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POSSIBLE ROLE OF Ca^{2+} AND MEMBRANE PHOSPHOLIPID IN HORMONAL CONTROL OF PROTEIN PHOSPHORYLATION*

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INDEXING WORDS

hormone; calcium; membrane phospholipid; protein kinase; phosphatidylinositol turnover; phosphatidylserine; diglyceride; membrane phosphorylation; histone phosphorylation

SYNOPSIS

A new species of cyclic nucleotide-independent multifunctional protein kinase has been found in many mammalian tissues. This enzyme is normally present in soluble fractions as an inactive form. In the presence of Ca^{2+}, the enzyme attaches to membrane to exhibit full enzymatic activity. The active membrane component has been identified as phospholipid plus diglyceride. In the absence of diglyceride relatively higher concentrations of Ca^{2+} (10^{-4} M range) are needed. However, a small quantity of diglyceride sharply decreases the Ca^{2+} concentration giving rise to the maximum enzyme activation to micromolar range. This effect is greatest for diglyceride which contains an unsaturated fatty acid at least at the position 2. Other neutral lipids are ineffective. In the presence of diglyceride and micromolar Ca^{2+}, phosphatidylserine is most relevant with the capacity to activate the enzyme, whereas other phospholipids are far less effective. Ca^{2+} can not be replaced by other divalent cations. Since diglyceride which possesses an unsaturated fatty

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* This article is the dissertation submitted by Yoshimi Takai to Kobe University School of Medicine for the requirement of Doctor of Medical Sciences.

** Abbreviations used are: SDS, sodium dodecyl sulfate; and EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

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acid seems to be generated from phosphatidylinositol, it is likely that this enzyme activation is intimately related to the phospholipid turnover which is provoked by various hormones. The enzyme thus activated phosphorylates several membrane proteins and histone, and shows multifunctional catalytic activities. Available evidence suggests that this enzyme plays an important role in hormone actions and that Ca^{2+} and various membrane phospholipids play a role cooperatively in the tranmembrane control of protein phosphorylation reactions.

INTRODUCTION

Ca^{2+} and cyclic nucleotides have been known to be involved in a variety of cellular activities including muscle contraction, exocytotic secretion, initiation of DNA synthesis and changes in metabolic states.[31] These cellular activities are modulated by various extracellular stimulators such as hormone and neurotransmitter, and Ca^{2+} is now considered to serve as an intracellular messenger for these stimulators in a manner analogous to cyclic nucleotides.[31] Although the principal mediators of the regulatory effects of cyclic AMP and cyclic GMP have been identified as cyclic AMP-dependent and cyclic GMP-dependent protein kinases, respectively,[25,42] the factors mediating the intracellular regulatory properties of Ca^{2+} are far less understood. Ca^{2+}- dependent modulator protein (Calmodulin) has been recently proposed to be one of the possible effectors responsible for a variety of Ca^{2+}-mediated processes.[44] This modulator protein has been first described as an activator for cyclic nucleotide phosphodiesterase,[3,20] and later shown to modulate a variety of enzymes such as adenylate cyclase,[2] Ca^{2+}-Mg^{2+} ATPase,[11,19] myosin light chain kinase,[5,45] muscle glycogen phosphorylase kinase[4] and glycogen synthetase.[32,34]

On the other hand, it has been well established that phosphatidylinositol of cell membranes turns over very rapidly in response to various extracellular stimulators, whose intracellular messenger has been proposed to be Ca^{2+}.[8,13,16,29,30] This response has been originally found in acetylcholine-sensitive tissues such as pancreas and brain, and a rapid increase in the incorporation of radioactive phosphate into this phospholipid has been described.[16] Such phosphatidylinositol turnover has been subsequently shown to be provoked in almost all tissues, and it is now clear that the phosphatidylinositol breakdown is initiated by a species of specific phospholipase C.[6,8,13,29,30]

Ca^{2+}, PHOSPHOLIPID AND PROTEIN KINASE

During analysis in this laboratory of cyclic AMP-dependent and cyclic GMP-dependent protein kinases, a new species of cyclic nucleotide-independent multifunctional protein kinase has been found in various mammalian tissues and organs.[18,37,38,46] This protein kinase is initially found as an unidentified protein kinase which may be activated by limited proteolysis with a Ca^{2+}-dependent protease.[18,37] The enzyme thus activated is able to phosphorylate not only histone but also muscle glycogen phosphorylase kinase and glycogen synthetase, resulting in the activation and inactivation of the respective enzymes.[21,22] Further studies have revealed that the protein kinase may be alternatively activated without limited proteolysis by a membrane-associated factor in the presence of a physiological concentration of Ca^{2+}.[39,40] This factor has been identified as membrane phospholipid such as phosphatidylinositol and phosphatidylserine.[39,40] More recently, it has been clarified that the activation of this protein kinase may be directly related to phosphatidylinositol turnover described above.[23,41] The present article will review breifly such roles of Ca^{2+} and membrane phospholipids, especially phosphatidylinositol and phosphatidylserine, in the activation and function of this new species of multifunctional protein kinase. The protein kinase to be described here will be referred to hereafter as protein kinase C, and cyclic AMP-dependent and cyclic GMP-dependent protein kinases as protein kinase A and protein kinase G, respectively.

