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A SINGLE FORM OF PROTEIN KINASE C IS EXPRESSED IN BOVINE ADRENOCORTICAL TISSUE, AS COMPARED TO FOUR CHROMATOGRAPHICALLY RESOLVED ISOZYMES IN RAT BRAIN

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Protein kinase C purified to apparent homogeneity from rat brain was resolved into four active moieties following chromatography over a hydroxyapatite high resolution system. By contrast, the same procedure applied to bovine adrenocortical protein kinase C revealed that a single protein kinase C isoform could be detected in this tissue, with a chromatographic behavior identical to that of one of the brain isoenzymes. Although the isolated protein kinase C isozymes were all activated to various degrees in the presence of phospholipids and calcium, quantitative differences were observed in their catalytic properties, especially with regard to their sensitivity to diacylglycerol and TPA and to their relative affinity for different protein substrates. These observations confirmed at the protein level the heterogeneity of protein kinase C predicted on the basis of cDNA cloning studies. They also suggest that the expression of a specific set of protein kinase C isoenzyme(s) in a given cell type deserves further attention, since it may reflect a functionnal significance with regard to the regulation of specific cellular processes.

The calcium and phospholipid-dependent protein kinase (protein kinase C) is widely accepted as a major transmembrane signalling system in the mechanism of action of a number of extracellular messengers (1, 2). This concept is mostly based on the fact that, in the presence of phospholipids, the enzyme is activated by diacylglycerol and calcium, both representing potential intracellular messengers in cell activation processes (3-5). A pivotal role of protein kinase C in the control of cell growth was further suggested by the observation that the enzyme represents the major receptor for the tumor promoter phorbol esters (6). Protein kinase C has been purified to apparent homogeneity by a number of laboratories (7-10) and the enzyme was thought to be a single entity until analysis of its cDNA clones revealed that multiple forms of the protein were to be expected. Three cDNA clones (α, β, γ) were obtained from bovine, rat, rabbit and human libraries (11-14) whereas two clones (I and II) from rat brain were shown to derive from alternative

ABBREVIATONS: DAG : 1,2-diacylglycerol ; DTT : dithiothreitol ; HPLC : high performance liquid chromatography ; TPA : 12-O-tetradecanoate phorbol-13-acetate

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splicing (15). Four isoforms of the protein may thus be predicted (α, β_1, β_2, γ). Use of specific cDNA probes revealed that different cell types may express different kinase isoforms at the mRNA level (16) and these data raise the question as to whether various forms of the kinase may be related to different biological functions. Although evidence for protein kinase C heterogeneity at the protein level have been suggested (17-19), no isolation procedure of the four expected isozymes has yet been reported.

In previous work, we have presented evidence that protein kinase C may be involved in the control of differentiated bovine adrenocortical cell functions, especially in response to specific hormonal stimuli such as angiotensin II (20). Protein kinase C is present in this cell type (21) but it was not known which isoenzyme(s) are expressed in this system.

This communication reports the separation of four isoforms of protein kinase C from an apparently homogeneous preparation of rat brain enzyme, using a high resolution chromatographic technique. By contrast, the same analytical procedure disclosed a single isoform for the adrenocortical protein kinase. Some catalytic properties of the isolated individual isoforms were examined, especially in term of relative sensitivity to activators and of protein substrate affinity. These observations suggest that the type of isozyme involved may be of significance when specific functions of protein kinase C in a given cell system are considered.

MATERIALS AND METHODS

. Chemicals

[γ-^{32}P]-ATP (10 Ci/mmol) was purchased from the Radiochemical Center (Amersham). DEAE-cellulose (DE 52) was obtained from Whatman. Phenyl Sepharose (Sepharose 6B) was from Pharmacia and ACA 44 gel was from LKB. Hydroxyapatite HPLC column (7.6x100 mm) was provided by Mitsui Toatso Chemicals, Inc (Tokyo, Japan). Histone H_1, protamine, EDTA, EGTA, bovine serum albumine, phosphatidylserine, TPA and diolein were obtained from Sigma. Casein was treated according to (22) before use.

