Vol. 152, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1988 Pages 336-343

CONSTITUTIVE ACTIVITY OF MEMBRANE-INSERTED PROTEIN KINASE C

Mohammad D. Bazzi and Gary L. Nelsestuen

Department of Biochemistry, University of Minnesota, St. Paul, MN, 55108

Received February 12, 1988

Summary. Incubation of purified protein kinase C (PKC) with phospholipid vesicles produced two populations of membrane-bound PKC: one population was dissociated by calcium chelation and the other was not. The second population appeared to be inserted into the membrane. The activity of membrane-inserted PKC was Ca^{2+}-independent and was only modestly sensitive to phorbol esters. Insertion was caused by high calcium concentrations or by phorbol esters plus low calcium. These conditions correlated with those needed to activate PKC; insertion into the membrane may be a primary mechanism of PKC activation. PKC may be a long-term cell regulator which becomes inserted into the membrane upon appearance of the second messengers, calcium and diacylglycerol, and remains in an active membrane-bound state when the second messengers have been removed.

The mechanisms of protein kinase C (PKC) activation and inhibition are of interest because of possible function of these processes in transduction of extracellular signals (1-3). With certain substrates (4) PKC activity requires Ca^{2+} and phospholipid. Activity at low Ca^{2+} concentrations is greatly stimulated by diacylglycerol (DAG) or phorbol esters (1, 5). Other structurally diverse compounds that have tumor-promoting activity are able to substitute for DAG or phorbol esters (6-9). The mechanisms of these activations are not understood.

Recent studies showed that calcium-dependent binding of PKC to membranes did not correlate with appearance of activity (10). Subsequent studies showed that binding of substrate to the membrane was also required for PKC activity (4, 11, 12). Calcium-dependent binding of PKC to vesicles was observed to be partially irreversible (10) although the properties of the irreversibly bound PKC were not documented. A study of PKC binding to phospholipid monolayers (13) has suggested that PKC is capable of inserting into the hydrocarbon region of the phospholipid, thereby forming a calcium-independent PKC-phospholipid complex. In this study, we examined the PKC-membrane insertion event and the influence of this process on PKC activity and its cofactor requirements. The results indicated a two-stage binding of PKC to phospholipid vesicles where the second stage consisted of insertion into the membrane. Phorbol 12, 13 dibutyrate (PDBu) enhanced formation of the irreversible complex. The activity of the inserted protein was no longer stimulated by calcium. The insertion process may explain the function of phorbol esters and diacylglycerol in PKC activation.

Materials and Methods

The sources of purified PKC, substrates, and other reagents have been described in detail previously (10). Phospholipid vesicles were formed by dissolving brain extract (Folch fraction III

0006-291X/88 $1.50

or VII, Sigma Chemical Co.) in chloroform, drying the solution with a stream of nitrogen, and suspending the phospholipids in 20 mM Tris buffer (pH 7.5) by brief, direct probe sonication. PKC activity was assayed in the presence and absence of Ca^{2+} as described by Kikkawa et al. (14).

To produce irreversible binding to phospholipids, PKC was incubated for specified times with phospholipid ± Ca^{2+} and ± 30 nM PDBu. One half of the sample was assayed in the presence of Ca^{2+}. EGTA (1.5 mM in excess over Ca^{2+}) was added to the other half of the sample before assay. Background activity was determined from protein incubated with phospholipid vesicles as above but with EGTA present at all times.

Other additions to the incubation mixtures included 30 nM PDBu. The extent of calcium-independent activity was determined as described above. Assays were conducted in the presence of either calcium or EGTA (as above). PDBu was added at the beginning of the assay time as needed to give identical assay conditions.

Results

The effect of incubating a PKC-phospholipid complex for 20 minutes on subsequent PKC activity is shown in Figure 1. Incubation under conditions were PKC was membrane-bound (high