EXPERIMENTAL PROCEDURES

Materials and Chemicals

Protein kinase C was purified partially from rat brain cytosol by DEAE-cellulose column chromatography followed by gel filtration on Sephadex G-150 and isolelectrofocusing electrophoresis as specified earlier.[18] The enzyme preparation employed for the present studies was essentially free of endogenous phosphate acceptor proteins and interfering enzymes. The catalytic fragment of protein kinase C was obtained by limited proteolysis with a Ca^{2+}- dependent protease as described previously.[18] Protein kinase A was prepared from bovine cerebellum as described earlier.[35] Rat brain synaptic membrane was prepared as described previously.[39] Human erythrocyte ghost was prepared by the method of Dodge *et al.*.[7] Calmodulin was a generous gift of Dr. S. Kakiuchi, Osaka University School of Medicine. An authentic sample of phosphatidylinositol (pig liver) was kindly donated by Dr. T. Yamakawa,

the University of Tokyo, Faculty of Medicine, and those of phosphatidylserine (bovine brain), phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin (human erythrocyte) were generous gifts of Dr. T. Fujii, Kyoto College of Pharmacy. Phosphatidic acid (egg yolk) was purchased from Sigma, and was further purified by two-dimensional thin layer chromatography as described previously.[39] Oleic acid, monoolein, diolein, triolein, stearic acid, monostearin, distearin, tristearin, palmitic acid, monopalmitin, dipalmitin, tripalmitin, arachidonic acid and cholesterol were purchased from Nakarai Chemicals, Kyoto. Triarachidonin was obtained from Gasukuro Kogyo, Tokyo. Lipase (Rhizopus delemar) was purchased from Seikagaku Kogyo, Tokyo. 1-Stearoyl-2-oleoyl diglyceride was purchased from Serdary Research Laboratories. Monoarachidonin and diarachidonin were prepared from triarachidonin by treatment with lipase and were purified by thin layer chromatography as described previously.[23] Unless otherwise specified each sample of diglyceride was a mixture of 1,2- and 1,3-diacyl derivatives as judged by thin layer chromatography. H1, H2A, H2B, H3 and H4 histones were prepared from calf thymus as described previously.[38] [γ-^{32}P] ATP and [γ-^{32}P]GTP were prepared by the method of Glynn and Chappell.[10] Other materials and chemicals were prepared as described previously.[18,21-23, 35-41,46]

Extraction and Fractionation of Lipid

Total lipid was extracted from human erythrocyte ghost and synaptic membrane with chloroform/methanol (2:1) as described by Folch et al..[9] Neutral lipid, glycolipid and phospholipid were fractionated from the total lipid on a silicic acid (Unisil, 100 to 200 mesh, Clarkson Chemical Co.) column as described by Rouser et al..[33] The phospholipid fraction employed for the present studies was free of diglyceride as judged by thin layer chromatography. Further fractionation of lipid was performed by thin layer chromatography on Silica Gel H (E. Merck) plates as described earlier.[39]

Enzyme Assays

Protein kinase C was assayed by measuring the incorporation of ^{32}Pi into H1 histone from [γ-^{32}P] ATP. All reactions were carried out in plastic tubes. The standard reaction mixture (0.25 ml) contained 5 μmol of Tris/HCl at pH 7.5, 1.25 μmol of magnesium nitrate, 50 μg of H1 histone, 2.5 nmol of [γ-^{32}P]ATP (5-15 x 10^4 cpm/nmol), 0.5 μg of protein kinase C, various concentrations of Ca^{2+} and lipid or membrane as indicated in each experiment. All reagents employed for the assay were

taken up in water which was prepared by a double distillation apparatus followed by passing through a Chelex-100 column to remove Ca^{2+} as much as possible as specified earlier.[23] When lipid was employed, it was suspended in 20 mM Tris/HCl at pH 7.5 by sonication with a Kontes sonifier K881440 for 5 min at 0°C. The incubation was carried out for 3 min at 30°C. The reaction was stopped by the addition of 25% trichloroacetic acid, and acid-precipitable materials were collected on a Toyo-Roshi membrane filter (pore size, 0.45 μm) as specified by Kumon et al..[24] Protein kinase A was assayed under conditions similar to those for protein kinase C except that, instead of Ca^{2+} and lipid, 250 pmol of cyclic AMP was added.

Determinations

The radioactivity of ^{32}p-samples was determined using a Nuclear Chicago Geiger Muller gas flow counter, Model 4338. Protein was determined by the method of Lowry et al.[27] with bovine serum albumin as a standard.

Other Procedures

Molecular weight of protein was calculated utilizing a sedimentation coefficient by the equation described by Martin and Ames.[28] The sedimentation coefficient and Stokes radius of protein were estimated by sucrose density gradient centrifugation and gel filtration analyse, respectively, as specified earlier.[36] Isoelectric point of protein was determined by isoelectrofocusing electrophoresis as described previously.[18] SDS**-polyacrylamide slab gel electrophoresis and autoradiography were carried out as described by Laemmli et al.[26] and Kumon et al.,[24] respectively.

RESULTS

Kinetic and Physical Properties of Protein Kinase C

Table 1 shows that protein kinase C was active in the presence of both Ca^{2+} and membrane, and either one of these factors alone was practically inactive. This activation was specific for Ca^{2+}, and other divalent cations such as Mg^{2+}, Mn^{2+}, Cd^{2+}, Ba^{2+}, Ni^{2+} and Co^{2+} were inactive except for Sr^{2+} which was less than 10% as active as Ca^{2+}. Ca^{2+} and membrane were needed for the activation but not for the catalytic activity of the enzyme, since the catalytic fragment which was obtained by limited proteolysis[18, 37, 38, 46] was fully active in the absence of these factors. Protein kinase C was independent of any cyclic

Table 1. Activation of protein kinase C by membrane and $CaCl_2$

Protein kinase C was assayed under the standard conditions except that either protein kinase C, membrane or $CaCl_2$ was omitted from the complete reaction mixture as indicated. In this experiment synaptic membrane (3 μg as protein) and 1 x 10^{-4} M $CaCl_2$ were employed.