. Purification and HPLC analysis of protein-kinase C

Cytosol fractions (100,000 g x 1 hour supernatant) were prepared from the dissected cortical tissue (30 g) of fresh bovine adrenal glands and from rat brain tissue (8 g) in 250 ml and 70 ml, respectively, of homogenization buffer (20 mM Tris-HCl, pH 7.5, containing 2 % glycerol, 10 mM EGTA, 10 mM EDTA, 1 mM DTT). The cytosol was then diluted into 8 volumes of buffer A (10 mM Tris-HCl, pH 7.5, containing 2 % glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT). The procedure started with a chromatography on DEAE cellulose (1.5x12 cm column, eluted with a 0 to 0.4 M NaCl gradient). The active kinase fractions were pooled and precipitated with 80 % ammonium sulfate, then processed through ACA-44 gel fitration followed by phenyl sepharose (0.9x2.5 cm column, eluted with a 1.5 to 0 M NaCl gradient). The kinase preparation was dialysed overnight against HPLC buffer (10 mM potassium phosphate, pH 7.5, containing 10 % glycerol, 1 mM DTT, 0.5 mM EDTA and 0.5 mM EGTA) before being transfered onto the HPLC column, previously equilibrated with the same buffer. The kinase preparation (about 5 ml) was applied to the column at a flowrate of 0.4 ml/hour. After washing until

disappearance of the absorbance at 280 nm, elution was carried out, using a curvilinear potassium phosphate gradient (10 to 180 mM) delivered by the HPLC Shimadzu programming system at a flow rate of 0.4 ml/hour. All chromatography steps were performed at 4°C.

Protein kinase C assay was performed by measuring the transfer of ^{32}P from [γ-^{32}P]-ATP to histone H_1, essentially as described by Kraft et al. (23). The reaction mixture (80 µl) contained 0.01 mM [^{32}P]-ATP (specific activity 1,000 cpm/pmol), 90 µg of histone, 10 mM $MgCl_2$, and 750 µM $CaCl_2$. Phosphatidylserine (26 µg) and diolein (0.8 µg) were included or not in the assay, as indicated, using a lipid suspension in 100 mM Tris/HCl pH 7.5, previously sonicated for 5 min. at 30°C. The reaction was carried out at 30°C for 10 min. and stopped upon addition of 12 % trichloroacetic acid in the presence of casein (900 µg) added as a carrier. The protein precipitate was dissolved in 1 N NaOH, and ^{32}P incorporation was measured by scintillation counting in Aquasol (New England Nuclear) with an Intertechnique SL-32 spectrometer. In some cases, as indicated, a mixed micelle assay was used, essentially as described by Bonni and Rando (24). A mixture of phosphatidyl choline (540 µg), phosphatidylserine (130 µg) and various amounts of diolein or TPA, as indicated, were dried under nitrogen. The dried residue was redissolved into 25 µl of 100 mM Tris-HCl buffer, pH 7.5, containing 0.2 mM EGTA and 100 mM octylglucoside. After vigorous vortexing at 30°C, the vesicle suspension was obtained by a ten fold dilution with 10 mM Tris-HCl buffer, pH 7.5. The phospholipid vesicle suspension (8 µl) was used in the protein kinase C assay mixture (total volume of 70 µl), containing all the reagents at the final concentrations as mentioned above.

RESULTS

1. Isoforms of protein kinase C from rat brain and bovine adrenocortical tissues.

Purified protein kinase C preparations obtained from rat brain tissue following DEAE-cellulose, ACA-44 and phenyl-sepharose chromatography were transfered onto the hydroxyapatite HPLC column. Various elution programs using a potassium phosphate concentration gradient were examined and the best resolution was obtained with a 10 to 180 mM, curvilinear salt concentration gradient, as shown in figure 1. Under these conditions, assay of histone kinase activity in the eluted fractions, in the absence and in the presence of phospholipids and calcium revealed four peaks of protein kinase C activity. These isoforms of the kinase eluted at 35 (form A), 45 (B), 60 (C) and 90 (D) mM potassium phosphate concentration, respectively (figure 1, upper pannel). When the isolated active fractions were self-phosphorylated in the presence of [γ-^{32}P]-ATP and analyzed by polyacrylamide gel electrophoresis, followed by autoratiography, they all exhibited a single band with a Mr of about 80 kDa, whose labeling was phospholipid and calcium dependent (not shown). This was in agreement with one of the previously described typical properties of protein kinase C (25).

