Rapid Microassay for Protein Kinase C Translocation in Swiss 3T3 Cells†

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ABSTRACT: The Ca^{2+}/phosphatidylserine-stimulated protein kinase C (PKC) appears to exist as interconvertible inactive, soluble and active, membrane-bound forms. Changes in the bimodal distribution of PKC induced by diacylglycerol or tumor-promoting phorbol esters have been proposed to regulate the activity of this kinase [Nishizuka, Y. (1984) *Nature (London) 308*, 693-698]. A rapid microassay for assessment of protein kinase C translocation between cytosol and membranes was developed. This procedure, which relied on the selective digitonin-mediated release of cytoplasmic proteins, eliminated potential homogenization and fractionation artifacts. PKC activity toward histone H1 was determined after limited trypsinolysis, which abolished the Ca^{2+}/phospholipid requirement of the enzyme and prevented interference by inhibitory proteins. Complete translocation of PKC to the membrane fraction and subsequent down-regulation of the kinase in response to 12-*O*-tetradecanoylphorbol-13-acetate treatment of Swiss 3T3 cells could be demonstrated by this method. Platelet-derived growth factor, insulin-like growth factor 1, vasopressin, and prostaglandin $F_{2\alpha}$ facilitated partial conversions of PKC to the membrane-bound form in quiescent 3T3 cells.

The Ca^{2+}- and phospholipid-stimulated enzyme protein kinase C (PKC)[1] plays a pivotal role in the regulation of cell metabolism and function in response to polyphosphoinositide turnover [for reviews, see Nishizuka (1983, 1984) and Berridge & Irvine (1984)]. A wide spectrum of hormones and other biologically active substances stimulates the phospholipase C catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (an intracellular mobilizer of Ca^{2+}) and diacylglycerol (an activator of PKC). Diacylglycerol appears to enhance the affinity of PKC for both Ca^{2+} and phosphatidylserine (Kishimoto et al., 1980; Parker et al., 1984). Active tumor-promoting phorbol esters, notably 12-*O*-tetradecanoylphorbol-13-acetate (TPA), can substitute for diacylglycerol in the activation of PKC, and so far, PKC is the only phorbol ester receptor that has been identified (Nishizuka, 1983, 1984). However, other non-phorbol ester tumor promoters with no obvious diacylglycerol-like structure, such as teleocidin and mezerein, also activate PKC (Couturier et al., 1984; Miyake et al., 1984).

Under resting conditions, PKC partitions with the cytosolic extract of cells that have been homogenized in the presence of calcium chelator (Katoh & Kuo, 1982). By contrast, when cultured cells are initially exposed to TPA, PKC is almost exclusively recovered with the particulate fraction upon cellular disruption (Kraft et al., 1982; Tapley & Murray, 1984; Wooten & Wrenn, 1984). Redistribution of PKC in TPA-treated cells apparently reflects the formation of a quaternary complex between the kinase, Ca^{2+}, and the membrane-associated phospholipid and phorbol ester (Wolf et al., 1985a; Ganong et al., 1986). Recent studies have implied that association of PKC with membranes, which is promoted by physiological levels of calcium and phosphatidylserine, is insufficient for full activation of PKC, but rather "primes" the kinase and renders it sensitive to the activators, diacylglycerol and phorbol esters (Wolf et al., 1985b; Ganong et al., 1986).

PKC may be partially translocated to the membrane subfraction of a wide variety of cultured cells in response to agents which accelerate PIP_2 hydrolysis and diacylglycerol formation (Drust & Martin, 1985; Farrar & Anderson, 1985; Hirota et al., 1985; Akers et al., 1986; Liles et al., 1986; Nel et al., 1986; Pontremoli et al., 1986). Conversely, extracellular Ca^{2+} deprivation of BALB/c 3T3 cells causes a reduction in the amount of particulate-associated PKC activity, and this was correlated with an inhibition of cell proliferation (Donnelly et al., 1985). Collectively, these studies indicate that PKC exists as interconvertible inactive, soluble and active, membrane-bound forms, although there may also be an intermediate inactive, membrane-bound form of primed PKC (Wolf et al., 1985b).