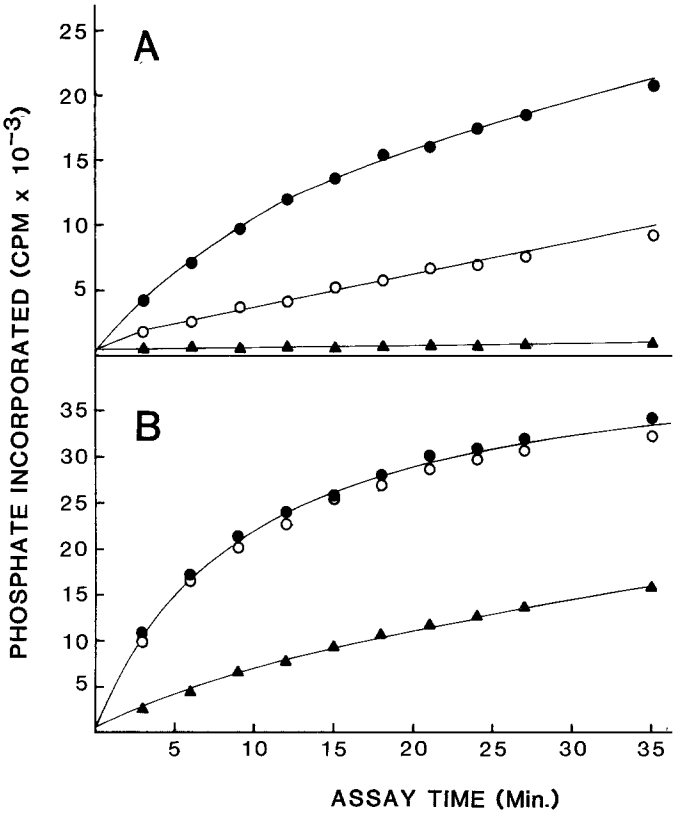

Figure 1. Generation of Ca^{2+}-independent activity from PKC. PKC (0.1 μg) was incubated with phospholipid vesicles (0.2 mg/ml) in the presence of either 0.5 mM Ca^{2+} or 1.0 mM EGTA. After a 20 minute incubation, samples were divided in half and EGTA was added to one half (final concentration of 2.0 mM). After an additional 10 minutes, substrate, $MgCl_2$, and radiolabeled ATP were added. The amount of phosphorylated product was determined at various times and the data are plotted. The results show phosphorylation by PKC that was incubated and assayed in the presence of Ca^{2+} (●), incubated in the presence of Ca^{2+} but assayed in the presence of EGTA (O), and PKC that was incubated and assayed in the presence of EGTA (▲). The incubation was carried out in the absence (panel A) or the presence (panel B) of 30 nM PDBu.

calcium, Fig. 1A) generated significant calcium-independent activity. This calcium-independent activity appeared to be induced by a PKC-membrane binding event since the enzyme preparations did not display significant Ca^{2+}-independent activity when PKC was not membrane-bound during the incubation (Fig. 1A). Production of Ca^{2+}-independent activity was enhanced by 30 nM PDBu in the incubation mixture (Fig. 1B). In this case, PKC activity was not significantly inhibited by EGTA. Phorbol esters alone produced some Ca^{2+}-independent activity (Fig. 1B).

Ca^{2+}-independent kinase activity is found in a proteolytic fragment of PKC (15-18) and this might account for the results in Figure 1. The preparations of PKC used in this study were stable for long periods of time and did not show this cleavage event. However, to establish that Ca^{2+}-independent activity was actually due to PKC-membrane insertion, an incubation mixture was subjected to gel filtration. The results (Fig. 2, solid line) showed that a substantial portion of the kinase activity eluted with the phospholipid vesicles, even in the presence of EGTA, and that a smaller portion eluted at the position of the free enzyme. PKC incubated under the same conditions but in the absence of phospholipid eluted at its expected position (Fig. 2, dashed line) showing that the PKC was monomeric and not aggregated. These results suggested that incubation conditions produced two populations of membrane-bound PKC, a population dissociated by EGTA, and an irreversibly-bound population.

The cofactor requirements of the membrane-inserted PKC were compared to those of the free enzyme (Table I). The original PKC preparation showed the expected properties and its activity was stimulated by phospholipids, phorbol esters, and Ca^{2+}. In contrast, membrane-inserted PKC was completely independent of Ca^{2+} or added phospholipids and was only modestly influenced by PDBu (Table I).

