Inactive membrane protein kinase Cs: a possible target for receptor signalling

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The activation of the multifunctional cell signalling enzymes, the protein kinase Cs (PKCs), is generally thought to result from the translocation of inactive cytosolic enzymes to activation sites in cell membranes. However, recent studies suggest that PKCs may also be stimulated in cells by processes independent of translocation. One possible mechanism is the modulation of the activity of PKCs already resident in membranes. A PKC assay that measures enzyme activity directly in isolated native membranes has revealed the presence of an activatable pool of PKCs resident in native membranes of various cells and tissues. In 3T3-L1 cells, some or all of this pool of membrane PKCs was stimulated within 10 min of exposing the cells to 10 ng/ml epidermal growth factor or 100 ng/ml fibroblast growth factor. Similar increases in PKC activity were observed in native membranes isolated from CTLL-2, WEHI-231 and S49 lymphoma cells that had been exposed to interleukin-2. These growth factors all stimulated membrane PKC activity without detectably translocating cytosolic enzymes to the membranes. In

intact WEHI cells, low concentrations (5–10 μ M) of a diacylglycerol, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), or low concentrations (2–10 nM) of phorbol 12-myristate 13-acetate sufficed to activate PKCs already resident in membranes, but much higher concentrations (50–100 μ M and 50–100 nM respectively) were needed to detectably stimulate the translocation of cytosolic PKCs. A phosphatidylcholine-specific phospholipase C also selectively stimulated membrane PKCs in WEHI cells at concentrations that were much less than those needed to induce the translocation of cytosolic enzymes. Furthermore, interleukin-2 and low concentrations of OAG both stimulated the phosphorylation of the 85 kDa PKC-selective substrate protein in intact WEHI cells in which translocation of PKCs was not evident. These results suggest that the membranes of some cells maintain a pool of activatable PKCs that respond to lower levels of extracellular stimuli than cytosolic PKCs, and that can be stimulated by signals which produce diacylglycerols through the hydrolysis of phospholipids other than polyphosphoinositides.

INTRODUCTION

The activation of protein kinase Cs (PKCs) in cells stimulated by various agents is commonly associated with a redistribution of cytosolic enzymes to membranes [1–8]. However, recent studies suggest that membrane-associated PKC activity may also be stimulated without a redistribution of enzymes from cytosol to membranes [9–14]. Experiments with purified PKCs and phospholipid vesicles [15] or erythrocyte membranes [16,17] have suggested that PKCs might associate with cell membranes in a Ca^{2+} -dependent (i.e. EGTA-sensitive) manner and remain there in an inactive, but still signal-responsive, state [18,19]. However, it has not been possible to demonstrate the presence of such an inactive, signal-responsive pool of membrane PKCs because of the inability of conventional PKC assays to distinguish such a membrane pool. Conventional methods require that the membranes first be solubilized and their associated PKCs then be reconstituted into artificial phospholipid membranes in the presence of a maximally effective concentration of a specific activator, the phorbol ester phorbol 12-myristate 13-acetate (PMA), and excess Ca^{2+} [20,21]. These manipulations would fully activate all PKCs, including any inactive enzyme(s) that might have been associated with the native cell membranes. Thus, conventional *in vitro* assays can measure changes only in the total amount of membrane-associated PKCs and not changes in the activity of pre-existing membrane-associated PKCs, except in cases where enzyme modification leads to activation [13]. In other words, the stimulation of a pre-existing pool of membrane

PKCs without the translocation of enzymes from the cytoplasm would go undetected by both the conventional activity assay and by any immunoblotting assay.

In this report, using a recently described ‘direct’ PKC assay [22] which measures enzyme activity directly in its native membrane-associated state, we provide direct evidence that some cells have a significant amount of activatable PKCs in their native membranes. We demonstrate that growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF) and interleukin (IL)-2 stimulate PKC activity in target cells not by inducing the translocation of cytosolic enzyme to membranes, but by activating the pre-existing pool of membrane PKCs. Evidence is also presented, using the synthetic diacylglycerol (DAG) 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), the phorbol ester PMA and a phosphatidylcholine-specific phospholipase C, that the stimulation of membrane PKCs in intact cells occurs at diacylglycerol concentrations much lower than that required to translocate cytosolic PKCs to membranes. These results strongly support the hypothesis [18] that membranes of unstimulated cells contain an inactive pool of PKCs which can be activated by physiological stimulators.