In all of the aforementioned studies, PKC translocation has been assayed on the basis of Ca^{2+}/phosphatidylserine activation of PKC in the presence of diacylglycerol or active phorbol ester using subcellular fractions from homogenized cells. A study by Shoji et al. (1986), which utilized immunoblotting techniques with antisera raised against PKC, confirmed that TPA causes PKC translocation to the plasma membrane of HL60 cells. However, these immunocytochemical localization experiments were performed with subcellular fractions obtained after homogenization. PKC translocation has not been evaluated by a method that avoids the possible generation of homogenization artifacts.

Assessment of the bimodal distribution of PKC on the basis of Ca^{2+}/phosphatidylserine/diacylglycerol-stimulated kinase activity toward histone H1 can be complicated by several factors. PKC associated with the particulate fraction may be fully active in the absence of exogenous Ca^{2+} or phospholipid

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[1] Abbreviations: CT, CTP:phosphocholine cytidylyltransferase; DMEM, Dulbecco's modified Eagle medium; EGF, epidermal growth factor; FCS, fetal calf serum; FGF, fibroblast growth factor; IGF-1, insulin-like growth factor 1; PDGF, platelet-derived growth factor; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKI-[5-24], cAMP-dependent protein kinase inhibitor peptide; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; kDa, kilodalton(s); TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Mops, 3-(*N*-morpholino)-propanesulfonic acid; HPLC, high-performance liquid chromatography.

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(Pontremoli et al., 1986), so it is usually necessary to solubilize and partially purify the membrane-bound PKC. Anion-exchange fractionation of cytosol is required to resolve soluble inhibitor proteins that interfere with the standard PKC assay (Schwantke & Le Peuch, 1984; McDonald & Walsh, 1985). Furthermore, PKC can be rendered Ca^{2+} and phospholipid independent by limited proteolysis to a 51-kDa form both before (Tapley & Murray, 1984) and after homogenization and subcellular fractionation (Kishimoto et al., 1983; Melloni et al., 1985, 1986; Mizuta et al., 1985; Tapley & Murray, 1985).

The aim of the present study was to develop a facile method to assay for PKC translocation in cultured cells exposed to hormonal stimuli. The procedure that we adopted avoids homogenization and fractionation and minimizes the influence of factors that could potentially interfere with a quantitative analysis of the subcellular distribution of PKC.

Experimental Procedures

Materials. Swiss 3T3-D1 cells and PDGF were generously donated by Dr. Elaine Raines and Dr. Russell Ross (Department of Biochemistry, University of Washington, Seattle). Acidic and basic FGFs were the gift of Dr. Bradley Olwin (Department of Biochemistry, University of Washington, Seattle). EGF was purified to homogeneity from adult mouse submaxillary glands (Pel-Freeze, San Francisco) as described (Savage & Cohen, 1972). IGF-1 was purchased from AM Gen Biologicals. The cAMP-dependent protein kinase inhibitor peptide PKI-[5–24] (Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp) was kindly provided by Dr. John Scott of this laboratory (Scott et al., 1986). TPA, phorbol-12,13-dibutyrate, 4α-phorbol, insulin, [Arg^8]vasopressin, prostaglandin $F_{2\alpha}$, histone H1 (type III-S), digitonin, phosphatidylserine, and diolein were purchased from Sigma. TPCK–trypsin and lima bean trypsin inhibitor were from Worthington. Buffalo rat liver 3A (BRL-3A) and A431 cells were obtained from the American Type Culture Collection. [γ-^{32}P]ATP was bought from New England Nuclear.

Cell Culture and Preparation of Extracts. Cells were grown to confluence in either 15-cm Falcon Integrid tissue culture dishes or Falcon 24-well tissue culture plates in 10% fetal calf serum (FCS) (KC Biological) and low-glucose Dulbecco's modified Eagle medium (DMEM) (Gibco) at 37 °C in 5% CO_2. Swiss 3T3-D1 cells were rendered quiescent by 24–48-h incubation in 0.33% FCS in DMEM. Unless stated otherwise, cells were incubated in the absence or presence of various factors for 15 min prior to preparation of extracts.

