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The interaction of protein kinase C and other specific cytoplasmic proteins with phospholipid bilayers

G.T. Snoek [a], I. Rosenberg [b], S.W. de Laat [a] and C. Gitler [b]

[a] *Hubrecht Laboratory, International Embryological Institute, Uppsalalaan 8, 3584 CT Utrecht (The Netherlands) and* [b] *Department of Membrane Research, Weizmann Institute of Science, Rehovot (Israel)*

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The role of lipid composition in the interaction of purified protein kinase C with large unilamelar vesicles was determined by the extent of photolabelling of the enzyme with 5-[^{125}I]iodonaphthalene 1-azide. The protein kinase C was only slightly labelled when exposed to phosphatidylcholine (PC) liposomes. The addition of phorbol 12-myristate 13-acetate (PMA) or of diacylglycerol to the PC liposomes enhanced significantly the labelling of the protein kinase C at low calcium concentrations. A further enhancement in the photolabelling of the protein kinase C was observed in liposomes containing 2% phosphatidylserine (PS). At low calcium concentrations, the binding of the enzyme to these liposomes increased in the presence of added PMA or diacylglycerol. Raising the levels of PS beyond 2% in the liposomes did not enhance the binding of the protein kinase C. However, when the enzymatic activity of the protein kinase C was measured using basic histones as substrates, maximum phosphorylation was obtained in liposomes with a PC to PS ratio of 1. The fact that the translocation of the protein kinase C from solution to the surface of the liposomes could be monitored by its labelling with 5-iodonaphthalene 1-azide prompted us to determine whether other cytoplasmic proteins might share this property. The interaction of cytoplasmic proteins from HeLa cells with PC liposomes gave trace labelling irrespective of whether calcium was added. When the HeLa cell cytoplasmic proteins were allowed to interact with liposomes containing PS, selective 5-iodonaphthalene-1-azide photolabelling was observed in distinct proteins. Addition of calcium and of PMA or diacylglycerol modified the labelling of some but not all of these proteins. These results suggest that the methodology developed might serve to identify proteins that move to the membrane during stimulation of cells by phorbol esters or by growth factors which induce the generation of diacylglycerol. These results also suggest a role for the phospholipid composition of the plasma membrane (or any intracellular membrane) in the modulation of the activation processes of specific phospholipid-dependent proteins, in particular protein kinase C.

Introduction

The phospholipid-dependent, Ca^{2+}-activated protein kinase (protein kinase C) has been implicated as a regulatory element in signal transduction [1] and is involved in regulation of growth, cellular differentiation, early embryonic development and tumour promotion [2]. During signal transduction, induced by ligand-receptor binding, phosphoinositides are hydrolysed in the plasma membrane [3,4]. Diacylglycerol is transiently produced in the plasma membrane and it induces the activation of protein kinase C. This activation probably is a two-step process: in cells, the stimu-

Abbreviations: PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulphonyl fluoride; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; INA, 5-iodonaphthalene 1-azide; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; FSBA, fluorosulphonylbenzoyladenosine.

lation of phosphoinositide hydrolysis or the addition of exogenous diacylglycerol induces association of protein kinase C with the plasma membrane (Refs. 5, 6; Clevers and Snoek, unpublished data). In addition, it has been demonstrated that diacylglycerol increases the affinity of protein kinase C for Ca^{2+} in vitro [7]. As a result, protein kinase C is fully activated by interaction with phospholipids at physiological Ca^{2+} concentrations. Furthermore, another product of phosphoinositide hydrolysis, inositol triphosphate (IP_3), is shown to increase the intracellular Ca^{2+} concentration by release from intracellular stores [8,9]. The tumour-promoting phorbol ester phorbol 12-myristate 13-acetate (PMA) has been shown to activate protein kinase C directly, bypassing the phosphoinositide hydrolysis [10]. In many cells, shortly after incubation with PMA, most of the cytoplasmic protein kinase C activity has disappeared and is found in the particulate fraction (Refs. 5, 6; Clevers and Snoek, unpublished data).

