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Ca^{2+}/PHOSPHOLIPID-DEPENDENT PROTEIN KINASE ACTIVITY CORRELATES TO THE ABILITY OF TRANSFORMED LIVER CELLS TO PROLIFERATE IN Ca^{2+}-DEFICIENT MEDIUM

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Extracellular Ca^{2+}-deprivation inhibited the proliferation of normal T51B rat liver cells and reduced (2-4 fold) the amount of EDTA/EGTA-extractable protein kinase C activity. By contrast, preneoplastic and neoplastic T51B rat liver cells were able to proliferate in Ca^{2+}-deficient medium and retained all of their extractable protein kinase C activity.

Many preneoplastic (i.e., not tumorigenic but expressing some neoplastic characteristics) and all neoplastic cells of epithelial and mesenchymal origin tested to date are able to proliferate in Ca^{2+}-deficient media that do not support the proliferation of their non-neoplastic counterparts (1,2). A key to understanding this common characteristic of neoplastic cells is the ability of the tumor promoter TPA (12-O-tetradecanoylphorbol-13-acetate) to bypass Ca^{2+} and enable non-neoplastic cells to proliferate in Ca^{2+}-deficient medium like many of their preneoplastic and all of their neoplastic derivatives (3-7). Thus, this characteristic might be related to the activity of the Ca^{2+}/phospholipid-dependent protein kinase, protein kinase C (8), because this enzyme appears to be linked to the TPA receptor and TPA can bypass Ca^{2+} and directly activate this normally Ca^{2+}-dependent enzyme even in the absence of Ca^{2+} (8-11).

In this communication we provide the first evidence of a relation between protein kinase C activity and the ability of preneoplastic and neoplastic cells to proliferate in Ca^{2+}-deficient medium.

MATERIALS AND METHODS

T51B rat liver cells were cultured as described previously (6). The cell density in T51B cultures was determined by suspending the cells and counting

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them with a hemacytometer. Colony formation was determined as described previously (1,2).

A preneoplastic (i.e., non-tumorigenic but having some neoplastic characteristics) clone, T51B-261A, originated spontaneously and a neoplastic clone, T51B-261B originated from a colony appearing in Ca^{2+}-deficient medium after exposure of T51B cells to aflatoxin B_1 (AFB_1; 2.0 µg/ml; [2]). Tumorigenicity of the cells of these clones was determined by injecting 5 x 10^6 cells subcutaneously into the dorsa of 3-10 weanling athymic nude BALB/c (nu/nu) mice. Cells which did not produce tumors in 6 months were designated non-tumorigenic.

DNA-synthetic activity was determined autoradiographically and by flow cytofluorometry. The autoradiographic procedure was that of Boynton and Whitfield (12). Cells were prepared for flow cytometry by first suspending them in 1 ml of 0.05% trypsin at 37°C. This suspension was cooled for 10 minutes in an ice-bath and if cells were clumped they were gently syringed 5 times through a 21-gauge hypodermic needle. The cells were pelleted by centrifugation at 600 xg in a Beckman RC-2 centrifuge and resuspended in 1 ml of ice cold GME solution (1.1 g glucose, 8.0 g NaCl, 0.4 g KCl, 0.39 g $Na_2HPO_4.12H_2O$, 0.15 g KH_2PO_4, 0.093 g EGTA, and 1 liter of distilled water; pH 7.0). If the cells were clumped at this stage, they were again gently syringed through a 21-gauge needle. The cells were then fixed by adding 3 ml of 95% ethanol to the 1 ml suspension. Before staining the DNA, the cells were pelleted at 600 xg and resuspended to a final concentration of 0.5 to 1.0 x 10^6/ml in 1 ml of GME solution. RNA was removed and the DNA stained by adding 0.2 ml of a 0.1% aqueous solution of ribonuclease (from Sigma Chemical Co.) and 50 µl of a 0.1% aqueous solution of propidium iodide and incubating the cells in this mixture for 30 minutes at 37°C with frequent and vigorous vortexing. The relative DNA content of the stained cells was measured with a model 50H cytofluorograph from Ortho Diagnostic Systems Inc. (Westwood, MA) equipped with an agron-ion laser tuned to an excitation wavelength of 488 nm. Red fluorescence (the indicator of relative DNA content) was measured and the data were stored in an Ortho Diagnostic System 2150 computer. The laser power and the photomultiplier gain were the same for all samples.