Cytosolic (150000*g* for 20 min) extracts were obtained from the 15-cm culture dishes as described (Pelech et al., 1986). The soluble and particulate extracts from the 24-well plate cultures were prepared as follows. The plates were placed on ice, and the medium from the individual wells was removed. Each well was washed twice with 1 mL of ice-cold phosphate-buffered saline. The soluble extract was produced when the cells were incubated at 0 °C for 5 min with 0.2 mL of buffer A (0.5 mg of digitonin/mL, 20 mM Mops, pH 7.2, 10 mM EGTA, and 5 mM EDTA). The cell remnants, after removal of the soluble extract, were solubilized in 0.2 mL of buffer A plus 0.5% Triton X-100 to obtain the particulate extract. The extracts were used immediately or stored at −70 °C for later use with equivalent results.

Enzyme Assays. The cellular extracts were incubated for 10 min at 30 °C in the absence or presence of 7.5 μg of trypsin/mL, and 0.15 mg of lima bean trypsin inhibitor/mL was subsequently added to terminate trypsinolysis. (Optimal protease stimulation of the H1 kinase activity was attained with 5–10 μg of trypsin/mL; higher concentrations of trypsin were inhibitory.) Unless stated otherwise, all kinase assays contained 800 nM PKI-[5-24], 50 μM [γ-^{32}P]ATP, 15 mM $MgCl_2$, 1 mg of histone H1/mL, and 10 μL of the soluble or particulate extract in a final volume of 25 μL. The kinase assays commenced upon addition of [γ-^{32}P]ATP and were of 10–20-min duration at 30 °C. At the conclusion of the reaction period, 20-μL aliquots were spotted onto 1.5 cm^2 pieces of Whatman P81 phosphocellulose paper and 30 s later washed 5 times for at least 2 min each wash in a solution of 10 mL of phosphoric acid/L of H_2O. The wet filter papers were transferred into 6-mL plastic scintillation vials that contained 5 mL of Aquasol scintillation fluid and analyzed for radioactivity in a Packard counter. The Ca^{2+}/phosphatidylserine/diolein-stimulated PKC activity was assessed in cellular extracts as described (Liles et al., 1986). Ca^{2+}/phosphatidylserine/diolein-stimulated PKC activity could not be detected in the digitonin extracts when two different PKC assay systems were employed (Liles et al., 1986; Hannun et al., 1985), presumably because of interference of the activation of PKC by digitonin.

Results and Discussion

Activation of PKC by Proteolysis. PKC activity in column-fractionated cell extracts is typically measured as the histone H1 kinase activity that is stimulated by a sonicated mixture of phosphatidylserine and diglyceride in the presence of calcium. Potential problems with this approach include interference by other components in the extracts and conversion of PKC to a Ca^{2+}/phospholipid-independent form by calcium-activated proteases (Kishimoto et al., 1983). Trypsinolysis of PKC can eliminate the Ca^{2+}/phospholipid requirement of the kinase, allowing expression of 60–90% of the original activity toward histone H1 with Ca^{2+}, phosphatidylserine, and diolein (Parker et al., 1984). For this study, we exploited trypsin activation of PKC as a method for quantitation of this kinase in cell extracts.

It was important to establish that other histone H1 kinases did not undergo simultaneous proteolytic activation if trypsinolysis was to be used to measure PKC activity. The activities of at least five other kinases can be increased following proteolysis: phosphorylase kinase (Hayakawa et al., 1973); myosin light chain kinase (Tanaka et al., 1980); protease-activated kinase I (Tahara & Traugh, 1981); protease-activated kinase II (Lubben & Traugh, 1983); protease-activated histone H4 kinase (Houssaye et al., 1983). However, with the exception of the latter two (Lubben & Traugh, 1983; Magnino et al., 1983), the others do not phosphorylate histone H1 (Chrisman et al., 1982; Adelstein & Klee, 1981; Tahara & Traugh, 1981).

When cytosol from quiescent Swiss 3T3 cells was fractionated on DEAE-Sephacel, only one peak of trypsin-stimulated histone H1 kinase activity was detectable (Figure 1). Similar results were obtained when the extracts were fractionated by gel filtration on Sephacryl-S300 or TSK-400 HPLC (data not illustrated). This activity comigrated with the Ca^{2+}/phosphatidylserine/diolein-stimulated histone H1 kinase activity in each of the three chromatographic systems.