The mechanism of activation of protein kinase C is far from resolved. In vitro, protein kinase C has been reported to be activated by exposure to liposomes containing negatively charged phospholipids such as phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidic acid (PA) in combination with calcium, not by liposomes composed of uncharged phospholipids like phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [11,12]. In addition, this activation is enhanced by diacylglycerol or PMA [10,1]. Cabot [13] has shown that in vivo binding of phorbol esters to human promyelocytic leukaemia cells was strongly influenced by the phospholipid composition of the plasma membrane. It must be concluded that the nature of the phospholipid bilayer is important for the association process of protein kinase C and its activation. Recently it has been demonstrated by Boni and Rando [14] that different physiological forms of synthetic phospholipid bilayers, i.e., small or large unilamellar vesicles or multilamellar vesicles, are differentially capable of complying with the phospholipid requirement of protein kinase C.

In the present study we report that the interaction of protein kinase C with liposomes can be determined by the extent of photolabelling of the enzyme with 5-[^{125}I]iodonaphthalene 1-azide [15]. In addition, we present methodology that should allow the identification of other cytoplasmic proteins that bind to the plasma membrane due to specific interactions with membrane lipids. [^{125}I]INA, a photoactivatable labelling reagent, has been shown to be restricted, by its insolubility in water, to the lipid core of the phospholipid bilayer. Therein, the reactive nitrene upon radiation attaches covalently to proteins embedded in the lipid bilayer. Using large unilamellar vesicles (LUV) as models, we have investigated the effect of phospholipid composition, Ca^{2+} concentration and the presence of diacylglycerol or PMA on the association of protein kinase C with phospholipid bilayers. We report here that the presence of negatively charged PS is essential for interaction of phospholipid bilayers with protein kinase C and that PMA and diacylglycerol are able to stimulate this interaction.

Besides protein kinase C, there are other known proteins that are activated by association with phospholipids, for example proteinases [16], α-actinin [17] and thrombin [18,19]. Using the same system as for protein kinase C we have identified a group of cytoplasmic proteins in HeLa cells which can be induced to associate with the phospholipid bilayer when the necessary phospholipids are present.

The results in this paper suggest that in the activating process, in which association of protein kinase C with the plasma membrane is essential for activation, the phospholipid composition of the membrane, the availability of Ca^{2+} and the presence of endogenous (diacylglycerol) or exogenous (PMA) activators are important factors determining the rate of association and activation of protein kinase C. This mechanism of activation and regulation is probably also effective for other specific proteins.

Methods

Preparation of HeLa cytoplasmic protein fraction

HeLa cells were grown to confluency in Dulbecco's modified Eagle's medium containing 7.5% foetal calf serum. Monolayers were washed twice with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline and suspended in 8 mM EGTA in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline. After

centrifugation, cells were resuspended in lysis buffer (20 mM Hepes (pH 7.4)/2 mM EDTA/0.5 mM EGTA/1 mM PMSF/330 mM sucrose/5 mM dithiothreitol), homogenized by 20 strokes in a Dounce homogenizer and centrifuged for 1 h at $100\,000 \times g$.

Purification of protein kinase C

Protein kinase C was partially purified from mouse brain as described by Inoue et al. [16]. Murine brains were homogenized in 20 mM Tris (pH 7.5)/250 mM sucrose/2 mM EDTA/10 mM EGTA and centrifuged for 90 min at 21 500 rpm in a Beckman SW 27 rotor. The supernatant was applied to a DE-52 column, equilibrated with 20 mM Tris (pH 7.5)/2 mM EDTA/5 mM EGTA/ 50 mM β-mercaptoethanol. The enzyme was eluted by application of a linear NaCl concentration gradient (0–0.4 M) in the same buffer. The fractions containing protein kinase C activity were pooled, concentrated and applied to a Sephadex G-100 column equilibrated in 20 mM Tris (pH 7.5)/0.5 mM EGTA/50 mM β-mercaptoethanol. Elution was carried out with the same buffer and the protein-kinase-C-containing fractions were pooled and concentrated. Before adding the protein kinase C preparation to lipid vesicles, the buffer was exchanged for a buffer containing 20 mM Hepes (pH 7.5)/2 mM EDTA/0.5 mM EGTA/5 mM dithiothreitol/330 mM sucrose by passage through a Sephadex G-25 column.

