Sheorain, V. S., Corbin, J. D., & Soderling, T. R. (1985a) *J. Biol. Chem. 260*, 1567-1572.
Sheorain, V. S., Ramakrishna, S., Benjamin, W. B., & Soderling, T. R. (1985b) *J. Biol. Chem. 260*, 12287-12292.
Smith, C. J., Rubin, C. S., & Rosen, O. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2641-2645.
Sommercorn, J., Mulligan, J. A., Lozeman, F. J., & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8834-8838.
Swergold, G. D., Rosen, O. M., & Rubin, C. S. (1982) *J. Biol. Chem. 257*, 4207-4215.
Vulliet, P. R., Hall, F. L., Mitchell, J. P., & Hardie, D. G. (1989) *J. Biol. Chem. 264*, 16292-16298.
Yu, K.-T., Khalaf, N., & Czech, M. P. (1987) *J. Biol. Chem. 262*, 16677-16685.
Yu, K.-T., Benjamin, W. B., Ramakrishna, S., Khalaf, N., & Czech, M. P. (1990) *Biochem. J.* (in press).

Protein Kinase C Interaction with Calcium: A Phospholipid-Dependent Process†

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Received December 19, 1989; Revised Manuscript Received April 26, 1990

ABSTRACT: The calcium-binding properties of calcium- and phospholipid-dependent protein kinase C (PKC) were investigated by equilibrium dialysis in the presence and the absence of phospholipids. Calcium binding to PKC displayed striking and unexpected behavior; the free proteins bound virtually no calcium at intracellular calcium concentrations and bound limited calcium (about 1 mol/mol of PKC) at 200 μM calcium. However, in the presence of membranes containing acidic phospholipids, PKC bound at least eight calcium ions per protein. The presence of 1 μM phorbol dibutyrate (PDBu) in the dialysis buffer had little effect on these calcium-binding properties. Analysis of PKC–calcium binding by gel filtration under equilibrium conditions gave similar results; only membrane-associated PKC bound significant amounts of calcium. Consequently, PKC is a member of what may be a large group of proteins that bind calcium in a phospholipid-dependent manner. The calcium concentrations needed to induce PKC–membrane binding were similar to those needed for calcium binding (about 40 μM calcium at the midpoint). However, the calcium concentration required for PKC–membrane binding was strongly influenced by the phosphatidylserine composition of the membranes. Membranes with higher percentages of phosphatidylserine required lower concentrations of calcium. These properties suggested that the calcium sites may be generated at the interface between PKC and the membrane. Calcium may function as a bridge between PKC and phospholipids. These studies also suggested that calcium-dependent PKC–membrane binding and PKC function could be regulated by a number of factors in addition to calcium levels and diacylglycerol content of the membrane.

The calcium- and phospholipid-dependent protein kinase C (PKC)[1] is an important regulatory enzyme believed to be critically situated in the signal transduction cascade (Nishizuka, 1986; Nikkawa & Nishizuka, 1986). The activation of PKC by diacylglycerols links this enzyme to the regulatory scheme known as the phosphatidylinositol cycle. PKC is also a phorbol ester receptor (Castagna et al., 1982), and many phorbol ester effects are attributed to PKC. Recently, PKC has been shown to consist of a family of closely related isozymes [for a review, see Nishizuka (1989)]. Despite some molecular heterogeneity, most PKC isozymes exhibit similar biochemical properties including activation by calcium and phospholipids.

Association of PKC with membranes showed a calcium requirement that was distinct from that of activation (Bazzi & Nelsestuen, 1987; Wolf et al., 1985). In addition, calcium alters the phorbol ester binding properties of membrane-associated PKC. Thus, calcium appears to play multiple roles in the various functions of PKC. While this evidence suggests that PKC is a calcium-binding protein, we are not aware of reports of direct PKC–calcium-binding measurements. The primary sequence of PKC did not reveal the presence of known calcium sites (Parker et al., 1986; Ohno et al., 1987), and a unique structure may be responsible for calcium binding. Since PKC is an intracellular protein, it would be expected to bind calcium with high affinity.

This study was initiated to examine the calcium-binding properties of protein kinase C. A surprising result was that free PKC bound virtually no calcium at intracellular calcium concentrations and bound very little calcium at much higher calcium concentrations. However, PKC bound a large number of calcium ions (at least 8) when acidic phospholipids were present. The presence of phorbol esters did not significantly influence the the calcium-binding properties of PKC.

Experimental Procedures

Materials. Bovine brain phosphatidylserine (PS), egg yolk phosphatidylcholine (PC), and dansyl-PE were purchased from the Sigma Chemical Co. [γ-^{32}P]ATP (3 Ci/mmol) and 1,2-dipalmitoyl-L-3-phosphatidyl[*N-methyl*-^{3}H]choline (73 Ci/mmol) were purchased from Amersham Corp. $^{45}CaCl_2$ (32.89

† Supported in part by Grant GM 38819 from the National Institutes of Health.

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PC, phosphatidylcholine; PS, phosphatidylserine; dansyl-PE, *N*-dansyl-L-α-dipalmitoylphosphatidylethanolamine; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); PKC, phospholipid- and calcium-dependent protein kinase C.