System	Protein kinase activity
	(cpm)
Complete system	16,900
- protein kinase C	0
- membrane	640
- $CaCl_2$	990

nucleotide. The regulatory subunit and protein inhibitor of protein kinase A did not inhibit the reaction. The Km values for ATP and Hl histone were about 6.6 x 10^{-6} M and 30 μg/ml, respectively. GTP was unable to serve as phosphate donor for this enzyme. The optimum pH was about 7.5 to 8.0 with 40 mM Tris/HCl as a test buffer. Mg^{2+} was essential for the catalytic activity with the optimum concentrations of about 5 to 10 mM. Protein kinase C showed a sedimentation coefficient of about 5.1 with a molecular weight of 7.7 x 10^4 and a Stokes radius of about 42Å. The isoelectric point of the enzyme was about pH 5.6. These properties are summarized in Table 2 in comparison with those of protein kinases A and G. It is clear that protein kinase C is distinguishable from protein kinases A and G in their physical and kinetic properties. Protein kinase C is independent of Calmodulin and is also different from Ca^{2+} and Calmodulin-dependent protein kinases such as myosin light chain kinase and glycogen phosphorylase kinase.

Identification of Active Components of Membranes

Membranes themselves obtained from various mammalian tissues such as liver, brain and erythrocyte were able to activate protein kinase C. Cytoplasmic membranes as well as other membranes appeared to be active. The membrane fraction carrying 5'-nucleotidase seemed to be most active.

Table 2. Comparison of properties of protein kinases C, A and G

Property	Protein kinase C	Protein kinase A*	Protein kinase G*
Activator	Membrane & Ca^{2+}	Cyclic AMP	Cyclic GMP
Molecular weight	77,000	180,000	140,000
Subunit structure	?	R_2C_2**	E_2***
km for ATP	6.6×10^{-6} M	6.7×10^{-6} M	1.0×10^{-5} M

* Data are taken from reference 35.

** R and C indicate regulatory and catalytic subunits, respectively.

*** E_2 indicates that protein kinase G is composed of two identical subunits.

Active component of membranes was stable in a boiling water bath at neutral pH. It resisted trypsin and pronase, and was quantitatively recovered in the lipid fraction when extracted with chloroform/methanol (2:1). Upon fractionation of total lipid on a silicic acid column, about 50% of the initial activity was recovered in the phospholipid fraction when assayed in the presence of higher concentrations of Ca^{2+} (10^{-4} M range) as shown in Fig. 1. However, when assayed in the lower concentrations of Ca^{2+} (10^{-6} M range), practically no activity was found in any of the lipid fractions including neutral lipid and glycolipid. It was soon revealed that the addition of neutral lipid to phospholipid markedly enhanced the reaction velocity at lower concentrations of Ca^{2+} as shown in Fig. 2. In other words, both phospholipid and neutral lipid were required for full activation of the enzyme at lower concentrations of Ca^{2+}. Kinetic analysis indicated that this enhancement of the reaction velocity by neutral lipid was due to the decrease in apparent Ka values for Ca^{2+} as well as for phospholipid. Glycolipid could not substitute for neutral lipid for this enhancement.

Analysis of the neutral lipid fraction indicated that diglyceride possessing an unsaturated fatty acid at least at the position 2 was the active component which could actually enhance and fortify the Ca^{2+} and phospholipid-dependent activation of protein kinase C. Effects of various synthetic diglycerides on Ka value for Ca^{2+} and reaction velocity of protein kinase C are summarized in Table 3. Diolein, diarachidonin and 1-stearoyl-2-oleoyl diglyceride were equally effective in this capacity.

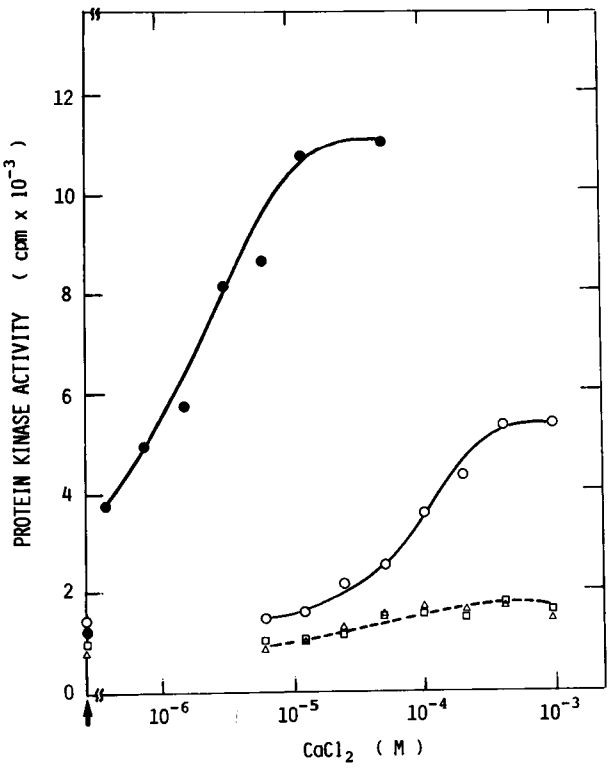

Fig. 1 Protein kinase C activity in the presence of total lipid, neutral lipid, glycolipid and phospholipid at various concentrations of $CaCl_2$. Various lipids which were prepared from erythrocyte ghost were employed in this experiment. Protein kinase C was assayed under the standard conditions except that lipid and $CaCl_2$ were added as indicated. Where indicated with an arrow, EGTA (0.5 mM at final concentration) was added instead of $CaCl_2$. (●——●), with total lipid (5.8 μg; this total lipid was composed of phospholipid (4.1 μg), neutral lipid (1.4 μg) and glycolipid (0.3 μg)); (○——○), with phospholipid (4.1 μg); (□——□), with neutral lipid (1.4 μg); and (Δ——Δ), with glycolipid (0.3 μg).