Protein kinase C assay

The Ca^{2+}- and phospholipid-dependent protein kinase activity was assayed by adding 0.1–1 μg of purified protein kinase C to a reaction mixture (final volume 125 μl) of 20 mM Tris (pH 7.5)/7.5 mM MgAc/10 $\mu g \cdot ml^{-1}$ leupeptin/200 $\mu g \cdot ml^{-1}$ histone IIIs/10 μM ATP/96 $\mu g \cdot ml^{-1}$ phosphatidylserine (PS)/3.2 $\mu g \cdot ml^{-1}$ diolein/1 mM $CaCl_2$. The control value was determined in the same reaction mixture without PS, diolein and/or $CaCl_2$, with 1 mM EGTA added. The mixtures were preincubated for 5 min at 30°C. The reaction was started by the addition of ATP and [γ-^{32}P]ATP (10 μM, $2 \cdot 10^6$ cpm per incubation) and allowed to proceed at 30°C for 4 min. The reaction was stopped by pipetting 100 μl of the reaction mixture into 1 ml ice-cold 25% trichloroacetic acid. The precipitated protein was collected on a Millipore filter (0.45 μm) and washed five times with 3 ml ice-cold 10% trichloroacetic acid/ 10 mM sodium pyrophosphate.

Labelling procedures

[^{125}I]INA was synthesized as described by Raviv et al. [20]. The partition coefficient into the liquid lipid phase of biological membranes is more than $1 \cdot 10^7$ and it has been demonstrated that [^{125}I]INA partitions more than 99.9% into different membranes [20]. All operations with [^{125}I]INA were under subdued light to prevent activation. Phosphatidylserine (PS) and phosphatidylcholine (PC) were mixed in various ratios (final phospholipid concentration 20 mg/ml) and dispersed in 25 mM Hepes (pH 7.0)/50 mM Na_2SO_4 and sonicated for 9 min (3×3 min with 1 min intervals). The liposomes were frozen in liquid nitrogen and after thawing sonicated for 30 s to yield large unilamellar vesicles (LUV). LUV (200 μg phospholipid) were incubated for 5 min at room temperature (in subdued light) with protein kinase C (40 μg) or the HeLa cytoplasmic fraction (100 μg protein), with or without Ca^{2+} ($CaCl_2$) in 25 mM Hepes (pH 7.5)/50 mM Na_2SO_4, final concentrations 10 μM[2] or 1 mM). The incubation volume was 200 μl and in all cases 5 mM glutathione was added. Reduced glutathione is a quencher of unreacted [^{125}I]INA [20]. [^{125}I]INA was added ($2 \cdot 10^6$ cpm in ethanol, final ethanol concentration 0.5%) and incubated at room temperature for 5 min. Then the suspension was irradiated for 5 min with the 314 nm line of a Mercury lamp (M-60). A filter (Melles Griot WG305) was used to remove light below 300 nm. Diolein (final concentration 32 μg/ml) or PMA (final concentration 10 μg/ml) were added to the LUV, dissolved in DMSO. An equivalent volume of DMSO (final concentration 0.5%) was added to the controls. After irradiation, 50 μl of each sample was mixed with 50 μl sample buffer and SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli [21] with minor modifications. The gels were stained with Coomassie brilliant blue R, dried and autoradiography of the gels was performed using XAR-5 film (Kodak). The autoradiograms were scanned with a Chromoscan 3 (Joyce Loebl). The 70 kDa areas (10×7 mm) were scanned and integrated.

[^{14}C]FSBA labelling of protein kinase C

Partially purified protein kinase C (50 μg protein) was incubated with 2 μl 5-*p*-fluorosulphonylbenzoyl[*adenine*-8-^{14}C]adenosine, ([^{14}C]-FSBA, 0.2 mCi/ml, 41.9 mCi/mmol, New England Nuclear), 1 mM $CaCl_2$, liposomes containing 96 μg/ml PS and 3.2 μg/ml diolein for 1 h at room temperature. Aspecific binding of [^{14}C]FSBA was determined in the presence of 5 μM unlabelled FSBA. The samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography was performed as described for the [^{125}I]INA-labelled samples.