By contrast, when the same analytical procedure was applied to a bovine adrenocortical protein kinase C preparation, a single peak of activity was detected following hydroxyapatite HPLC chromatography (figure 1, lower

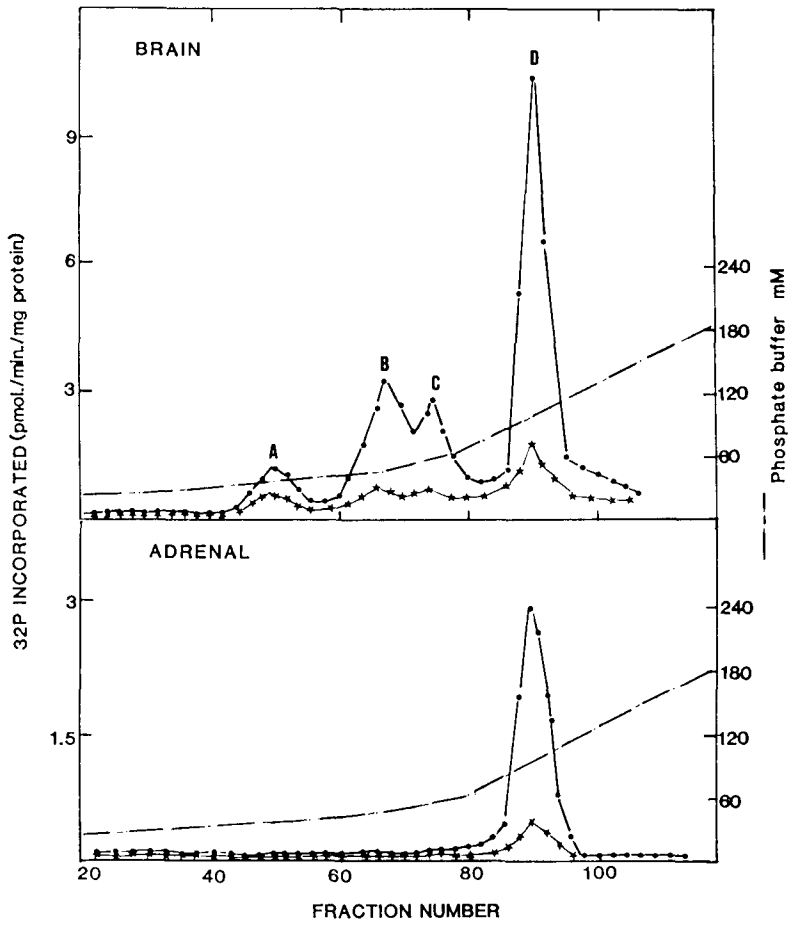

Figure 1 .
Hydroxyapatite HPLC analysis of purified cytosolic rat brain (upper panel) and bovine adrenal cortex (lower panel) protein kinase C.
Elution was performed with a 10 to 180 mM curvilinear potassium phosphate gradient (dotted line...). Aliquots (5 µl) of the collected fractions (0.7 ml each) were assayed for protein kinase activity using histone H_1 as protein substrate, either in the absence (✶- ✶) or in the presence (● - ●) of a phospholipid/diolein/Ca^{2+} mixture, as described under "Materials and Methods". The four isolated peaks of brain protein kinase activities were designated A,B,C and D isoforms, respectively.

panel). This phospholipid and calcium-dependent histone kinase activity eluted at 90 mM phosphate concentration, thus exhibiting the chromatographic properties of the D isoform of brain protein kinase C. Whereas variable phospholipid and calcium dependence was observed in the case of the A, B, C and D brain isoforms, as detected in the chromatographic fraction (figure 1, upper pannel), the activation factor due to the presence of phospholipids was similar for the brain D isoform and the adrenocortical enzyme. This suggested that the single form of protein kinase detected in bovine adenocortical tissue may be similar if not identical to one of the brain isoform, namely the D moeity as defined in figure 1.

2. Catalytic properties of the isolated protein kinase C isoforms.

a/ Activation by DAG and a phorbol ester

The four isolated rat brain protein kinase C isoforms, as well as the adrenocortical moiety, were compared with regard to their sensitivity to diacylglycerol (diolein) and an active phorbol ester (TPA), using a mixed

