Role of Water in Protein Kinase C Catalysis and Its Binding to Membranes[†]

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ABSTRACT: The role of hydration in the catalytic activity and membrane binding of rat brain protein kinase C (PKC) was investigated by modulating the activity of water with polyethylene glycols with molecular weights of 1000−20000 and dextran with a molecular weight of 20 000. These polymers create an osmotic stress due to their exclusion from hydration shells and crevices on proteins, causing dehydration. Polymers larger than 1000 caused an activation of the PKC-catalyzed phosphorylation of histone, while PEG 1000 had no significant effect. The extent of activation by PEG and dextran 20000 was larger than that of PEG 6000 or 8000 when vesicles were composed of 1:1 POPS/POPC, suggesting the presence of at least two distinct regions of exclusion on PKC: one inaccessible to PEGs larger than 1000 and the other inaccessible only to PEGs of >10000. The extent of activation was dependent on the composition of the vesicles used. If basal activity (without PEG) was low (e.g. with low PS content in membranes), then the extent of activation was similar for all polymers larger than 1000. Binding of PKC to membranes containing 50 mol % PS was unaffected by PEG 6000 but was inhibited by PEG 20000. At a low PS content of 10%, both PEG 6000 and 20000 inhibited binding. This suggests that PKC becomes hydrated upon binding to membranes. Under conditions in which all of the enzyme is membrane-bound, both K_m and V_{max} for the phosphorylation of histone increased linearly with osmotic stress induced by PEG 6000. Thus, PKC becomes hydrated with 2311 ± 476 water molecules upon binding of histone and is dehydrated by 1349 ± 882 water molecules in going to the transition state. K_m and V_{max} for phosphorylation of the MARCKS peptide also increase with osmotic stress induced by PEG 6000. When protamine sulfate was used as a substrate (cofactor-independent), V_{max} for the reaction was unaffected, but K_m decreased with osmotic pressure (with PEG 6000), suggesting that PKC becomes dehydrated upon binding protamine. Similar results were found with a peptide substrate derived from the pseudosubstrate site of PKCϵ. Since dextran, a polymer unrelated in structure to PEG, could cause a similar activation of PKC, the effects seen are likely due to osmotic stress and not to specific binding of PEG to PKC. Also, results obtained with PE-linked PEG were opposite to those with free PEG. PE-linked PEGs of 2000 and 5000 caused an inhibition of PKC-catalyzed phosphorylation of histone when present in membranes. If a specific interaction occurred with PEG, this would be expected to occur even with PE−PEG. The effects observed with free PEG are also independent of ionic strength. Free PEG had no effect on the bilayer to hexagonal phase transition temperature of DEPE membranes, suggesting that the effects on PKC activity are not a consequence of changes in membrane properties at the osmotic pressures used.

Water is known to play a role in the conformational changes that occur between different functional states of macromolecules. Recent attention has been given to the role of hydration in the functioning of ion channels, in membrane phase transitions, in the binding of enzymes and substrates, in interactions between DNA molecules, and in the activation of enzymes in solution [reviewed in Parsegian et al. (1995)]. Polyethylene glycols (PEGs)[1] are routinely used as osmotic stressing agents, to alter the water activity in a solution. These polymers are preferentially excluded from the hydration shells around proteins or other macromolecules. This exclusion creates an osmotic stress, which draws water away from the protein surface or out of a polymer-inaccessible crevice. If an enzyme's environment is osmotically stressed, there will be a change in catalytic activity if the number of bound waters is changed between the enzyme and substrate and the enzyme−substrate complex (change in K_m) or between the ground state and transition state of the later complex (change in V_{max}).

In this study, we have investigated the role of hydration in the functioning of rat brain protein kinase C. While most studies using osmotic stress focus on water soluble enzymes, few discuss membrane-dependent activation (McGee & Teuschler, 1995). The classical isoforms of PKC used in this study are of particular interest because they require lipid and calcium for activation. The activity of PKC is influenced

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[1] Abbreviations: PKC, protein kinase C; PE, phosphatidylethanolamine; PEG, polyethylene glycol (if followed by a number, it represents the molecular weight); PE-linked PEG, 1-palmitoyl-2-oleoylphosphatidylethanolamine-linked polyethylene glycol (if followed by a number, it represents the molecular weight of the PEG without PE); POPS, 1-palmitoyl-2-oleoylphosphatidylserine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PS, phosphatidylserine; PC, phosphatidylcholine; DG, 1,2-dioleoyl-*sn*-glycerol; H_{II}, inverted hexagonal phase; T_H, bilayer to hexagonal phase transition temperature; LUVs, large unilamellar vesicles; SLVs, sucrose-loaded large unilamellar vesicles; MARCKS peptide, acetyl-FKKSFKL-amide; pseudosubstrate site peptide, acetyl-ERMRPRKRQGSVRRRV-amide.

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by the physical properties of bilayers [reviewed in Epand (1992)]. Osmotic stress is known to have an effect on phase transitions of membranes (Rand & Parsegian, 1997), and therefore, it is of interest to determine the effects of hydration on PKC's activity and binding to membranes. Also, PKC is an example of an amphitropic enzyme. PEGs of various sizes and dextran were used as osmotic stressing agents to determine the number of water molecules which are associated with the activation of PKC. The large number of water molecules found to be involved in this process reveals that activation must involve a substantial conformational change in the enzyme and that the extent of enzyme hydration is an important factor in determining its state of activation.