Results

Characterization of purified protein kinase C

The purified protein kinase C had a very high Ca^{2+}- and phospholipid-dependent protein kinase activity (specific activity was 16000 pmol phosphate per min per mg protein). No cAMP- or cGMP-dependent protein kinase activity was detectable. Protein staining of the SDS polyacrylamide gels of the protein kinase C preparation showed one strongly stained 70 kDa band (Fig. 1A, lane 1). To determine whether this band represented protein kinase C, we labelled the protein kinase C preparation with the ATP analogue, [^{14}C]FSBA. During this procedure the [^{14}C]FSBA is covalently bound to the ATP-binding site of a kinase [22,23]. In Fig. 1B, lane 2, it can be seen that the 70 kDa band is labelled strongly by [^{14}C]FSBA. The labelling is kinase-specific, because in the presence of a high concentration of unlabelled FSBA (5 μM) no proteins are labelled (Fig. 1B, lane 1). We concluded from these results that the 70 kDa band represents protein kinase C.

Protein kinase C– phospholipid interaction

Effect of diacylglycerol or PMA and Ca^{2+} on the interaction of protein kinase with PC liposomes. The incubation, under subdued light, of [^{125}I]INA-containing PC liposomes with the purified protein kinase C, followed by photolysis, resulted in minor incorporation of the label into the enzyme (Fig. 2). Addition of calcium did not increase the labelling. However, addition of PMA to the PC liposomes resulted in an enhanced binding of the enzyme as evidenced by a rise in the incorporation of

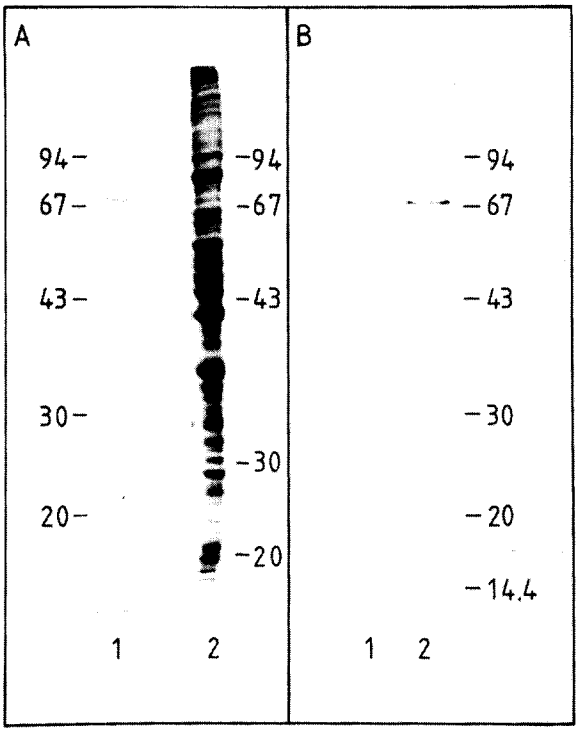

Fig. 1. (A) SDS-polyacrylamide gel electrophoregrams of purified protein kinase C (lane 1) and HeLa cytoplasmic proteins (lane 2). Coomassie blue staining. (B) Autoradiogram of SDS-polyacrylamide electrophoregram of protein kinase C labelled with [^{14}C]FSBA in the presence (lane 1) or absence (lane 2) of 5 μM FSBA as described in Methods. The gel has been autoradiographed for 14 days.

[^{125}I]INA into the protein kinase C. The binding was highest in the presence of 10 μM Ca^{2+}. Significant labelling of the protein kinase C also resulted from the addition of diacylglycerol to the PC liposomes at 0 or 10 μM calcium. The presence of 1 mM Ca^{2+} resulted in inhibition of the incorporation of [^{125}I]INA into the protein kinase C (Fig. 2).

