Ca^{2+}-activated and phospholipid-dependent hepatic protein kinase (protein kinase C): A standardized method for the determination of enzyme activity

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Overview

Ca^{2+}-activated and -phospholipid-dependent protein kinase (protein kinase C), a serine/threonine protein kinase, plays a pivotal regulatory role in signal transduction, cellular regulation, tumor promotion, and differentiation.[1,2] The enzyme appears to be unbiquitous in mammalian cells, having been found in virtually every tissue examined, although the actual levels of enzyme activity vary considerably among different tissues.[3,4] It has been localized in both cystolic and particulate fractions and is known to exist in isoenzymic forms.[5-7]

Protein kinase C requires the simultaneous presence of Ca^{2+} and a negatively charged phospholipid such as phosphatidylserine for its maximal activity.[1,2] Diacylglycerol (DAG) also modulates protein kinase C activity by increasing the affinity of the enzyme for both Ca^{2+} and phospholipid, and thus allows activation of the enzyme under physiological conditions.[1,2,8,9] Physiologically, DAG is transiently generated via phospholipase C–catalyzed breakdown of polyphosphoinositides in response to various growth factors, hormones, or other stimuli.[10-12] Tumor-promoting agents (TPA) such as phorbol esters can substitute for DAG both in vivo and in vitro.[1,2,9] In this capacity protein kinase C also serves as a receptor for TPA.[13] Additionally, both DAG and TPA normally cause a translocation of protein kinase C from the cytosol to particulate fractions of the cell.[14] This process presumably represents the mechanism by which protein kinase mediates its biological action. Other agents such as unsaturated fatty acids,[15] long-chain bases, sphingosine,[16] lysophospholipids,[17] and short-chain phosphatidylcholines[18] can also modulate protein kinase C physiologically.

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Protein kinase C activity is most conveniently assayed by measuring the transfer of [^{32}P] phosphate from [γ-^{32}P] ATP to acceptor proteins such as histone. To assay protein kinase C from particulate and cytosolic fractions of tissues, the enzyme is usually extracted using a combination of detergent and metal ion chelators. It is increasingly evident, however, that the optimal conditions for the extraction and assay of protein kinase C vary with the source and method of preparation of subcellular fractions, the lipid composition of membranes, the latency of the enzyme, and the susceptibility of the enzyme to proteolytic degradation. Large errors can, therefore, easily occur in protein kinase C activity values if no attempts are made to standardize conditions for enzyme extraction, to prevent degradation and/or inactivation of the enzyme, or to measure its activity under optimum assay conditions. A notable example is the rat liver, which was initially thought to be a poor source of protein kinase C.[3,4] By using optimum assay conditions we were able to report recently that rat liver contains protein kinase C activity that is at least three- to fivefold higher than previously thought.[5] It is, therefore, advisable to evaluate critically the optimum protein kinase C assay conditions (if not known) for any given tissue under study, prior to carrying out planned biochemical/physiological studies.

The purpose of this article is to describe details of the procedure currently used in our laboratory to measure accurately and quantitatively the protein kinase C activity in crude homogenates or isolated subcellular fractions from rat liver. It can be easily adapted to measure protein kinase C activity in livers from a variety of animal models.[19]

Reagents

Use distilled and deionized water for preparations of all solutions.

Homogenization buffer (20 mM Tris HCl, pH 7.4, 2 mM EDTA, 0.5 mM EGTA, 50 mM 2-mercaptoethanol, 0.1 mM TLCK, 0.1 mM TPCK, 0.5 mM benzamidine, 5 μg/ml leupeptin, pepstatin, chymostatin, antipain, 10 μg/ml aprotinin, 0.2 mM PMSF, and 0.25 M sucrose). Add 2.42 g Tris base, 0.744 g EDTA, 0.190 g EGTA, 3.49 ml 2-mercaptoethanol, 37 mg TLCK, 35 mg TPCK, 78 mg benzamidine, 0.5 ml (1 mg/ml) each of leupeptin, pepstatin, chymostatin, antipain, 0.5 ml (2 mg/ml) aprotinin, and 85.5 g sucrose to ~800 ml of water. Adjust pH to 7.4 with 1 N HCl. Make up volume to 1000 ml. Make fresh or store refrigerated for no more than a few days. Immediately before use, add 1 μl of 200 mM PMSF (34.84 mg/ml in isopropanol) per ml of homogenization buffer. (Note that the half-life of PMSF in aqueous solution is only 30 min; it is, therefore, important to add stock PMSF to the buffer at the time of homogenization).