Since protease-activated kinase II and protease-activated H4 kinase share somewhat similar DEAE and gel filtration chromatographic properties with PKC (Lubben & Traugh, 1983; Magnino et al., 1983), the contribution of these kinases toward the total trypsin-stimulated histone H1 kinase activity in 3T3 cell extracts could not be readily discounted. However, as shown in the next section, in experiments with TPA, greater

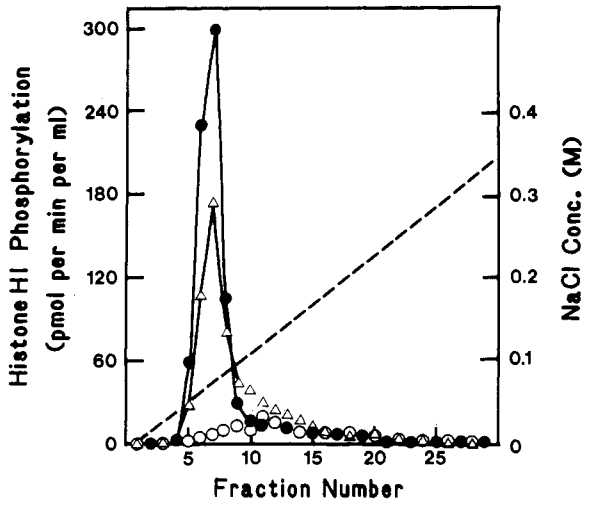

FIGURE 1: DEAE-Sephacel chromatography of the trypsin-activated and Ca^{2+}/phosphatidylserine/diolein-activated histone H1 kinases in Swiss 3T3 cell cytosol. The 100000*g*, 1-h supernatant from four 15-cm dishes of confluent and quiescent Swiss 3T3 cells was applied to a 1 × 2.5 cm DEAE-Sephacel column equilibrated with buffer B (10 mM Mops, pH 7.2, 2 mM EGTA, and 2 mM EDTA). The column was washed with 20 mL of buffer B and developed with 36 mL of a linear 0–400 mM NaCl gradient in buffer B. The collected fractions (≃1 mL) were assayed with 1.0 mg of histone H1/mL and 50 μM [γ-^{32}P]ATP for basal (○), Ca^{2+}/phosphatidylserine/diolein-stimulated (●), and trypsin-stimulated (△) kinase activities as described under Experimental Procedures.

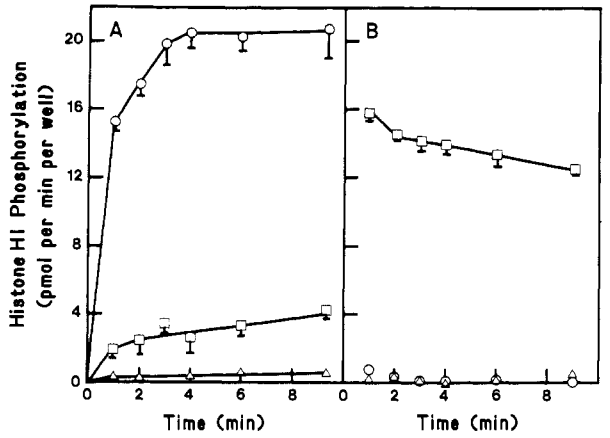

FIGURE 2: Time dependence of digitonin-mediated release of PKC from TPA-treated Swiss 3T3 cells. Confluent and quiescent Swiss 3T3 cells in 24-well plates were incubated at 37 °C in the absence (○) or presence of 200 nM TPA for 15 min (□) or 5 μM TPA for 15 h (△). Cells were subsequently exposed to buffer A (0.5 mg of digitonin/mL, 20 mM Mops, pH 7.2, 10 mM EGTA, and 5 mM EDTA) for 1–9 min. Following removal of the soluble cell extract (panel A), the cell remnants were solubilized in buffer A plus 0.5% Triton X-100 to obtain the particulate cell extract (panel B). Histone H1 kinase activity was assessed in these fractions after limited trypsinolysis as described under Experimental Procedures. Each value is the mean ± standard deviation of determinations from triplicate wells.

than 95% of the trypsin-stimulated histone H1 kinase activity appeared to originate from PKC.