MATERIALS AND METHODS

Materials

EGF, basic FGF, recombinant IL-2 and pan-PKC antibody were from Upstate Biotechnology Inc. (Lake Placid, NY,

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FGF, fibroblast growth factor; IL, interleukin; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethanesulphonyl fluoride.

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U.S.A.). Isozyme-specific antibodies were purchased from GIBCO BRL (Burlington, ON, Canada). OAG, phosphatidylcholine-specific PLC (*Bacillus cereus*) and PMA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Protein reagent was from Bio-Rad Laboratories (Richmond, CA). DAG kinase inhibitors I and II were from Calbiochem. [γ - ^{32}P]ATP (6000 Ci/mmol) and $^{32}\text{P}_i$ (9000 Ci/mmol) were purchased from New England Nuclear (Du Pont Canada Inc., Mississauga, ON, Canada). The PKC-selective peptide substrate Ac-FKKSFKL-NH₂ (Ac, acetyl) was synthesized in this laboratory as described previously [22].

Cells and cell culture

S49 T-lymphoma, *cyc*⁻ S49 T-lymphoma, human myeloid (HL-60), murine erythroleukaemia (MEL) and human erythroleukaemia (HEL) cells were grown in suspension in 90 % (v/v) RPMI medium and 10 % (v/v) fetal bovine serum as described [22]. Cytotoxic T-cells (CTLL-2) were cultured in the same medium supplemented with 100 units/ml rIL-2 and 0.05 mM 2-mercaptoethanol. WEHI-231 B-lymphoma cells were grown in 90 % (v/v) high-glucose Dulbecco's modified Eagle's medium (DMEM), 10 % fetal bovine serum and 0.05 mM 2-mercaptoethanol. 3T3-L1 fibroblasts, NRK rat kidney cells, and Balb/MK-2 keratinocytes were cultured in monolayers as described previously [9,22,23]. Cells were used when the density of the erythroid and lymphoid suspension cultures reached $(1\text{--}1.5) \times 10^6/\text{ml}$, and when the NRK, Balb/MK-2 and 3T3-L1 monolayers attained confluence. Rat tissues were removed immediately after killing the animal by cervical dislocation and rinsed in ice-cold PBS. Thymocytes and spleen cells were prepared as described [24]. Platelets were isolated from rat blood obtained by heart puncture [25].

Cell lysis

Cells were washed twice with ice-cold PBS, suspended in 1 ml of ice-cold hypotonic lysis medium [1 mM NaHCO₃, 5 mM MgCl₂ and 100 μM phenylmethanesulphonyl fluoride (PMSF)] for 2 min and lysed by vortexing vigorously for 2 min. Note that the lysis medium does not contain metal ion chelators such as EDTA or EGTA. Whole rat brain and hippocampus were homogenized in a Potter–Elvehjem homogenizer with 20 up-and-down strokes in lysis medium. All subsequent steps were carried out at 4 °C. Nuclei and unlysed cells were sedimented at 600 *g* for 5 min and membrane and cytosol fractions were separated by centrifugation at 100 000 *g* for 10 min in a Beckman TL-100 ultracentrifuge.

Conventional PKC assay

The amounts of PKC present in cytosol and membrane (in some cases nuclear) fractions were measured by the conventional PKC assay as described previously [20,21]. Briefly, membrane PKC was solubilized by suspending membranes in 50 mM Tris/HCl buffer, pH 7.5, containing 2 mM EGTA, 0.5 mM EDTA and 0.1 % (v/v) Triton X-100 and incubating them for 15 min on ice with periodic vortexing. Under these conditions > 95 % of membrane-associated PKC was extracted. The membrane extract was clarified at 100 000 *g* for 10 min. The cytosolic fraction was adjusted to 50 mM Tris/HCl, pH 7.5, 2 mM EGTA, 0.5 mM EDTA, and 0.1 % Triton X-100. PKC in the membrane extract and cytosol fraction was measured in a reaction mixture (100 μl) containing 5–10 μg of protein, 75 μM PKC-selective peptide substrate (see below), 50 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 2 mM CaCl₂, 10 μg of phosphatidylserine, 1 μM PMA, 100 μM sodium vanadate, 100 μM sodium pyrophosphate, 1 mM sodium

