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A NOVEL METHOD FOR MEASURING PROTEIN KINASE C ACTIVITY IN A NATIVE MEMBRANE-ASSOCIATED STATE

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SUMMARY: Physiological activation of protein kinase C (PKC) is believed to occur by redistributing soluble enzyme to the phospholipid environment of membranes. Currently available *in vitro* methods of measuring PKC activation all involve prior extraction of membrane-associated enzyme and its reconstitution in an artificial phospholipid environment or modification (such as partial trypsinization) of the enzyme itself. Here we report a novel method which, for the first time, allows measurement of active PKC still in its native, membrane-associated state using a specific, physiological substrate. Thus, with this new method PKC activity can be measured while still in an environment that approximates the *in vivo* situation.

Protein kinase C (PKC), a family of Ca^{2+}/phospholipid dependent phosphotransferases, associates with cell membranes where it is activated and appears to be involved in stimulating a wide variety of cellular processes including tumor promotion (1). Three ways of measuring PKC activity *in vitro* have been devised. In the first method, the enzyme(s) is extracted from membranes with detergents, partially purified, reconstituted with phosphatidylserine, and its activity measured with a histone substrate, histone IIIS (2,3). In the second method, the enzyme(s) is also extracted with detergents, but the extracted enzyme activity is measured directly without partial purification using a modified histone H1 which is believed to be a specific PKC substrate (4). In the third method, the cells are permeabilized with digitonin to release soluble PKC, both the soluble and residual membrane-associated enzyme are partially trypsinized, and their activities assayed using histone H1 substrate in the presence of a cyclic AMP-dependent protein kinase inhibitor (5). Clearly lacking is a way of measuring PKC activity directly in its native membrane-associated state without prior extraction. In this communication, we describe the development of a simple and universally applicable method of directly measuring PKC activity *in vitro* while it is still associated with its native membrane, using a murine lymphoma cell extract containing a PKC-specific 85kDa protein substrate.

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METHODS

Cell Culture: Murine *cyc*⁻S49T-lymphoma cells were grown in suspension culture in RPMI medium supplemented with 10% horse serum. Cultures were seeded routinely at a density of 2-4x10^5 cells/ml and grown for 3 days in T-75 flasks at 36°C in a humidified atmosphere of 95% air and 5% CO_2. The cells were harvested by centrifugation at 600xg. Stock cultures of NRK, BALB/3T3, and T51B rat liver cells were grown in DME medium containing 10% bovine calf serum. MRC-5 human diploid fibroblasts were cultured in DME medium containing 10% fetal bovine serum.

Cell lysis: Lymphoma cells (4x10^7) in suspension culture were washed with ice-cold phosphate-buffered saline (PBS) and lysed by vortexing for 2 min at room temperature in 1.2 ml of an ice-cold hypotonic medium containing 1 mM $NaHCO_3$, 5 mM $MgCl_2$, and 100 μM phenylmethyl sulfonyl fluoride (PMSF), pH 7.5. 100 μl ice-cold Tris-HCl buffer (500 mM, pH7.5) was added to the cell lysate to 50 mM, and the nuclei removed by centrifugation at 500xg for 5 min at 4°C.

Cell monolayers were washed twice with PBS and incubated on ice in 1 ml of hypotonic lysis medium for 5 min. The swollen cells were scraped from the dish and lysed by vortexing at room temperature for 2 min. A post-nuclear fraction was prepared by centrifuging the cell lysate at 500xg for 5 min at 4°C.