Digitonin-Mediated Release of Cytosolic PKC from TPA-Treated Mouse Fibroblasts. When cells are permeabilized with digitonin, most cytoplasmic proteins rapidly diffuse into the medium, while membrane-associated proteins tend to be retained in the particulate cell remnants (Mackall et al., 1979; Pelech et al., 1984a,b). This phenomenon was exploited to examine the distribution of PKC in quiescent and TPA-treated Swiss 3T3 cells. The histone H1 kinase activity detectable

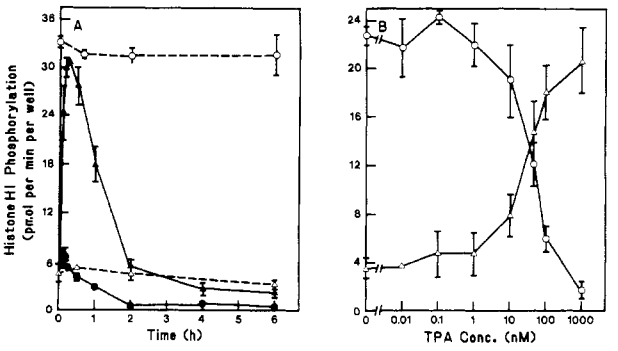

FIGURE 3: Translocation of PKC in TPA-treated Swiss 3T3 cells as a function of time and phorbol ester concentration. (Panel A) Confluent and quiescent Swiss 3T3 cells in 24-well plates were incubated without (○, △) and with 500 nM TPA (●, ▲) for 5 min–6 h. (Panel B) Cells were incubated with 0–1 μM TPA for 15 min. Soluble extracts (○, ●) were obtained upon 5-min exposure of the cells to buffer A, and particulate extracts (△, ▲) were prepared from the cell remnants with buffer A plus Triton X-100. Histone H1 kinase activity was assessed in these extracts after limited trypsinolysis as described under Experimental Procedures. Each value is the mean ± standard deviation of determinations from triplicate wells.

Table I: Translocation of PKC in Swiss 3T3 Cells Exposed to Phorbol Ester Derivatives and Various Mitogens[a]

substance	histone H1 kinase act. (pmol·min^{-1}·well^{-1}) particulate fraction	soluble fraction
untreated	2.69 ± 0.53	35.2 ± 4.1
phorbol dibutyrate (500 nM)	18.1 ± 1.81	13.4 ± 0.4
4α-phorbol (500 nM)	2.03 ± 0.18	37.5 ± 1.2
PDGF (10 ng/mL)	4.82 ± 0.77	32.1 ± 4.4
acidic FGF (10 ng/mL)	2.54 ± 0.90	37.1 ± 1.1
basic FGF (10 ng/mL)	2.32 ± 0.61	36.0 ± 0.6
EGF (100 nM)	2.58 ± 0.64	38.7 ± 1.0
insulin (100 nM)	2.45 ± 0.65	40.4 ± 1.1
IGF-1 (100 nM)	6.34 ± 1.36	33.2 ± 2.8
vasopressin (400 nM)	3.72 ± 0.40	33.7 ± 2.0
$PGF_{2\alpha}$ (400 nM)	4.10 ± 0.58	35.0 ± 2.5

[a] Confluent and quiescent Swiss 3T3 cells in 24-well plates were incubated for 20 min with various factors at the shown concentrations. Soluble and particulate extracts were prepared, and the histone H1 kinase activity was assessed in these extracts after limited trypsinolysis as described under Experimental Procedures. Each value is the mean ± standard deviation of determinations from triplicate wells.

after trypsinolysis was tentatively taken to represent PKC. Within 3–4-min exposure of quiescent cells to digitonin, all of the releasable PKC had leaked into the medium (soluble extract), and essentially no PKC activity could be detected in the Triton X-100 solubilized cell remnants (particulate extract) (Figure 2). By contrast, when cells that had been preincubated for 15 min with 200 nM TPA were treated with digitonin, only about 15% of the PKC appeared in the soluble extract, and the remainder was quantitatively recovered in the particulate extract (Figure 2). As shown in Figure 3A, maximal translocation of PKC to the particulate fraction occurred within 10-min exposure to 200 nM TPA. Half-maximal translocation of PKC in 15 min was achieved with ≃30 nM TPA (Figure 3B). Only active tumor-promoting phorbol esters (TPA and phorbol dibutyrate) facilitated membrane association of PKC, since 4α-phorbol was ineffective (Table I).