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mCi/mg) was purchased from New England Nuclear and was diluted with standard $CaCl_2$ solutions as needed to produce the desired calcium concentration and specific activity. The latter was manipulated to provide radioactivity levels that gave <1% counting error in all experiments. Polycarbonate filters (0.1-μm diameter) were purchased from Nucleopore Corp. Indo-1, a fluorescent calcium-binding dye, was purchased from Molecular Probes. Other chemicals and reagents were of the highest grade available. PKC was purified to homogeneity from bovine brain according to a previously published procedure (Bazzi & Nelsestuen, 1987).

Calcium-Binding Measurements. Standard calcium solutions were made by dissolving anhydrous $CaCO_3$, that had been heated at 140 °C overnight to remove traces of water, in dilute HCl (the pH of the final solution was adjusted to 5.5). Prior to calcium-binding measurements, the proteins and phospholipids were dialyzed extensively against several changes of buffer (20 mM Tris, pH 7.8, 10% glycerol, 100 mM NaCl, and 0.5 mM DTT) to remove EGTA, EDTA, and β-mercaptoethanol that were present during purification. Contaminating calcium was detected by a fluorescent dye (see below). Radioactive PC (mixed with PC to give 6 μCi of ^3H/mg) was added to the samples so that the initial dialysis sample contained 0.33 mg/mL. These vesicles do not bind calcium or PKC and served as an internal standard to detect sample dilution that occurred during dialysis. That is, protein and phospholipid concentrations at various stages of dialysis were estimated from the ^3H content of the sample assuming that the ratio of PC to other macromolecules remained the same throughout the experiment. Various dialysis samples also contained 0.52 mg/mL PKC (6.8 μM) and/or 1.7 mg/mL phospholipid vesicles (PS/PC, 25:75).

The samples were dialyzed at 4 °C for 5–12 h against large volumes (buffer to sample ratios of ≥500:1) of calcium-containing buffer. After each dialysis step, dual label counting procedures were used to determine the concentrations of ^{45}Ca and ^3H in the sample. The calcium level was then increased by addition of $^{45}CaCl_2$ to the buffer. The half-time for attaining dialysis equilibrium, determined in a separate experiment (20 μM calcium), was slightly less than 30 min.

Estimation of calcium that was actually bound to the PKC–membrane complex required subtraction of calcium that was bound to the phospholipid alone. This was accomplished by simple subtraction of calcium bound to an identical amount of phospholipid that did not contain PKC. Unfortunately, this subtraction will overestimate the background and will underestimate the number of calcium ions bound to the PKC–membrane complex. This error arises from the fact that some of the PS molecules will be bound to PKC and will not contribute to the background calcium binding. In effect, the sample containing PKC has fewer PS molecules that contribute to the background than does the same amount of phospholipid alone. The magnitude of this background subtraction error is discussed below.

Calcium binding to PKC was also measured by the method of Hummel and Dreyer (1962). BSA was added to the buffer to prevent adsorption of PKC to the column matrix, and to stabilize the free protein in the presence of calcium (Bazzi & Nelsestuen, 1989a). The concentration of calcium in each fraction was estimated by sampling the radioactivity present, and the PKC was located by its kinase activity toward protamine sulfate.

Protein–Phospholipid Binding. Association of PKC with phospholipid vesicles was measured by fluorescence energy transfer methods as described in detail previously (Bazzi & Nelsestuen, 1987). Fluorescence energy transfer from tryptophan in the protein (excitation at 284 nm) to dansyl groups in the phospholipid (emission at 520 nm, with a 500-nm cutoff filter) was used to monitor the association of the protein–phospholipid complex as a function of calcium in the medium. Protein (41 μg) and phospholipid vesicles (12 μg) were initially mixed in 1.6 mL of buffer containing 50 mM Tris, pH 7.5, and 100 mM NaCl, and the calcium concentration was increased by successive additions of calcium. Direct excitation of the dansyl group at 284 nm produced a reference emission intensity (I_0). Fluorescence energy transfer was expressed as a percentage change in emission intensity, $(I - I_0)100/I_0$, where I is the fluorescence intensity of the protein–lipid complex and I_0 is the intensity of the phospholipid alone.

Fluorescence measurements were made at 25 °C in a Hitachi–Perkin-Elmer Model MPF 44A fluorescence spectrophotometer.

Estimation of Contaminating Calcium. The calcium concentrations at which most of the observed binding occurred were relatively high, and contaminating calcium levels should have relatively little effect. Nevertheless, contaminating calcium was monitored to ensure that it did not influence the major conclusions of this study. Distilled water was deionized and charcoal-filtered by a Nanopure water system (Barnstead) before use. All the materials used in dialysis measurements were reagent grade. Dialysis tubing (Spectra/por 4) was prepared by heating at 80 °C for 1 h in a solution containing 1 mM EDTA and 1 mM $NaHCO_3$, and was stored and thoroughly rinsed in Nanopure water before use. The level of contaminating calcium in the dialysis buffer (contained 20 mM Tris, pH 7.8, 10% glycerol, 0.5 mM dithiothreitol, and 100 mM NaCl) was estimated by using indo-1. This fluorescent probe is sensitive to calcium concentrations in the nanomole range (Grynkiewicz et al., 1985) and was used at a concentration of 1 μM.