Protein kinase C activity in the post-nuclear fraction (PNF): 100 μl of reaction buffer was added to 1 ml of PNF to give a final concentration of 1 mM $NaHCO_3$, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), 10 μM $CaCl_2$, 100 μM sodium vanadate, 100 μM sodium pyrophosphate,1 mM sodium fluoride, and 100 μM PMSF. 100 μl of this PNF (100-150 μg protein) was incubated with the indicated additions (see Fig.1) plus [^{32}P]-ATP (10 μM,4000 cpm/ pmol) at 37°C for 10 min in a final reaction volume of 115 μl. The reaction was stopped by adding EGTA to a concentration of 1 mM, and membranes were rapidly sedimented by centrifugation at 280,000xg for 5 min at 4°C in a TL-100 ultracentrifuge (Beckman Instruments Inc.). Proteins in the high-speed supernatant were separated on a 10% polyacrylamide gel(6). Gels were dried and autoradiographed on Kodak SB X-ray film for 48 hours.

Protein kinase C activity in isolated membranes: The post-nuclear supernatant from S49T-lymphoma cells or their *cyc*⁻ variants (4x10^7 cells, 1.3 ml) was centrifuged at 280,000xg for 10 min at 4°C in a TL-100 ultracentrifuge. The supernatant was heated in a boiling water bath for 5 min, cooled to room temperature for 30 min, and the precipitated proteins were removed by centrifugation at 16,000xg in a microfuge. The supernatant, termed heat-treated cytosol (HT-cytosol), was used as a source of the 85 kDa protein substrate for PKC assays. HT-cytosol could be lyophilized and then stored at -80°C.

To assay membrane-associated PKC activity the test cells were hypotonically lysed and the PNS centrifuged at 280,000xg for 10 min at 4°C in a TL-100 ultracentrifuge. The resulting membrane pellet was washed twice with 0.5 ml of lysis buffer and suspended in 50 μl of assay buffer consisting of 1 mM $NaHCO_3$, 50 mM Tris-HCl(pH 7.5), 5 mM $MgCl_2$, 200 μM sodium vanadate, 200 μM sodium pyrophosphate, 2 mM sodium fluoride, 200 μM PMSF, and 2 μM $CaCl_2$. The reaction was started by adding 50 μl (5 μg protein) of the lymphoma cell HT-cytosol containing (^{32}P)-ATP (20 μM, 4000 cpm/ pmol). Following incubation for 10 min at 37°C, the reaction was stopped by adding EGTA to a final concentration of 1 mM. Membranes were removed by centrifugation at 280,000xg for 5 min, and the supernatant proteins were separated on a 10% polyacrylamide gel. The gels were dried and autoradiographed on Kodak SB5 X-ray film for 48 hours.
Protein contents were measured according to Bradford (7).

RESULTS AND DISCUSSION

Phorbol esters, such as 12-O-tetradecanoyl phorbol-13-acetate (TPA), are potent PKC stimulators which promote the redistribution of inactive enzyme from the cytosol to membranes where it is activated by phospholipids such as phosphatidylserine (1). It has been demonstrated, using these phorbol esters, that certain endogenous proteins, such as a 47 kDa in platelets (9) and 80-87 kDa proteins in brain and other cells (10-12) are specific cellular substrates for PKC.

Initially, we found that S49T-lymphoma cells and their *cyc*$^-$ variants also had a PKC-specific 85 kDa protein substrate. If this 85 kDa protein were to be used as the substrate in an *in vitro* assay for membrane-associated PKC activity, it should be phosphorylatable in a cell-free system in response to PKC stimulators. Indeed, adding an active phorbol ester such as TPA or phorbol dibutyrate to the post nuclear fraction (PNF) of S49T-lymphoma cells or their *cyc*$^-$ variants greatly stimulated the phosphorylation of this cytosolic 85kDa protein (Fig.1). In fact, this was the only protein visibly phosphorylated in response to the phorbol esters. Its phosphorylation was also stimulated by other PKC activators such as diolein and 1-oleyol-2 acetyl glycerol, and, most importantly, by purified PKC (data not shown). Phorbol ester-induced phosphorylation of this protein was suppressed by H-7 (Fig.1), a known PKC inhibitor (13), and by the Ca^{2+}-chelating EGTA (1 mM) (data not shown). Also, the phosphorylation of the 85 kDa protein was not stimulated by either calcicalmodulin or cyclic AMP (Fig.1). The 85 kDa phosphoprotein was not autophosphorylated PKC because two-dimensional electrophoresis revealed that its pI was far more acidic than that of PKC (data not shown). Finally, this protein was heat-stable and able to serve as a PKC substrate after being incubated at 100°C for 5 min.