MATERIALS AND METHODS

Materials. Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Polyethylene glycol 6000 was from J. T. Baker (Phillipsburg, NJ). PEG 1000, 8000, and 20000 were from Sigma (St. Louis, MO). Histone H1 was from GIBCO/BRL (Grand Island, NY). Protamine sulfate, bovine serum albumin fraction V, and ATP sodium salt were from Sigma. $CaCl_2$ was from Fisher, and Tris was from Boehringer Mannheim. [γ-^{32}P]ATP was from ICN, and [9,10-^{3}H]dipalmitoylphosphatidylcholine was from NEN. The MARCKS peptide, acetyl-FKKSFKL-amide, was purchased from NRC (Ottawa, ON), and the pseudosubstrate site peptide, acetyl-ERMRPRKRQGSVRRRV-amide, was custom synthesized by the Indiana University Biotechnology Facility (Indianapolis, IN).

PKC Purification. Rat brain PKC was purified by a modification of the procedure of Huang et al. (1986) as described elsewhere (Mosior & Epand, 1993). Purified PKC displayed a single band on a silver-stained electrophoresis gel.

Phospholipid Vesicles. Lipid films were made by dissolving phospholipids in 2:1 (v:v) chloroform/methanol and drying under a stream of nitrogen followed by desiccation under vacuum for 2 h. Films were suspended in sucrose buffer (0.170 M sucrose/5 mM $MgCl_2$/20 mM Tris-HCl at pH 7.0) for binding assays, or standard buffer (100 mM KCl/5 mM $MgCl_2$/20 mM Tris-HCl, at pH 7.0) for activity assays, and subjected to five freeze−thaw cycles. Vesicles were extruded through two 0.1 μm pore polycarbonate filters in a microextruder. For binding assays, the sucrose-loaded vesicles were suspended in standard buffer and centrifuged at 100000*g* to dilute out the sucrose.

PKC Binding Assays. The sucrose-loaded vesicle (SLV) assay was modified from the procedure of Rebecci et al. (1992) as described elsewhere (Mosior & Epand, 1993). PKC was incubated with SLVs and then centrifuged at 100000*g* for 30 min at 25 °C to separate the membrane-bound enzyme. Solutions of free PEG in the assay buffer were added prior to centrifugation. For assays using PE−PEG, the additive was incorporated at the stage of making the dry lipid film from the chloroform/methanol solution. In assays using PEG 20000, the vesicles and PKC were centrifuged at 150000*g* for 2 h to account for the higher viscosity of the solution. The percentage of vesicles sedimented with the highest PEG concentration was typically about 70% of those sedimented without PEG. This was taken into account when calculating the percentage of enzyme bound. The pellet and supernatant were assayed under identical conditions for activity toward protamine sulfate. PEG was added to the bottom fractions so that the final amount of PEG was equal to that in the top fraction. The buffer contained 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.0), 200 μM $CaCl_2$, and 0.3 mg/mL BSA.

PKC Activity Assays. The activity of PKC toward histone or protamine sulfate was determined as previously described (Mosior & Epand, 1993; Kaibuchi et al., 1981). Histone or protamine sulfate was added to a final concentration of 0.2 mg/mL (in a 150 μL total volume), and acetyl-FKKSFKL-amide was used at a final concentration of 90 μM. Phospholipid was 100 μM in the form of large unilamellar vesicles (LUVs). [γ-^{32}P]ATP (0.2 mCi/mL) was 20 μM, and PKC was 750 ng/mL. The reaction temperature was 37 °C.

The activity toward peptide substrates was determined in a similar manner; however, in this case, the reaction was stopped with 5% acetic acid, and a portion of this was spotted onto P-81 Whatman ion exchange paper which was then washed with 0.04% phosphoric acid.

Differential Scanning Calorimetry (DSC). Bilayer to hexagonal phase transition temperatures were measured as described previously (Ling et al., 1995). An MC-2 high-sensitivity calorimeter was used (Microcal Co., Amherst, MA) with a scan rate of 41 K/h. Lipid films of dielaidoylphosphatidylethanolamine (DEPE) were suspended in buffer to a final concentration of 5 mg/mL. For experiments using PE−PEG, the lipid-linked polymer was added directly as a component of the films. For experiments with free PEG, the PEG was added to the hydration buffer.

Kinetics. Activity assays were performed as above, using either histone, protamine sulfate, MARCKS peptide, or the pseudosubstrate site peptide as substrates. K_m and V_{max} were determined from initial rates using the program GraFit 3.0 (R. J. Leather-Barrow; Erithacus Software).

PEG Purification. A solution of PEG 6000 (4 g per 10 mL of H_2O) was dialyzed extensively with Spectra/Por tubing with a 1000 MW cutoff. The remaining solution was lyophilized for ∼24 h. The PEG was recrystallized according to Lentz et al. (1992). Briefly, PEG was dissolved in chloroform, and an 8-fold excess of diethyl ether was added to recrystallize it. The PEG was filtered and left to dry.

Results using this purified PEG were similar to those with PEG used as supplied by the manufacturer, indicating that the effects were not caused by the presence of contaminants.