Despite the precautions given above, the buffers contained calcium as indicated by indo-1 fluorescence emission at 400 nm (Figure 1A). However, this signal was eliminated by 2 μM EGTA (Figure 1B) and was reduced by 80% by 1 μM EGTA (Figure 1B). This indicated that the original buffer solutions contained slightly less than 1 μM calcium. For reference, EGTA titration of buffer containing 4 μM added calcium is given in Figure 1B. Buffer that had been manipulated in the manner of a typical equilibrium dialysis experiment, except that no exogenous calcium was added, showed contaminating metal ions that were substantially less than 2 μM (Figure 1B). Metal ion contamination in the phospholipid preparations was similar to the amount in buffer that had been exposed to dialysis conditions (Figure 1B).

These levels of calcium contamination were low relative to the calcium added in most experiments. Therefore, the free and bound calcium levels reported in this study were calculated from the added calcium without correction for contaminating metals. This method of calculation will produce significant error for the results at the lowest concentrations of added calcium. However, these errors were substantially offsetting; contaminating calcium will increase free calcium levels and decrease its specific activity. Calculations that do not consider this calcium will underestimate free and bound calcium concentrations to similar extents so that the slope of the resulting binding curve (bound vs free calcium) will be unaffected. Overall, the bound and free calcium concentrations reported at the lowest calcium concentrations used are viewed as nominal values that accurately document the slope and trend of the calcium-binding curve.

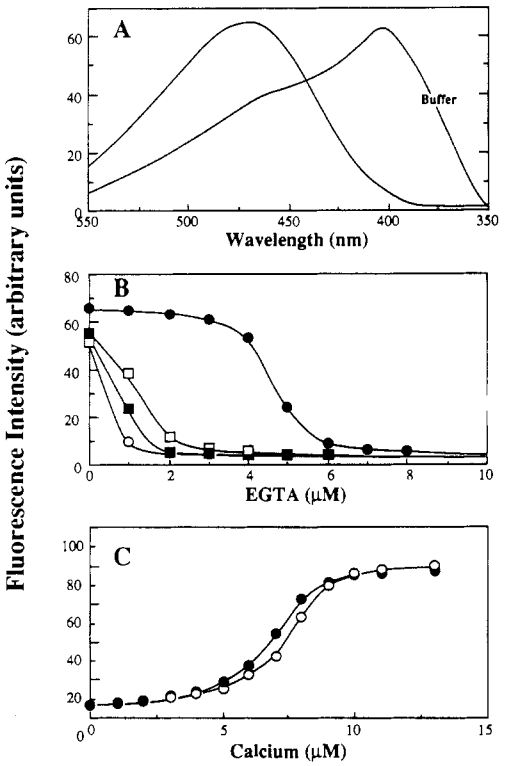

FIGURE 1: Estimation of contaminating calcium. Panel A shows the fluorescence spectrum of indo-1 (1 μM) mixed in the dialysis buffer used for calcium-binding measurements, before (spectrum with high-intensity emission at 400 nm) and after (spectrum with low-intensity emission at 400 nm) the addition of 2 μM EGTA. Panel B shows the magnitude of indo-1 (1 μM) fluorescence emission at 400 nm as function of added EGTA. The titrations shown include freshly prepared buffer (○), buffer plus 4 μM Ca (●), buffer plus 0.15 mg/mL phospholipid vesicles (PS/PC, 25:75) (□), and buffer that had been subjected to dialysis conditions for 7 h (■). Panel C shows the titration of buffer containing 10 μM EGTA either with standard 200 μM calcium (○) or with the 200 μM calcium dialysis buffer after equilibrium dialysis was completed (●).

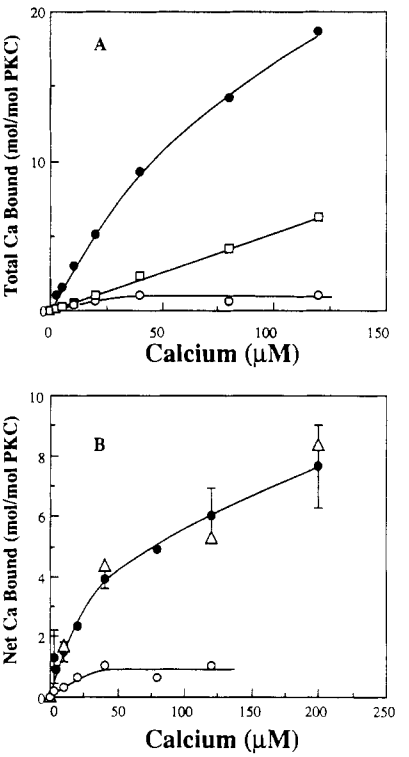

FIGURE 2: Calcium binding to PKC measured by equilibrium dialysis. Panel A shows total calcium binding to free PKC (○), PKC plus phospholipid vesicles (●), and phospholipid vesicles alone (□). Panel B shows specific calcium binding to PKC in the presence (●) or the absence of phospholipid vesicles (○). The error bars represent standard deviations for at least four separate values. Values given without error bars represent single determinations. The nature of preliminary or incomplete data sets that are not included in the graph is discussed in the text. Panel B also shows the of the results of calcium binding conducted in buffer containing 1 μM PDBu (△). In both panels, the dialysis buffer contained 20 mM Tris, pH 7.8, 10% glycerol, 100 mM NaCl, 0.5 mM DTT, and the indicated concentration of calcium. The phospholipid vesicles were composed of PS/PC (25:75).