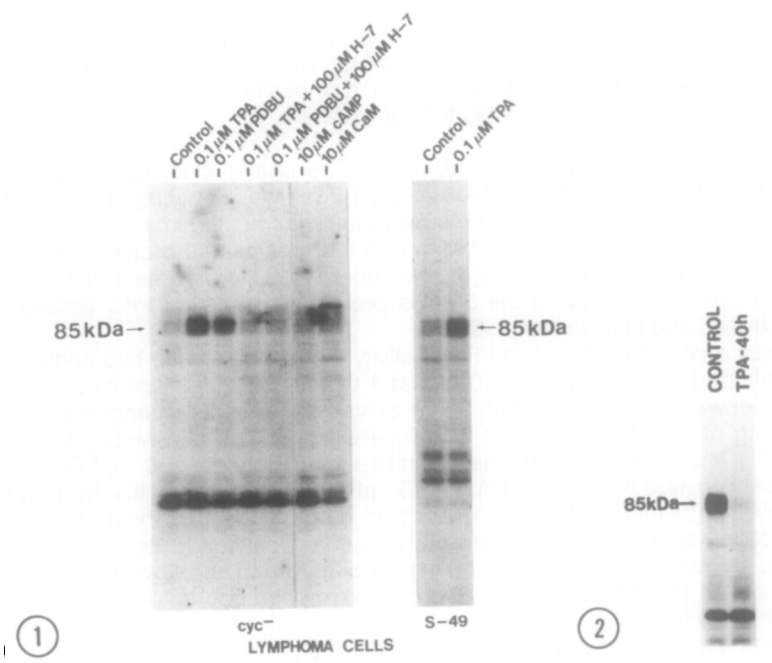

FIG.1: PHORBOL ESTERS SPECIFICALLY STIMULATE THE PHOSPHORYLATION OF A 85KDA PROTEIN IN LYMPHOMA CELL LYSATE: S49 and *cyc*$^-$S49 T-lymphoma cells were washed once with phosphate-buffered saline and lysed as described in METHODS. Membrane-associated PKC activity in the post nuclear fraction (130 μg) was measured as described in the METHODS. Phorbol esters were dissolved in dimethyl sulfoxide(Me_2SO). Controls received the same amount (0.5%) of Me_2SO.

FIG.2: EFFECT OF PKC DOWN-REGULATION ON 85 KDA PHOSPHORYLATION: NRK rat kidney cells ($5x10^5$ cells/ 100 mm dish) were grown to confluence in 90% DME and 10% calf serum and rendered quiescent by incubation in serum-free DME medium for 48 h. Cells were treated either with 0.1% Me_2SO (control) or 300 nM TPA in 0.1% Me_2SO for 40 h. Both control and TPA-treated cells were then challenged with 1 μM TPA for 15 min. before the cells were harvested and membrane-PKC activity was measured as described in METHODS.

Prolonged treatment with TPA induces cells to selectively degrade (down-regulate) PKC (14-16). Therefore, if the 85 kDa protein from S49T-lymphoma cells is a specific substrate for PKC, then membranes prepared from PKC-depleted, TPA-challenged cells should not phosphorylate the 85 kDa protein in lymphoma cell PNF. This was shown to be case using membranes from PKC-depleted, TPA-challenged NRK rat kidney cells (Fig.2). Therefore, the heat-stable, 85 kDa cytosolic protein is clearly a specific physiological substrate for PKC.