Extended treatment (≥30 min) with TPA appeared to down-regulate PKC (Figure 3A). There was a rapid decline in the total trypsin-stimulated histone H1 kinase activity in the soluble and particulate extracts of 3T3 cells incubated for 30–120 min with 200 nM TPA (Figure 3A). Prolonged ex-

posure for 15 h of 3T3 cells to 5 μM TPA led to virtually the complete disappearance of the protease-activated histone H1 kinase (Figure 2).

The TPA-induced translocation of the trypsin-activated histone H1 kinase was entirely consistent with the behavior of PKC (see the introduction). As observed here with the trypsin-activated histone H1 kinase, PKC has been shown to undergo essentially complete down-regulation upon extended exposure of various cell lines, including 3T3 fibroblasts, to phorbol esters (Rodriguez-Pena & Rozengurt, 1984; Ballester & Rosen, 1985; Blackshear et al., 1985; Wickremasinghe et al., 1985; Fabbro et al., 1986; Katakami et al., 1986). These findings, which have not been reported for protease-activated kinase II and protease-activated histone H4 kinase, provide further evidence that the trypsin-activated histone H1 kinase was PKC.

While this paper was in preparation, we became aware of a similar study by TerBush and Holz (personal communication) in which the effects of phorbol esters on the subcellular distribution of PKC were examined in digitonin-permeabilized adrenal chromaffin cells. These investigators relied on Ca^{2+}/phospholipid/diolein sensitivity to assay for PKC activity and obtained results similar to those of our study with respect to the selective digitonin-mediated release of the soluble form of PKC from cells.

PKC Translocation in Mitogen-Treated Cells. Since the combination of digitonin extraction and protease activation proved to be a rapid and sensitive means for assaying TPA-mediated redistribution of PKC within Swiss 3T3 cells, this approach was adopted to test the effect of various mitogens on PKC translocation (Table I). Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), vasopressin, and PDGF each induced 1.4–1.8-fold increases in the particulate-associated trypsin-stimulated histone H1 kinase activity. All of these hormones promote polyphosphoinositide breakdown and diacylglycerol production (MacPhee et al., 1984; Hasegawa-Sasaki, 1985; Siess et al., 1986). No effect was evident with the acidic and basic fibroblast growth factors which also initiate PIP_2 hydrolysis in 3T3 cells (Tsuda et al., 1985) nor with insulin and EGF which do not appear to cause rapid polyphosphoinositide turnover in other fibroblasts (Coughlin et al., 1985; Besterman et al., 1986; L'Allemain & Pouyssegur, 1986). It is unknown whether insulin-like growth factor 1 (IGF-1), which also increased the level of membrane-associated trypsin-activated histone H1 kinase, stimulates PIP_2 hydrolysis in these or other cells. Due to the low level of PKC translocation induced by PDGF, vasopressin, $PGF_{2\alpha}$, and IGF-1, it was not possible to detect significant decreases in the soluble PKC activity (Table I).

PKC translocation in 3T3 cells in response to PDGF, vasopressin, $PGF_{2\alpha}$, and IGF-1 was investigated further (Figure 4). Saturating concentrations of these mitogens produced maximal increases in the membrane-associated PKC activity within 5 min, but these effects became reversed after 10 min (Figure 4A). The most potent inducer of PKC translocation was PDGF (Figure 4B). The concentrations of vasopressin, $PGF_{2\alpha}$, and IGF-1 that caused half-maximal translocation of PKC by these hormones were approximately 200 times greater than the corresponding concentration of PDGF ($ED_{50} \simeq 0.05$ nM). This was consistent with the relative affinities of these hormones for their respective receptors.