At the higher calcium concentrations where most binding occurred, contaminating calcium concentrations were not significant. For example, after equilibrium dialysis had been completed, the calcium concentration of the dialysis buffer was compared to the standard $CaCl_2$ solution by titration of an EGTA/indo-1 solution (Figure 1C). The midpoint for this titration differed from the standard by less than 6%.

Other Methods. Small unilamellar vesicles of defined compositions were prepared by sonication and gel filtration chromatography (Huang, 1969; Bazzi & Nelsestuen, 1987). Phospholipid concentrations were determined from organic phosphate (Chen et al., 1956) using a phosphorus to phospholipid weight ratio of 1:25. Membranes of PS/PC are stable in calcium solutions and do not undergo detectable calcium-dependent fusion or aggregation under the conditions used in this study (Nelsestuen & Lim, 1977; Schwalbe et al., 1989). Protein concentration was determined by using the Bradford reagent with BSA as a standard, and comparable results were obtained, when possible, by the BCA method (Pierce Chemical Co.). PKC activity was measured by using protamine sulfate as a substrate (Bazzi & Nelsestuen, 1989a).

RESULTS

Phospholipid-Dependent Calcium Binding. Calcium binding to PKC was examined in the presence or the absence of vesicles containing acidic phospholipids, and typical results are shown in Figure 2A. In the absence of phospholipid vesicles, PKC bound little or no calcium at intracellular calcium concentrations (<10 μM, Figure 2A). Furthermore, PKC bound only about 1 calcium ion/protein at the highest concentration of calcium tested (200 μM). In five out of six separate experiments, free PKC bound less than 1.0 calcium ion below 50 μM free calcium (data not shown). In contrast, the PKC–phospholipid complex bound large amounts of calcium, reaching an uncorrected total of 17 calcium ions per PKC molecule at 200 μM calcium (Figure 2A).

Determination of net calcium bound to the PKC–membrane complex required measurement of calcium bound to phospholipid alone. This represented nonspecific binding and was subtracted as a background. The curve drawn through the data for the phospholipid alone (Figure 2A) fit an apparent K_d for the calcium–PS interaction of 0.53 mM assuming that one calcium bound per two exposed PS molecules and that 67% of the PS are exposed on the outer surface of the vesicles. This appeared very consistent with earlier reports of an apparent K_d for calcium binding to PS/PC (50:50) of 0.44 mM (Nelsestuen & Lim, 1977) and an apparent K_d for calcium binding to pure PS of 0.26 mM (Portis et al., 1979). This trend suggested that the affinity of calcium for PS is influenced by the charge density of the membrane.

The net amount of calcium bound per PKC was estimated by simple subtraction of calcium bound to the phospholipid sample from calcium bound to the PKC–phospholipid sample (Figure 2B). Net calcium binding reached about 8 calciums per PKC and was half of that value at about 40 μM free calcium. For reasons outlined under Experimental Procedures,

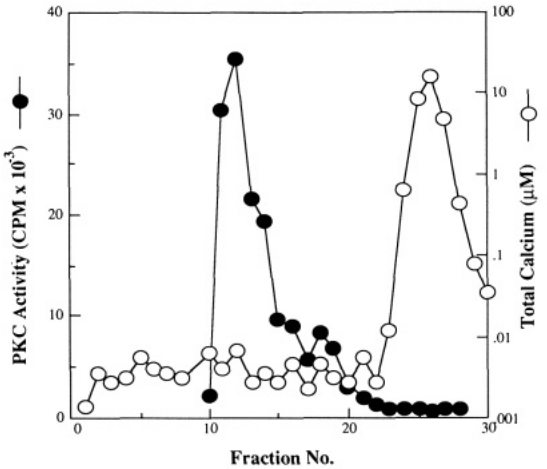

FIGURE 3: Dissociation of calcium and PKC from the membrane. PKC–Ca–phospholipid complex (sample that was dialyzed in the presence of 1 μM PDBu, see Figure 2) was made 2.0 mM in EGTA and was applied on a Sephacryl S-300 column (1.0 × 30 cm). The column was equilibrated and eluted (0.75 mL/fraction) with a buffer containing 20 mM Tris, pH 7.5, 100 mM NaCl, 10% glcyerol, 30 mM β-mercaptoethanol, 1 mM EDTA, and 1 mM EGTA. Protein kinase activity (●) toward protamine sulfate and calcium concentration (○) are shown.