Extraction buffer. Extraction buffer is the same as homogenization buffer but contains 2% CHAPSO (20 mg/ml, Pierce Chemical Co.), instead of 0.25 M sucrose and the concentration of EGTA is raised to 10 mM (3.8 mg/ml).

Dilution buffer. Same as the extraction buffer but without CHAPSO and EGTA.

Column buffer. The composition of column buffer is identical to that of extraction buffer, except CHAPSO is omitted and the concentration of EGTA is kept at 0.5 mM (0.19 mg/ml).

Column elution buffer. Same as the column buffer containing 0.15 M NaCl (8.7 mg/ml).

0.5 M PIPES buffer, pH 6.8. Dissolve 15.12 g of Pipes (Research Organics Inc.) in 60 ml of water. Adjust pH to 6.8 with 1 N NaOH. Make up volume to 100 ml. Store refrigerated. The buffer is stable for at least one month.

0.4 M Magnesium acetate. Dissolve 8.576 g magnesium acetate tetrahydrate [$(CH_3\ COO)_2$ Mg. $4H_2O$; Fluka Chemical Corp.) in water for a final volume of 100 ml. Store refrigerated. The solution is stable for at least one month.

4 mg/ml Lysine rich histone. Add 10 mg lysine rich calf thymus histone (Type III-S; Sigma Chemical Co.) in 1.5 ml of water, stir to dissolve, and dilute to 2.5 ml with water. This may be kept in refrigerator for several weeks.

0.2 M 2-Mercaptoethanol. Dilute 1.4 ml of 2-mercaptoethanol (d:1.1168 g; Sigma Chemical Co.) to 100 ml with distilled water. Store refrigerated. The solution is stable for a few weeks.

0.4 M Sodium fluoride. Dissolve 1.168 g sodium fluoride (NaF; Mallinckrodt) in water for a final volume of 100 ml. Store refrigerated. The solution is stable for at least one month.

7.5 mM Calcium chloride. Dissolve 110.3 mg calcium chloride ($CaCl_2 \cdot 2H_2O$, E.M. Science) in 100 ml of water. Store refrigerated. The solution is stable for at least 1 month.

10 mM ATP. Dissolve 30 mg of ATP (disodium salt, Sigma Chemical Co.) in ~4 ml of water. Adjust to about neutral pH range (pH 6.5 to 7.5) with 1 N NaOH (use indicator paper). Read absorbance of suitably dilute solution (e.g., 0.02 ml to 20 ml) at 259 nm. (Note: a quartz cuvette must be used to measure ATP absorbancy). The concentration of ATP (in mM) is then computed by the formula $A_{259} \times 1000/15.4$. Adjust entire stock solution of ATP to 10 mM (A_{259} for 10 mM ATP = 154). This may be kept frozen at −20° C in small aliquots (0.2 to 0.3 ml). Under these conditions APT is stable for at least several months.

1 mM ATP. Dilute one volume of stock ATP (10 mM) with nine volumes of water. This solution should be prepared fresh each day of use and remains stable for at least 24 h at 4° C.

Mixture I (for 100 assays). Add the following solutions while the tube is on ice: 0.50 ml of 0.5 M Pipes buffer, pH 6.8; 0.25 ml of 0.4 M magnesium acetate; 2.00 ml of 4 mg/ml histone; 0.25 ml of 0.2 M 2-mercaptoethanol and 1.50 ml water. Mix well and store on ice.

Mixture II (for 100 assays). Dry 250 µl (2.5 mg) of 10 mg/ml phosphatidylserine in chloroform (from beef brain, Serdary Research Laboratories, Inc.) plus 40 µl (100 µg) of 2.5 mg/ml dioleoyl glycerol in chloroform under a stream of N_2 at 40° C. Remove traces of solvent under vacuum drying. Add 1 ml of 10 mM Pipes pH 6.8 (dilute 0.02 ml of 0.5 M Pipes pH 6.8 to 1.00 ml with water) and sonicate the tube in a bath type sonicator for 5 min under N_2. Flush the tube with fresh N_2, cap and store at room temperature. Prepare fresh daily.