Whereas, dipalmitin and distearin were far less effective. None of other triacyl- and monoacylglycerols was effective irrespective of the fatty acyl moieties including palmitic, stearic, oleic and arachidonic acids. Cholesterol and free fatty acids were also ineffective under the similar conditions.

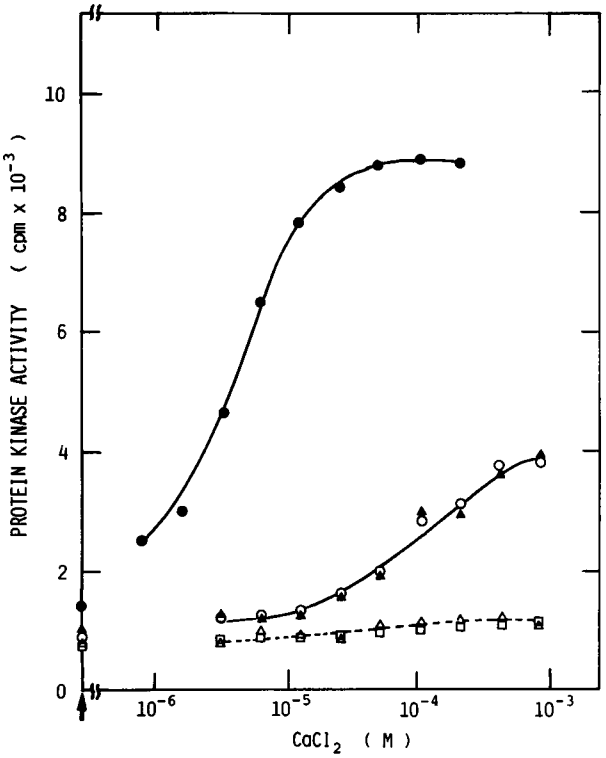

Fig. 2 Effects of neutral lipid and glycolipid on reaction velocity of protein kinase C in the presence of phospholipid at various concentrations of $CaCl_2$. Various lipids which were prepared from erythrocyte ghost were employed in this experiment. Phospholipid was first mixed with either neutral lipid or glycolipid in a small volume of chloroform. After chloroform was removed in vacuo, each mixture was suspended in 20 mM Tris/HCl at pH 7.5 by sonication as described under EXPERIMENTAL PROCEDURES. Protein kinase C was assayed under the standard conditions except that lipid and $CaCl_2$ were added as indicated. Where indicated with an arrow, EGTA (0.5 mM at final concentration) was added instead of $CaCl_2$. (o——o), with phospholipid (4.1 μg) alone; (□——□), with neutral lipid (1.4 μg) alone; (Δ——Δ), with glycolipid (0.3 μg) alone; (●——●), with phospholipid (4.1 μg) plus neutral lipid (1.4 μg); and (▲——▲), with phospholipid (4.1 μg) plus glycolipid (0.3 μg).

Table 3. Effects of various diglycerides on Ka value for Ca^{2+} and reaction velocity of protein kinase C

The reaction mixture contained phospholipid (4.1 μg), diglyceride indicated (0.2 μ g) and varying concentrations of $CaCl_2$. Other conditions were the same to those described in Fig. 2. The protein kinase activity at 6.4×10^{-6} M $CaCl_2$ is given.

Diglyceride added	Ka for Ca^{2+}	Protein kinase activity
	(μM)	(cpm)
None	70	980
Diolein	4	6,500
Diarachidonin	4	6,220
1-Stearoyl-2-oleoyl diglyceride	8	4,610
Dipalmitin	30	1,760
Distearin	70	950

In the next set of experiments, active phospholipid was analyzed on a Silica Gel H plate. When the enzyme was assayed in the absence of unsaturated diglyceride at higher concentrations of Ca^{2+} (10^{-4} M range), activity was localized on the spots of phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine. In fact, in the absence of unsaturated diglyceride authentic samples of the three phospholipids could support the enzymatic activity of protein kinase C, only when assayed at higher concentrations of Ca^{2+} as shown in Fig. 3, A to C. At lower concentrations of Ca^{2+} (10^{-6} M range) these phospholipids were all ineffective. If, however, the assay was made in the presence of diolein and phosphatidylserine, the enzyme was highly active at extremely lower concentrations of Ca^{2+}, and such effect of phospholipid was specific for phosphatidylserine as shown in Fig. 3A. Phosphatidylinositol and phosphatidylethanolamine were inactive at lower concentrations of Ca^{2+} both in the presence and absence of unsaturated diglyceride as shown in Fig. 3, B and C. Phosphatidylcholine was totally inactive over a wide range of Ca^{2+} concentrations irrespective of the presence and absence of diglyceride as shown in Fig. 3D. Phosphatidic acid and

