $Ca^{2+}$-dependent group A PKCs is retarded, phorbol ester binding to the regulatory domain is not affected. The current study demonstrates that the interaction of cationic PKC substrates with PS/DG/$Ca^{2+}$ is required for the stimulation of the kinase, yet aggregation of these components in the presence of nonionic detergent progressively hinders the catalytic domain of the $Ca^{2+}$-dependent PKC from phosphorylating the cationic substrate. The current findings may be applicable for selective measurement of the $Ca^{2+}$-independent PKC activities by using cationic substrates aggregated with PS/$Ca^{2+}$/NP-40 mixed micelles.

## EXPERIMENTAL PROCEDURES

*Materials.* Commercial products utilized were as follows: histone III-S, myelin basic protein, kemptide, protamine sulfate, and cAMP-dependent protein kinase (beef heart) from Sigma Chemical; [$\gamma$-$^{32}$P]ATP (28 Ci/mmol) and [$^3$H]phorbol 12,13-dibutyrate (PDBu) from DuPont−New England Nuclear; phorbol 12,13-dibutyrate from LC Services (Woburn, MA); phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylinositol (PI), 1,2-dioleoylglycerol, sphingomyelin (SM), and phosphatidylethanolamine (PE) from Avanti Polar Lipids (Alabaster, AL); protein determination reagent from Bio-Rad; GF/C filters (25 mm) and P81 phosphocellulose paper from Whatman; rat neurogranin peptide analog (NG(F-W)$_{29-47}$), AAAKIQASWRGHMARKKIK, and myelin basic protein peptide$_{4-14}$ (MBP$_{4-14}$), QKRPSQRSKYL, from Research Genetics (Huntsville, AL); Nonidet P−40 (NP−40) from Pharmacia-LKB; octyl glycoside, phospholipase C (*Bacillus cereus*) and diacylglycerol kinase (*Escherichia coli*) from Calbiochem; histone H1 and [Ser$^{25}$]-PKC-$\alpha$ pseudosubstrate peptide$_{19-31}$ from Gibco/BRL; and the catalytic subunit of cAMP-dependent protein kinase from Promega. All other chemicals were reagent grade or of higher purity.

*Methods.* PKC $\alpha$, $\beta$, and $\gamma$, corresponding to PKC types III, II, and I, respectively, were purified from rat brain as described (Huang et al., 1987). Recombinant PKC $\delta$ and $\epsilon$ were expressed in Sf9 insect cells and purified as described (Kazanietz et al., 1993). M-kinase, the catalytic domain of PKC, was prepared from PKC $\beta$ (Huang & Huang, 1986). PKC activity was assayed using mixed micelles containing NP-40, PS, and DG or octyl glucoside, PS, and DG or lipid vesicles containing PS and DG or PC/PE/SM/PI/PS and DG. It should be noted that NP-40 and Triton X-100, which has been used in many previous studies, are both *p-tert*-octylphenol poly(ethylene glycol ether)$_n$ with NP-40 $n$ = 9 and Triton X-100 $n$ = 9−10. PKC assays contained 30 mM Tris HCl (pH 7.5), 6 mM magnesium acetate, 25 $\mu$M [$\gamma$-$^{32}$P]-ATP (1000-3000 cpm/pmol), variable substrate, 400 $\mu$M $CaCl_2$ or 1 mM EGTA, 0.5−1 $\mu$g/mL PKC $\beta$, and one of the following mixtures: 829 $\mu$M NP-40, 14.5 mol % PS, and 0.5 mol % DG (Huang et al., 1988); 4.8 mM NP-40, 8 mol % PS, and 1 mol % DG (Hannun et al., 1985); 40 mM octyl glycoside, 8 mol % PS, and 1 mol % DG (Hannun et al., 1986); 40 $\mu$g/mL PS and 2 $\mu$g/mL DG; or a phospholipid composition mimicking that of the cell membrane, PC/PE/SM/PI/PS (247, 127, 120, 47, and 40 $\mu$g/mL) and 80 $\mu$g/mL DG (Mato, 1990), unless otherwise noted. Reactions were carried out at 22 °C for 2−6 min, unless otherwise noted, in a final volume of 25 $\mu$L, and $^{32}P_i$ incorporation was determined, as previously described, using P81 phosphocellulose paper (Roskoski, 1983). Stocks of phospholipid/DG were prepared by drying mixtures in $CHCl_3$ under a $N_2$ stream; lipids were resuspended in 10-fold concentrated detergent or 20 mM Tris HCl (pH 7.5), vortexed, and sonicated (2 min, bath sonicator). Light scattering measurements were carried out using a Hewlett-Packard 8452A photo-diode array spectrophotometer at 300 nm. Neurogranin was purified from rat brain as described (Huang et al., 1993). DG species were generated from brain PC and liver PI using phospholipase C (*B. cereus*) in an ether/aqueous two-phase system and were isolated as previously described (Mavis et al., 1972). The averaged molecular weights of the PC- and PI-derived DGs were estimated as 620. No significant oxidation of the generated DGs was observed (Klein, 1970) and 1,2→1,3-DG isomerization was determined before use (Go et al., 1987). Any stock solution of 1,2-DG with significant isomerization was not used. DG