To determine protein kinase C activity, 600,000 cells were plated in each of several 100 mm Petri dishes containing 10 ml of a complete medium consisting of 90% (v/v) BME (Eagle's basal medium with Earle's salts mixture; GIBCO [Grand Island, NY]) and 10% (v/v) BCS (bovine calf serum from Colorado Serum Co., Denver, CO) which contained the antibiotic Gentamicin (25 µg/ml; Microbiological Associates, Bethesda, MD). After 24 hours of incubation in this high-calcium (1.8 mM Ca^{2+}) medium at 37°C in an atmosphere of 95% air/5% CO_2, the cultures were either left in the original medium, or the plating medium was discarded and replaced with Ca^{2+}-deficient BME-BCS medium containing only 0.025 mM Ca^{2+}. The Ca^{2+}-deficient medium was prepared by mixing low-calcium BME (from GIBCO) with BCS, the Ca^{2+} content of which was reduced by chelation with EGTA (ethylene-glycol-bis-(β-aminoethyl ether)-N,N'-tetraacetic acid) according to Borle and Briggs (13).

Twenty-four hours later (48 hours after the initial plating), the cells from eight Petri dishes were removed and suspended in ice-cold buffer consisting of 2 mM EDTA, 2 mM EGTA, 50 mM 2-mercaptoethanol, 2 mM phenylmethyl sulfonyl fluoride and 20 mM TRIS-HCL (pH 7.5). They were then homogenized using 15 up-down strokes with a Potter-Elvehjem homogenizer and the homogenate was centrifuged at 105,000 xg for 1 hour at 4°C. Protein kinase C activity was determined either in the crude 105,000 xg supernatant or after partial purification on 0.6 x 8.0 cm DEAE-52 cellulose columns which were previously equilibrated with EDTA/EGTA buffer. Protein kinase C was eluted from the columns with a 10 ml linear gradient (0-250 mM) and 1 ml fractions collected. A 40 µl sample of each fraction was assayed for protein kinase C activity.

Total cellular protein kinase C activity was determined by solubilizing and extracting whole cells in EDTA/EGTA buffer containing 1% NP-40 for 1 hour at 4°C. The solubilized extract was centrifuged at 105,000 xg for 1 hour at 4°C and the protein kinase C in the supernatant was partially purified on DEAE-52 cellulose columns as described above. Protein kinase C activity in a 40 µl sample of each fraction was determined and the activities in these fractions were added together to obtain total enzyme activity.

Protein kinase C activity was assayed by adding 40 µl of either the crude extract or the partially purified enzyme preparation to an assay mixture (final volume 270 µl) containing 5 µmoles TRIS-HCL (pH 7.5), 1.25 µmoles $MgCl_2$, 50 µg histone H1 substrate, 2.5 nmoles [γ-^{32}P]-ATP ($1.0-1.5 \times 10^5$ cpm/nmole), 1.0 µg 1,2-diolein, and 1.5 mm $CaCl_2$. The reaction was allowed to proceed in the presence or absence of 10 µg phosphatidylserine (15) for 10 minutes at 30°C and was stopped by adding 2.0 ml of 20% trichloroacetic acid (TCA) and 50 µg of bovine serum albumin. The TCA-precipitable material containing phosphorylated substrate was pelleted by centrifugation. The pellet was dissolved in 1.0 N NaOH, re-precipitated with 20% TCA, centrifuged again, and the final pellet was resuspended in 1N NaOH and added to 1 ml of water and 10 ml of liquid scintillation cocktail 968 (New England Nuclear Corp., Boston, MA). The radioactivity from the phosphorylated histone H1 substrate was measured with a Beckman LS255 liquid scintillation counter.