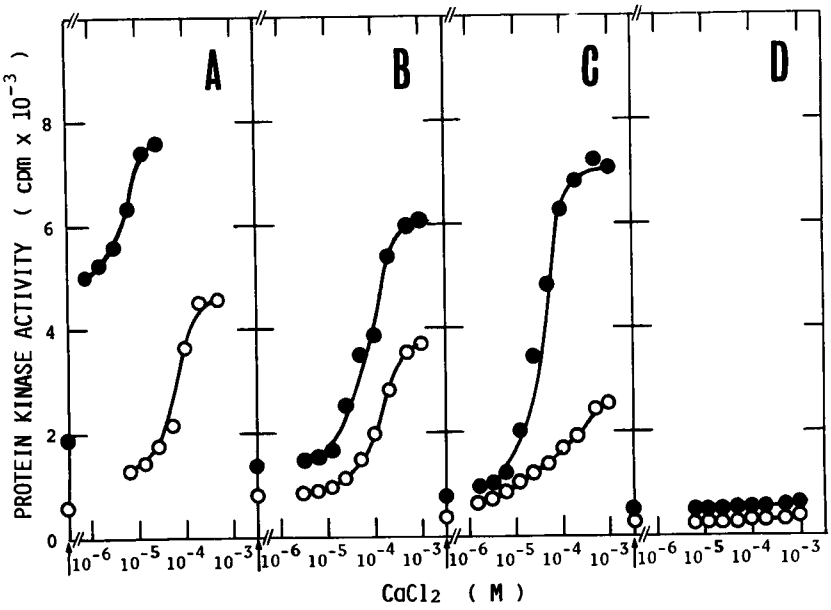

Fig. 3 Effect of diolein on reaction velocity of protein kinase C in the presence of various phospholipids at various concentrations of $CaCl_2$. Mixtures of each phospholipid and diolein were prepared as described in Fig. 2. Protein kinase C was assayed under the standard conditions except that lipid and $CaCl_2$ were added as indicated. Each phospholipid (2 μg) and diolein (0.2 μg) were employed. Where indicated with an arrow, EGTA (0.5 mM at final concentration) was added instead of $CaCl_2$. A, with phosphatidylserine; B, with phosphatidylinositol; C, with phosphatidylethanolamine; and D, with phosphatidylcholine. (●——●), assayed in the presence of diolein; and (o——o), assayed in the absence of diolein.

sphingomyelin were also practically inactive (data not shown). Diolein alone showed a little or no effect. These results indicate that at physiological concentrations of Ca^{2+} (10^{-6} M range), phosphatidylserine and diglyceride possessing an unsaturated fatty acid at least at the position 2 play a role of crucial importance to activate this unique protein kinase.

Possible Mode of Activation of Protein Kinase C

When protein kinase C was mixed with membranes such as synaptic membrane or erythrocyte ghost in the absence of Ca^{2+} and then the mixture was centrifuged to separate again the enzyme from membranes, the

enzyme remained soluble as an inactive form. However, when protein kinase C was mixed with membranes in the presence of Ca^{2+} and then centrifuged, the enzyme was precipitated with membranes as an active form. This activation process appeared to be reversible since the enzyme was inactivated again by removing Ca^{2+} with EGTA.

It appears to be established that phosphatidylinositol turnover, which is provoked by various extracellular stimulators, is initiated by hydrolysis of the phosphodiester linkage in a manner of phospholipase C. Since phosphatidylinositol in mammalian tissues is composed of unsaturated fatty acids such as arachidonic and oleic acids particularly at the position 2,[17] the primary product of this turnover is expected to be unsaturated diglyceride. It is conceivable, therefore, that the extracellular stimulator-induced activation of phospholipase C may initiate phosphatidylinositol turnover on one hand and, on the other hand, the resulting unsaturated diglyceride may serve as a messenger which in turn activates protein kinase C in the presence of Ca^{2+} and phosphatidylserine.

Catalytic Properties of Protein Kinase C

Table 4. Phosphorylation of five species of histones by protein kinases C and A

Protein kinase C was assayed in the presence of 35 μg of erythrocyte total lipid and 1×10^{-5} M $CaCl_2$ under the standard conditions except that 50 μg each of the acceptor protein was added. Protein kinase A (1.5 μg of protein) was assayed under the standard conditions except that 50 μg each of the acceptor protein was added.

Histone	Protein kinase C	Protein kinase A
	(cpm)	(cpm)
H1 histone	17,400	3,300
H2A histone	2,700	2,100
H2B histone	4,000	17,400
H3 histone	1,000	400
H4 histone	200	300

Protein kinase C activated as described above was able to phosphorylate five species of histone as described for protein kinase A. However, the substrate specificity of protein kinase C was different from that of protein kinase A as shown in Table 4. Protein kinase C preferentially phosphorylated H1 histone, whereas protein kinase A rapidly phosphorylated H2B histone. Protein kinase C did not phosphorylate bovine casein, egg yolk phosvitin, human serum albumin, human γ-globulin and human serum fibrinogen. In addition to histone protein kinase C appeared to favor the endogenous proteins associated with membranes rather than soluble proteins as phosphate acceptors. Fig. 4 shows autoradiography of SDS-polyacrylamide slab gel electropherogram which illustrates brain endogenous substrate proteins of protein kinases C and A. Both protein kinases reacted several proteins recovered in a particulate fraction containing synaptic membrane and myelin. It was very characteristic that two proteins with molecular weight of around 20,000, most likely associated with membranes, were very heavily phosphorylated by protein kinase C. Although it is not known at this time whether these substrate proteins described above are indeed phosphorylated *in vivo* by protein kinase C, it is likely that protein kinase C is a multifunctional protein kinase as described for protein kinases A and G.

Tissue Distribution of Protein Kinase C

Table 5 shows the tissue distribution and relative activities of protein kinase C in some mammalian tissues in comparison with those of protein kinase A. The two classes of enzymes were assayed under comparable conditions, therefore, these enzymatic activities may be directly compared with each other. It is evident that protein kinase C is distributed in many tissues and organs in amounts that are comparable to protein kinase A, and that the enzyme is present in large quantities particularly in brain and lymphocyte.