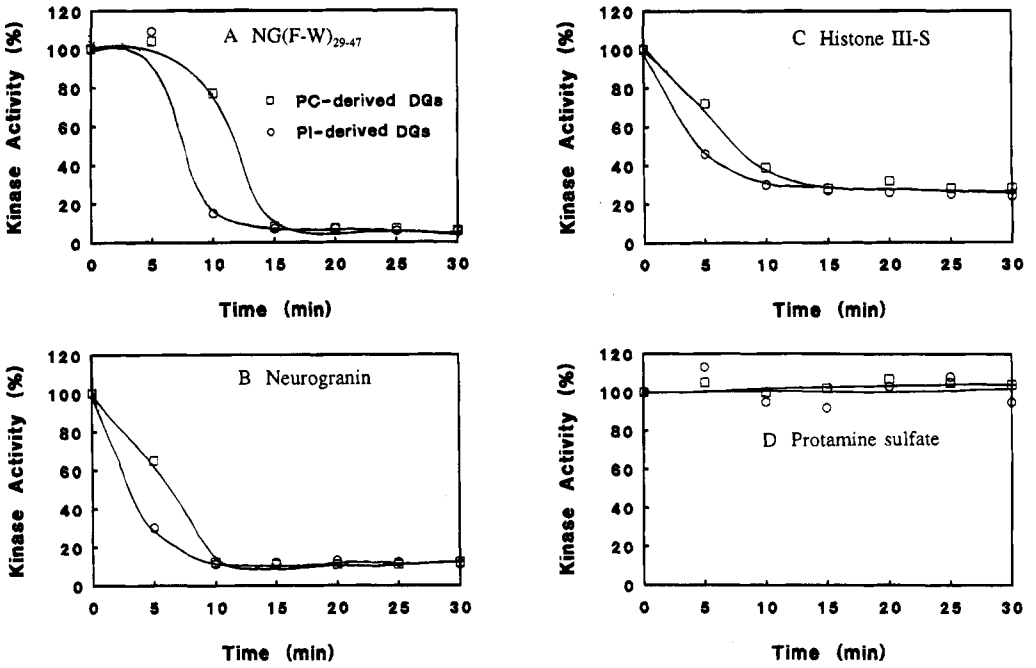


FIGURE 1: Time-dependent loss of substrate phosphorylation with PKC using mixed micelles containing NP-40, PS, and DG. PKC assay mixture containing (A) NG(F-W)$_{29-47}$ peptide (10 $\mu$M), (B) neurogranin (10 $\mu$M), (C) histone III-S (0.2 mg/mL), or (D) protamine sulfate (1 mg/mL) in the presence of 400 $\mu$M $CaCl_2$ was preincubated (22 °C) with mixed micelles containing 829 $\mu$M NP-40, 14.5 mol % PS, and 0.5 mol % DG for the indicated times, after which reactions were started by addition of PKC $\beta$ (0.8 $\mu$g/mL). Diacylglycerols derived from brain phosphatidylcholine (squares) or liver phosphatidylinositol (circles) were employed. In all experiments, similar results were obtained in several independent experiments.

was quantitated by using diacylglycerol kinase in a NP-40 mixed micelle assay (Preiss et al., 1986, 1987). SDS–polyacrylamide gel electrophoresis was performed as described (Laemmli, 1970). [$^3$H]PDBu binding was determined using a modified poly(ethylene glycol) precipitation method (Huang & Huang, 1986). [$^3$H]PDBu binding was performed by preincubating mixed micelles containing 829 $\mu$M NP-40 and 14.5 mol % PS with assay mixture containing 30 mM Tris HCl (pH 7.5), 6 mM MgAc, 25 $\mu$M ATP, 0.5 mg/mL BSA, 400 $\mu$M $CaCl_2$, 0.2 mg/mL histone III-S, and 27 nM [$^3$H]PDBu (100 $\mu$L final) for the indicated times (22 °C). [$^3$H]PDBu binding was initiated by addition of PKC $\beta$ (50–200 ng) and incubation was carried out at 4 °C (30 min). Protein was then precipitated by addition of 30% poly(ethylene glycol) 8000 (0.5 mL) followed by incubation at 4 °C (30 min). Reaction mixtures were vacuum-filtered on GF/C filters, and the filters were counted in the presence of liquid scintillation cocktail. The data presented are representative of results obtained in several independent experiments.

## RESULTS

Measurement of PKC $\beta$ activity with mixed micelles containing NP-40, PS, and DG previously preincubated at 22 °C with substrate histone III-S, neurogranin, or NG(F-W)$_{29-47}$ peptide in the presence of $CaCl_2$ (400 $\mu$M) resulted in a time-dependent loss in substrate phosphorylation with a $t_{1/2}$ ~3–12 min, depending on the substrate (Figure 1, panels A, B, and C). In contrast, under the same preincubation conditions with protamine sulfate (1 mg/mL) as substrate, there was no time-dependent decrease in the kinase activity (Figure 1D). It is apparent that during preincubation selective PKC substrates interact with lipid cofactors, nonionic detergent, and $Ca^{2+}$ to form complexes that either are poorly recognized by PKC or inhibit the kinase. Since oxidation of polyunsaturated fatty acids in DG is a potential hazard in causing a loss of the kinase activity, we compared the effects of DGs derived from PC and PI, which contain low and high levels of arachidonic acid, respectively, in forming the inhibitory complexes. DGs derived from PC and PI yielded slightly different time-dependent loss of substrate phosphorylation curves under these conditions; DGs derived from PI were more potent than those from PC. Unsaturated 1,2-dioleoylglycerol yielded similar results as the DGs derived from PI, and saturated 1,2-dioctanoylglycerol resembled those DGs from PC (data not shown). In spite of the minor differences in the effectiveness of these various DGs in forming the inhibitory complexes, oxidation of unsaturated fatty acid is not likely the prime cause of the observed responses.

Inhibition of substrate phosphorylation as determined by using substrate preincubated with mixed micelles containing NP-40, PS, DG, and $Ca^{2+}$ is temperature- and substrate concentration-dependent (Figure 2). Assay of PKC at 30 °C (2 min) with histone III-S preincubated with mixed micelles containing 829 $\mu$M NP-40, 14.5 mol % PS, and 0.5 mol % DG or 4.8 mM NP-40, 8 mol % PS, and 1 mol % DG (Hannun et al., 1985) in the presence of 400 $\mu$M of $CaCl_2$ at 4 °C resulted in little or no time-dependent loss of substrate phosphorylation (Figure 2A,B). In contrast, under identical conditions except that preincubation was carried out at 22 °C, a time- and substrate concentration-dependent loss of substrate phosphorylation was observed (Figure 2C,D). A more rapid loss of substrate phosphorylation was observed at the lowest histone III-S concentration (0.2 mg/mL), and increasing the concentration to 3 and 6 mg/mL for the low or high [NP-40] conditions, respectively, completely eliminated the effect (Figure 2C,D). In the case of high [NP-40] (4.8 mM NP-40, 8 mol % PS, and 1 mol % DG), it took higher concentrations of substrate to reverse