While TPA dramatically stimulated the 85 kDa protein phosphorylation in lymphoma cell PNFs (Figs. 1 , 3A), it did not detectably increase the phosphorylation of this protein in the PNFs of several other cells (Fig.3A). But TPA had stimulated membrane-associated PKC activity in these other PNFs, because membranes from these preparations strikingly phosphorylated the 85 kDa substrate in lymphoma cell extracts (Fig.3B). Therefore, the 85 kDa protein from *cyc*⁻S49T-lymphoma cells was a specific and highly sensitive substrate for measuring *in situ* PKC activity in membranes from other cells.

Ca^{2+} is known to modulate the membrane association and activation of PKC (1,8,13,17,18). Changes in the membrane-PKC activity measured by this assay were not due to subtle variations in the Ca^{2+} concentration of the membrane-isolation buffer, because even Ca^{2+} concentrations as high as 10 μM did not change the basal membrane-associated PKC activity (data not shown). .

The ability of this method to detect responses of membrane-associated PKC activity to more physiological agents was tested with two mitogens, bombesin and EGF, which are known

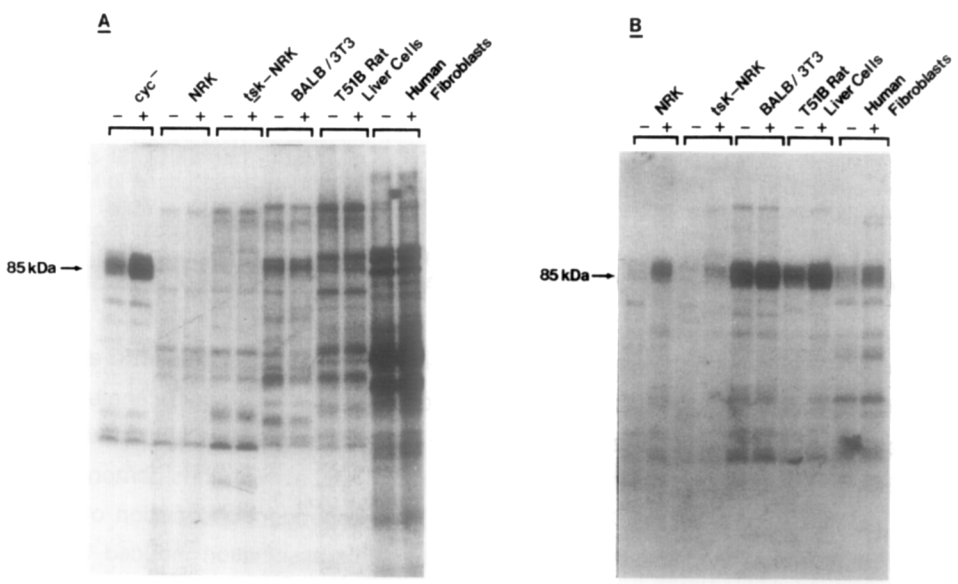

FIG.3: PHOSPHORYLATION OF THE 85 KDA PROTEIN IN DIFFERENT CELL TYPES: **A**.The cells were grown to confluence and rendered quiescent as in Fig.2. Whole post-nuclear fractions from various cell types were prepared as described in METHODS. The PNFs (100 μl: 100-150 μg protein) were incubated for 15 min. with (+) or without (-) 0.1 uM TPA and then the phosphorylation of 85 kDa protein measured as described in METHODS. **B**.Post-nuclear fractions were first treated with either Me_2SO (-) or 0.1 μM TPA (+) for 10 min, membranes isolated and incubated with heat-treated cytosol from *cyc*⁻S49T-cells, and 85 kDa protein phosphorylation determined as described in METHODS.