fluoride, 100 μM PMSF and 50 μM [γ - ^{32}P]ATP (220 c.p.m./pmol). After incubation for 10 min at 25 °C, the reaction was stopped by adding 10 μl of 5 % (v/v) acetic acid and the samples were clarified by centrifugation at 16000 *g* for 5 min in a microfuge. A 90 μl sample of each supernatant was applied to P81 Whatman paper (2 cm²) and the papers were washed twice for 10 min in 5 % acetic acid (10 ml/cm² of paper). The radioactivity bound to the washed papers was determined by liquid scintillation counting.

Direct assay for measuring membrane-associated PKC activity

Membrane-associated PKC activity was measured directly in isolated native membranes [22], without prior extraction and reconstitution of the enzymes in artificial phospholipid membranes, by the incorporation of ^{32}P into the PKC-selective peptide substrate Ac-FKKSFKL-NH₂ [22,26–28]. This peptide corresponds to residues 160–166 of the phosphorylation site domain (151–175) of the Myristoylated Alanine Rich C Kinase Substrate (MARCKS) protein, a specific cellular substrate of PKC [28,29]. It, and the MARCKS protein's phosphorylation site domain to which it belongs, are highly selective substrates for PKC: their phosphorylation is not significantly catalysed by other kinases such as cyclic AMP- and cyclic GMP-dependent protein kinases, or phosphorylase kinase or the calmodulin-dependent protein kinases I, II and III [22,26–28]. Moreover, the substrate peptide is equally phosphorylated by the several PKC isoforms [27]. Isolated native membranes were suspended in the assay buffer (consisting of 50 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 1 μM CaCl₂, 100 μM sodium vanadate, 100 μM sodium pyrophosphate, 1 mM sodium fluoride and 100 μM PMSF) using an Eppendorf digital pipette and vigorously vortexed to get a homogeneous suspension. The reaction mixture (100 μl) consisted of 4–6 μg of membrane protein, 75 μM peptide substrate, 50 mM Tris/HCl buffer, pH 7.5, 5 mM MgCl₂, 1 μM CaCl₂, 100 μM sodium vanadate, 100 μM sodium pyrophosphate, 1 mM sodium fluoride, 100 μM PMSF and 50 μM [γ - ^{32}P]ATP (220 c.p.m./pmol). The reaction was carried out as described above for the conventional assay. In all cases PKC activity is expressed as the amount of ^{32}P (c.p.m.) incorporated into the peptide substrate.

^{32}P -labelling of cells

WEHI-231 cells ($4 \times 10^7/4\text{ ml}$) were incubated with $^{32}\text{P}_i$ (200 μCi) in phosphate-deficient (10 % of normal), serum-free medium for 4 h at 37 °C to label the intracellular ATP pool. The cells ($10^7/\text{ml}$) were then incubated with various agents (see Figure legends) for 10 min at 37 °C. They were then rapidly sedimented, solubilized in 50 mM Tris/HCl buffer, pH 7.5, containing 0.1 % Triton X-100, 100 μM sodium vanadate, 100 μM sodium pyrophosphate, 1 mM sodium fluoride and 100 μM PMSF. The cell lysate was clarified in a microfuge and proteins (30 μg) were separated on an SDS/10 % (w/v) polyacrylamide gel [30]. Phosphorylation of the PKC-specific substrate, the 85 kDa MARCKS protein, was visualized by autoradiography using Kodak XAR film.

Immunoblotting of PKC

Immunoblotting was performed according to Towbin et al. [31]. Proteins from cytosol and solubilized membranes were separated on SDS/10 % polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. The nitrocellulose membrane was then incubated with either a polyclonal pan-PKC rabbit antibody or isozyme-specific rabbit antibodies (1 $\mu\text{g}/\text{ml}$)

overnight at room temperature. The PKC-antibody complexes were visualized with an alkaline-phosphatase-conjugated goat anti-(rabbit IgG). To determine the specificity of the isozyme-specific antibodies towards PKC, the antibodies were pre-incubated for 30 min at room temperature with the corresponding isozyme-specific peptides against which they were raised before incubating with nitrocellulose papers.

Total protein concentration determination

Protein contents were measured according to Bradford [32].