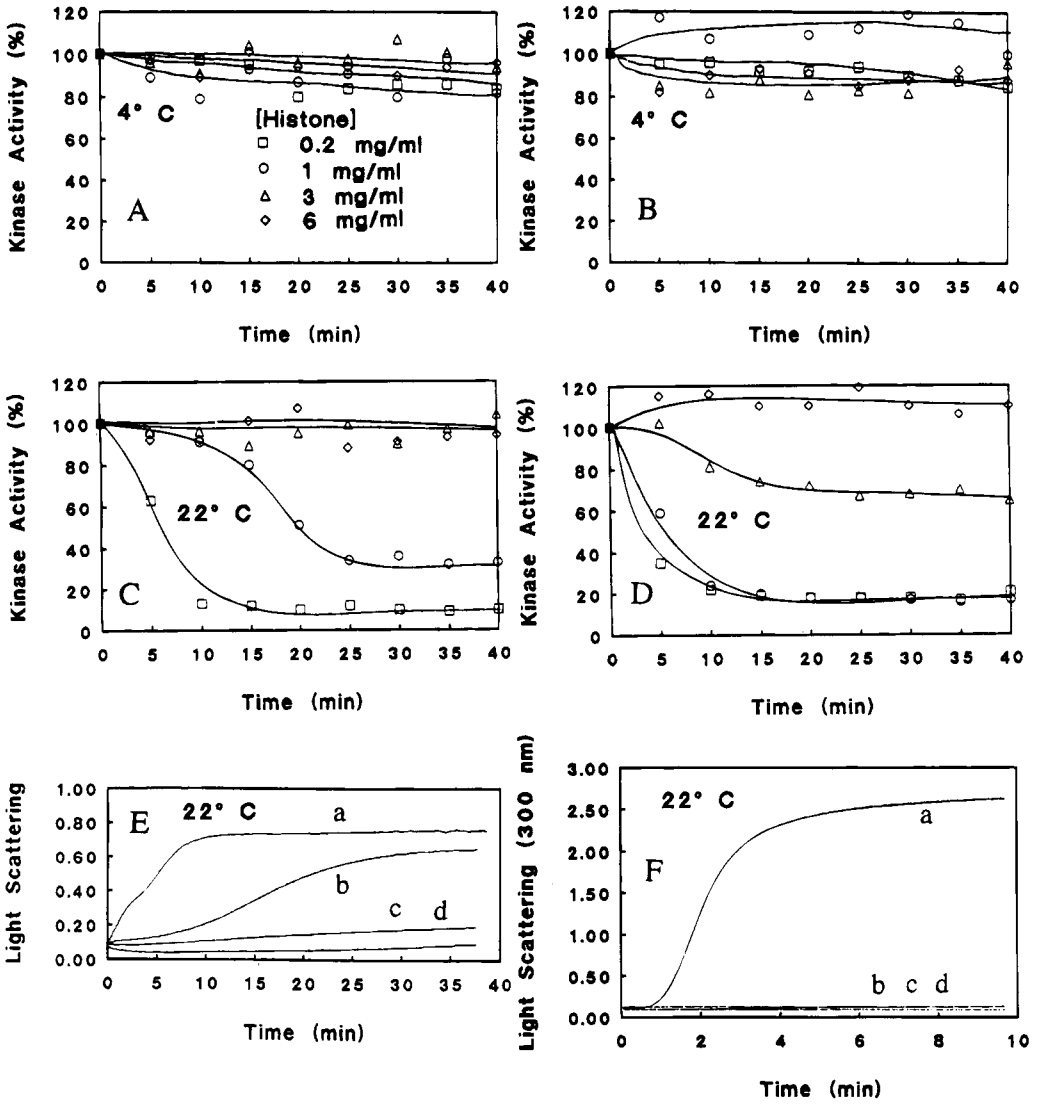


FIGURE 2: Temperature and substrate concentration dependence of loss of substrate phosphorylation with PKC using mixed micelles containing NP-40, PS, and DG. (A–D) Kinase reactions. PKC assay mixture containing 0.2 mg/mL (squares), 1 mg/mL (circles), 3 mg/mL (triangles), or 6 mg/mL (diamonds) histone III-S in the presence of 400 $\mu$M $CaCl_2$ was preincubated with mixed micelles containing 829 $\mu$M NP-40, 14.5 mol % PS, and 0.5 mol % 1,2 dioleoylglycerol (A, C) or 4.8 mM NP-40, 8 mol % PS, and 1 mol % 1,2 dioleoylglycerol (B, D) at 4 °C (A, B) or 22 °C (C, D) for the indicated times. Kinase reactions were started by addition of PKC $\beta$ (0.8 $\mu$g/mL) and carried out for 2 min at 30 °C. (E, F) Parallel light scattering measurements. (E) PKC assay mixture containing 0.2 mg/mL (a), 1 mg/mL (b), 3 mg/mL (c), or 6 mg/mL (d) histone III-S in the presence of 400 $\mu$M $CaCl_2$ was incubated with mixed micelles containing 829 $\mu$M NP-40, 14.5 mol % PS, and 0.5 mol % 1,2 dioleoylglycerol for the indicated times at 22 °C. (F) PKC assay mixture containing 0.2 mg/mL histone III-S and 400 $\mu$M $CaCl_2$ (a), 0.2 mg/mL histone III-S and 1 mM EGTA (b), 1 mM EGTA (c), or 400 $\mu$M $CaCl_2$ (d) was incubated with mixed micelles containing 4.8 mM NP-40, 8 mol % PS, and 1 mol % 1,2 dioleoylglycerol for the indicated times at 22 °C. Note the different time and light scattering scales in panels E and F.

the effect as compared to low [NP-40] conditions (829 $\mu$M NP-40, 14.5 mol % PS, and 0.5 mol % DG) (Figure 2C,D). Similar experiments conducted at 30 °C yielded similar yet more rapid loss in substrate phosphorylation compared to that shown in Figure 2C,D (data not shown). Light scattering experiments conducted on PKC assay mixture containing histone III-S and $CaCl_2$ preincubated with mixed micelles containing NP-40, PS, and DG, at 22 °C, indicated a similar time course for aggregation as that for loss of substrate phosphorylation (Figure 2E,F). Similar to the loss of substrate phosphorylation, aggregation was substrate concentration-dependent. At high histone III-S levels (3 and 6 mg/mL) aggregation was essentially totally inhibited (Figure 2E). Aggregation of the mixed micelles in the presence of cationic substrate and $CaCl_2$ proceeded with an initial lag phase followed by rapid aggregation; this suggests a mechanism of slow nucleation followed by rapid elongation/aggregation (Figure 2E,F). In the case of high [NP-40] (4.8 mM NP-40, 8 mol % PS, and 1 mol % DG), aggregation was supported synergistically by $CaCl_2$ (400 $\mu$M) and histone III-S (0.2 mg/mL); no aggregation was observed with $CaCl_2$ (400 $\mu$M), 1 mM EGTA, or histone III-S (0.2 mg/mL) in the presence of 1 mM EGTA (Figure 2F). Similar results were obtained with low [NP-40] (829 $\mu$M NP-40, 14.5 mol % PS, and 0.5 mol % DG) with the exception that histone III-S in the presence of EGTA supported weak aggregation (data not shown). Both aggregation and loss of substrate phosphorylation were more rapid at the higher [NP-40] (essentially complete in 5 min) relative to the lower [NP-40] conditions (essentially complete in 10 min) with 0.2 mg/mL histone III-S as substrate (Figure 2C–F). Light scattering experiments identical to those of Figure 2F, except