Protein kinase C activity was the difference between the picomoles of ^{32}P incorporated into histone H1 per mg of protein during the 10-minute reaction period in the presence and absence of phosphatidylserine (15). Protein contents of the 105,000 xg supernatants were determined by the method of Lowry et al. (14).

RESULTS

Non-neoplastic T51B rat liver cells cannot proliferate in Ca^{2+}-deficient BME-BCS medium, but they remain viable and capable of proliferating again if enough Ca^{2+} be added to the medium (1). Thus, during the first 24 hours after the normal BME-BCS medium containing 1.8 mM Ca^{2+} was replaced with BME-BCS medium containing only 0.025 mM Ca^{2+}, the proportion of cells making DNA (assessed by autoradiography and flow cytolfuorometry) dropped from about 42% to about 8% while the proportion of G1 cells rose from about 55% to 84%, and there was little or no increase in cell number during the next 5 days (Fig. 1). Furthermore, most of the cells in a culture of normal T51B cells could not form colonies in Ca^{2+}-deficient medium (Table 1). By contrast, the preneoplastic and neoplastic derivatives of T51B cells were able to proliferate indefinitely and form colonies in Ca^{2+}-deficient BME-BCS medium (Fig. 1; Table 1).

Protein kinase C was extracted from homogenates of T51B cells by an EDTA/EGTA buffer and its activity was determined in either a crude 105,000 xg supernatant of this extract or after partial purification by DEAE-52 cellulose

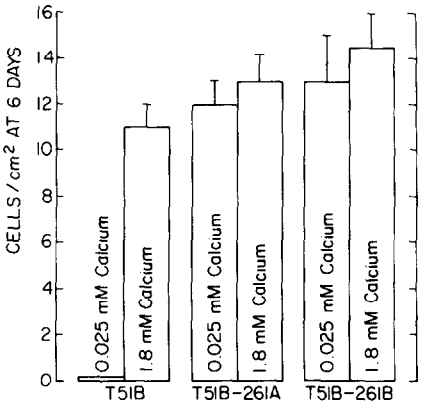

Fig. 1. The different abilities of T51B, T51B-261A and T51B-261B rat liver cells to proliferate in normal (1.8 mM) and calcium-deficient (0.025 mM) BME-BCS medium. T51B, non-tumorigenic; T51B-261A, non-tumorigenic, spontaneously transformed clone which originated from a colony formed by an untreated T51B cell in calcium-deficient medium; T51B-261B, tumorigenic clone which originated from a colony formed by an AFB_1-treated cell in calcium-deficient medium. Cells were plated at a density of 357/cm^2 in high-calcium 90% BME-10% BCS medium in 60 mm petri dishes. Twenty-four hrs later the medium was replaced with one consisting of 90% BME-10% BCS containing either 1.25 mM Ca^{2+} or 0.025 mM Ca^{2+}. The number of cells per cm^2 was determined 5 days later using a hemactyometer after removing them from the petri dish surface in a 0.05% trypsin solution. The values are means ± S.E.M. of the values from four separate cultures.

column chromatography (Figs. 2-4; Table 2). Its calcium-, diacylglycerol- and phosphatidylserine-dependence are illustrated in Figs. 2 and 3. The protein kinase C activity in the EDTA/EGTA extracts represented 42% of the total

TABLE 1

The Ability of Normal T51B Rat Liver Cells and Preneoplastic and Neoplastic Derivatives of T51B Cells to Proliferate and Form Colonies in Calcium-Deficient Medium and Produce Tumors in Athymic Nude Mice

Cell or Clone	Treatment	Colony Formation in Low-Calcium Medium[a] (Colonies per Dish)	Tumor Formation in Nude Mice
T51B (Non-Neopl.)	None	2	0/10
T51B-261A (Preneopl.)	Spontaneous	93	0/5
T51B-261B (Neopl.)	2.0 μg/ml AFB_1	90	4/5

a. Cells were plated at a density of 35 per cm^2 in 90% BME-10% BCS containing 1.8 mM calcium and 24 hrs later the medium replaced with fresh 90% BME-10% BCS containing only 0.025 mM calcium. The cultures were incubated for 4 weeks at which time they were fixed, stained (1) and colonies counted.