DISCUSSION

Since the first discovery of phosphatidylinositol turnover by Hokin and Hokin [16] in 1955, this unique phenomenon has been extensively studied by many investigators. [8, 13, 29, 30] Nevertheless, all attempts to clarify the physiological significance of this phospholipid turnover have been thus far uniformly unsuccessful. Available evidence described in this paper strongly suggests that phosphatidylinositol turnover may be directly coupled with the activation of a new species of multifunctional protein kinase, that is protein kinase C. Thus, phospholipid and

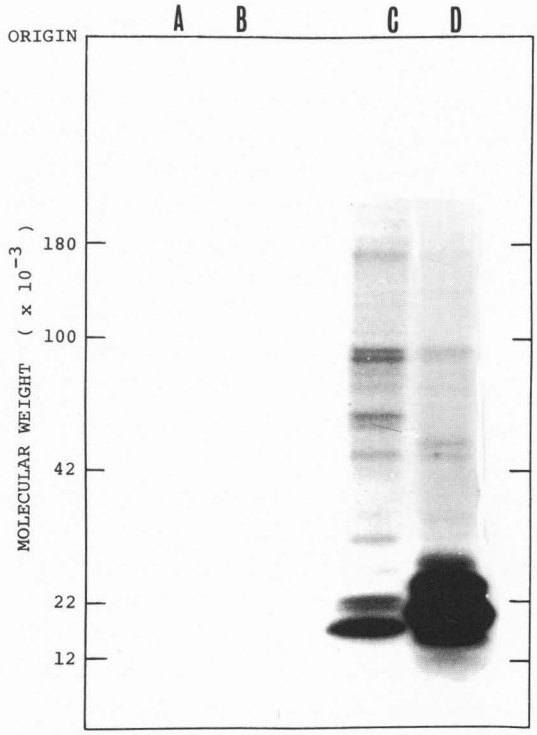

Fig.4 Autoradiography of SDS-polyacrylamide slab gel electropherogram of rat brain endogenous proteins phosphorylated by protein kinases C and A. Rat brain (1 g wet weight) was homogenized in a Potter-Elvehjem Teflon-glass homogenizer with 3 ml of 20 mM Tris/HCl at pH 7.5 containing 0.25 M sucrose. The homogenate was centrifuged for 60 min at 100,000 x g. The precipitate was suspended in 3 ml of the same buffer. The supernatant and precipitate were boiled for 5 min to kill endogenous enzymatic activities and employed as soluble and particulate fractions, respectively. The soluble and particulate fractions (200 μg of protein each) were phosphorylated fully by protein kinase C (4 μg of protein) in the presence of 35 μg of erythrocyte total lipid and 5 x 10^{-4} M $CaCl_2$ and by protein kinase A (12 μg of protein) under the respective standard assay conditions except that 12.5 nmol of [γ-^{32}P]ATP (8 x 10^5 cpm/nmol) was employed and that incubation was perfomed for 60 min. Each reaction was terminated by the addition of 125 μl of 9% SDS containing 6% 2-mercaptoethanol, 15% glycerol, 186 mM Tris/HCl at pH 6.7 and a small amount of bromophenol blue dye. Each radioactive proteins preparation was boiled for 3 min and a 50-μl aliquot each was subjected to SDS-polyacrylamide slab gel electrophoresis as described under EXPERIMENTAL PROCEDURES. A and B, soluble proteins phosphorylated by protein kinases A and C, respectively; and C and D, particulate proteins phosphorylated by protein kinases A and C, respectively.

Table 5. Relative activities of protein kinases C and A in various tissues and organs

Brain, lung, kidney, liver and adipose tissue (1 g wet weight each) were homogenized in a Potter-Elvehjem Teflonglass homogenizer with 3 volumes of 20 mM Tris/HCl at pH 7.5 containing 0.25 M sucrose, 2 mM EDTA and 10 mM EGTA. Heart and skeletal muscle (1 g wet weight each) were homogenized in a Waring blendor for 2 min with 3 volumes of the same buffer. Lymphocytes (5×10^7 cells) were suspended in 1 ml of the same buffer and disrupted by sonication with a Kontes sonifier K881440 for 2 min. Each homogenate was then centrifuged for 60 min at 100,000 x g. These procedures were performed at 0-4°C. The supernatant thus obtained was employed for protein kinase assay. Protein kinase C was assayed in the presence of 35 μg of erythrocyte total lipid and 5×10^{-4} M $CaCl_2$, and protein kinase A was assayed in the presence of 1×10^{-6} M cyclic AMP under each standard assay condition.

Tissue	Protein kinase C	Protein kinase A
	(pmoles/min/mg of protein)	
Brain	3,270	250
Lymphocyte	1,060	320
Lung	360	290
Kidney	280	150
Liver	180	130
Adipose tissue	170	270
Heart	110	230
Skeletal muscle	80	110

Ca^{2+} play a role of crucial importance in hormonal control of protein phosphorylation reactions through protein kinase C. It may be emphasized that in the mechanism described in this paper protein kinase C may be activated without a net increase in the intracellular Ca^{2+} concentrations, since a small quantity of diglyceride which results from the phosphatidylinositol hydrolysis would sharply increase the affinity of the newly found protein kinase system for this divalent cation.