Effect of diacylglycerol or PMA and Ca^{2+} on the interaction of protein kinase C with PS, PC liposomes. The addition of increasing levels of PS to PC liposomes resulted in a marked enhancement in the labelling of the protein kinase C. The dependence on PS reached a maximum at 2% of PS. Thereafter, raising the level of PS did not significantly increase the degree of labelling. For this reason, we report only the effect of 2% PS. It can be observed (Fig. 2) that the presence of PS resulted in labelling that was much higher than that

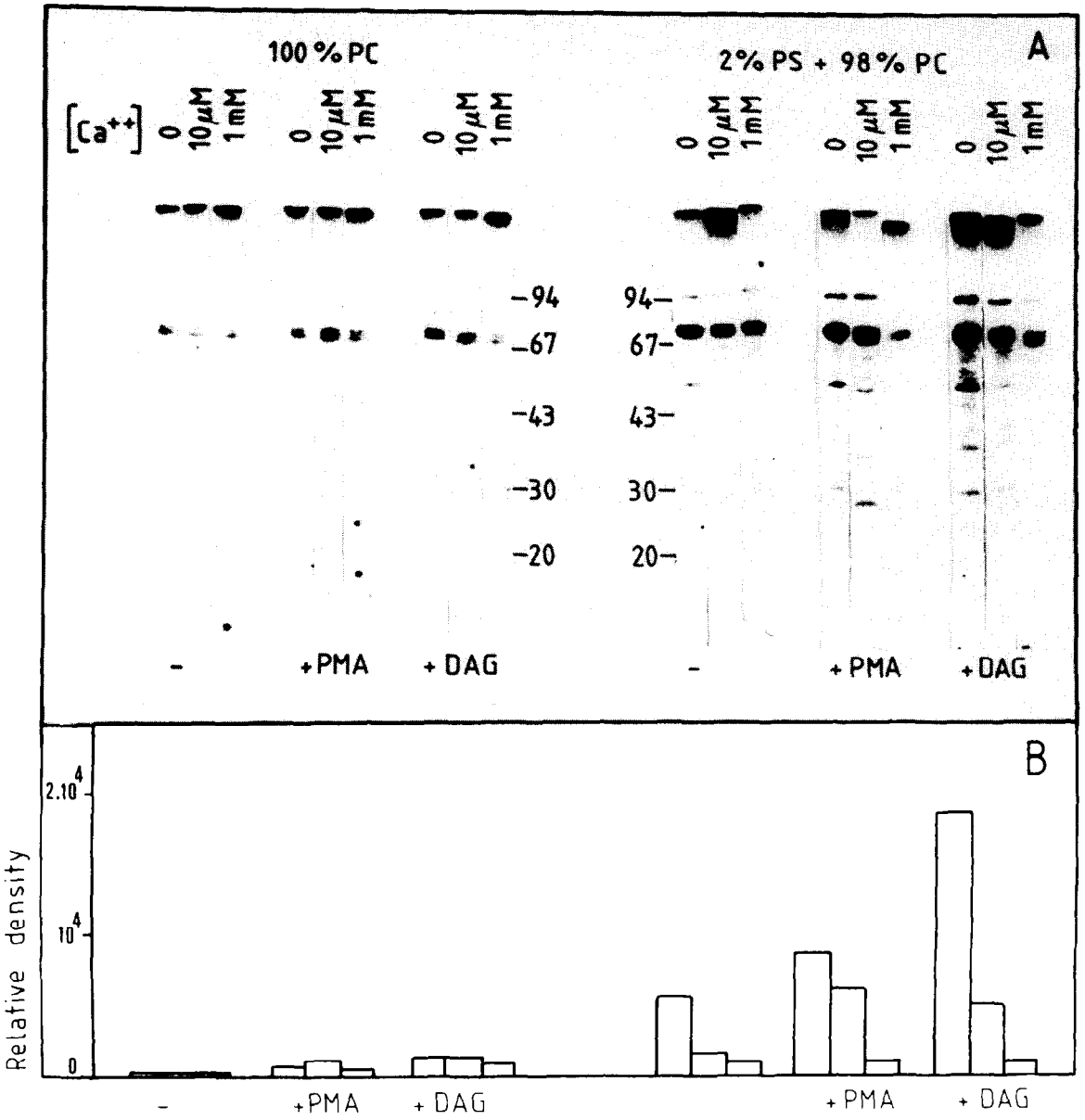

Fig. 2. (A) Autoradiogram of SDS polyacrylamide electrophoregram of protein kinase C incubated with LUV of variable composition (100% PC and 98% PC/2% PS) and labelled with [^{125}I]INA as described in Methods. Ca^{2+}, PMA or diacylglycerol (DAG) were added as shown in the figure. The gels have been autoradiographed for 5 days. (B) Relative quantification of the 70 kDa bands on the autoradiograms in (A). The films were scanned as described in Methods. The integrated area is 10×7 mm.

recorded in the presence of PC liposomes. The degree of [^{125}I]INA incorporation into the protein kinase C was equivalent in the absence or presence of 10 μM calcium.