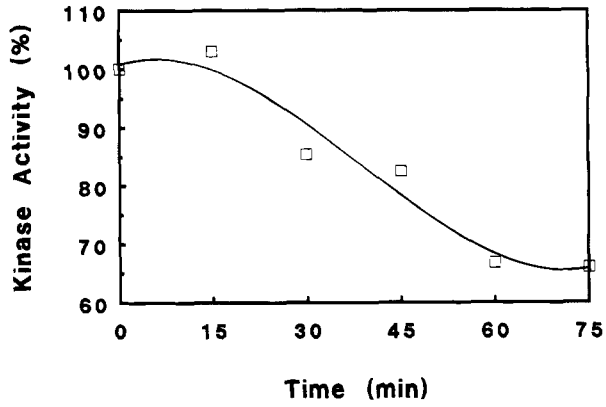


FIGURE 3: Time-dependent loss of substrate phosphorylation with PKC using mixed micelles containing octyl glucoside, PS, and DG. PKC assay mixture containing 1 mg/mL histone III-S and 400 $\mu$M $CaCl_2$ was preincubated with mixed micelles containing 40 mM octyl glucoside, 8 mol % PS, and 1 mol % 1,2 dioleoylglycerol for the indicated times at 22 °C. Kinase reactions were started by addition of PKC $\beta$ (0.8 $\mu$g/mL) and carried out for 6 min (22 °C).

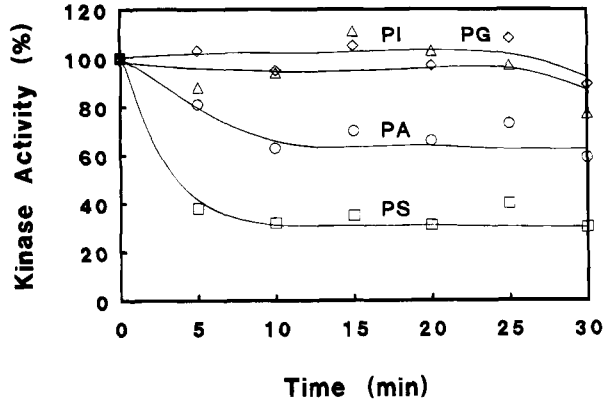


FIGURE 4: Phospholipid dependence of the time-dependent loss of substrate phosphorylation with PKC using mixed micelles containing NP-40, phospholipid, and DG. PKC assay mixture containing 0.2 mg/mL histone III-S and 400 $\mu$M $CaCl_2$ was preincubated (22 °C) with mixed micelles containing 829 $\mu$M NP-40, 4 mol % DG, and 14.5 mol % PS (squares), PA (circles), PI (triangles), or PG (diamonds) for the indicated times after which reactions were started by addition of PKC $\beta$ (0.8 $\mu$g/mL). The relative kinase activity with different phospholipids without preincubation was PS, 100%; PA, 58%; PG, 42%; and PI, 29%.

that preincubation was at 4 °C, indicated little to no time-dependent aggregation for both low and high [NP-40] (829 $\mu$M NP-40, 14.5 mol% PS, and 0.5 mol% DG and 4.8 mM NP-40, 8 mol % PS, and 1 mol % DG, respectively) under these conditions (data not shown). Hence, the data indicate that the time-dependent loss of substrate phosphorylation results from aggregation of PS/DG/$Ca^{2+}$/NP-40 mixed micelles with substrate. Both aggregation and the resulting loss of substrate phosphorylation were dependent on the ratio of cationic substrate to the number of mixed micelles.

To examine whether the loss of substrate phosphorylation resulting from aggregation of substrate and mixed micelles was specific to the NP-40, similar experiments were carried out with mixed micelles containing octyl glucoside as the nonionic detergent. Under these conditions, preincubation of cationic substrate and $CaCl_2$ (400 $\mu$M) with mixed micelles containing octyl glucoside, PS, and DG (40 mM, 8 mol %, and 1 mol %, respectively) at 22 °C resulted in a similar time-dependent loss of substrate phosphorylation, although maximal inhibition was less (~65% of control) and was slower (~60 min for maximal inhibition), as compared to mixed micelles containing NP-40, PS, and DG (Figure 3). Hence, loss of substrate phosphorylation by the aggregation of cationic polypeptide and mixed micelles is not specific to NP-40 but appears to be a general property of mixed micelles containing non-ionic detergent, PS, and DG.

The phospholipid dependence of the formation of the aggregates was examined by preincubation (22 °C) of histone III-S (0.2 mg/mL), $CaCl_2$ (400 $\mu$M), and mixed micelles containing 829 $\mu$M NP-40, 4 mol % DG, and either 14.5 mol % PS, PA, PI, or PG and then tested with PKC $\beta$. Under these conditions PS yielded strong, PA intermediate, and PI and PG no significant loss of substrate phosphorylation (Figure 4). Light scattering experiments under similar conditions indicated that PS-, PI-, or PG-containing mixed micelles formed aggregates with a similar time course, whereas PA did not (data not shown).