It has been generally accepted that some hormones which increase cellular concentrations of cyclic AMP do not produce phosphatidylinositol turnover.[29] In addition, among a variety of hormones there appears to be a wide spread correlation between abilities to increase cellular cyclic GMP concentrations and to cause phosphatidylinositol turnover.[29] Guanylate cyclase has been proposed to be activated in vitro by arachidonic acid hydroperoxide as well as prostaglandin endoperoxide.[12,14] Majerus and his associates have recently reported that this fatty acid may be derived from diglyceride which is produced from phosphatidylinositol turnover.[1] A specific diglyceride lipase which catalyzes such generation of arachidonic acid has also been identified.[1] Therefore, it seems likely that phosphatidylinositol turnover may be related to the activation of guanylate cyclase and, thus diglyceride may play roles in controlling not only protein kinase C but also protein kinase G.

Axelrod and his associates have recently proposed that enzymatic methylation of membrane phosphatidylethanolamine is stimulated by hormone which may increase cellular cyclic AMP, and that this enzymatic methylation may be coupled with the activation of adenylate cyclase.[15] Since it has been well established that phosphatidylserine is converted to phosphatidylethanolamine by its decarboxylation,[43] it is possible that this species of phospholipid may be involved in the regulation of both protein kinases C and A. Although the exact correlation between these three species of protein kinases and membrane phospholipid metabolism has remained to be exlored further, it is conceivable that various phospholipids, especially phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine, seem to play specific roles in the transmembrane control of protein phosphorylation reactions catalyzed by the three species of protein kinases mentioned above.

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REFERENCES

1. Bell, R.L., Kennerly, D.A., Stanford, N., and Majerus, P.W. Proc. Natl. Acad. Sci. USA. 1979. 76. 3238/3241. Diglyceride lipase: A pathway for arachidonate release from human platelets.
2. Brostrom, C.O., Huang, Y.C., Breckenridge, B. McL., and Wolff, D.J. Proc. Natl. Acad. Sci. USA. 1975. 72. 64/68. Identification of a calcium-binding protein as a calcium-dependent regulator of brain adenylate cyclase.
3. Cheung, W.Y. Biochem. Biophys. Res. Commun. 1970. 38. 533/538. Cyclic 3',5'-nucleotide phosphodiesterase: demonstration of an activator.
4. Cohen, P., Burchell, A., Foulkes, J.G., and Cohen, P.T.W. FEBS Lett. 1978. 92. 287/293. Identification of the Ca^{2+}-dependent modulator protein as the fourth subunit of rabbit skeletal muscle phosphorylase kinase.
5. Dabrowska, R., Sherry, J.M.F., Aromatorio, D.K., and Hartshorne, D.J. Biochemistry 1978. 17. 253/258. Modulator protein as a component of the myosin light chain kinase from chicken gizzard.
6. Dawson, R.M.C., Freinkel, N., Jungalwala, F.B., and Clarke, N. Biochem. J. 1971. 122. 605/607. The enzymic formation of myoinositol 1:2-cyclic phosphate from phosphatidylinositol.
7. Dodge, J.T., Mitchell, C., and Hanahan, D.J. Arch. Biochem. Biophys. 1963. 100. 119/130. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes.
8. Durell, J., Garland, J.T., and Friedel, R.O. Science 1969. 165. 862/866. Acetylcholine action: biochemical aspects.
9. Folch, J., Lees, M., and Stanley, G.H.S. J. Biol. Chem. 1957. 226. 497/509. A simple method for the isolation and purification of total lipids from animal tissues.
10. Glynn, I.M., and Chappell, J.B. Biochem. J. 1964. 90. 147/149. A simple method for the preparation of ^{32}P-labelled adenosine triphosphate of high specific activity.

11. Gopinath, R.M., and Vincenzi, F.F. Biochem. Biophys. Res. Commun. 1977. 77. 1203/1209. Phosphodiesterase protein activator mimics red blood cell cytoplasmic activator of Ca^{2+}-Mg^{2+}ATPase.

12. Graff, G., Stephenson, J.H., Glass, D.B., Haddox, M.K., and Goldberg, N.D. J. Biol. Chem. 1978. 253, 7662/7676. Activation of soluble splenic cell guanylate cyclase by prostaglandin endoperoxides and fatty acid hydroperoxides.

13. Hawthorne, J.N., and White, D.A. Vitam. Horm. (New York) 1975. 33. 529/573. Myo-inositol lipids.

14. Hidaka, H., and Asano, T. Proc. Natl. Acad. Sci. USA. 1977. 74. 3657/3661. Stimulation of human platelet guanylate cyclase by unsaturated fatty acid peroxides.

15. Hirata, F., Strittmatter, W.J., and Axelrod, J. Proc. Natl. Acad. Sci. USA. 1979. 76. 368/372. β-adrenergic receptor agonists increase phospholipid methylation, membrane fluidity, and β-adrenergic receptor-adenylate cyclase coupling.

16. Hokin, L.E., and Hokin, M.R. Biochim. Biophys. Acta 1955. 18. 102/110. Effects of acetylcholine on the turnover of phosphoryl units in individual phospholipids of pancreas slices and brain cortex slices.

17. Holub, B.J., Kuksis, A., and Thompson, W. J. Lipid Res. 1970. 11. 558/564. Molecular species of mono-, di-, and triphosphoinositides of bovine brain.

18. Inoue, M., Kishimoto, A., Takai, Y., and Nishizuka, Y. J. Biol. Chem. 1977. 252. 7610/7616. Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain.

19. Jarrett, H.W., and Penniston, J.T. Biochem. Biophys. Res. Commun. 1977. 77. 1210/1216. Partial purification of the Ca^{2+}-Mg^{2+}ATPase activator from human erythrocytes: its similarity to the activator of 3':5'-cyclic nucleotide phosphodiesterase.

20. Kakiuchi, S., and Yamazaki, R., Biochem. Biophys. Res. Commun. 1970. 41. 1104/1110. Calcium dependent phosphodiesterase activity and its activating factor from brain.