The addition of PMA or diacylglycerol to the PS, PC liposomes resulted in a further enhancement in the photolabelling of the protein kinase C (Fig. 2). Highest labelling was observed at 0 and 10 μM Ca^{2+}. The presence of 1 mM Ca^{2+} inhibited the labelling to a greater extent in the presence of added PMA. Some other proteins were labelled under various conditions. They might

represent minor contaminants present in the protein kinase C preparation or partial breakdown products derived from protein kinase C.

Effects of liposome composition on the activity of protein kinase C. The protein kinase C catalysed phosphorylation of histone IIIs increased linearly with PS content of the liposomes until the ratio of PS to PC was 1 (Fig. 3). The activity decreased slightly when pure PS was used. The activity measured at 2% of PS in the presence of 10-fold less diacylglycerol than that used in the labelling experiments was found to be about 3% of that at the optimal PS concentration.

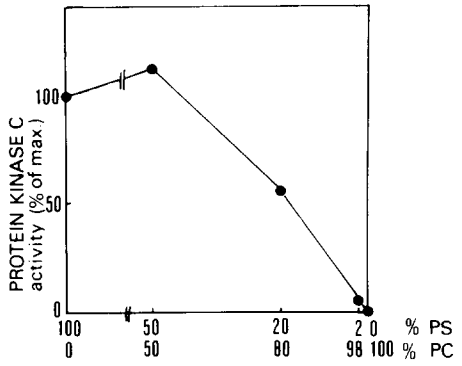

Fig. 3. Activation of a purified protein kinase C preparation with LUV of variable composition. The total phospholipid concentration is 96 μg/ml; 3.2 μg/ml diolein is added. The protein kinase C activity is determined as described in Methods.

The interaction of phospholipids with cytoplasmic proteins

Effect of diacylglycerol or PMA and Ca^{2+} on the interaction of HeLa cytoplasmic proteins with PC liposomes. The incubation, under subdued light, of [^{125}I]INA-containing PC liposomes with HeLa-cell-derived cytoplasmic proteins, followed by photolysis, resulted in minor incorporation of the label into bands with apparent molecular weights of 57, 31 and 33 kDa (Fig. 4). The addition of calcium decreased this labelling slightly. When, in addition to PC, the liposomes contained PMA, one band of 44 kDa was labelled in the absence of Ca^{2+}. With 10 mM Ca^{2+}, strong labelling was observed in the 57 kDa band only. Addition of diacylglycerol to the PC liposomes resulted in decreased labelling of all bands (Fig. 4), irrespective of whether Ca^{2+} was present.

Effect of diacylglycerol or PMA and Ca^{2+} on the interaction of HeLa cytoplasmic proteins with PS, PC liposomes. The addition of 2% PS to the PC liposomes resulted in a marked labelling of HeLa cytoplasmic proteins of apparent molecular masses