1 mM [γ-^{32}P] ATP (50 µCi/ml). Vacuum-dry (or dry under N_2) 50 µCi of [γ-^{32}P] adenosine 5-triphosphate tetra (triethylammonium) salt (30 Ci; 11.1 TBq; NEN, DuPont Co.) in a siliconized 13 × 100 mm glass tube. Add 1.0 ml of 1 mM unlabeled ATP, vortex briefly, store well shielded at 4° C until used. This reagent should be freshly prepared on the day of use.

Mixture III (for 100 assays). Mix 1 ml of 1 mM [γ-^{32}P] ATP with 0.5 ml of 0.4 M sodium fluoride (NaF), vortex, and store well shielded at room temperature. Use immediately. To determine specific activity, dilute 15 µl of mixture III to 1.5 ml of water; count 15 µl for radioactivity determination. Specific activity = DPM × 100/10,000 pmol ATP = DPM/pmol ATP [Note: the stock [γ-^{32}P] obtained from supplier contains a negligible amount of ATP mass which is not taken into consideration when calculating the specific activity of [γ-^{32}P] ATP.]

2.5 mM EGTA. Add 95 mg of EGTA (Gallard-Schlesinger Industries, Inc.) in 80 ml of water. Adjust pH to 7.0 with 0.1 N NaOH. Make up volume to 100 ml. Store refrigerated. The solution is stable for at least one month.

75 mM Phosphoric acid. Dilute 20 ml of phosphoric acid (85%, Fisher Scientific) to 4000 ml with water.

Acetone (J.T. Baker Chemical Co., Phillipsburg, NJ).

Ion-exchange paper. Phosphocellulose paper (Whatman P81). Cut square pieces of ~2 cm^2.

Scintillation fluid. Dissolve 10 g Omnifluor (NEN, DuPont Co.) in 2.5 liter of toluene. Store in dark bottles. A solution of 0.45 g 2, 5-diphenyloxa-

zole and 0.2 g 2, 2′-p-phenylene bis-(5-phenyloxazole) in 2.5 liter toluene can be substituted for Omnifluor based scintillation fluid.

Procedure

Preparation of subcellular fractions of rat liver. Sprague-Dawley male rats (200–200 g) are killed by decapitation. The livers are rapidly excised and perfused with phosphate-buffered saline, weighed, cut into small pieces, and chilled in homogenization buffer. All further operations are carried out at 0–5° C. Twenty to 30 g of pooled liver tissue is then homogenized three times in homogenization buffer (four volumes) using a Potter-Elvehjem type homogenizer with a loose fitting Teflon pestle rotating at 1300 rpm for 20 second at 4° C. Subcellular fractionation of homogenate is carried out by differential centrifugation as described previously.[5,19]

The nuclear (N) fraction (10,000 g-min.), the mitochondrial (M) fraction (66,000 g-min.) and the light mitochondrial-lysosomal (L) fraction (25,000 g-min) are obtained using a JA-20 fixed angle rotor and Beckman JS-21 refrigerated centrifuge (Beckman Instruments). Separation between the microsomal (P) and the cytosol is achieved by centrifugation at (4×10^6 g/min.) using a 60 Ti (or Type 50.1 Ti) rotor and a Beckman L8-M ultracentrifuge. All sedimented fractions are washed an additional two times in Tris-based homogenization buffer and finally resuspended to a desired volume in the same buffer. All these fractions are processed and assayed immediately for protein kinase C or "marker enzymes." [Note: if desired, highly purified liver fractions (e.g., plasma membranes, Golgi, endoplasmic reticulum, nuclei, mitochondria, etc.) can be used. For additional details, readers may consult earlier publications—refs. 20,21].