RESULTS

Effect of Polyethylene Glycol-Linked Phosphatidylethanolamine on the Activity and Membrane Binding of PKC. Polyethylene glycol-linked PEs, when added to LUVs containing 50 mol % PS, were found to inhibit PKC-catalyzed phosphorylation of histone (Figure 1). PE−PEG 5000 was a more potent inhibitor than PE-PEG 2000 on a molar basis and even somewhat more potent on a weight basis.

The inhibition of PKC activity caused by PE−PEG was not due to an inhibition in the amount of PKC which bound to the membrane (Figure 1). The percentage of PKC bound at each PE−PEG concentration was actually greater in the presence of PE−PEG, especially PE−PEG 5000. Similar results were found with lipophosphoglycan (LPG) from *Leishmania donovani*, which inhibited the membrane-bound form of the enzyme (Giorgione et al., 1996). It has been

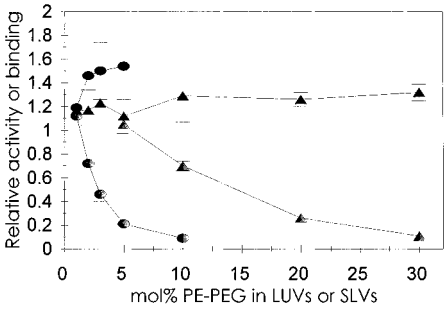

FIGURE 1: PE−PEG 2000 and 5000 inhibit the PKC-catalyzed phosphorylation of histone and its binding to SLVs. (Activity) The LUVs were composed of 50 mol % POPS, 0−30 mol % PE−PEG 2000 or 5000, and POPC. The activity is expressed relative to the activity obtained using LUVs without PE−PEG: shaded ovals (PE−PEG 5000) and shaded triangles (PE−PEG 2000). (Binding) The sucrose-loaded vesicles were similar to LUVs. The binding is expressed as a percentage of the binding obtained with vesicles without PE−PEG: solid ovals (PE−PEG 5000) and solid triangles (PE−PEG 2000). Data are expressed as the mean of triplicate determinations ± SD (some error bars omitted for clarity).

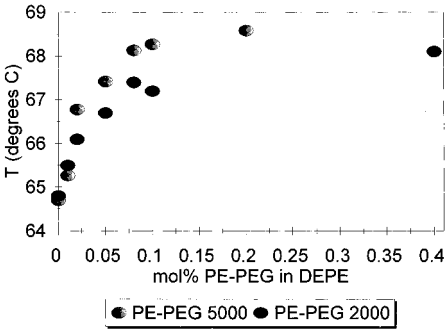

FIGURE 2: PE−PEG 2000 and 5000 raise the bilayer to the hexagonal phase transition temperature of DEPE multilamellar vesicles. T_H is plotted against the mole percent of PE−PEG in MLVs.

shown that LPG inhibits a conformational change in PKC which is required for the activation of the enzyme. It is possible that this is a general mechanism by which membrane-anchored hydrophilic polymers inhibit the activity of membrane-bound PKC. Both PE−PEG and LPG have potent effects in raising the bilayer to the hexagonal phase transition temperature at low mole fractions (Figure 2). It has also been recently shown that DOPE−PEGs stabilize the bilayer phase in mixtures of lipids which normally form hexagonal phases (Holland et al., 1996). Compounds with this property are often found to be inhibitors of PKC [reviewed in Epand (1992)], although LPG is an unusual example of an anionic inhibitor of PKC. LPG is a polymer, with a large negative charge over its entirety, while PE−PEGs contain only one negative charge localized at the phospholipid head group. It is unlikely that PKC is binding directly to PE−PEG, since no binding occurred when Ca^{2+} was removed from the assay (data not shown).

Effects of Free PEG on PKC Activity and Membrane Binding. PEG is a hydrophilic polymer that is impermeable to membranes, and because of its large size, it may also be excluded from crevices in proteins as well sites at which proteins bind to membranes. A consequence of the exclusion of PEG from certain compartments will be creation of an osmotic gradient which will cause dehydration of the regions to which PEG is not accessible. PEG can then affect the activity of membrane-bound enzymes by shifting the position

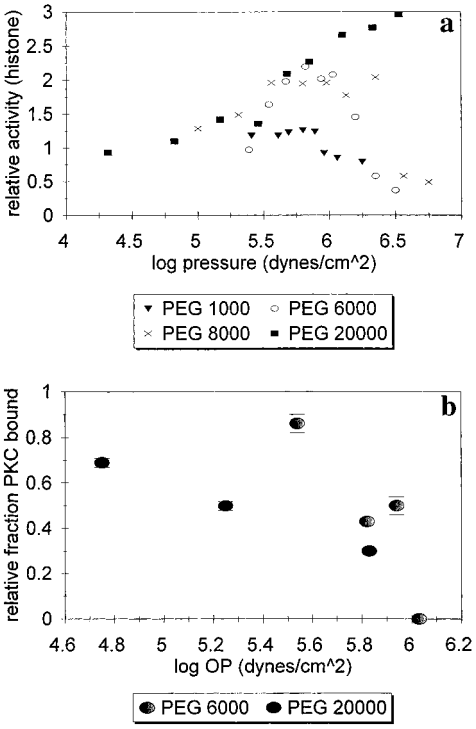

FIGURE 3: (a) Effects of osmotic pressure created by PEGs of various sizes on the PKC-catalyzed phosphorylation of histone. The LUVs were composed of 50 mol % POPS and 50 mol % POPC. PEGs were added to the reaction buffer as in Materials and Methods. The activity is expressed as a fold increase in the activity obtained without PEG and plotted as a function of osmotic pressure. The SD (not shown) did not exceed ±9% in all but one case. (b) PEG 6000 and 20000 inhibit the binding of PKC to SLVs containing 10 mol % POPS. The SLVs were composed of 10 mol % POPS and 90 mol % POPC. PEGs were added to the reaction buffer as in Materials and Methods. The binding is expressed as a fraction of that obtained in the absence of PEG and plotted as a function of osmotic pressure. Data are expressed as the mean of triplicate determinations ± SD.