Lipid vesicles (40 $\mu$g/mL PS and 2 $\mu$g/mL DG or PC/PE/SM/PI/PS, 247, 127, 120, 47, and 40 $\mu$g/mL, and 80 $\mu$g/mL DG) in the absence of detergent were also examined for their ability to cause time-dependent aggregation and loss of substrate phosphorylation when preincubated with PKC assay mixture containing cationic substrate and $CaCl_2$. PKC assay mixture containing 1 mg/mL histone III-S and $CaCl_2$ (400 $\mu$M) when preincubated with lipid vesicles containing 40 $\mu$g/mL PS and 2 $\mu$g/mL DG at 22 °C showed no time-dependent loss of substrate phosphorylation in spite of a rapid increase in aggregation (order of seconds) (Figure 5A,C), in contrast to the case of nonionic detergent mixed micelles containing PS and DG. Under these conditions, DGs derived from PC or PI showed no significant difference in the kinase activity (Figure 5A). When PKC assay mixture containing variable concentration (0.2−6 mg/mL) of histone III-S in the presence of $CaCl_2$ (400 $\mu$M) was preincubated with lipid vesicles containing a phospholipid composition mimicking the cell membrane, PC/PE/SM/PI/PS (247, 127, 120, 47, and 40 $\mu$g/mL) and DG (80 $\mu$g/mL DG) (Mato, 1990), there was aggregation of these components but no time-dependent loss of substrate phosphorylation was observed (Figure 5B,D). Hence, not all polypeptide/lipid aggregates are inhibitory to the kinase activity; mixed micelles containing nonionic detergent, PS, and DG in the presence of cationic substrate and $CaCl_2$ can be inhibitory, whereas lipid vesicles are not, under the conditions tested.

Group A PKCs $\alpha$, $\beta$, and $\gamma$ exhibited similar time-dependent loss in substrate phosphorylation when assayed with histone III-S (0.2 mg/mL) preincubated with mixed micelles containing 829 $\mu$M NP-40, 14.5 mol % PS, 0.5 mol % DG, and 400 $\mu$M $CaCl_2$ (Figure 6A). In contrast, the group B PKC, PKC $\delta$, displayed no significant time-dependent loss of substrate phosphorylation when assayed under similar conditions with either $MBP_{4-14}$ (3 $\mu$M), MBP (1 mg/mL), $NG(F\text{-}W)_{29-47}$ (10 $\mu$M), or neurogranin (10 $\mu$M) as substrates in the presence of $CaCl_2$ (400 $\mu$M) (Figure 6B). Similar to PKC $\delta$, the PKC $\epsilon$ activity remained the same when assayed with complexes prepared by preincubation (at 22 °C) of 829 $\mu$M NP-40, 14.5 mol % PS, 0.5 mol % DG, 400 $\mu$M $CaCl_2$, and $MBP_{4-14}$ (3 $\mu$M), MBP (1 mg/mL), or [$Ser^{25}$]PKC $\alpha$ $pseudosubstrate_{19-31}$ (10 $\mu$M) over a 30-min period (data not shown). PKC $\delta$ and $\epsilon$ exhibit poor activity with histone III-S as a substrate and thus this protein was not used for the formation of complexes. However, when

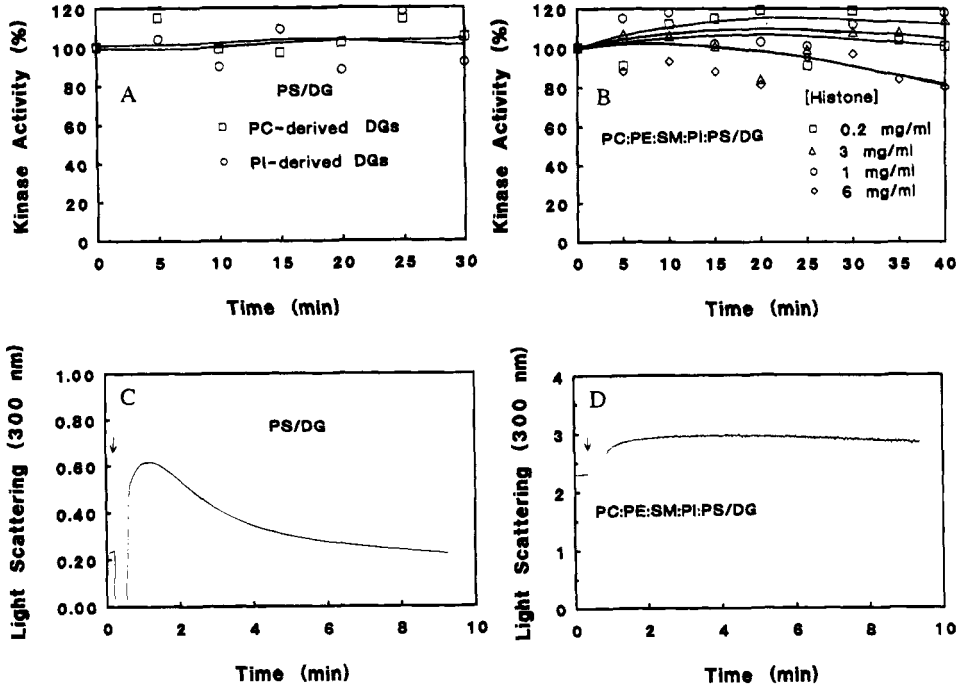


FIGURE 5: Stable substrate phosphorylation with PKC using lipid vesicles without detergent. (A, B) Kinase reactions. (A) Lipid vesicles containing 40 $\mu$g/mL PS and 2 $\mu$g/mL DG were preincubated with PKC assay mixture containing 1 mg/mL histone III-S and 400 $\mu$M $CaCl_2$ for the indicated times, and reactions were started by addition of PKC $\beta$ (0.8 $\mu$g/mL); DGs were derived from brain PC (squares) or from liver PI (circles). (B) Lipid vesicles containing PC/PE/SM/PI/PS (247, 127, 120, 47, and 40 $\mu$g/mL) and 80 $\mu$g/mL 1,2 dioleoylglycerol were preincubated with PKC assay mixture containing 400 $\mu$M $CaCl_2$ and 0.2 mg/mL (squares), 1 mg/mL (circles), 3 mg/mL (triangles), or 6 mg/mL (diamonds) histone III-S for the indicated times, and reactions were started by addition of PKC $\beta$ (0.6 $\mu$g/mL). (C, D) Parallel light scattering measurements. (C) Lipid vesicles containing 40 $\mu$g/mL PS and 2 $\mu$g/mL DG were incubated with PKC assay mixture; 1 mg/mL histone III-S and 400 $\mu$M $CaCl_2$ were added at the arrow. (D) Lipid vesicles containing PC/PE/SM/PI/PS (247, 127, 120, 47, and 40 $\mu$g/mL) and 80 $\mu$g/mL 1,2 dioleoylglycerol were incubated with PKC assay mixture; 0.2 mg/mL histone III-S and 400 $\mu$M $CaCl_2$ were added at the arrow. All preincubations and light scattering measurements were carried out at 22 °C; note the different light scattering and time scales.