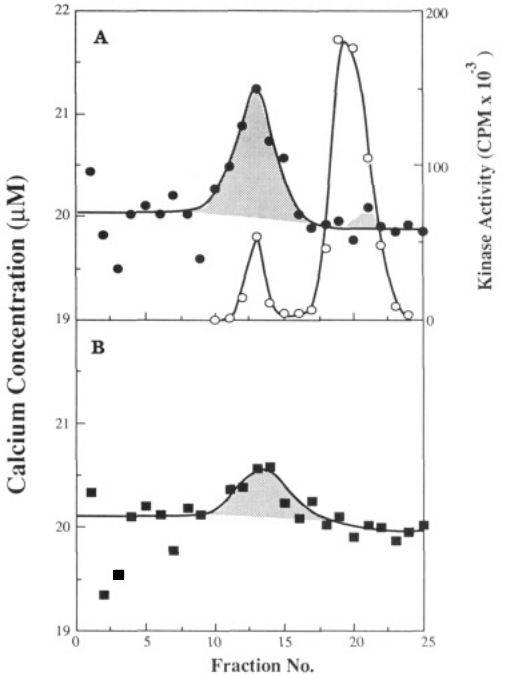

FIGURE 4: Determination of calcium binding to PKC protein by gel filtration. Samples containing either 54.5 μg of PKC plus 177 μg of phospholipids (panel A) or 177 μg of phospholipid vesicles only (panel B) were incubated in 0.6 mL of equilibration buffer for 20 min. The samples were applied on Sephacryl S-300 columns (1.0 × 30 cm) equilbrated and eluted (0.75 mL/fraction) with a buffer containing 20 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, 0.5 mM ditheothreitol, 0.5 mg/mL BSA, and 20 μM ^{45}Ca. Protein kinase activity (○) toward protamine sulfate and calcium concentration (●, ■) are shown.

this background subtraction will be too high. The magnitude of this error can be estimated by a simple calculation that assumes that each PKC molecule bound eight PS molecules and that nonspecific calcium binding was directly proportional to the available PS. If 67% of the phospholipid is accessible on the outside of the vesicles, the concentration of total PS in the sample was 380 μM, and 15% would be complexed with PKC. Overestimation of the background by 15% would correspond to about one more calcium ion specifically bound per PKC. An error of this magnitude would not alter the major conclusions which are that calcium binding to PKC consists of a large number of sites that are dependent almost entirely upon the presence of phospholipid.

The high standard deviation for calcium binding at 2.5 μM calcium (Figure 2B) arose from 2 anomalous data points (of a total of 12 experiments). The actual values for these anomolous determinations were 2.5 and 3.7 calcium ions bound per PKC. This high calcium binding appeared to be artifactual for several reasons. It was not maintained through the course of dialysis, and the second point in the calcium titration curve returned to normal values. Immediate repetition of these experiments with the same protein and phospholipid preparations did not replicate the high values. Overall, while these two anomalously high values appeared to be artifactual and could not be repeated, they were included in the averaged results since the nature of the artifact could not be determined.

Phorbol esters activate PKC at low concentrations of calcium (Blumberg, 1988; Ashendel, 1985), suggesting that phorbol esters may interact with PKC and increase the enzyme's affinity for calcium. However, we found that phorbol esters exerted little or no effect on the calcium-binding properties of PKC. The results in Figure 2B show single determinations. However these results were consistent with two other experiments that measured calcium binding only up to 30 μM calcium (data not shown). This result was consistent with previous direct PKC–phospholipid-binding measurements (Bazzi & Nelsestuen, 1987) where phorbol esters showed no detected effect on PKC–membrane binding.

However, one effect of PDBu on PKC was illustrated by the fact that PKC, dialyzed in the presence of PDBu, could not be dissociated from the membrane by calcium chelation (Figure 3). Similar experiments conducted in the absence of phorbol esters gave 30–80% protein dissociation after calcium chelation (data not shown). The results in Figure 3 also showed that calcium dissociated from the PKC–phospholipid complex. While about 23% of the calcium present in the sample loaded onto the column (Figure 2B, △) was bound to the PKC–phospholipid complex, no detectable calcium (<0.07% of that eluted from the column) eluted with PKC–membrane complex in Figure 3.

Calcium binding to PKC was also examined by gel filtration chromatography (Hummel & Dryer, 1962). The calcium concentration used (20 μM) was insufficient to bind all of the protein to the membrane, and nearly 85% of the applied protein eluted in the position of free protein (Figure 4A). The calcium elution pattern showed that calcium was bound to the protein–membrane complex. Excess calcium eluting with the PKC–phospholipid complex (Figure 4A) was considerably greater than that eluting with the phospholipid alone (Figure 4B). This result contrasted greatly with the case of free PKC where no excess calcium was associated with the peak of free protein (Figure 4A). Integration of the excess calcium eluting with the protein peaks (shaded areas in Figure 4) suggested that the amount of calcium bound to membrane-associated PKC was at least 12 times greater than that bound to free PKC. When combined with the fact that there was more than 5 times as much free PKC, the estimated calcium-binding stoichiometry for the free protein was ≤1.7% of the calcium bound to membrane-bound PKC.