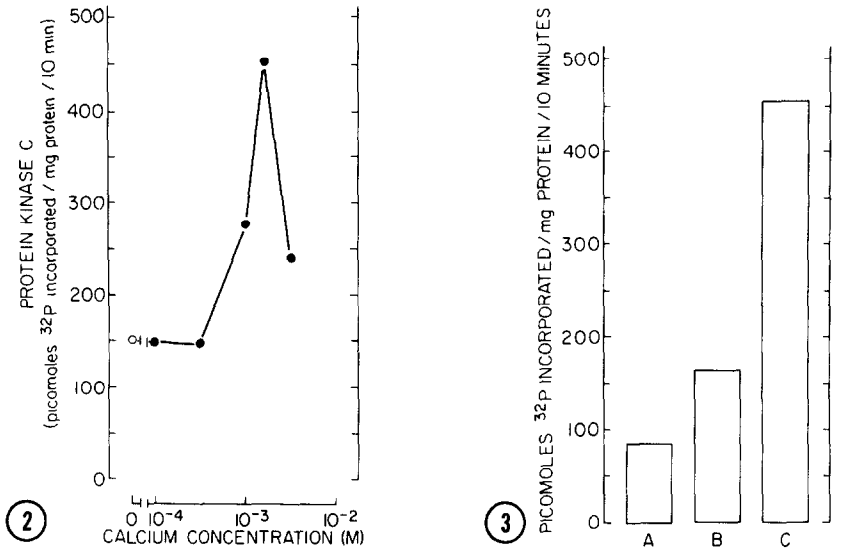

Fig. 2. Effects of different $CaCl_2$ concentrations on protein kinase C activity extracted with EDTA/EGTA-buffer from non-neoplastic T51B rat liver cells which had been proliferating in BME-BCS medium containing 1.8 mM Ca^{2+}. It should be noted that the $CaCl_2$ concentration is the total amount added and does not represent the free Ca^{2+} in the reaction mixture. The zero value for $CaCl_2$ represents protein kinase C activity in the presence of excess EDTA/EGTA (0.32 mM). Each point was determined in triplicate.

Fig. 3. Effects of 1,2-diolein and phosphatidylserine on protein kinase C activity extracted with EDTA-EGTA-buffer from non-neoplastic T51B rat liver cells which had been proliferating in BME-BCS medium containing 1.8 mM Ca^{2+}. The reaction mixture contained: (A), 1.0 µg 1,2-diolein and 1.5 mM $CaCl_2$; (B), 10 µg phosphatidylserine and 1.5 mM $CaCl_2$; (C), 1.0 µg 1,2-diolein, 10 µg phosphatidylserine and 1.5 mM $CaCl_2$.

cellular activity. Protein kinase C activity in the crude 105,000 xg supernatant or in the partially purified enzyme preparation of T51B cells, which had been proliferating in normal BME-BCS medium before homogenization and extraction, was between 2- and 4-fold greater than in T51B cells whose proliferation had been inhibited by incubation for 24 hours in BME-BCS medium containing only 0.025 mM calcium (Fig. 4; Table 2). It should be noted that the reduced protein kinase C activity of Ca^{2+}-deprived T51B cells could not have been due to dephosphorylation of substrate by increased phosphoprotein phosphatase activity because the activity was also reduced in the partially purified enzyme preparation (Fig. 4).