the results with $NG(F\text{-}W)_{29-47}$ or neurogranin as a substrate for both PKC $\beta$ (Figure 1A,B) and PKC $\delta$ (Figure 6B) are compared, it becomes evident that the loss of substrate phosphorylation is selective for the PKC isozymes. Recombinant PKC $\beta_1$ and $\gamma$, expressed in and purified from Sf9 insect cells (Kazanietz et al., 1993), yielded a similar time-dependent inactivation as PKC $\alpha$, $\beta$, and $\gamma$ purified from rat brain (data not shown and Figure 6). Under similar conditions, a time-dependent loss of substrate phosphorylation with M-kinase, derived from trypsinized PKC $\beta$, was observed upon assaying with mixed micelles containing 829 $\mu$M NP-40, 14.5 mol % PS, and 0.5 mol % DG that were preincubated in the presence of 400 $\mu$M $CaCl_2$ and 0.2 mg/mL histone III-S (Figure 7).

It was of interest to know if the mixed micelles, under similar conditions, had an effect on phorbol ester binding to PKC. In contrast to the kinase activity, [$^3$H]PDBu binding to PKC $\beta$ in the presence of mixed micelles containing 829 $\mu$M NP-40 and 14.5 mol % PS when preincubated in the presence of 0.2 mg/mL histone III-S and $CaCl_2$ (400 $\mu$M) was unaffected (Figure 8). Specificity of the inhibitory complexes was also examined by testing with cAMP-dependent protein kinase (PKA). In contrast to group A PKCs, holo-PKA and the catalytic subunit, when assayed with mixed micelles containing 829 $\mu$M NP-40, 14.5 mol % PS, and 0.5 mol % DG that were preincubated with either kemptide (10 $\mu$M), histone III-S (0.2 mg/mL), histone H1 (0.2 mg/mL), or MBP (0.2 mg/mL) in the presence of $CaCl_2$ (400 $\mu$M), displayed no time-dependent loss in substrate phosphorylation (Figure 9). These data indicate that the mixed micelle/cationic substrate/$CaCl_2$ aggregates cause a selective loss of substrate phosphorylation with the group A PKCs.

The formation of inhibitory complexes requires $Ca^{2+}$, PS, cationic polypeptide, and nonionic detergent. These complexes once formed were very stable and were not readily reversed by the addition of excess EGTA or cationic polypeptide. In both cases, incubation with either 2 mM EGTA or 6 mg/mL histone III-S for up to 15 min with mixed micelles containing 829 $\mu$M NP-40, 14.5 mol % PS, and 0.5 mol % DG preaggregated in the presence of 0.2 mg/mL histone III-S and 400 $\mu$M $CaCl_2$ resulted in no reversal of aggregation as monitored by light scattering (data not shown). The question, can inhibition of group A PKC activity by the mixed micelle/cationic substrate/$CaCl_2$ aggregates be overcome by freshly prepared mixed micelles, was examined. To test this, aggregates were formed by preincubating a PKC assay mixture containing histone III-S (0.2 mg/mL) or $NG(F\text{-}W)_{29-47}$ (10 $\mu$M) and $CaCl_2$ (400 $\mu$M) with mixed micelles containing 829 $\mu$M NP-40, 14.5 mol% PS, and 0.5 mol% DG for 30 min (22 °C). Equal concentrations of mixed micelles, cationic substrate, and $CaCl_2$ were then added to these solutions, PKC $\beta$ was immediately added, and activity was determined in the 1−7-min time range. Addition of fresh mixed micelles, cationic substrate, and $CaCl_2$ resulted in no significant substrate phosphorylation (data not shown), suggesting that these newly added components immediately form similar aggregates under these conditions. These aggregates, however, do not inhibit the PKC-catalyzed phosphorylation of protamine sulfate when

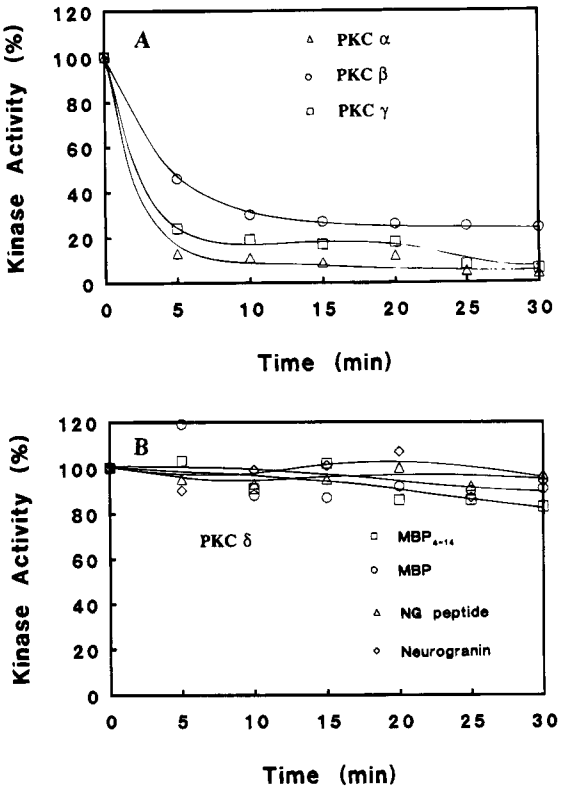


FIGURE 6: Time-dependent loss of substrate phosphorylation with PKC α, β, and γ, but not PKC-δ, using mixed micelles containing NP-40, PS, and DG. (A) PKC assay mixture containing 829 μM NP-40, 14.5 mol % PS, and 0.5 mol % 1,2 dioleoylglycerol in the presence of 400 μM $CaCl_2$ was preincubated with 0.2 mg/mL histone III-S for the indicated times at 22 °C. Kinase reactions were started by addition of PKC α (0.6 μg/mL) (triangles), β (0.8 μg/mL) (circles), or γ (1.3 μg/mL) (squares). (B) PKC assay mixture containing 829 μM NP-40, 14.5 mol % PS, and 0.5 mol % DG in the presence of 400 μM $CaCl_2$ was preincubated with 3 μM myelin basic $peptide_{4-14}$ (squares), 1 mg/mL myelin basic protein (circles), 10 μM $NG(F\text{-}W)_{29-47}$ (triangles), or 10 μM neurogranin (diamonds) for the indicated times at 22 °C. Kinase reactions were started by addition of PKC δ. The activity of the recombinant PKC δ used in the assay was equated to that of PKC β with protamine sulfate as a substrate.