Solubilization of particulate protein kinase C. One of 2 ml aliquots of N, M, L or P fractions (4 mg protein/ml) are mixed with an equal volume of extraction buffer and sonicated (i.e., 15 seconds × 3 using a bath type sonicator at 4° C). After incubation at 4° C for 60 min, the entire suspension is diluted fivefold with dilution buffer, centrifuged at 105,000*g* for 60 min and the supernatant is saved. Twenty μl aliquots in duplicate are used for protein kinase C assays. However, to reduce the contribution by other kinases (i.e., non-protein kinase C type enzymes), all membrane and cytosolic extracts should be subjected to DEAE-cellulose column chromatography as described below. Such treatment also results in partial purification and enrichment of protein kinase C.

DEAE Cellulose column chromatography. The detergent extracted particulate fractions (supernatant fractions) or cytosolic fractions (1 ml) are applied to small DEAE-cellulose columns (DE 52, Whatman, 0.9 × 2 cm) previously equilibrated with column buffer. The column is washed with the same buffer (~20–25 ml) until the effluent has an absorbancy of 0.01–0.02 at 380 nM. The protein kinase C activity is then eluted from the column with column elution buffer. Twenty fractions of one ml each are collected. Each fraction is immediately assayed for enzyme activity. Most of the protein kinase C activity elutes between the fractions 2 and 4. Average recovery of protein kinase C is in the range of 80–85%.

Assay method. Rat liver protein kinase C activity is assayed by measuring the transfer of [^{32}P] phosphate from [γ-^{32}P] ATP to acceptor protein such as lysine rich histone (ATP + histone→phosphohistone + ADP). Unreacted ATP and its metabolite (ADP) are removed by adsorption of [^{32}P] phosphohistone on phosphocellulose strips under acidic conditions, followed by extensive washing.

Unless otherwise noted, all operations are carried out at 30° C with solutions that have been equilibrated to 30° C.

1. Add 45 μl mixture I to a series of disposable 12 × 75 mm glass test tubes.
2. To measure total protein kinase C activity add 10 μl mixture II plus 10 μl of 7.5 mM $CaCl_2$. Likewise, to measure basal activity add 20 μl of 2.5 mM EGTA.

3. Add 20 μl of the appropriate buffer (blank) or enzyme (sample) solution into the tubes, vortex and incubate reaction mixtures at 30° C for 2 min.
4. Initiate protein kinase C assays by adding 15 μl of mixture III to the reaction tubes.
5. After incubation for 5 min at 30° C, 50 μl aliquots are spotted onto 2.0 cm^2 pieces of Whatman P81 phosphocellulose paper (number with no. 2 lead pencil). Fifteen to 20 seconds later the strips are dropped into a wire basket positioned in an apparatus containing 75 mM phosphoric acid. This apparatus (FBN Scientific) is designed to continuously remove the unreacted ATP. Normally two such pieces of equipment are used in tandem. This allows a major (~98%) portion of the unreacted ATP to be released into the phosphoric acid solution of the first apparatus during the first 15–30 min following termination of the assay, after which the wire basket and papers are transferred to the second apparatus to rinse overnight.
6. A third phosphoric acid wash is carried out, followed by a 5 min wash in acetone to extract water.
7. The filters are then dried, transferred to 7 ml scintillation vials (containing 6 ml scintillation fluid) and counted for [^{32}P] radioactivity in a scintillation spectrometer (e.g., Beckman Model LS-3801).

Calculation

Protein kinase C activity (expressed as pmol of [^{32}P] phosphate transferred from [γ-^{32}P] to histone per minute per mg protein) is computed from the following formula:

$$\frac{(DPM_{Sample}\text{-}DPM_{Blank}) \times 2}{Sa \times \Delta t \times \text{mg protein in sample}}$$

where Sa = Specific activity of [γ-^{32}P] ATP in DPM per pmol and Δt = Incubation time (e.g., 5 min).

Protein kinase C activity is determined by subtracting the amount of [^{32}P] incorporation into histone (in the presence of 0.5 mM EGTA) from the amount of [^{32}P] incorporation noted in the presence of $CaCl_2$, phosphatidylserine, and diolein.

Discussion

This report represents a quantitative, reproducible, and accurate assay for measuring hepatic protein kinase C activity in either crude homogenates or partially purified, subcellular fractions of rat liver. The method takes into consideration several factors that profoundly affect the activity of protein kinase C: these include (1) the particulate nature of the enzyme, (2) the existence of the latent form of the enzyme, and (3) the extreme susceptibility of the enzyme to proteolytic degradation. Specific assay conditions have been designed to take these issues into consideration.