To evaluate the general applicability of this method, PKC translocation was examined in the A431 human cervical carcinoma cell line and Buffalo rat liver, BRL-3A, cells (Table II). TPA (200 nM) treatment for 20 min facilitated redistribution of the trypsin-activated histone H1 kinase in subconfluent and proliferating cultures of both A431 and BRL-3A cells. EGF, which has been reported to stimulate polyphosphoinositide turnover, diacylglycerol production, and PKC activation in A431 cells (Sawyer & Cohen, 1981; Sahai et al., 1982; Smith et al., 1983), produced a 1.7-fold increase in the particulate trypsin-stimulated histone H1 kinase activity and a corresponding decrease in the soluble kinase activity (Table II). IGF-1 also appeared to induce a modest PKC translocation in BRL-3A cells.

Throughout this study, the inclusion of EDTA/EGTA in the digitonin buffer ensured that only the membrane-bound form of PKC which was active remained associated with the cell remnants after the first extraction. It is likely that the membrane-bound but inactive form of PKC, i.e., the primed form, was released in the presence of Ca^{2+} chelators. Therefore, in the strictest sense, the digitonin-soluble PKC activity in these experiments may be generated from both the soluble and membrane-associated inactive forms of PKC in the intact cell. Parallel studies in which the Ca^{2+} levels are controlled in the digitonin buffers may permit estimation of the amount of soluble, inactive; membrane-bound, inactive

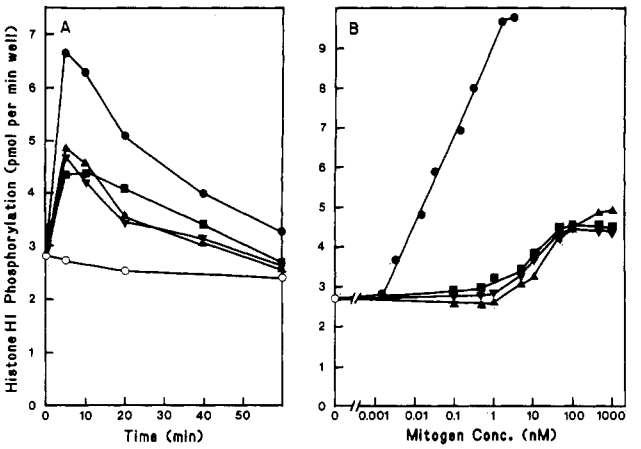

FIGURE 4: Particulate PKC activity in Swiss 3T3 cells incubated with PDGF, IGF-1, vasopressin, and $PGF_{2\alpha}$. (Panel A) Confluent and quiescent Swiss 3T3 cells in 24-well plates were incubated for 5–60 min without (○) or with 10 ng/mL PDGF (●), 100 nM IGF-1 (■), 250 nM vasopressin (▲), and 250 nM $PGF_{2\alpha}$ (▼). (Panel B) Cells were incubated for 15 min in the presence of 0–100 ng/mL PDGF (●), 0–1 μM IGF-1 (■), 0–1 μM vasopressin (▲), and 0–1 μM $PGF_{2\alpha}$ (▼). Soluble and particulate extracts were prepared, and histone H1 kinase activity was determined after limited trypsinolysis of these extracts as described under Experimental Procedures. Each value is the mean of duplicate determinations. The experiments were performed on three separate occasions with similar results.

Table II: PKC Translocation in A431 and BRL-3A Cells[a]

	histone H1 kinase act. (pmol·min^{-1}·well^{-1})	
	particulate fraction	soluble fraction
A431 cells		
untreated	1.64 ± 0.26	3.80 ± 0.31
TPA (200 nM)	4.79 ± 0.41	1.58 ± 0.25
EGF (100 nM)	2.72 ± 0.59	2.65 ± 0.01
BRL-3A cells		
untreated	0.46 ± 0.32	6.73 ± 0.89
TPA (200 nM)	4.85 ± 0.31	1.90 ± 0.31
IGF-1 (100 nM)	1.13 ± 0.65	6.55 ± 0.13

[a] Subconfluent cultures of A431 and BRL-3A cells in 24-well plates were incubated at 30 °C for 20 min with 100 nM EGF, 100 nM IGF-1, or 200 nM TPA. Soluble and particulate extracts were prepared, and the histone H1 kinase activity was evaluated in these extracts after limited trypsinolysis as described under Experimental Procedures. Each value is the mean ± standard deviation of four determinations.