The time-dependence of generating Ca^{2+}-independent activity was also examined. In the absence of PDBu, 0.5 mM Ca^{2+} caused time-dependent formation of Ca^{2+}-independent activity

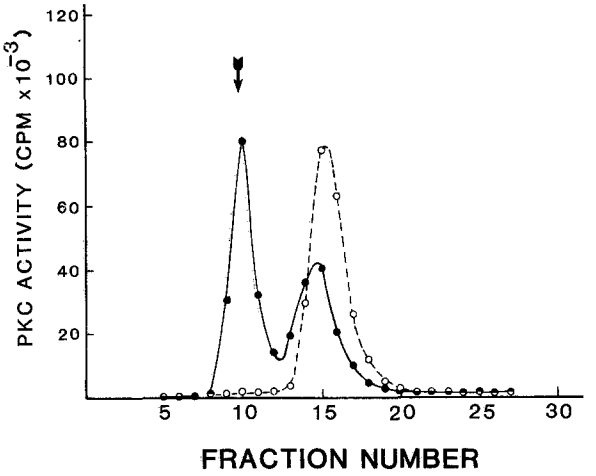

Figure 2. Separation of irreversibly membrane-bound PKC by gel filtration. PKC (2.0 μg) was incubated in 0.5 mM Ca^{2+} in the presence (●) or absence (O) of phospholipid vesicles (1 mg/mL). After 20 minutes, EGTA was added (final concentration of 2.0 mM) and the sample was applied to a Sephacryl S300 column (25 x 1.5 cm) equilibrated with buffer containing 20 mM Tris (pH 7.5), 1.0 mM EDTA, 1.0 mM EGTA, and 30 mM ß-mercaptoethanol. The arrow shows the exclusion volume of the column which was determined separately. Each fraction was approximately 2.3 mL.

Table I

Cofactor Requirements of Membrane-Inserted and Free PKC

Additions			PKC Activity			
Ca^{2+}	Phospholipid	PDBu	Free PKC		Membrane-Inserted[a]	
+	+	+	22050	(100)[b]	35164	(100)[b]
-	+	+	3879	(17.6)	32610	(92.7)
+	-	+	2980	(13.5)	35688	(101.5)
-	-	+	2753	(12.5)	30521	(86.8)
+	+	-	4659	(21.1)	24092	(68.5)
-	+	-	2489	(11.3)	21215	(60.3)
+	-	-	2648	(12.0)	21734	(61.8)
-	-	-	2622	(11.9)	19740	(56.1)

The activity of PKC was measured in the presence of 20 mM Tris buffer (pH 7.5), 0.2 mg/mL histone III-S, 10 mM $MgCl_2$, 10 μM γ-^{32}P ATP, with or without 1.5 μM $CaCl_2$, 30 nM PDBu, and/or 0.2 mg/mL phospholipid vesicles. The concentration of calcium (1.5 μM) represents the free calcium concentration calculated as described previously (4).

[a] Membrane-inserted PKC was from fractions 8, 9 and 10 of the column elution shown in Figure 2. This preparation contained 0.25 mg/mL phospholipid.

[b] The values in parentheses represent the percentage of the activity observed in the presence of all cofactors.

(Fig. 3A) and PDBu gave a small enhancement of this process. However, at low calcium concentrations (Fig. 3B), the effect of PDBu was pronounced. Since formation of Ca^{2+}-independent activity appeared to be the result of PKC-membrane insertion (see above), it followed that phorbol esters functioned by facilitating this process. These results suggested that, at low Ca^{2+} concentrations, PKC could not penetrate phospholipid vesicles. Addition of PDBu rendered this insertion possible.

Discussion

Previous direct measurement of calcium-dependent PKC-membrane binding revealed a population of protein molecules that were irreversibly bound. This population was minor when vesicles were used (10) but became a major component with phospholipid monolayers (13). While the function of the irreversibly bound protein was not investigated, it was shown that appearance of PKC activity did not correlate with the reversible calcium-dependent membrane binding event and that substrate-membrane binding was also crucial to activity (4, 11, 12). However, it is possible that still other membrane-related events are important for generating PKC activity. The study presented here examined the activity and cofactor requirement of the irreversible membrane-bound form of PKC. The results and an hypothesis for PKC activation are summarized schematically in Figure 4.

PKC-membrane binding is proposed to consist of two steps. The first step involves PKC-membrane binding, a process which requires lower calcium concentrations than are needed