RESULTS AND DISCUSSION

It has been reported [9,33] that EGF and FGF stimulate the phosphorylation of PKC-specific substrates such as the 80–85 kDa MARCKS protein in 3T3-L1 cells without a detectable translocation of cytosolic PKCs to membranes. To find out whether this response is attributable to changes in the activity of PKCs already associated (i.e. prior to stimulation) with the membranes, 3T3-L1 cells were exposed to EGF (10 ng/ml), FGF (100 ng/ml) or PMA (1 μ M) for 10 min, and membrane PKC activity was measured by both the conventional and the direct assays. For the conventional assay, isolated native cell membranes were solubilized in Tris/HCl buffer, pH 7.5, containing 2 mM EGTA, 0.5 mM EDTA and 0.1 % Triton X-100, and the total PKC in the membrane extract was measured as described in Materials and methods section. Treating the cells with PMA strongly increased total PKC activity in membrane extracts and dramatically reduced cytosolic enzyme activity, suggesting translocation of cytosolic PKCs to membranes (Table 1). However, neither EGF nor FGF caused a detectable redistribution of cytosolic PKCs to membranes (Table 1), which was in agreement with the previous report [9]. By contrast, when PKC activity was measured in intact native membranes using the direct assay (i.e. without treating the membranes with Ca^{2+} chelators or solubilizing them and reconstituting their PKCs in artificial membranes), both EGF and FGF were found to have triggered a 1.8-fold increase in membrane PKC activity despite there having been no detectable translocation of cytosolic PKCs (Table 1). It was unlikely that the increases in PKC activity were attributable to post-translational modification of pre-existing

Table 1 Stimulation of membrane PKC activity without translocation in intact 3T3-L1 cells

Cells, grown to confluence in 90% DMEM and 10% fetal calf serum, were washed twice in serum-free DMEM and maintained in that medium for 4 h before treatment without (control) or with 10 ng/ml EGF, 100 ng/ml FGF or 1 μ M PMA for 10 min at 37 °C. After treatment, cells were immediately placed on ice, washed with ice-cold PBS and harvested by scraping into PBS. PKC activity in isolated membranes was measured by the direct method in the presence and absence of 1 μ M PMA in the reaction mixture and by the conventional assay as described in the Materials and methods section. Cytosolic PKC was measured by the conventional method. PKC activity is expressed as pmol of ^{32}P incorporated into the peptide substrate/min per mg of membrane or cytosolic protein. Data represent means $\pm$ S.D. of 3–5 experiments performed in triplicate.

Treatment	Direct assay		Conventional assay	
	Membranes	Membranes + PMA	Membrane extract	Cytosol
Control	203.8 $\pm$ 31.5	412.7 $\pm$ 103.2	390.7 $\pm$ 49.2	187.1 $\pm$ 27.9
EGF	364.7 $\pm$ 28.2	448.0 $\pm$ 77.2	444.1 $\pm$ 71.5	213.5 $\pm$ 8.2
FGF	350.2 $\pm$ 8.2	432.2 $\pm$ 52.4	447.4 $\pm$ 52.5	205.1 $\pm$ 16.7
PMA	598.5 $\pm$ 107.5	575.7 $\pm$ 126.7	756.8 $\pm$ 63.6	66.6 $\pm$ 15.2

Table 2 Subcellular distribution of PKCs

Washed cells were hypotonically lysed in the absence of any Ca^{2+} chelating agents, and nuclear, membrane and cytosol fractions were separated. The nuclear and membrane fractions were solubilized and the distribution of PKCs in all three fractions was determined by the conventional method as described in the Materials and methods section. PKC activity recovered in subcellular fractions was 85–95% of that in the cell homogenate. The distribution of enzyme activity is expressed as the percentage of total recovered activity.

Cell fraction	Activity (% of total cellular PKC activity)		
	Cell type ... 3T3-L1	WEHI 231	Platelets
Membranes	50.4	53.8	45.9
Cytosol	16.5	35.5	31.5
Crude nuclei	33.1	10.7	22.5*

* Represents activity in cell debris.

membrane PKCs, because such modifications leading to enzyme activation would be detected by the conventional assay [11,13].