Thus, both the gel filtration and the equilibrium dialysis techniques supported the conclusion that free PKC bound extremely little calcium and the results only differed in the limit of detection. Of the two methods used, the gel filtration technique (Figure 4A) indicated a lower amount of calcium

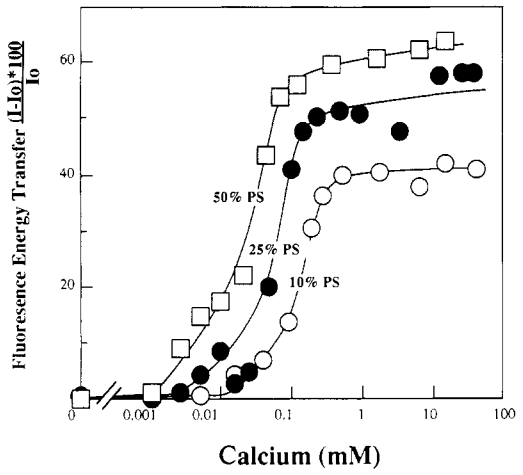

FIGURE 5: Calcium requirements for protein–membrane binding as a function of membrane composition. The binding of PKC to phospholipid vesicles was measured by fluorescence energy transfer (verticle axis, see Experimental Procedures) for membranes containing 10% dansyl-PE and either 10% (○), 25% (●), or 50% (□) PS. The remaining phospholipid (to 100%) was PC. The measurements were performed in 1.6 mL of buffer containing 20 mM Tris (pH 7.5) and 100 mM NaCl using 41 μg of protein plus 12 μg of phospholipid vesicles. Calcium concentrations in the sample were varied by sequential addition of standard calcium solutions. The signal for each calcium addition became stable within seconds and did not change over a time period needed to complete the entire titration. All signal changes were reversed with EDTA.

bound to free PKC. This technique may give the more reliable result. For equilibrium dialysis, the protein was sampled from a solution that was still in contact with the dialysis membrane as well as with any impurities that the membrane may contain. Impurities may enhance calcium binding. The gel filtration techniques, on the other hand, measured protein that eluted from a column in a continuous manner and was separated from large and small contaminants.

Protein–Phospholipid Binding. The association of PKC with phospholipids was studied to test whether its behavior correlated with calcium-binding properties. A simple comparison is whether or not the calcium concentration required for PKC–membrane binding correlated with calcium binding to complex. Indeed, for membranes containing 25% PS (the same composition used for dialysis measurements), the midpoint of the membrane-binding curve (Figure 5) compared favorably with that of the calcium-binding curve (Figure 2B). These results suggested that both measurements, protein–membrane binding and calcium binding, measured coincident events.

PKC–membrane binding showed other characteristics that could be important to PKC function. Since PKC bound calcium in a phospholipid-dependent manner, calcium binding to the membrane should be influenced by the charge density of the membrane. The results in Figure 5 showed that the calcium requirement for binding was quite dependent on the composition of membranes. For example, membranes containing 50% PS required approximately 10-fold less calcium than those containing 10% PS. It is clear that, at limiting calcium, PKC will show high preference for membranes of high acidic phospholipid content.

Discussion

The results obtained in this study showed that PKC bound calcium in a phospholipid-dependent manner; the free protein bound little or no calcium, but at least eight calcium ions were associated with the protein–membrane complex. While other models cannot be ruled out, it seems probable that the calcium-binding sites are generated at the interface between the proteins and the membrane so that calcium effectively forms the "bridge" that holds the protein and phospholipid complex together. This mechanism of calcium binding is similar to that proposed for the vitamin K dependent calcium-binding proteins where various models for protein–membrane interaction have been discussed [see Schwalbe et al. (1989) and references cited therein]. The calcium-binding properties of PKC may enhance understanding of several other properties of PKC.

In the presence of calcium, PKC has a very high affinity for membranes containing acidic phospholipids, with an estimated dissociation constant of ≤5 nM (Bazzi & Nelsestuen, 1987). Such a tight interaction between protein and membrane might be generated by many points of contact provided by a large number of calcium ions between the components. It has been proposed that the PKC–membrane contact might consist of bridging by as few as one calcium ion (Ganong et al., 1986; Hannun et al., 1986). However, additional stabilizing factors would appear necessary to explain the high affinity of the PKC–membrane interaction. It should be emphasized that the bridging mechanism for generating a high-affinity PKC–membrane complex applies exclusively to the reversible PKC–phospholipid complex. PKC also forms an irreversible complex with phospholipid vesicles that was stable in the absence of calcium (Bazzi & Nelsestuen, 1988; also see Figure 3 above).

Phorbol ester binding has a major feature in common with calcium binding to PKC which is that only the membrane-associated form of the enzyme bound either calcium or phorbol esters to a detected extent. It is possible that both calcium and phorbol esters bind at the protein–membrane interface so that each contains partial binding sites. The half-sites provided by the protein may be too weak to be detected by themselves. The more highly active phorbol esters have an essential lipophilic arm (Wender et al., 1986), which might from part of a membrane interaction. Much further work will be needed to precisely locate the binding sites for calcium and phorbol esters.