of equilibrium between different states on the basis of their extent of hydration.

This analysis is dependent on the assumption that there is no direct interaction between PEG and the enzyme or membrane (Rand et al., 1993; Parsegian et al., 1995). This is indicated by the fact that the effects of PE−PEG on PKC activity are opposite to those of free PEG. The free polymer (PEG 6000) activates the PKC-catalyzed phosphorylation of histone when it is present in solution at low concentrations (Figure 3a). This suggests that the effects are not a consequence of the specific structure of PE−PEG interacting with PKC but rather an effect on membrane properties. At higher concentrations (>15 mM), PEG 6000 shows less activation and even causes a slight inhibition at 20 mM and above. This inhibition may be due to the polymer's ability to aggregate or precipitate protein, or to the increased viscosity produced at higher PEG concentrations. This latter explanation is likely a factor. When glycerol was used in a similar assay to increase the viscosity without creating an osmotic stress, the only effect was an inhibition at higher concentrations which correlated with the inhibition seen at higher PEG concentrations. Glycerol increases the viscosity without being excluded from crevices or precipitating proteins. A similar trend to that which is seen in Figure 3a with PEG 6000 was observed when the peptide acetyl-FKKSFKL-amide was used as the substrate (not shown),

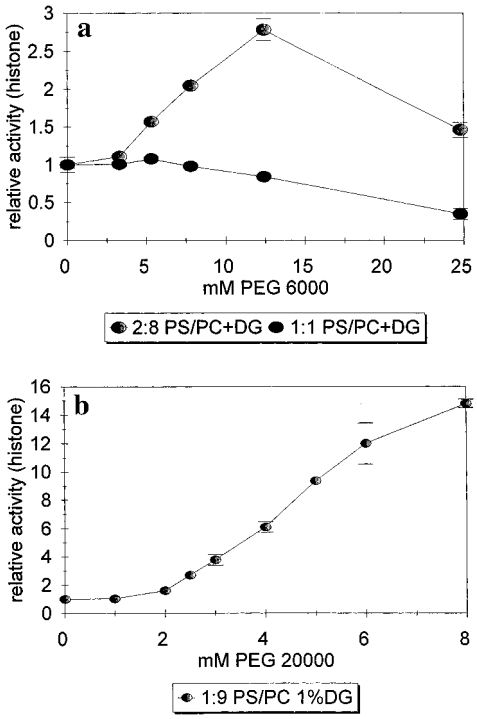

FIGURE 4: Effect of PEG 6000 and 20000 on the PKC-catalyzed phosphorylation of histone. (a) (PEG 6000) The LUVs were composed of either 20 mol % POPS and 1 mol % DG with POPC or 50 mol % POPS and 1 mol % DG with POPC. The activity is expressed relative to that obtained in the absence of PEG. Data are expressed as the mean of triplicate determinations ± SD. (b) (PEG 20000) The LUVs were composed of 10 mol % POPS, 1 mol % DG, and POPC. PEG was added to the reaction buffer. The activity is expressed relative to the activity obtained without PEG. Data are expressed as the mean of triplicate determinations ± SD.

which represents a phosphorylation site of the MARCKS protein, a physiological substrate of PKC.

When the activity is plotted against the log of the osmotic pressure, the effects of PEGs of different sizes on the phosphorylation of histone can be compared. Activation of PKC was seen for both PEG 6000 and PEG 8000, while PEG 1000 had no significant effect on PKC activity (Figure 3a). This suggests that PEG 1000 is not excluded from the compartment that is dehydrated by the larger PEG species. There is no biphasic effect seen with PEG 20000, even at the highest osmotic pressure used. The activation by either PEG 6000 or PEG 20000 reaches almost 25-fold if the concentration of PS in the vesicles is decreased from 50 to 10% so that basal PKC activity is lowest (not shown).

Activation of PKC phosphorylation of histone is seen with PEG 6000 (Figure 4a) and PEG 20000 (Figure 4b) when the PS concentration is 10 or 20%, even when diacylglycerol is present in the membrane, which normally would increase the amount of PKC that binds. However, at 50 mol % PS with DG, the maximal activation by PEG 20000 is only 1.4-fold (not shown). The activation seen at any particular vesicle composition is usually higher with PEG 20000 than with PEG 6000 or 8000 (unless the vesicles already support maximal PKC activity, in which case PEG of any size has no additional effect; or if basal activity is minimal, then PEGs of both sizes show maximal activation). The differing effects on the activation of PKC which were obtained with PEGs of various sizes indicate the presence of at least two aqueous compartments around PKC. One is inaccessible to PEGs of around 6000, while the other is inaccessible only to those of 20000. It is likely that PEG 6000 is also excluded from a hydration shell around PKC, but this shell has a smaller volume than that excluded from PEG 20000. There is no significant activation by PEG 1000, even though basal activity is at a minimum (not shown). This supports the conclusion that PEG 1000 is not excluded from a crevice on PKC.