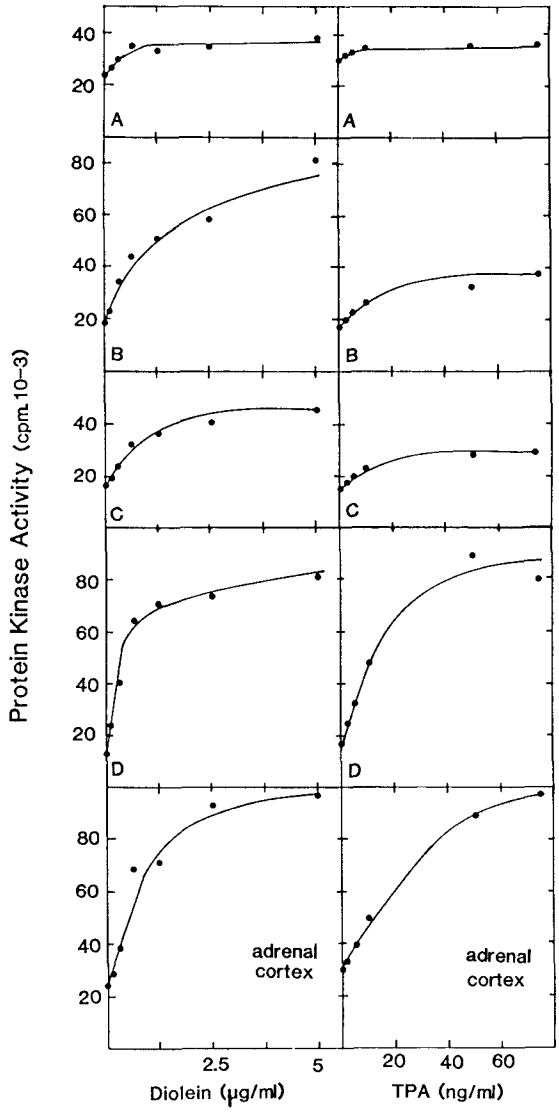

Figure 2 .
Concentration dependent activation of the isozymic forms of protein kinase C by Diolein and TPA.
PKC activity of the four isolated brain isozymes and of the bovine adrenal cortex moiety were measured, using the mixed micelle assay as described in the experimental section, in the presence of various amounts of diolein (left panel) or TPA (right panel). These graphs were used to determine half maximal activating concentrations (Ka) and activation factors, for diolein and TPA (Table I).

TABLE I

Half maximal effective concentrations (Ka) and optimal activation factors of diolein and TPA on the activity of the different protein kinase C isoforms isolated from brain and adrenocortical tissues

	BRAIN PROTEIN KINASE C SUBTYPE				PROTEIN KINASE C EXPRESSED IN ADRENAL CORTEX
	A	B	C	D	
Ka diolein (μg/ml)	1.25	0.8	2.5	0.47	2.5
Activation factor by diolein (5 μg/ml)	1.5	4.6	2.8	5.6	4.2
Ka TPA (ng/ml)	5	40	16	22	50
Activation factor by TPA (75 ng/ml)	1.2	2.3	1.9	5.8	3.5

micelle assay and histone H_1 as the protein substrate. Figure 2 illustrates the dose-response curves obtained with increasing amounts of the activator(s) in the assay. The effective concentrations were in the μM range for diolein and roughly a thousand fold less for the phorbol ester, as expected (26). As illustrated in figure 2 and Table I, the individual isozyme activities exhibited different activation ratio in the presence of DAG, the highest activation factor being observed with the B and D isoforms (4.6 to 6 fold), while the isozyme A was activated by less than two fold. When the half maximal effective dose (Ka) of activator was considered, the B and D isozymes appeared also the most sensitive to diacylglycerol (Table I). When TPA was introduced as a DAG-like activator in the protein kinase C assay, the brain D isozymic form appeared activated the most (about 6 fold). In the case of the A form activity, the very weak effect of TPA paralleled the poor activation elicited by DAG. By contrast, a striking discrepancy was observed in the case of the B and C isoforms. In both cases, activation by TPA was much less effective than that induced by DAG (Table I). With both DAG or TPA activators, the brain D isozyme and the adrenocortical enzyme were similarly maximally activated (by an average of 5 fold). Although slight differences appeared when the activation constants (Ka) of DAG and TPA were calculated for the two enzymes (Table I), these data suggested that the D isozyme from brain and the enzyme expressed in adrenocortical tissue were very similar

with regard to their response to DAG and phorbol ester activations. On the other hand, it may be noticed that, although TPA is expected to mimic the action of DAG on protein kinase C activity (26), a clear discrepancy was observed between the B and C brain isozyme response to the two activators (figure 2, Table I).