The primary structure of PKC did not reveal known calcium-binding sites such as the EF-hand or calelectrin-like sequence (Parker et al., 1986; Ohno et al., 1987). This study suggested that PKC probably should not be formally classified as an intracellular calcium-binding protein at all. Free PKC had such low affinity for calcium that it would not bind significant levels of calcium at intracellular calcium concentrations. Endogenous calcium in the buffer would displace the binding curve somewhat (see above) but would not mask high-affinity binding sites on free PKC, if they were present. The results showed little calcium binding to the free protein under any conditions, and we conclude that PKC had few if any calcium-binding sites. If protein kinase C contains calcium ions that do not exchange at all during this procedure, they will appear as a part of the intrinsic protein properties and would not respond to added calcium.

Some observations have suggested that high calcium concentrations enhance the interaction of PKC with column matrices (Bazzi & Nelsestuen, 1989a) or hydrophobic affinity columns (Walsh et al., 1984). It is possible that this property arises from the low levels of calcium binding to the free protein. Alternatively, calcium sites may be generated at the interface between PKC and the synthetic materials. Further work will be needed to define this calcium-dependent property.

A group of proteins that display qualitatively similar calcium-binding properties are the lipocortins or calpactins [for a review, see Klee (1988)]. For example, lipocortin II binds

2 mol of calcium in the presence of acidic phospholipids but has very low affinity for calcium in the absence of phospholipids (Glenney, 1986). Similarly, lipocortin I binds 3–4 mol of calcium in a phospholipid-dependent manner (Schaelpfer & Haigler, 1987). In this case, it was proposed that phospholipids functioned by increasing the affinity of these proteins for calcium. However, it seems likely that the lipocortins are qualitatively similar to PKC and that they belong to the same general family of calcium-interacting proteins. A major difference is that the lipopcortins bind many fewer calcium ions per protein. This may require different types of interaction with membranes. For example, the lipocortins either may have lower membrane-binding affinity due to a smaller number of contacts or may have other types of protein–membrane contacts. We have isolated other intracellular calcium-dependent phospholipid-binding proteins. Two of these proteins have been found to display interaction with calcium that is very similar to that shown here for PKC (Bazzi & Nelsestuen, 1990). Thus, the general property of phospholipid-dependent calcium binding may be common to a large group of intracellular proteins.

A potentially troubling property of calcium and membrane binding by PKC is the high calcium requirement. These results may initially seem to contradict reports that PKC can be activated by micromolar concentrations of calcium. However, the results are not necessarily in conflict. Many studies on activation of PKC utilize conditions that are far removed from those used in this study. For example, kinase activity is usually measured in a medium containing low ionic strength. In contrast, this study utilized ionic strength that was close to the physiological level. Activity measurements are often conducted with membranes of 100% acidic phospholipid. This study utilized membranes containing an acidic phospholipid content that approximated the average for intracellular membranes. Activation of PKC using membranes of low PS content and physiological ionic strength does require calcium concentrations similar to those required in this study (Bazzi & Nelsestuen, 1987). Another factor is that the in vitro assay utilizes high phospholipid to protein ratios (typically more than 1000:1) while this study utilized much lower ratios. At an extremely high phospholipid levels, mass action might allow virtually quantitative PKC–membrane binding at calcium concentrations far below the midpoint for the titrations in this study. In fact, detectable levels of PKC–membrane binding did occur at micromolar levels of calcium, especially with membranes containing 50% PS (Figure 5). Thus, as normally designed, the in vitro assay conditions may allow activation of PKC at micromolar calcium concentrations despite an overall calcium-binding affinity such as that observed in this study.

Despite the high calcium requirement for calcium– and membrane–PKC binding, it is likely that these interactions are important intracellular events that might be regulated by multiple conditions. In this respect, it is important to note that calcium binding at the interface between two components will differ greatly from calcium binding to a monomeric protein. In the latter case, the free calcium concentration needed for calcium binding is independent of protein concentration. In contrast, calcium binding at the interface between a protein and a membrane will be influenced by many factors including the absolute concentration of each component, the ratio of the two, and the composition of the membrane component (e.g., see Figure 5). For example, if regions of the cell membrane are enriched in acidic phospholipids, PKC and other proteins should concentrate at that site, and some PKC–membrane binding may occur at intracellular calcium concentrations. Alternatively, chemical derivatization of PKC may reduce the calcium required for PKC–Ca–membrane interaction. For example, phosphorylation and partial proteolysis (Schaelpfer & Haigler, 1987; Ando et al., 1989; Chuah & Pallen, 1989) have been shown to reduce the calcium requirements for lipocortin I–membrane binding, and such chemical modifications may be necessary for PKC to function under intracellular conditions. Thus, PKC response to micromolar increases in intracellular calcium and DAG appearance may require other conditions or factors.