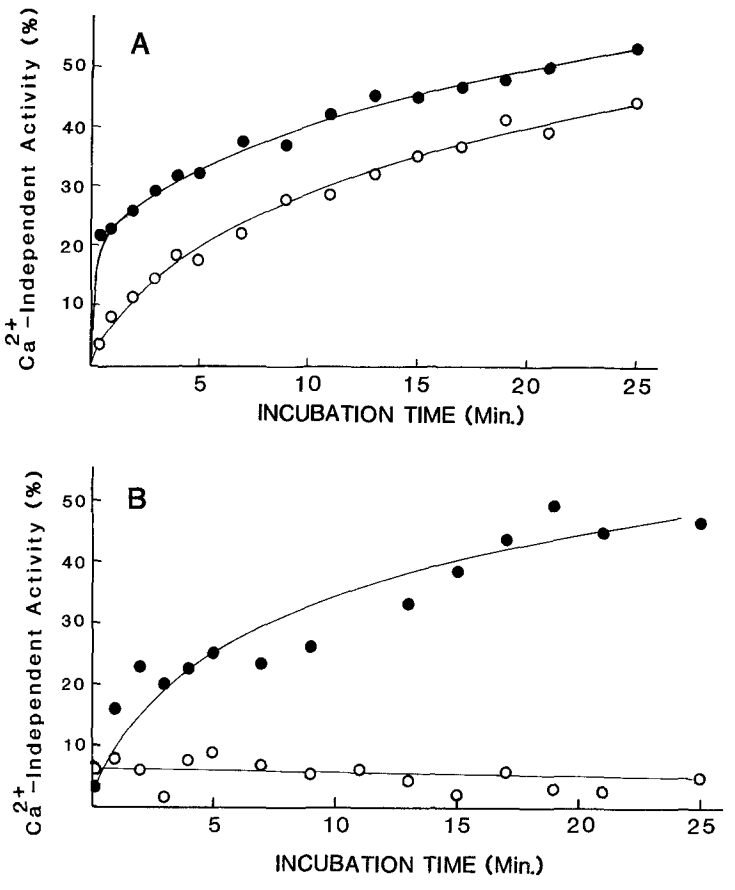

Figure 3. Time-dependent formation of Ca^{2+}-independent PKC activity. In panel A, PKC (25 ng/mL) was incubated with phospholipid vesicles (0.2 mg/mL) in the presence of 0.5 mM Ca^{2+} plus (●) or minus (○) 30 nM PBDu. The mixture was incubated for the indicated time before an aliquot (215 µL) was withdrawn and added to buffer or to EGTA-containing buffer (final EGTA concentration of 2.0 mM). $MgCl_2$, histone, and ATP were then added to start the activity measurements. The Ca^{2+}-independent activity is expressed as a percentage of the activity observed in the presence of Ca^{2+}. Panel B shows a similar experiment except that the free calcium concentration was 1.5 µM (4).

for activity (10, 19). The second step, insertion into the hydrocarbon region of the membrane, is stimulated by either high calcium or by phorbol esters. The activity of the inserted protein is no longer dependent on calcium (Table I). Thus, it is possible that biologically active phorbol esters function by inserting PKC into the membrane thereby eliminating the calcium requirement of PKC activity. However, low concentrations of Ca^{2+} are still needed to induce the initial PKC-membrane binding. This scheme may provide explanations for many observed properties of PKC and could provide a better understanding of the biochemical basis of PKC regulation under in vivo and in vitro conditions.

Activation of PKC by DAG displays stereospecificity, and only 1,2 sn diglycerides are active (20-23). Similarly, inactive stereoisomers of phorbol esters are known (5, 24, 25). Recently, new classes of PKC activators, which appear to be structurally unrelated to phorbol esters, have been described (6-9). Key structural features common to all of these compounds have been indentified (9, 26). The concept of specific ligand-protein interaction, however, does not account