In rodent liver, the majority (80–85%) of protein kinase C is associated with the particulate fraction. Interestingly, most of this activity is latent and can be visualized only after disruption of the architecture of hepatic membranes through the use of a detergent. Although a variety of non-ionic detergents can solubilize and unmask particulate protein kinase C, the best results are obtained when CHAPSO is used in a final concentration of 1% (ref. 5). This concentration of CHAPSO along with metal chelators results in three to fourfold stimulation and 80–85% solubilization of protein kinase C activity.

In extracting protein kinase C from subcellular fractions, a major concern is the degradation of the enzyme during sample preparation. Degradation would result in an overall decrease in activity. The degradation of protein kinase C that can result from exposure to endogenous proteases is circumvented by including a variety of proteolytic inhibitors and Ca^{2+} chelators in the homogenization buffer. Under these conditions, protein

kinase C is quite stable in crude extracts; nevertheless, my recommendation is to perform the enzyme assay as quickly as possible.

Hepatic protein kinase C can be assayed over a pH range of 6.6 to 7.2, but pH 6.8 is preferred. Also, relatively high concentrations of phosphatidylserine, Ca^{2+}, and diolein are required for the hepatic enzyme. All assays are carried out under optimum conditions of time and enzyme concentration and in the presence of saturating concentrations of substrate (ATP and histone) and activators (Ca^{2+}, phosphatidylserine, and diolein). In order to minimize the action of ATPases, which are normally present in high concentration in hepatic subcellular fractions, saturating concentrations of ATP are utilized, and assays are performed in the presence of NaF (inhibitor of ATPase).

In our experience a variety of histone substrates can be used with good results. Maximum and identical results are obtained with lysine-rich histone (Sigma type IIIs) or histone H_1 (Boehringer Mannheim), although type IIIs is much more economical to use. Protamine, another commonly used phosphate acceptor substrate, is unacceptable because Ca^{2+} and phosphatidylserine-stimulated enzyme activity is not detectable with this substrate.

One disadvantage of using histone as a phosphate acceptor for protein kinase C is that histones are good substrates for other protein kinases as well, and these are normally present in large amounts in crude liver extracts. Thus, in some instances, investigators may encounter a high basal enzyme activity (measured in the presence of EGTA) due to a combined action of Ca^{2+} phospholipid-independent protein kinase C and non-protein kinase C type protein kinases. The problem of high basal activity can be overcome by (1) partially purifying the enzyme through DEAE cellulose column chromatography or (2) by using a synthetic peptide substrate. The DEAE cellulose column chromatography effectively separates protein kinase C from the majority of other protein kinases, and this results in lower basal values. One drawback in using column chromatography is that it requires relatively high concentrations of starting material, but fortunately this is not a limiting factor when dealing with liver subcellular fractions.

On the other hand, synthetic peptide substrates (e.g., glycogen synthase peptide) have been demonstrated to be highly specific substrates for protein kinase C (ref. 22) and, as expected, very little basal hepatic protein kinase C activity is observed. In addition, peptide substrates have the added benefit of showing little or no background activity observed in protein kinase C assays.[22] However, the cost of these substrates is high when measuring a large number of samples.

In conclusion, this report presents a standardized technique for the measurement of hepatic protein kinase C in crude homogenates or isolated subcellular fractions of rat liver. The method is easily adaptable to liver of other animals, and, with a few modifications, the method can also be employed to measure protein kinase C activity in non-hepatic tissues.

Abbreviations

CHAPSO	3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate
EDTA	ethylenediaminetetra acetic acid
EGTA	ethyleneglycolbis (amino ethyl ether) tetra acetic acid or 1,2 di (2-aminoethoxy) ethane NNN′N′ tetra acetic acid
PIPES	piperazine N,N′-bis (2-ethane sulfonic acid)
TLCK	N^{α}-tosyl-L-lysine chloromethyl ketone
TPCK	N^{6}-tosyl-L-phenylalanine chloromethyl ketone

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