In contrast to normal cells, preneoplastic and neoplastic T51B cells which continued to proliferate in Ca^{2+}-deficient medium (Table 1), did not reduce their protein kinase C activity in this medium (Table 2). In fact, the

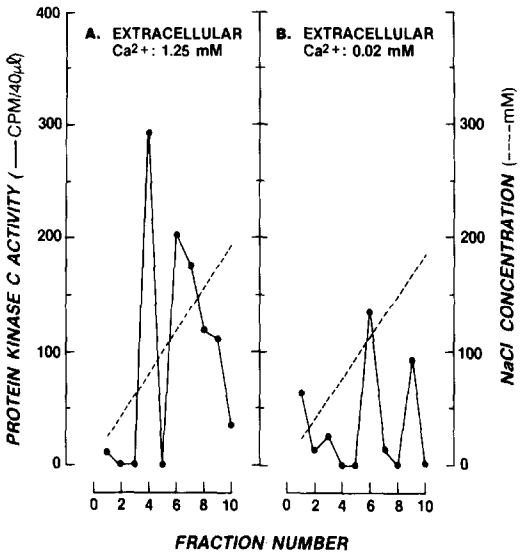

Fig. 4. Demonstration of the 2- to 4-fold reduction in the partially purified (by DEAE-52 cellulose) protein kinase C activity from 105,000 xg supernatants from EDTA/EGTA extracts of T51B cells which were (A) proliferating in medium containing 1.25 mM calcium or (B) proliferatively inactive in medium containing 0.025 mM calcium. 1.25 mg of 105,000 xg supernatant protein was loaded onto DEAE-52 cellulose columns in both A and B which were pre-equilibrated with EDTA/EGTA buffer. The protein kinase C activity was eluted with a 10 ml linear (0-250 mM) NaCl gradient and 1 ml fractions collected. Protein kinase C activity of a 40 μl sample from each fraction was assayed and was the difference between the cpm of ^{32}P incorporated into histone H1 per 40 μl sample during the 10-minute reaction period in the presence and absence of phosphatidylserine (15). It should be noted that total protein kinase C activity of the calcium-deprived T51B cells in this experiment was 1/3 of the activity of cells incubated in normal medium.

EDTA/EGTA extractable protein kinase C activities in Ca^{2+}-deprived preneoplastic and neoplastic cells were higher than the activities in cells growing in normal medium.

DISCUSSION

The facts that a large drop in the level of EDTA-EGTA extractable protein kinase C activity accompanied the greatly reduced proliferative activity of Ca^{2+}-deprived non-neoplastic T51B rat liver cells and that the continued proliferation of preneoplastic and neoplastic T51B cells in Ca^{2+}-deficient medium was accompanied by an increase in the level of protein kinase C activity, suggest that this Ca^{2+}-dependent enzyme may be the effector component of a normally Ca^{2+}-dependent proliferative control mechanism. This suggestion is supported by the facts that the tumor-promoting TPA can both

TABLE 2

Protein Kinase C Activity Extracted from Non-Neoplastic T51B Rat Liver Cells and Preneoplastic and Neoplastic Derivatives of T51B Cells

Cell or Clone	ECC[a]	Protein Kinase C Activity[b]	PkC-High Ca^{2+} Cells / PkC-Low Ca^{2+} Cells
T51B (Non-Neopl.)			
Experiment 1	1.8	489	3.5
	0.025	138	
Experiment 2	1.8	684	3.0
	0.025	226	
Experiment 3	1.8	718	3.1
	0.025	231	
T51B-261A (Preneopl.)	1.8	462	0.89
	0.025	517	
T51B-261B (Neoplastic)	1.8	653	0.83
	0.025	789	

[a]ECC: Extracellular calcium concentration (mmoles/l) in the BME-BCS medium in which the cells were cultivated before homogenization and extraction with EDTA/EGTA buffer.

[b]Difference between number of picomoles of ^{32}P (from [γ-^{32}P]-ATP) incorporated into Histone H1/mg of extracted protein/10 minutes in the presence and absence of phosphatidylserine.

bypass Ca^{2+} and directly stimulate protein kinase C (9) and enable non-neoplastic T51B and other non-neoplastic cells to proliferate like neoplastic cells in Ca^{2+}-deficient medium (1,3-7). This action of TPA and the present observations combine to suggest that the ability to proliferate in Ca^{2+}-deficient medium, a common property of neoplastic cells, may be due to a permanent activation or functioning of protein kinase C regardless of the extracellular Ca^{2+} level.

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