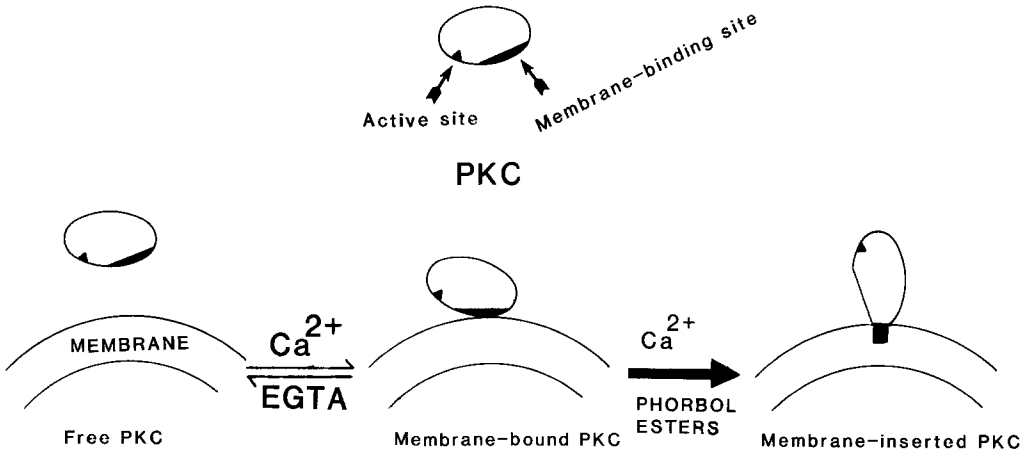

Figure 4. Schematic representation of PKC-phospholipid interaction steps. The PKC molecule with an active site and membrane-binding domain is depicted. PKC initially binds to the membrane in a calcium-dependent manner and can be dissociated with EGTA. Phorbol esters and/or high Ca^{2+} concentrations induce insertion of PKC into the hydrocarbon region of the membrane. The latter process is irreversible and produces a Ca^{2+}-independent kinase.

for the observation that high calcium concentration can replace the need for any of these activators, despite the fact that the latter are known to remain bound to PKC at high calcium concentrations. In addition to the possible spatial similarity around the points of attachment to PKC (9, 26), a key structural feature of the activators is a lipophilic arm which may suggest a membrane-related event. Unesterified phorbol esters do not activate PKC. Thus, it is possible that the activation of PKC by many agents is accomplished via the single mechanism of inserting PKC into the membrane. The resulting kinase activity is independent of cofactors and is also independent of the reagent used to cause insertion.

Boccklno and Exton (27) gave a preliminary report suggesting that Ca^{2+} was necessary to initiate PKC activity, but was not needed for sustained activity. The development of Ca^{2+}-independent activity of PKC did not appear to be due to proteolytic degradation of PKC. This result may also be due to insertion of PKC into the membrane, thereby producing a cofactor-independent kinase.

The concentration of phorbol esters used in these studies (30 nM PDBu) was adequate to activate PKC, but was not high enough to induce changes in the membrane structure (2, 28). Thus, phorbol esters appeared to promote the insertion of PKC into the membrane via specific binding to PKC and not via disruption of the membrane bilayer itself. This irreversible association of PKC with membrane is usually referred to as translocation of PKC to the membrane. A common explanation for this event is that binding of probol esters to PKC increases the enzyme affinity for calcium. However, phorbol esters and diacylglycerol did not reduce the Ca^{2+} needed to form the reversible Ca^{2+}-dependent PKC-membrane complex (10). In contrast, phorbol esters clearly stimulated PKC activity, and the studies presented here show that they also promoted membrane insertion. Therefore, an attractive explanation for translocation of PKC to the membrane as well as for PKC activation consists of a cofactor-enhanced membrane insertion event.

Insertion of PKC into the membrane and stimulation of this process by phorbol esters may be consistent with a number of previous observations in whole cells. It is well established that PKC

can be isolated from either the particulate or soluble fractions of many tissues. However, it is not always possible to extract the enzyme with calcium chelators. For example, fractionation of brain tissues in the presence of EGTA revealed that only about one third of the PKC is recovered in the soluble fraction; the remaining activity is recovered in mitochondrial and microsomal fractions (14, 29). In another case, PKC is recovered in the soluble fraction when platelets are homogenized in the presence of EGTA. However, pretreatment of platelets with phorbol esters renders PKC membrane-bound, even in the presence of EGTA (30). Dissociation of PKC from erythrocyte vesicles displays similar characteristics (31); in the absence of phorbol esters, association and dissociation of PKC from the membranes is regulated by Ca^{2+}. In the presence of phorbol esters, however, PKC was not dissociated by Ca^{2+} chelators.

Constitutive activity of membrane-inserted PKC provides a new type of regulation event that might be important for long term effects in the cell. That is, the second messengers, Ca^{2+} and diacylglycerol (the latter is presumed to act in a manner similar to phorbol esters), may function by causing insertion of PKC into the membrane. The second messengers may then be degraded but the inserted PKC remains active until it is degraded by the protein turnover processes. This obviously provides a possible mechanism for cell memory events. Messenger-induced protein insertion into a membrane might be a more general process for regulation and could apply to other proteins as well. Further studies are needed to determine if these mechanisms function in vivo.

Acknowledgement This work was supported in part by grant GM 38819 from the National Institutes of Health.