These results were consistent with EGF and FGF having stimulated a pool of PKCs already associated with the native membranes of 3T3-L1 cells. Because the stimutable PKCs were already membrane-associated, it was likely that the phospholipid and Ca^{2+} (for Ca^{2+} -dependent PKC) requirements of the enzymes would have been satisfied and that the only limiting factor for PKC activation would be the physiological activator, DAG. Thus, if an activatable pool of membrane PKCs were present under these conditions, a DAG analogue such as PMA would be expected to stimulate this PKC pool when added to isolated membranes during the reaction. Indeed, 1 μ M PMA added to the reaction mixture increased the PKC activity $\sim$ 2.2-fold in native membranes from unstimulated 3T3-L1 cells, but it did not significantly affect the already elevated activity in native membranes from growth-factor-stimulated cells (Table 1).

These results indicated that the growth factors must have stimulated most of the activatable pool of PKCs resident in the native membranes *in vivo* and that membranes isolated from these cells had very little activatable PKC to respond to PMA added to the reaction mixture. Thus 3T3-L1 cells appeared to have $\sim$ 50% of their membrane PKCs in a stimutable state, and both EGF and FGF increased PKC activity in these cells, not by translocation, but by activating a large fraction ($\sim$ 80%) of this resident membrane pool. This EGF- and FGF-induced increase in PKC activity of native membranes would have represented the activation of a substantial fraction of the cells' complement of PKCs because, as in agreement with a previous report [9], only 15–20% of the unstimulated 3T3-L1 cells' total PKCs was cytosolic, whereas 50% was associated with membranes (Table 2).

The presence of a PMA-responsive pool of PKCs in native membranes was not unique to 3T3-L1 cells. As shown in Table 3, the basal PKC activity in membranes isolated from various cells and tissues increased 1.7–2.3-fold when 1 μ M PMA was added to the reaction mixture. Similar increases in PKC activity were obtained when the native membranes were treated with 100 μ M OAG, a DAG, instead of 1 μ M PMA (Table 4). Dose dependence studies revealed that as little as 10 nM PMA or 10 μ M OAG was sufficient to stimulate PKCs resident in the native membranes (Figure 1), indicating that the increases in membrane PKC activity at high concentrations of PMA (1 μ M) or OAG (100 μ M) were not caused by any non-specific membrane perturbations. Another indication that the stimutable pool of

Table 3 Presence of activatable PKCs in membranes isolated from various cells and tissues

Membranes from cells and tissues were prepared and PKC activity in isolated membranes was determined by the direct method in the presence and absence of $1 \mu\text{M}$ PMA in the reaction mixture as described in the Materials and methods section. Data represent means $\pm$ S.D. of 3–5 experiments performed in triplicate. Numbers in parentheses indicate the ratio of total to active membrane PKCs.

Cells	PKC activity (pmol/min per mg of protein)	
	Membranes (Active)	Membranes + PMA (Total)
S40 T-lymphoma	191.6 $\pm$ 3.8	350.0 $\pm$ 33.3 (1.8)
cyc — S49 T-lymphoma	264.1 $\pm$ 26.5	573.5 $\pm$ 70.9 (2.2)
WEHI 231 B-lymphoma	522.3 $\pm$ 67.5	1000.0 $\pm$ 105.3 (1.9)
CTLL-2 T-lymphoma	297.0 $\pm$ 24.5	539.6 $\pm$ 40.1 (1.8)
Murine erythroleukaemia	433.5 $\pm$ 48.8	742.1 $\pm$ 47.3 (1.7)
Human erythroleukaemia	209.6 $\pm$ 43.9	418.6 $\pm$ 30.5 (2.0)
HL-60 human myeloid	552.8 $\pm$ 52.4	937.7 $\pm$ 67.5 (1.7)
3T3-L1 fibroblasts	209.5 $\pm$ 19.3	377.2 $\pm$ 40.1 (1.8)
NRK rat kidney	704.5 $\pm$ 14.5	664.8 $\pm$ 6.8 (0.9)
Balb/MK2 keratinocytes	640.6 $\pm$ 81.3	599.7 $\pm$ 87.7 (0.9)
Rat thymus	382.9 $\pm$ 5.0	837.4 $\pm$ 80.1 (2.2)
Rat spleen	947.9 $\pm$ 68.2	1693.6 $\pm$ 194.5 (1.8)
Rat platelets	7330.6 $\pm$ 734.5	16570.2 $\pm$ 930.6 (2.3)
Rat brain (whole)	713.8 $\pm$ 92.9	1439.3 $\pm$ 90.5 (2.0)
Rat brain (hippocampus)	239.4 $\pm$ 15.1	250.2 $\pm$ 18.7 (1.0)

Table 4 Effect of DAG on PKC activity in isolated membranes

Membranes from WEHI cells, rat brain and platelets were prepared and PKC activity was measured by the direct method in the presence and absence of $100 \mu\text{M}$ OAG.