(primed); and membrane-bound, active forms of PKC in cells.

The results of the present study support the concept that PKC represents a translocatable enzyme, the activity of which is partly regulated by its location in the cell. Wilson (1978) proposed the term "ambiquitous" to describe proteins that are subject to this type of control. A growing list of enzymes may be classified as ambiquitous, but the most striking parallel with PKC is seen with CTP:phosphocholine cytidylyltransferase (CT) [for reviews, see Pelech & Vance (1984) and Vance & Pelech (1984)]. In particular, active phorbol esters stimulated phosphatidylcholine biosynthesis in HeLa cells via translocation of CT from the cytosol to the microsomes, and this was substantiated by the digitonin release method used here (Pelech et al., 1984b). Diacylglycerol (a substrate in the reaction catalyzed by the next enzyme in the pathway for phosphatidylcholine biosynthesis) also promotes CT binding to microsomes (Choy et al., 1979).

In summary, we have described a novel method for assessment of PKC translocation. This procedure required neither cell homogenization nor partial purification of PKC. Using this method, we have demonstrated that a number of hormones and factors can induce the membrane association of PKC in cultured cells. The application of the digitonin release method to other model systems may facilitate further identification of substances which translocate PKC and perhaps as yet unknown ambiquitous proteins.

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Iron(II)-Substituted Metallothionein: Evidence for the Existence of Iron–Thiolate Clusters†

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ABSTRACT: Metallothioneins (MT's) are unique low molecular weight (M_r 6000–7000) metal- and cysteine-rich proteins characterized by two tetrahedral tetrathiolate clusters containing three and four metal ions. Naturally occurring proteins usually contain the diamagnetic metal ions Zn(II) and/or Cd(II). We have now succeeded in substituting these ions by paramagnetic Fe(II). Rabbit liver MT-1 in which all seven metal binding sites were occupied by Fe(II) ions displays absorption features typical of tetrahedral tetrathiolate Fe(II) coordination. This is documented by the presence of a ligand field $^5E \rightarrow {}^5T_2$ transition in the near-infrared region centered at about 1850 nm ($\epsilon_{Fe} \approx 100\ M^{-1}\ cm^{-1}$) and a broad charge-transfer absorption in the UV region with a shoulder at 314 nm. A metal–thiolate cluster structure is inferred from the 7 to 20 ratio of metal ions to cysteine residues and from spectral studies in which successive increments of Fe(II) were incorporated into the metal-free protein. Thus, to about 4 equiv, the charge-transfer absorption and magnetic circular dichroism (MCD) features of the complexes formed resemble closely those of reduced rubredoxin from *Desulfovibro gigas* in which tetrahedral tetrathiolate Fe(II) coordination is documented. However, upon further addition of Fe(II) ions, the charge-transfer absorption bands undergo a progressive red-shift until the full metal occupancy of seven Fe(II) ions per molecule is reached. The bathochromic shift which is also manifested in the MCD spectra can be ascribed to the transformation of some of the terminal thiolate ligands to bridging when the full complement of Fe(II) is bound. The concomitant loss in amplitude of the MCD bands above 4 equiv is thought to arise from exchange coupling of vicinal Fe(II) via the thiolate bridges.

Metallothioneins (MT's)[1] constitute a class of low molecular weight (M_r 6000–7000) metal- and cysteine-rich proteins widely distributed in nature (Kägi et al., 1984) for which physiological functions in metal storage and/or heavy-metal detoxification were proposed (Nordberg & Kojima, 1979). The best characterized mammalian forms contain a single polypeptide chain of 61 amino acids, out of which 20 residues are cysteine (Kojima et al., 1976). Typical of all MT's is the occurrence of a number of unique Cys-X-Cys sequences (X = amino acid residues other than Cys) in their primary structures. The naturally occurring MT's usually bind seven

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[1] Abbreviations: MT, metallothionein; apoMT, apometallothionein; MCD, magnetic circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris, tris(hydroxymethyl)aminomethane; 2D, two dimensional; 3D, three dimensional; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance.

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