b/ Affinity of the protein kinase C isoforms for various protein substrates

Three different potent protein substrates of protein kinase C (namely histone H_1, protamine and the purified adrenocortical cholesterol side chain cleavage cytochrome P-450 (P-450_{scc}) (27) were examined as to their relative affinity for the four isolated brain isozymes as well as for the adrenocortical kinase. Various concentrations of the protein substrates were used in the kinase assay and respective affinities (Km) were calculated using Lineweaver-Burk plots. The corresponding data illustrated in Table II disclosed that, whereas all isolated enzymatic forms exhibited a very similar affinity for histone H_1, rather large differences appeared with protamine and P-450_{scc} as substrates. Although the order of decreasing affinity was P-450_{scc} histone H_1 protamine for all enzymatic forms, it may be noticed that the highest affinity for the adrenocortical cytochrome P-450 was exhibited by the protein kinase form isolated from adrenocortical tissue. These

TABLE II

Relative affinity of the four brain and the adrenocortical protein kinase C isoforms for different phosphorylatable protein substrates

PROTEIN SUBSTRATE	Km (μM)				
	RAT BRAIN PROTEIN KINASE C ISOZYMES				PROTEIN KINASE C FROM BOVINE ADRENAL CORTEX
	A	B	C	D	
Histone H_1	2.47	2.4	2	2.47	2.6
Cyt.P-450_{scc}	2.39	1.4	0.9	1.1	0.4
Protamine	34	180	20	100	18

Assays of protein kinase C were performed under standard conditions with different concentrations of each of the protein substrate : histone H_1, protamine and cytochrome P-450_{scc}. Km values were calculated from Lineweaver-Burk plots of the data.

observations suggest that the various isoforms of protein kinase C may not be equivalent as to their phosphorylation potential toward specific endogenous target(s) in a given cell type.

DISCUSSION

Development of a procedure based on the use of a high performance hydroxyapatite column allowed the resolution of an apparently homogeneous rat brain protein kinase C preparation into four isozymic forms. This confirmed at the protein level the heterogeneity of protein kinase C for which four possible different proteins were predicted on the basis of cDNA cloning studies (11-16). When these data were being obtained, a similar approach has been used by Nishizuka et al., who succeeded in the isolation of three isozymic forms of brain protein kinase C (28). The HPLC system used in the present work revealed four isozymes, most probably due to an improved resolution resulting from an optimization of the elution procedure using a curvilinear salt concentration gradient instead of a linear one. Further work is obviously required to identify the four enzymatic proteins characterized in this work with the four isozymes predicted on the basis of cDNA studies (28).

A striking point of the present work was that the same analytical procedure applied to protein kinase C from bovine adrenocortical tissue revealed that a single form of the enzyme was expressed in this tissue. The adrenocortical enzymatic protein exhibited a chromatographic behavior on hydroxyapatite identical to that of the D isoform from brain tissue. The two enzymatic activities were also similar with regard to their activation by DAG and TPA ; however, some quantitative differences appeared when their affinities for various protein substrates were compared. It remains to be examined whether the D isoform of brain protein kinase C is indeed identical to the isozyme expressed in bovine adrenal cortex or whether species difference or some post translational modification may alter in a subtle fashion the properties of a protein kinase C isozyme, as expressed in two different cell types.

One intriguing point raised by the present data concerns the lack of consistant correlation between the sensitivity of the enzyme activity toward diacylglycerol and TPA, depending upon the protein kinase C subtype. This supports previous observations suggesting that activation of protein kinase C by DAG was not functionally identical to that elicited by a tumor promoter depending upon the protein substrate involved (29). In addition, different protein kinase C isoforms exhibited different sensitivities to TPA ; it remains to be examined whether this behavior would correlate with different binding affinity for phorbol ester structures. This would be an interesting point in view of the previously observed heterogeneity in the population of cell phorbol ester receptors (6, 30).

Protein kinase C thus appears as a far more complex system than originally thought. Its ubiquitous distribution in mammalian tissues may in fact be expressed by different sets of isoenzymatic forms, specific of a given cell type. It remains to be seen whether these different forms are functionally equivalent or whether their substrate specificity and sensitivity to physiological activators may explain different functions in various tissues. In this context, it will be of special interest to examine whether the expression of a single isoform in an endocrine tissue such as adrenal cortex corresponds to specific functions of protein kinase C in the regulation of adrenocortical steroidogenic and secretory processes. This type of information may help in the elucidation of the biological significance of the protein kinase C system and its family subtypes in cell regulation processes.

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