21. Kishimoto, A., Takai, Y., and Nishizuka, Y. J. Biol. Chem. 1977. 252. 7449/7452. Activation of glycogen phosphorylase kinase by a calcium-activated, cyclic nucleotide-independent protein kinase system.

22. Kishimoto, A., Mori, T., Takai, Y., and Nishizuka, Y. J. Biochem. 1978. 84. 47/53. Comparison of calcium-activated, cyclic nucleotide-independent protein kinase and adenosine 3':5'-monophosphate-dependent protein kinase as regards the ability to stimulate glycogen breakdown in vitro.

23. Kishimoto, A., Takai. Y., Mori, T., Kikkawa, U., and Nishizuka, Y. J. Biol. Chem. 1980. in press. Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover.

24. Kumon, A., Nishiyama, K., Yamamura, H., and Nishizuka, Y. J. Biol. Chem. 1972. 247. 3726/3735. Multiplicity of adenosine 3',5'-monophosphate-dependent protein kinases from rat liver and mode of action of nucleoside 3', 5'-monophosphate.

25. Kuo, J.F., and Greengard, P. J. Biol. Chem. 1970. 245. 2493/2498. Cyclic nucleotide-dependent protein kinases. VI. Isolation and partial purification of a protein kinase activated by guanosine 3', 5'-monophosphate.

26. Laemmli, U.K. Nature 1970. 227. 680/685. Cleavage of structural proteins during the assembly of the head of bacteriophage T4.

27. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. J. Biol. Chem. 1951. 193. 265/275. Protein measurement with the folin phenol reagent.

28. Martin, R.G., and Ames, B.N. J. Biol. Chem. 1961. 236. 1372/1379. A method for determining the sedimentation behavior of enzymes: application to protein mixtures.

29. Michell, R.H. Biochim. Biophys. Acta 1975. 415. 81/147. Inositol phospholipids and cell surface receptor function.

30. Michell, R.H. Trends. Biochem. Sci. 1979. 4. 128/131. Inositol phospholipids in membrane function.

31. Rasmussen, H., and Goodman, D.B.P. Physiol. Rev. 1977. 57. 421/509. Relationships between calcium and cyclic nucleotides in cell activation.

32. Rylatt, D.B., Embi, N., and Cohen, P. FEBS Lett. 1979. 98. 76/80. Glycogen synthase kinase-2 from rabbit skeletal muscle is activated by the calcium-dependent regulator protein.

33. Rouser, G., Kritchevsky, G., and Yamamoto, A. in Lipid Chromatographic Analysis, 1967. Vol.1, Marcel Dekker Inc., New York.

34. Srivastava, A.K., Waisman, D.M., Brostrom, C.O., and Soderling, T.R. J. Biol. Chem. 1979. 254. 583/586. Stimulation of glycogen

synthase phosphorylation by calcium-dependent regulator protein.

35. Takai, Y., Nishiyama, K., Yamamura, H., and Nishizuka, Y. J. Biol. Chem. 1975. 250. 4690/4695. Guanosine 3',5'-monophosphate-dependent protein kinase from bovine cerebellum. Purification and characterization.

36. Takai, Y., Nakaya, S., Inoue, M., Kishimoto, A., Nishiyama, K., Yamamura, H., and Nishizuka, Y. J. Biol. Chem. 1976. 251. 1481/1487. Comparison of mode of activation of guanosine 3':5'-monophosphate-dependent and adenosine 3':5'-monophosphate-dependent protein kinases from silkworm.

37. Takai, Y., Yamamoto, M., Inoue, M., Kishimoto, A., and Nishizuka, Y. Biochem. Biophys. Res. Commun. 1977. 77. 542/550. A proenzyme of cyclic nucleotide-independent protein kinase and its activation by calcium-dependent neutral protease from rat liver.

38. Takai, Y., Kishimoto, A., Inoue, M., and Nishizuka, Y. J. Biol. Chem. 1977. 252. 7603/7609. Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. I. Purification and characterization of an active enzyme from bovine cerebellum.

39. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., and Nishizuka, Y. J. Biol. Chem. 1979. 254. 3692/3695. Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids.

40. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., Nishizuka, Y., Tamura, A., and Fujii, T. J. Biochem. 1979. 86. 575/578. A role of membranes in the activation of a new multifunctional protein kinase system.

41. Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T., and Nishizuka, Y. Biochem. Biophys. Res. Commun. 1979. 91. 1218/1224. Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated, phospholipid-dependent protein kinase system.

42. Walsh, D.A., Perkins, J.P., and Krebs, E.G. J. Biol. Chem. 1968. 243. 3763/3765. An adenosine 3',5'-monophosphate-dependent protein kinase from rabbit skeletal muscle.

43. Wilson, J.D., Gibson, K.D., and Udenfriend, S. J. Biol. Chem. 1960. 235. 3539/3543. Studies on the conversion in vitro of serine to ethanolamine by rat liver and brain.

44. Wolff, D.J., and Brostrom, C.O. Adv. Cyclic Nucleotide Res. 1979. 11. 27/88. Properties and function of the calcium-dependent regulator protein.

45. Yagi, K., Yazawa, M., Kakiuchi, S., Ohshima, M., and Uenishi, K. J. Biol. Chem. 1978. 253. 1338/1340. Identification of an activator protein for myosin light chain kinase as the Ca^{2+}-dependent modulator protein.

46. Yamamoto, M., Takai, Y., Inoue, M., Kishimoto, A., and Nishizuka, Y. J. Biochem. 1978. 83. 207/212. Characterization of cyclic nucleotide-independent protein kinase produced enzymatically from its proenzyme by calcium-dependent neutral protease from rat liver.