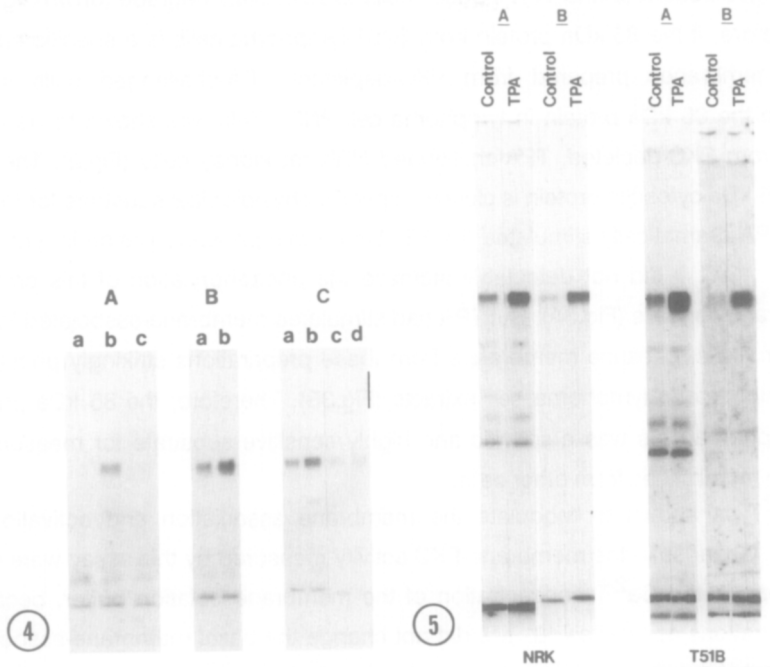

FIG.4: STIMULATION OF MEMBRANE-PKC ACTIVITY BY GROWTH FACTORS: Bombesin (**A**) or EGF (**B**) was added to quiescent NRK cultures for 10 min. at 37°C and membranes then prepared and assayed for PKC activity using heat-treated *cyc*-S49T-cell cytosol as described in METHODS. **A.** (*a*) and (*c*) No addition, (*b*) 1 μM bombesin. **B.** (*a*) No addition, (*b*) 10 ng/ mL EGF. **C.** Membranes from unstimulated NRK cells (*a,c*) or bombesin-stimulated NRK cells (*b,d*) were treated with either Tris buffer alone (*a,b*) or with EGTA in Tris buffer (*c,d*) as described in the legend for Fig.5, and PKC activity was measured as described in METHODS.

FIG.5: EFFECT OF EGTA ON TPA-STIMULATED MEMBRANE-PKC ACTIVITY: Quiescent NRK cells were treated with either 0.1% DMSO (control) or 0.1 μM TPA for 10 min. and membranes prepared as described in METHODS. One-half of the membrane fraction was suspended in (**A**) 500 μl Tris-HCl buffer (pH7.5), and the other half (**B**) was suspended in Tris buffer containing 2 mM EDTA and 10 mM EGTA and vortexed for 1 min. Membranes were pelleted and washed three times with a solution containing 1 mM $NaHCO_3$, 5 mM $MgCl_2$, 100 μM PMSF and 50 mM Tris buffer (pH7.5). The membranes were assayed for PKC activity as described in METHODS. Autoradiograms were scanned using a Bio-Rad video densitometer (Model 200) and incorporation of ^{32}P into the 85 kDa protein was measured from the peak area. In control cells EGTA treatment reduced the basal level of membrane-PKC activity by about 75% whereas it only marginally reduced (15-20%) the TPA-stimulated PKC activity.

to stimulate PKC (10,11). Indeed, the stimulation of membrane-associated PKC activity by these two agents was readily demonstrable with this method (Figs.4A,lane b; 4B,lane b). Interestingly, extracting the bombesin-treated (Fig.4C, lane d) or EGF-treated (data not shown) membranes with EGTA greatly reduced the membrane-associated PKC activity, thus demonstrating for the first time that these mitogens induce a loose, Ca^{2+}-dependent association of PKC with cell membranes rather than the tight, detergent-extractable association induced by TPA (Fig.5; ref.1). Thus, this novel method allows the direct measurement of PKC activity in its native membrane-associated form and can provide new insights into the enzyme's interaction with cell membranes in response to various stimulators.

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