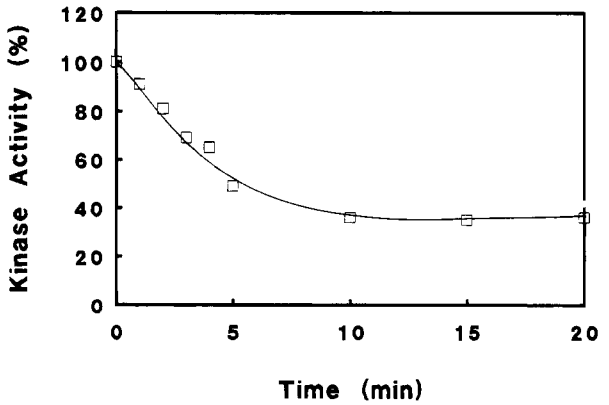


FIGURE 7: Time-dependent loss of substrate phosphorylation with M-kinase using mixed micelles containing NP-40, PS, and DG. PKC assay mixture containing 829 μM NP-40, 14.5 mol % PS, and 0.5 mol % 1,2-dioleoylglycerol in the presence of 400 μM $CaCl_2$ was preincubated with 0.2 mg/mL histone III-S for the indicated times at 22 °C. Kinase reactions were started by addition of M-kinase (0.7 μg/mL).

this latter protein (0.2 mg/mL) was added to preaggregated mixed micelles/histone III-S (0.2 mg/mL)/$CaCl_2$ (400 μM).

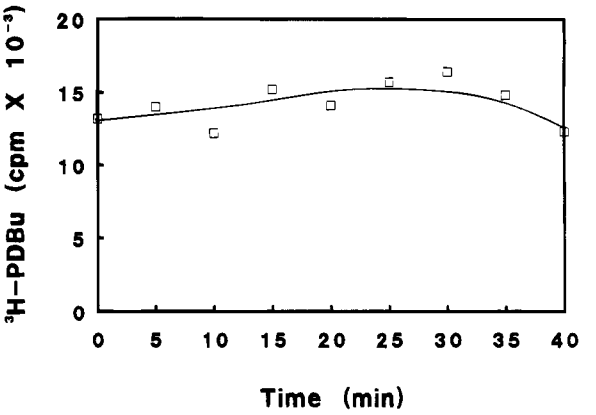


FIGURE 8: [$^3$H]Phorbol ester binding to PKC β using mixed micelles containing NP-40 and PS. PKC assay mixture containing 829 μM NP-40, 14.5 mol % PS, and 27 nM [$^3$H]phorbol 12,13-dibutyrate (PDBu) in the presence of 400 μM $CaCl_2$ was preincubated with 0.2 mg/mL histone III-S for the indicated times at 22 °C. [$^3$H]-PDBu binding was initiated by the addition of PKC β (1.25 μg/mL). Nonspecific binding (presence of 1 μM PDBu) was subtracted to present specific binding.

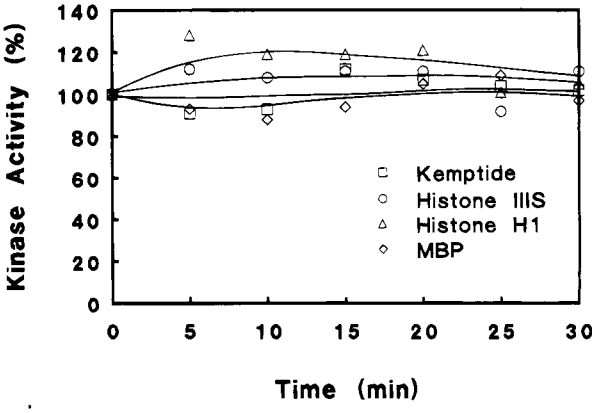


FIGURE 9: Lack of an effect by mixed micelles containing NP-40, PS, and DG on cAMP-dependent protein kinase. PKC assay mixture containing 829 μM NP-40, 14.5 mol % PS, and 0.5 mol % DG in the presence of 400 μM $CaCl_2$ and 2 μM cAMP was preincubated with 10 μM kemptide (squares), 0.2 mg/mL histone III-S (circles), 0.2 mg/mL histone H1 (triangles), or 0.2 mg/mL myelin basic protein (diamonds) for the indicated times (22 °C). Kinase reactions were initiated by addition of holo-PKA. Similar results were obtained with the catalytic subunit of PKA.

These results indicate that the cationic substrates aggregated with PS/DG/$Ca^{2+}$/NP-40 are not recognized by the catalytic domain of the $Ca^{2+}$-dependent group A PKCs and these aggregates do not inhibit the kinase-catalyzed phosphorylation of protamine sulfate.

## DISCUSSION

Acidic phospholipid such as PS activates group A PKCs in the presence of $Ca^{2+}$, yet in contrast, in the absence of divalent metal ion, activity is inhibited in an isozyme-selective manner (Huang & Huang, 1990). In the presence of $Ca^{2+}$, the regulatory domain, but not the catalytic domain, of group A PKCs associates with PS, allowing for activation, whereas in the absence of divalent metal ion, both the regulatory and catalytic domains interact with PS, which results in inhibition of activity (Huang & Huang, 1990). Here, it is demonstrated that mixed micelles containing PS and nonionic detergent in the presence of $Ca^{2+}$ and cationic substrate can form aggregates which selectively prevent the phosphorylation by the group A PKCs. Lipid vesicles

without nonionic detergent did not form such aggregates. Hence, the catalytic domains of group B PKCs, PKC $\delta$ and $\epsilon$, and the group A isozymes, although structurally homologous, are distinguishable by their interaction with the polypeptide/phospholipid/nonionic detergent/$Ca^{2+}$ aggregates. These findings may be applicable for selective measurement of the group B PKCs.