The activation of PKC by free PEG was specific for histone phosphorylation, while having little effect on the phosphorylation of protamine (not shown). The phosphorylation of histone requires the presence of Ca^{2+}, and PS for a structural rearrangement, while the phosphorylation of protamine is independent of these factors (Bazzi & Nelsestuen, 1987). Thus, PEG affects the membrane-dependent activation process of PKC. In the case of Arg-rich substrates, the activation of PKC can be accomplished by the substrate itself (Bruins & Epand, 1995). Activation of PKC occurs whether the PEG is present on the outside or both sides of the LUVs (not shown).

However, despite this activation, PEG 20000 inhibits the binding of PKC to the membrane (Figure 3b). There is essentially no activity of PKC in the absence of lipid. Therefore, the surprisingly small fraction of PKC that is actually bound must have an even higher fold activation. This suggests that a hydration step may be involved when the enzyme binds to the membrane. Binding is also inhibited by PEG 6000 if the concentration of PS in the vesicles is lowered to 10% (Figure 3b), while PEG 20000 inhibits binding even in the presence of 50% PS and 1% DG (not shown). No effect is observed with PEG 1000 because it likely has access to all compartments.

Although PEG 6000 inhibits binding, this is not observed if there is sufficient PS (50 mol %) in the membrane (not shown). There is slight inhibition of binding at higher concentrations of PEG, where the inhibition in activity is also seen, and is most likely due to precipitation of protein or solution viscosity inhibiting the attainment of equilibrium at these higher concentrations. The lack of an effect on membrane binding with high PS content due to PEG 6000 suggests that there is no change in hydration of PKC upon binding to SLVs and that the effect of PEG 6000 is only on catalysis.

Both K_m and V_{max} were determined for phosphorylation of histone by PKC at various osmotic pressures, using PEG 6000 with high PS where all of the PKC is bound (Figure 5). Both constants increased with increasing concentrations of PEG 6000. The change in the number of bound water molecules upon activation of PKC was determined (equations in the Appendix). Measuring the change in ln V_{max} with the change in osmotic pressure (Figure 6) revealed that 1349 ± 882 water molecules are removed per PKC molecule in going to the transition state. The dehydration step involved in the activation of PKC must be in the membrane-dependent conformational change, since there is an increase in V_{max} with increasing osmotic pressure. The number of water molecules associated with PKC's rearrangement to the catalytically active form is reasonable when compared to the volume of 95 700 Å^3 for PKC itself as calculated from its specific volume and molecular weight, which is equivalent to 3190 water molecules. Similarly, from the K_m data, 2311 ± 476 water molecules are "added" in binding of histone to the active site (Figure 6).

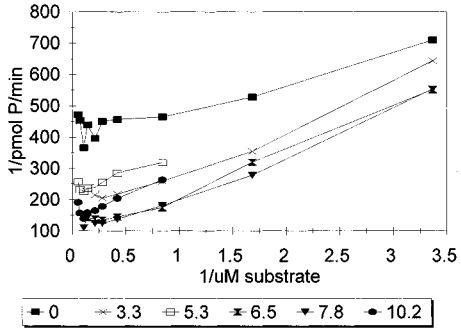

FIGURE 5: Lineweaver−Burke plots for the phosphorylation of histone by PKC in the presence of PEG 6000. LUVs were composed of 50 mol % POPS and 50 mol % POPC. PEG was added to the reaction buffer. K_m and V_{max} values were calculated using GraFit 3.0. Data are expressed as the mean of triplicate determinations of at least two separate experiments. The legend under the graph gives the concentration of PEG in millimolar units.

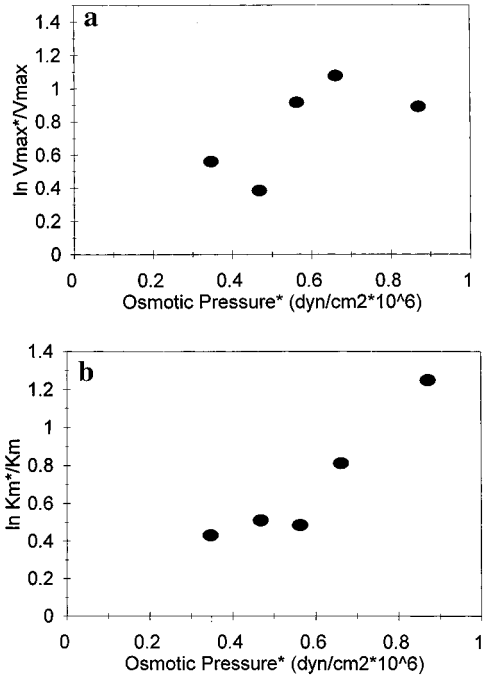

FIGURE 6: (a) Change in ln V_{max} with the increase in osmotic pressure with PEG 6000. V_{max} values were those obtained in Figure 5. The change in V_{max} was measured relative to that obtained without PEG. Linear regression and the equations in the Appendix were used to calculate the number of water molecules removed from PKC in going to the transition state (1349 ± 882). (b) Change in ln K_m with the increase in osmotic pressure with PEG 6000. K_m values were those obtained in Figure 5. Calculations were as in part a. The number of water molecules added to PKC upon histone binding was found to be 2311 ± 476.