References

1) Nishizuka, Y. (1986) *Science (Washington D.C.) 233*, 305-312.
2) Nishizuka, Y. (1986) *JCNI, J. Natl. Cancer Inst.76*, 363-370.
3) Kikkawa, U. & Nishizuka, Y. (1986) *Ann. Rev. Cell Biol., 2*, 149-178.
4) Bazzi, M. D. & Nelsestuen, G. L. (1987) *Biochemistry 26*, 1974-1982.
5) Ashendel, C. L. (1985) *Biochem. Biophys. Acta 822*, 219-242.
6) Fujiki, H., Tanaka, Y., Miyake, R., Kikkawa, U., Nishizuka, Y., & Sugimura, T. (1984) *Biochem. Biophys. Res. Commun. 120*, 339-343.
7) Couturier, A., Bazgar, S. & Castagna, M. (1984) *Biochem. Biophys. Res. Commun. 121*, 448-455.
8) Miyake, R., Tanaka, Y., Tsuda, T., Kaibuchi, K., Kikkawa, U., & Nishizuka, Y. (1984) *Biochem. Biophys. Res. Commun. 121*, 649-656.
9) Wender, P. A., Koehler, K. F., Sharkey, N. A., Dell'Aquila, M. L., & Blumberg, P. M. (1986) *Proc. Natl. Acad. Sci. USA 83*, 4214-4218.
10) Bazzi, M. D. & Nelsestuen, G. L. (1987) *Biochemistry 26*, 115-126.
11) Bazzi, M. D. & Nelsestuen, G. L. (1987) *Biochemistry 26*, 5002-5008.
12) Bazzi, M. D., Lampe, P. D., Strasburg, G. M., & Nelsestuen, G. L. (1987) *Biochem. Biophys. Acta 931*, 339-346.
13) Bazzi, M. D. & Nelsestuen, G. L. (1988) Submitted for publication.
14) Kikkawa, U., Minakuchi, R., Takia, Y., & Nishizuka, Y. (1983) *Methods Enzymol. 99*, 288-298.
15) Kishimoto, A., Kajikawa, N., Shiota, M., & Nishizuka, Y. (1983) *J. Biol. Chem. 258*, 1156-1164.
16) Tapley, P. M. & Murray, A. W. (1985) *Eur. J. Biochem. 151*, 419-423.
17) Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B., Salamino, F., & Horcker, B. L. (1985) *Proc. Natl. Acad. Sci. USA 82*, 6435-6439.
18) Mizuta, K., Hashimoto, E., & Yamamura, H. (1985) *Biochem. Biophys. Res. Commun. 131*, 1262-1268.

19) Wolf, M., LeVine, H., May, W.S. Jr., Cuatrecasas, P. & Sahyoun, N. (1985) *Nature (London) 37,* 546-549.
20) Rando, R. R., & Young, N. (1984) *Biochem. Biophys. Res. Commun. 122,* 818-823.
21) Nomura, H., Ase, K., Seikiguchi, K., Kikkawa, U., & Nishizuka, Y. (1986) *Biochem. Biophys. Res. Commun. 140,* 1143-1151.
22) Ganong, B. R., Loomis, C. R., Hannun, Y. A., & Bell, R. M. *Proc. Natl. Acad. Sci. USA 83,* 1184-1188.
23) Go, M., Seikiguchi, K., Nomura, H., Kikkawa, U., & Nishizuka, Y. (1986) *Biochem. Biophys. Res. Commun. 144,* 598-605.
24) Blumberg, P. M. (1980) *Crit. Rev. Toxicol. 8,* 153-198.
25) Blumberg, P. M. (1980) *Crit. Rev. Toxicol. 8,* 199-234.
26) Brasseur, R., Cabiaux, V., Huart, P., Castagna, M., Baztar, S., Ruysschaert, J. M. (1985) *Biochem. Biophys. Res. Commun. 127,* 969-976.
27 Bocckino S. & Exton, J. H. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45,* Abstr. # 1865
28) Nishizuka, Y. (1984) *Science (Washington D.C.) 255,* 1365-1370.
29) Kikkawa, U., Takai, Y. Minakuchi, R., Inohara, S., & Nishizuka, Y. (1982) *J. Biol. Chem. 257,* 13341-13348.
30) Takai, Y., Kaibuchi, K., Tsuda, T., & Hoshijima, M. (1985) *J. Cell. Biochem. 29,* 143-155.
31) Wolf, M., Cuatrecasas, P. & Sahyoun, N. (1985) *J. Biol. Chem. 260,* 15718-15722.