Histone III-S, neurogranin, and NG(F-W)$_{29-47}$ peptide in the presence of $CaCl_2$ and mixed micelles containing NP-40, PS, and DG form aggregates in a time-dependent manner with concomitant loss of substrate phosphorylation, whereas protamine sulfate as substrate did not. The former are all cationic substrates and their phosphorylations by PKC are dependent on the activators PS, DG, and $Ca^{2+}$. In contrast, protamine sulfate, which is a very good substrate for PKC, is a poly(cationic−anionic) compound and its phosphorylation by PKC is activator-independent; i.e., it can release the inhibitory pseudosubstrate domain from the active site of PKC in the absence of PS, DG, and $Ca^{2+}$, allowing for constitutive activity (Orr & Newton, 1994). Although there was no time-dependent loss of protamine sulfate phosphorylation in the presence of $CaCl_2$ (400 $\mu$M) and mixed micelles containing NP-40, PS, and DG (Figure 1), under the same conditions, aggregation was induced in a similar time-dependent manner as with the cationic PKC substrates (data not shown). These data suggest that the polyanionic character of protamine sulfate, as compared to the cationic basic character of the other PKC substrates, histone III-S, NG, and NG(F-W)$_{29-47}$ peptide, leads to aggregates with different surface structure which can be recognized by the catalytic domain as an active substrate.

Induction of aggregation of mixed micelles containing Triton X-100, PS, and DG, and lipid vesicles by PKC cationic substrates has been reported (Bazzi & Nelsestuen, 1987a,b) and formation of the complexes has been correlated with PKC activation (Bazzi & Nelsestuen, 1992), yet to our knowledge, there have been no reports concerning time-, substrate concentration-, or temperature-dependent formation of aggregates that results in diminishing substrate phosphorylation catalyzed by PKC. The aggregation of mixed micelles containing NP-40, PS, and DG in the presence of cationic substrate and $CaCl_2$ proceeds with an initial lag phase followed by rapid elongation/aggregation (Figure 2E,F). During the initial lag phase, complexes formed among the various PKC activators and the substrates stimulate the kinase activity and subsequent aggregation forms complexes that shield the substrate from phosphorylation by the $Ca^{2+}$-dependent PKCs. An analogous slow nucleation followed by a rapid elongation is evident in actin polymerization (Kasai et al., 1962; Estes et al., 1992). Both aggregation of the mixed micelles in the presence of cationic substrate and $Ca^{2+}$ and loss of substrate phosphorylation can be prevented by increased cationic substrate levels, indicating that the ratio of polycationic substrate to the number of polyanionic mixed micelles is a critical parameter (Figure 10). This is analogous to the antigen/antibody precipitation reaction, in which substoichiometric, stoichiometric, and excess levels of antigen relative to a fixed amount of bivalent antibody results in low, high, and low amounts of antigen/antibody complex/precipitant, respectively.

Mixed micelles containing NP-40, PS, and DG have less surface charge density than lipid vesicles without detergent. In the case of the mixed micelle system, the nonionic NP-40, which comprises >80% of the micelle on a molar basis, substantially dilutes the anionic surface charge density. In the case of lipid vesicles without detergent, a high surface charge density appears to permit aggregates in the presence of cationic substrate and $Ca^{2+}$ with a different surface structure. The differences in the surface charge density among the aggregates formed in the presence or absence of nonionic detergent may influence the interaction of the various kinases with the substrate. The phosphorylation sites of the cationic polypeptide associated with the aggregates apparently are not buried as neurogranin and NG(F-W)$_{29-47}$, each of which contains a single phosphorylation site, are readily phosphorylated by PKC $\delta$ but not by PKC $\alpha$, $\beta$, and $\gamma$ nor by M-kinase. Similarly, histone III-S aggregated with mixed micelles is readily phosphorylated by cAMP-dependent protein kinase. In addition to surface charge density, the head groups of phospholipids also play important roles in defining the surface structure of the aggregates. PS is most effective in forming such aggregates, whereas PI and PG are less effective. This specificity resembles that for the activation of the kinase. The PS molecule associated with the aggregates can fully support the binding of phorbol ester to group A PKCs, suggesting that the regulatory domain of PKC interacts properly with these complexes. On the basis of all these observations, we conclude that the catalytic domain of group A PKCs cannot recognize the phosphorylation site of the cationic polypeptide when they are aggregated with PS/$Ca^{2+}$/NP-40 mixed micelles.

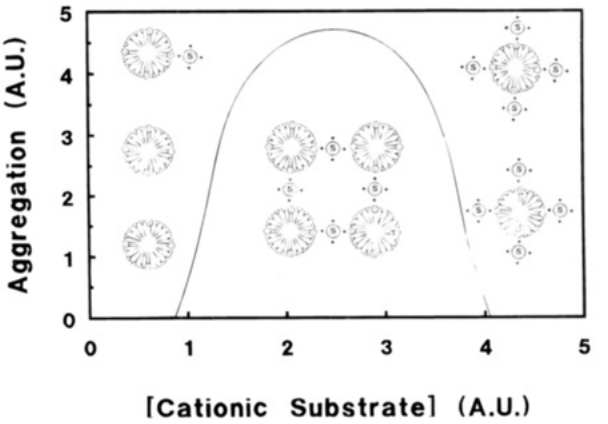


FIGURE 10: Schematic model of mixed micelle aggregation. Both mixed micelle aggregation and loss of substrate phosphorylation with group A PKC isozymes in the presence of cationic substrate and $CaCl_2$ are dependent on the ratio of cationic substrate to the number of mixed micelles. At excess cationic substrate levels relative to a fixed number of mixed micelles, there is little to no aggregation due to saturation of the polyanionic mixed micelles with cationic substrate, similar to the antigen/antibody complex/precipitation reaction. Nonionic detergent (triangles with single tails), anionic phosphatidylserine (small circles enclosing a negative sign with double tails), and cationic substrate (circles enclosing an S with positive signs) are as represented.

The interaction of acidic phospholipids and substrates with the catalytic and regulatory domains of group A and B PKCs is complex and dependent on the cofactors present and their mode of presentation. In assaying PKC activity, mixed micelles containing NP-40, PS, and DG have generally been thought to be advantageous relative to lipid vesicles, since the nonionic detergent is thought to create a well-defined micelle with small numbers of lipid molecules per micelle being easily inserted and adjusted and with the lipids being maintained in a well-solubilized form. As demonstrated here,

however, in using mixed micelles, as compared to lipid vesicles, additional factors need to be considered. Anecdotal reports have indicated that a rapid PKC assay is essential to obtain good activity. A major reason for this is likely because of the additional factors presented herein that need to be regulated in working with mixed micelles and PKC.

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