K_m and V_{max} were also found to increase when 8 mM PEG 20000 was used to increase osmotic pressure (K_m of 3.8 ± 1.9 to 5.1 ± 2.7 μM and V_{max} of 0.0050 ± 0.0014 to 0.0078 ± 0.0026 pmol of phosphate/min). A similar trend was seen when the MARKS peptide (which is lysine-rich, like histone) was used as a substrate, although the percent of PS of LUVs used was lower. When protamine or the pseudosubstrate peptide (another arginine-rich substrate) is the substrate, K_m decreases, while V_{max} is unchanged with PEG 6000. For the pseudosubstrate peptide, V_{max} = 0.0139 ± 0.0016 pmol of phosphate/min and K_m = 34.6 ± 9.8 μM without PEG, and V_{max} = 0.0137 ± 0.0007 pmol of phosphate/min, K_m = 2.2 ± 1.3 μM with 10.2 mM PEG 6000.

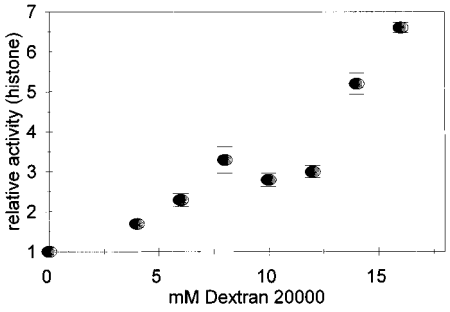

FIGURE 7: Dextran 20000 activates the PKC-catalyzed phosphorylation of histone. LUVs were composed of 1:1 POPS/POPC. Dextran was added to the reaction buffer. Activity is expressed as the fold increase in activity relative to that obtained without dextran. Data are expressed as the mean of triplicate determinations ± SD.

Dextran with a molecular weight of 20000 was also found to activate PKC phosphorylation of histone (Figure 7). However, binding could not be tested due to sedimentation of the dextran upon centrifugation.

PEG 6000 and PEG 20000 had no effect on the bilayer to hexagonal phase transition temperature of DEPE, even when present in the highest concentrations used for PKC assays (not shown).

We also verified that the effects observed with PEG were not a consequence of an increased ionic strength. PKC activity toward histone was measured with increasing NaCl concentrations in the presence and absence of 12.4 mM PEG 6000 (not shown). Increasing the ionic strength decreased the activity of PKC both with and without PEG. The extent of inhibition was the same in both cases. If the effects of PEG were due to an increase in ionic strength, one would expect the combined effects to be additive and in the opposite direction. Therefore, this variable can be ruled out.

DISCUSSION

PE-linked polyethylene glycols are used extensively in liposome-mediated drug delivery. Since they enter the bloodstream, understanding their effects on cellular enzymes is useful. These compounds were found to inhibit the membrane-bound form of PKC. The most significant finding was that these PE-linked PEGs have opposite effects on PKC as does free PEG, suggesting that there is no direct interaction between the polymer and the enzyme which causes a change in activity. This is an important prerequisite for the analysis of the effects of osmotic stress on a protein, enzyme, or channel (Parsegian et al., 1995). In addition, PEG has no effect on lipid polymorphism, and the observed effects are independent of ionic strength, demonstrating that the effects are specific for PKC. Further support that the observed effects result from changes in hydration comes from the findings obtained with dextran as the stressing agent. Similar levels of activation of PKC-catalyzed phosphorylation of histone were obtained with this polymer, which is unrelated in structure to polyethylene glycol. If there were a specific interaction between the enzyme and PEG, then similar effects should not be seen with dextran.

Large variations have been found in the numbers of water molecules associated with conformational changes of enzymes and proteins. Studies with hemoglobin found that 65−72 water molecules were associated with the binding of four oxygen molecules (Colombo & Bonilla-Rodriguez, 1996; Colombo et al., 1992), and binding of glucose to

hexokinase resulted in the release of 65 water molecules (Rand et al., 1993). Different results have been found for the role of water in the generation of coagulation factor Xa in the aqueous phase vs the role in generation of the intermediates on phospholipid membranes (McGee & Teuschler, 1995). Without membranes present, over 5000 water molecules were found to be removed from the protein surface during the reaction. When the reactions occurred on phospholipid membranes, about 800 water molecules were found to be added to the surface. Therefore, differences in hydration can exist depending on whether the proteins are membrane-bound. This may be the case for the K_m values found with protamine vs those found with histone, which change oppositely in the presence of osmotic stress, a possible consequence of the fact that histone binds to the active form of PKC while protamine binds to the inactive form. Thus, effects of PEG on the binding of histone to membrane-bound PKC indicate that hydration is increased when substrate binds to an activated form of PKC. Protamine binding itself also likely requires hydration, but this process is counterbalanced by a dehydration of the enzyme resulting from a conformational change that exposes hydrophobic groups (Bruins & Epand, 1995). Thus, the overall effect of PEG on the K_m of protamine is to lower it, indicating a dehydration upon forming the enzyme−substrate complex. V_{max} is increased by PEG in the case of histone but is unchanged for protamine. Since the conversion of the enzyme−substrate complex to the transition state is likely to be similar for both substrates, the greater effect of PEG on the V_{max} of histone is likely caused by a dehydration resulting from a greater insertion of the complex into the membrane upon formation of the transition state with histone. This conclusion is strengthened by the finding that a small lysine-rich peptide substrate shows trends similar to those of histone, while an Arg-rich peptide behaves in a manner similar to that of protamine. These may represent general effects of osmotic stress on the kinetics of the two types of substrates.