The PKC preparation used in this study consisted of a mixture of isozymes, and it is possible that each has somewhat different calcium-binding characteristics. However, it is likely that each isozyme will conform to the general, striking behavior demonstrated in this study so that all PKC isozymes will bind a large number of calcium ions in a phospholipid-dependent manner. Some comparisons of PKC isozymes suggest quantitative differences but do not show major qualitative differences. for example, nPKC has been reported to bind phorbol esters in a calcium-independent manner which constituted a large difference from other isozymes (Ohno et al., 1988; Akita et al., 1990). However, under certain conditions, other isozymes will associate with membranes in an irreversible manner in response to phorbol esters (Bazzi & Nelsestuen, 1989b), and the irreversible PKC–membrane complex binds phorbol esters in a calcium-independent manner (Bazzi & Nelsestuen, 1988). Thus, most PKC isozymes may bind phorbol esters in a calcium-independent manner but with differences in the conditions needed to generate this interaction. Ultimately, while different forms of PKC may show somewhat different metal ion binding properties, it seems likely that most or all forms will show the feature of phospholipid-dependent calcium binding that is demonstrated in this study.

Registry No. PKC, 9026-43-1; Ca, 7440-70-2.

References

Akita, Y., Ohno, S., Konno, Y., Yano, A., & Suzuki, K. (1990) *J. Biol. Chem. 265*, 354.

Ando, Y., Imamura, S., Hong, Y.-M., Owada, M. K., Kakunaga, T., & Kannagi, R. (1989) *J. Biol. Chem. 264*, 6948.

Ashendel, C. L. (1985) *Biochim. Biophys. Acta 822*, 219.

Bazzi, M. D., & Nelsestuen, G. L. (1987) *Biochemistry 26*, 115.

Bazzi, M. D., & Nelsestuen, G. L. (1988) *Biochemistry 27*, 7589.

Bazzi, M. D., & Nelsestuen, G. L. (1989a) *Biochemistry 28*, 3577.

Bazzi, M. D., & Nelsestuen, G. L. (1989b) *Biochemistry 28*, 9317.

Bazzi, M. D., & Nelsestuen, G. L. (1990) *FASEB J. 4*, 1785 (Abstract).

Blumberg, P. M. (1988) *Cancer Res. 48*, 1.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.

Castagna, M., Takai, Y., Kiabuchi, K., Sano, K., Kikkawa, U., & Nishizuka, Y. (1982) *J. Biol. Chem. 257*, 7847.

Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756.

Chuah, S. Y., & Pallen, C. J. (1989) *J. Biol. Chem. 264*, 21160.

Ganong, B. R., Loomis, C. R., Hannun, Y. A., & Bell, R. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1184.

Glenney, J. (1986) *J. Biol. Chem. 261*, 7247.

Gopalakrishna, R., Barsky, S. H., Thomas, T. P., & Andreson, W. B. (1986) *J. Biol. Chem. 261*, 16438.

Grynkiewics, G., Poenie, M., & Tsien, R. Y. (1985) *J. Biol. Chem. 260*, 3440.
Hannun, Y. A., Loomis, C. R., & Bell, R. M. (1986a) *J. Biol. Chem. 261*, 7184.
Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55.
Huang, C. (1969) *Biochemistry 8*, 344.
Hummel, J. P., & Dreyer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530.
Kikkawa, U., & Nishizuka, Y. (1986) *Annu. Rev. Cell Biol. 2*, 149.
Klee, C. B. (1988) *Biochemistry 27*, 6645.
Nelsestuen, G. L., & Lim, T. K. (1977) *Biochemistry 16*, 4164.
Nishizuka, Y. (1986) *Science 233*, 305.
Nishizuka, Y. (1989) *Nature (London) 334*, 661.
Ohno, S., Kawasaki, H., Imajoh, S., Suzuki, K., Inagaki, M., Yokokura, H., Sakoh, T., & Hidaka, H. (1987) *Nature (London) 325*, 161.
Ohno, S., Akita, Y., Konno, Y., Imajoh, S., & Suzuki, K. (1988) *Cell 53*, 731.
Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stable, S., Waterfield, M. D., & Ullrich, A. (1986) *Science (Washington, D.C.) 233*, 853.
Portis, A., Newton, C., Pangborn, W., & Papahadjopoulos, D. (1979) *Biochemistry 18*, 780.
Schlaepfer, D. D., & Haigler, H. T. (1987) *J. Biol. Chem. 262*, 6931.
Schwalbe, R. A., Ryan, J., Stern, D. M., Kisiel, W., Dahlbäck, B., & Nelsestuen, G. L. (1989) *J. Biol. Chem. 264*, 20288.
Walsh, M. P., Valentine, K. A., Nga, P. K., Carruthers, C. A., & Hollenberg, M. D. (1984) *Biochem. J. 224*, 117.
Wender, P. A., Koehler, K. F., Sharkey, N. A., Dell'Aquila, M. L., & Blumberg, P. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4214.
Wolf, M., LeVine, H., May, S., Cuatrecases, P., & Sahyoun, N. (1985) *Nature 37*, 546.