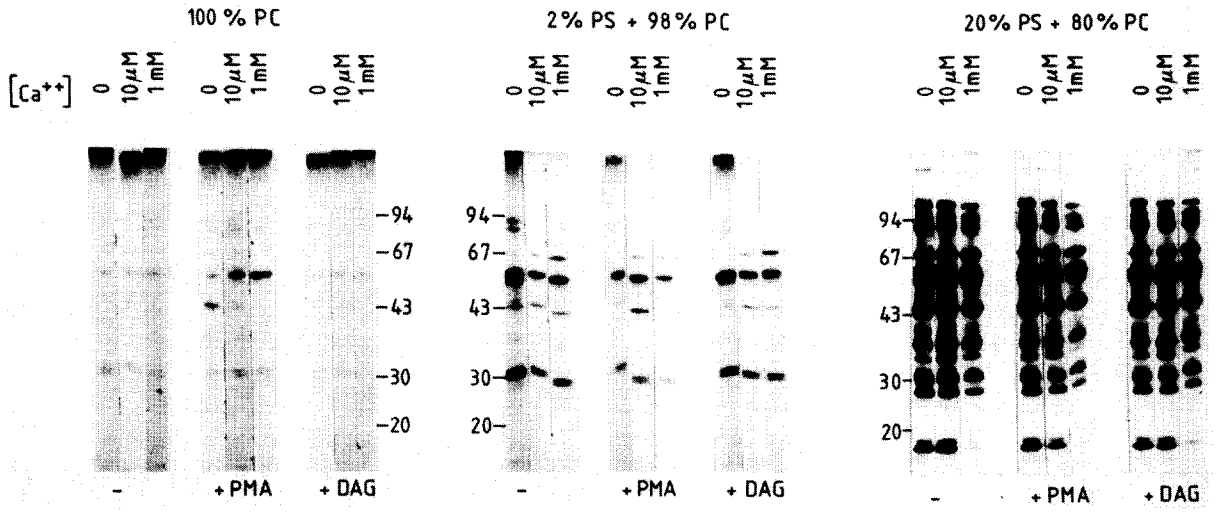

Fig. 4. Autoradiograms of SDS-polyacrylamide gel electrophoregram of HeLa cytoplasmic proteins incubated with LUV of variable composition (100% PC, 98% PC/2% PS and 80% PC/20%PS) and labelled with with [^{125}I]INA as described in Methods. (A) 100% PC; (B) 98% PC/2% PS; (C) 80%PC/20%PS. Ca^{2+}, PMA or diacylglycerol (DAG) was added as shown in the figure. The gels have been autoradiographed for 4 days.

of 92, 88, 57, 44, 33 and 31 kDa in the absence of Ca^{2+} (Fig. 4). At 10 μM Ca^{2+} the intensity of the labelling decreased and the 92 and 88 kDa proteins were not labelled.

The addition of 20% PS to the PC liposomes resulted in an increased number of labelled proteins, including those which were labelled at 2% PS. Ca^{2+} at 10 μM only had a slight effect on the labelling of several bands (Fig. 4).

In the presence of PMA in the 2% PS, PC liposomes, the number of labelled bands was less than those labelled with PS, PC liposomes alone. In addition, the degree of labelling was less, with the exception of the 44 kDa band, which was strongly labelled when the Ca^{2+} concentration was 10 μM (Fig. 4). The addition of diacylglycerol to 2% PS/PC liposomes did not affect the labelling.

In 20% PS/PC liposomes, PMA and diacylglycerol specifically affected labelling of some proteins. For example, the labelling of the 92, 88, 31 and 33 kDa proteins was inhibited by PMA in the presence of 10 μM Ca^{2+}. Diacylglycerol had the same effect as PMA but to a lesser extent. The high Ca^{2+} concentration inhibits labelling under all conditions.

Discussion

The involvement of the breakdown of polyphosphoinositides in the signal transduction induced by agonist–receptor interaction has been generally accepted. As a result of the interaction, second messengers are produced, including diacylglycerol, which causes the activation of protein kinase C. The association of protein kinase C with the plasma membrane is an essential step in the activation process. So far, the molecular mechanisms involved in the association of protein kinase C with the plasma membrane are only poorly understood. Knowledge of these mechanisms is of great importance, since the tumour-promoting phorbol esters also act through binding to protein kinase C, causing its redistribution from the cytoplasm to the plasma membrane, directly followed by its activation.

Protein kinase C is dependent on interaction with phospholipids for its activation and it has been demonstrated that the activation is dependent on the type of phospholipid present in the liposomes [11]. Only negatively charged phospholipids, such as PS, PI and PA, are able to activate protein kinase C in vitro; neutral phospholipids such as PC and PE are unable to activate protein kinase C. The protein kinase C activation in vitro can also be manipulated by varying the composition and structure of the lipid bilayers. Boni and Rando [14] have demonstrated that protein kinase C activation is more efficient by large unilamellar vesicles (LUV) when compared with activation by small unilamellar vesicles (SUV). On the other hand, Cabot [13] has shown that binding of phorbol dibutyrate to promyelocytic leukaemia cells can be manipulated by changing the phospholipid composition of the plasma membrane. The nature of the interaction between phospholipids and protein kinase C is not clear. It has been demonstrated that there is a variation in the reversibility of the association of protein kinase C with the plasma membrane. The association induced by hydrolysis of phosphoinositides is completely reversible within 30 min, while the PMA-induced association seems to be irreversible [6]. Chelating agents like EGTA are not able to interrupt the protein kinase C–plasma membrane/LUV association, but detergents like Nonidet P-40 or Triton X-100 are able to separate the phospholipids from protein kinase C [6], indicating a strong interaction between protein kinase C and phospholipids in the bilayer.