Although the equations used to calculate the number of water molecules associated with PKC activation involve the equilibrium constant K, our analysis assumes that K_m and V_{max} are a close approximation if the reaction follows Michaelis−Menten kinetics. K_m is a ratio of $(k_2 + k_3)/k_1$ and will be close to a dissociation constant if $k_2 > k_3$ according to the scheme

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

Additional complexities occur with interfacial catalysis because of the exchange rates of enzyme, substrate, and product between the membrane and aqueous phase. For example, it has been shown recently that the apparent K_m for the prothrombinase reaction depends on whether the substrate comes to the active site from the membrane rather than from the solution (Lu & Nelsestuen, 1996). However, in the present study, there is evidence to suggest that the measured K_m approximates an equilibrium dissociation constant for the enzyme−substrate complex. For peptide substrates (e.g. the MARKS peptide), Lineweaver−Burke plots are linear over a wide range of concentrations (Giorgione et al., 1996). Also, different substrates show the same effects with PEG 6000. The activation is to a similar extent, as well as the inhibition at higher concentrations of PEG. Protamine and histone have similar K_m values, even though one involves interfacial catalysis and the other does not. The difference in the direction of the change in K_m with PEG is a consequence of protamine binding to inactive PKC and histone binding to active PKC.

PEG inhibits the binding of PKC to membranes (Figure 3b). This indicates that the binding process is accompanied by increased hydration. There are two kinds of morphological features that can account for this increase in water binding. One is the creation of new or deeper crevices in the membrane-bound form of PKC. The crevices would be inaccessible to PEG, and therefore, osmotic stress would inhibit their formation and reduce the binding of PKC to membranes. In addition, the aqueous volume between PKC and the membrane, close to the point of membrane attachment of PKC, may also be inaccessible to PEG. However, in this case, unlike the conventional explanation for osmotic stress effects, removal of water from this region will increase PKC binding to the membrane. A similar phenomenon occurs for PEG-induced vesicle aggregation. We thus favour PEG-induced osmotic stress on the hydration of membrane-bound PKC, to explain the inhibition of binding.

The differences observed in the binding of PKC to membranes of 50 mol % PS in the presence of PEG 6000 vs PEG 20000 may be a consequence of PKC not having access to the membrane in the presence of PEG 20000. It has been shown that PEG 20000 absorbs to the bilayer surface and prevents the close approach of two bilayers because of its size. It does not have the same ability to draw water out from between the bilayers as PEGs of less than 10000 do, which stabilize interactions between membranes (Kuhl et al., 1996). If PEG 20000 is not allowing PKC access to the membrane, this would inhibit binding.

The general conclusion about changes in hydration can be summarized as follows (where E = PKC, L = lipid vesicle, P = protamine, H = histone, and * = transition state):

$$E + L + H_2O \rightleftarrows EL$$

$$E + P \rightleftarrows EP + H_2O$$

$$E + H + H_2O \rightleftarrows EH$$

$$EP \rightleftarrows EP^*$$

$$EH \rightleftarrows EH^* + H_2O$$

These findings are important for the understanding of the molecular mechanisms of PKC-dependent processes. It is the first example of a study of hydration changes in a membrane-bound allosteric enzyme.

The physiological relevance of these findings is uncertain. However, PKC has been shown to play a role in osmotically induced cellular signaling *in vivo*. Activation of PKC has been found to inhibit collecting duct osmotic water permeability (Han et al., 1994; Ando et al., 1992). In GH_4C_1 cells, PKC was found to be involved in Ca^{2+} influx and prolactin secretion caused by cell swelling (Sato et al., 1992). *Saccharomyces cervisiae* mutants lacking the PKC1 gene product were found to be viable when grown on osmotically supported medium, but lysed at restrictive temperatures on hypotonic medium (Paravicini et al., 1992; Levin & Bartlett-Heubusch, 1992). PKC has been shown to phosphorylate

and inhibit the activity of the Na/K-ATPase in intact renal epithelium (Middleton et al., 1993). Since this pump is involved in transport of solutes across epithelial cells, it may regulate the osmotic balance as well (since water would be transported in response to solutes). It remains to be proven if some of these relationships are a consequence of the sensitivity of PKC activity on hydration pressure.

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APPENDIX

Determination of the Number of H_2O Molecules Associated with PKC's Active Site and Transition State. Studies on hexokinase used changes in K_{diss} and K_m with osmotic pressure to determine the number of H_2O molecules associated with binding of glucose to hexokinase (Rand et al., 1993).

$$\Delta G = -RT \ln K_d + nRT \ln W$$

where W = water activity

$$nRT \ln W = \Pi \Delta V_w$$

where ΔV_w = the volume of water removed

$$\Delta G = -RT \ln K_d + \Pi \Delta V_w$$

so

$$\Delta V_w = RT\, \Delta \ln K_d / \Delta \Pi = RT \ln(K_d^\circ / K_d^\pi)/\Delta \Pi$$

You can also substitute K_m for K_d. We determined K_m and V_{max} using histone as the substrate, at different osmotic pressures. The plot of $\ln(K_m^\circ/K_m^\pi)$ vs Π should yield a straight line with a slope of $\Delta V_w/RT$ from

$$\ln(V_{max}^\pi / V_{max}^\circ) \text{ vs } \Pi \text{ (Figure 6a)}$$

$$\text{slope} = 0.945 \pm 0.618$$

$$\text{slope} = 0.945 = \Delta V_w/RT \quad RT = 2{,}577 \text{ J/mol}$$

The units for the slope are $cm^2 \times 10^{-6}$/dyn. Since 10 dyn/cm^2 = 1 J/m^3, then the slope = 9.45 $m^3 \times 10^{-6}$/J.

$$9.45\ m^3 \times 10^{-6}/J = \Delta V_w/2577 \text{ J/mol}$$

$$0.0243\ m^3/\text{mol} = \Delta V_w$$
$$= 4.047 \times 10^{-26}\ m^3/\text{molecule}$$
$$= 40\ 470\ Å^3/\text{molecule}$$

$$1\ H_2O = 30\ Å^3$$

Therefore, 1349 ± 882 water molecules were removed per PKC histone complex in going to the transition state; similarly, from K_m data, 2311 ± 476 water molecules were added in binding of histone to the active site of PKC.

REFERENCES

Ando, Y., Jacobson, H. R., & Breyer, M. D. (1992) *J. Clin. Invest. 81*, 1578−1584.
Bazzi, M. D., & Nelsestuen, G. L. (1987) *Biochemistry 26*, 1974−1982.
Bruins, R. H., & Epand, R. M. (1995) *Arch. Biochem. Biophys. 324*, 216−222.
Colombo, M. F., & Bonilla-Rodriguez, G. O. (1996) *J. Biol. Chem. 271*, 4895−4899.
Colombo, M. F., Rau, D. C., & Parsegian, V. A. (1992) *Science 256*, 655−659.
Epand, R. M. (1992) in *Protein Kinase C: Current concepts and future perspectives* (Lester, D. S., & Epand, R. M., Eds.) pp 135−156, Ellis Horwood, Chichester, England.
Giorgione, J. R., Turco, S. J., & Epand, R. M. (1996) *Proc. Natl. Acad. Sci. U.S. A. 93*, 11634−11639.
Han, J. S., Maeda, Y., Ecelbarger, C., & Knepper, M. A. (1994) *Am. J. Physiol. 266*, F139−F146.
Holland, J. W., Cullis, P. R., & Madden, T. D. (1996) *Biochemistry 35*, 2610−2617.
Huang, K.-P., Chan, K. F., Singh, T. J., Nakabayashi, H., & Huang, F. L. (1986) *J. Biol. Chem. 261*, 12134−12140.
Kaibuchi, K., Takai, Y., & Nishizuka, Y. (1981) *J. Biol. Chem. 256*, 7146−7149.
Kuhl, T., Guo, Y., Alderfer, J. L., Berman, A. D., Leckband, D., Israelachvili, J., & Hui, S. W. (1996) *Langmuir 12*, 3003−3014.
Lentz, B. R., McIntyre, G. F., Parks, D. J., Yates, J. C., & Massenburg, D. (1992) *Biochemistry 31*, 2643−2653.
Levin, D. E., & Bartlett-Heubusch, E. (1992) *J. Cell Biol. 116*, 1221−1229.
Ling, M., Stafford, A., Nir, S., Turco, S. J., Flanagan, T. D., & Epand, R. M. (1995) *Biochemistry 34*, 4676−4683.
Lu, Y., & Nelsestuen, G. L. (1996) *Biochemistry 35*, 8201−8209.
McGee, M. P., & Teuschler, H. J. (1995) *J. Biol. Chem. 270*, 15170−15174.
Middleton, J. P., Khan, W. A., Collinsworth, G., Hannun, Y. A., & Medford, R. M. (1993) *J. Biol. Chem. 268*, 15958−15964.
Mosior, M., & Epand, R. M. (1993) *Biochemistry 32*, 66−74.
Paravicini, G., Cooper, M., Friedli, L., Smith, D. J., Carpentier, J., Klig, L. S., & Payton, M. A. (1992) *Mol. Cell. Biol. 12*, 4896−4905.
Parsegian, V. A., Rand, R. P., & Rau, D. C. (1995) *Methods Enzymol. 259*, 43−94.
Rand, R. P., & Parsegian, V. A. (1997) in *Lipid Polymorphism and Membrane Properties* (Epand, R. M., Ed.) Academic Press, Inc., San Diego (in press).
Rand, R. P., Fuller, N. L., Butko, P., Francis, G., & Nicholls, P. (1993) *Biochemistry 32*, 5925−5929.
Rebecchi, M., Peterson, A., & McLaughlin, S. (1992) *Biochemistry 31*, 12742−12747.
Sato, N., Wang, X., & Greer, M. A. (1992) *Mol. Cell. Endocrinol. 86*, 137−142.

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