We have studied the physical interaction between protein kinase C and phospholipid bilayers by specifically labelling the part of the protein that is embedded in the bilayer. For this purpose we have used the photoactivated labelling agent [^{125}I]iodonaphthalene azide [15]. Direct labelling by [^{125}I]INA of these minor proteins in intact cells was found to be difficult because many intrinsic membrane proteins were heavily labelled. It was decided, therefore, to determine whether protein kinase C could be labelled by [^{125}I]INA upon interaction with LUV under conditions known to lead to an enhancement in its enzymatic activity. In LUV composed of the neutral phospholipid PC, protein kinase C is not inserted into the bilayer and thus not labelled by [^{125}I]INA. This result also indicates that [^{125}I]INA does not label proteins outside a phospholipid bilayer. Incorporation of PS into the LUV greatly increased the

protein kinase C–phospholipid bilayer interaction, even at a very low PS concentration. Addition of PMA or diacylglycerol to the LUV further increased the interaction of protein kinase C with the lipid bilayer. Both in the activation of protein kinase C and the formation of phorbol dibutyrate-protein kinase C-phospholipid complexes in vitro, the presence of Ca^{2+} is essential [10]. However, the association of protein kinase C with LUV does not require exogenous calcium. On the contrary, the association is inhibited by Ca^{2+}. Our own experience indicates that commercial PS preparations may be contaminated with Ca^{2+} and that this ion is not easily removed by water-soluble chelating agents like EGTA. This might be a reason why no exogenous Ca^{2+} is needed to insert protein kinase C into the lipid bilayer. On the other hand, addition of exogenous Ca^{2+} might affect inhibition of association of protein kinase C with the lipid bilayer by ionic interaction between the negatively charged PS and Ca^{2+}, thus shielding PS from protein kinase C.

We have found a discrepancy between PS dependency of protein kinase C activation and association of a lipid bilayer with protein kinase C. For optimal activity, a relatively high percentage (50%) of PS in the LUV is necessary, while the association of protein kinase C with a lipid bilayer is already optimal when 2% PS is incorporated in the LUV. These results suggest that association of protein kinase C with a phospholipid bilayer and activation of protein kinase C are dissociable processes, indicating that association without immediate activation is possible. Such a mechanism has also been suggested for the Ca^{2+}-induced protein kinase C binding to membranes by Wolf et al. [24]. These findings indicate that, for activation of protein kinase C, a phospholipid bilayer, an activator, Ca^{2+} and cytoplasmic protein kinase C are necessary factors; however, a more detailed regulation of the protein kinase C activation is possible by variations in the composition of the lipid bilayer. Together with changes in the intracellular Ca^{2+} concentration and the intramembrane diacylglycerol concentration induced by hydrolysis of phosphoinositides, the composition of the lipid bilayer might be an important regulatory factor in association and activation of protein kinase C.

Besides protein kinase C, several other proteins are known to be activated by association with a lipid bilayer, e.g., prothrombin, α-actinin [17,18,25]. These proteins are dependent on the presence of PS (prothrombin) or diacylglycerol or fatty acids (α-actinin) in the lipid bilayer as shown in experiments with [^{125}I]INA labelling [17,19,26]. In view of these results, we were interested in the presence of a class of proteins in cellular cytoplasm able to associate with the plasma membrane upon stimulation of the cell. They may function as activated enzymes, like kinases, phosphatases, proteinases, or as substrate molecules for membrane-bound, activated enzymes. In the present paper, we have shown that there is indeed a class of proteins in the cytoplasm from HeLa cells that is associated with a lipid bilayer when PS is present. We have also found some specific effects of diacylglycerol, PMA and Ca^{2+}, both stimulatory and inhibitory. The nature of these proteins remains to be resolved.

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