Cell type	PKC activity (pmol/min per mg of protein)	
	Membranes	Membranes + DAG
WEHI B-lymphoma	564.4 $\pm$ 47	1150.5 $\pm$ 21.5
Rat brain	451.0 $\pm$ 57.3	782.5 $\pm$ 82.0
Rat platelets	6986.7 $\pm$ 288.9	15569.4 $\pm$ 305.5

PKCs in the native membranes was not a non-specific effect of PMA was the inability of PMA to increase PKC activity in native membranes isolated from cells such as Balb/MK-2 mouse keratinocytes, NRK rat kidney cells, and rat hippocampal cells (Table 3).

Several lines of evidence indicated that the activatable pool of membrane PKCs was not a non-physiological product of the manipulations and the unconventional lysis conditions (i.e. the absence of metal ion chelators and the presence of Ca^{2+} and high concentrations of Mg^{2+} ions in the lysis medium). For example, adding DAG kinase inhibitors I and II to the lysis buffer and/or the reaction mixture failed to reduce the level of PMA-stimulatable PKCs in the isolated native membranes (results not shown), indicating that the stimulatable membrane PKCs pool was not produced by a DAG-kinase-mediated depletion of PKC-activating DAGs during cell lysis and membrane preparation. In addition, the stimulatable membrane PKCs pool was not a result of the Ca^{2+} or Mg^{2+} -dependent association of cytosolic PKCs with the membranes during cell fractionation, because lysing cells in media containing Ca^{2+} concentrations ranging from $0.05 \mu\text{M}$ to $5 \mu\text{M}$ with or without 5 mM MgCl_2 did not significantly affect the ratio of active/total PKC, a measure of

Figure 1 Dose-dependent effect of PMA and OAG on the activation of PKCs in isolated native membranes

Membranes from untreated (a) WEHI-231 cells or (b) rat platelets were prepared and PKC activity was measured in the presence of indicated concentrations of PMA (a) or OAG (b) in the reaction mixture as described in the legend for Tables 3 and 4. Data represent mean of two independent experiments done in triplicate.

Table 5 Effect of various concentrations of Ca^{2+} in lysis medium on activatable PKCs in membranes

Washed rat platelets were hypotonically lysed in medium containing indicated concentrations of Ca^{2+} (Ca^{2+} concentrations were determined by atomic emission spectroscopy). Membranes were isolated, washed twice with 50 mM Tris/HCl, pH 7.5, and suspended in the assay buffer containing $1 \mu\text{M}$ Ca^{2+} as described in the Materials and methods section. PKC activity in isolated membranes was determined in the presence and absence of $1 \mu\text{M}$ PMA in the reaction mixture as described in the Materials and methods section. Data represent means $\pm$ S.D. of 3 experiments performed in triplicate. Numbers in parentheses indicate the ratio of total to active membrane PKC.

Lysis medium	$[\text{Ca}^{2+}]$ (μM)	PKC activity (pmol/min per mg of protein)	
		Membranes (Active)	Membranes + PMA (Total)
Deionized H_2O	< 0.05	3.4 $\pm$ 0.1	7.6 $\pm$ 0.6 (2.3)
1 mM NaHCO_3	0.09	3.5 $\pm$ 0.1	7.3 $\pm$ 0.3 (2.1)
1 mM NaHCO_3 , 5 mM MgCl_2	4.40	2.8 $\pm$ 0.1	5.5 $\pm$ 0.1 (2.0)
1 mM NaHCO_3 , 5 μM CaCl_2	5.00	3.9 $\pm$ 0.2	8.9 $\pm$ 0.7 (2.3)

activatable membrane PKCs (Table 5). Similar results were obtained when PKC activity was measured in membranes isolated from WEHI cells lysed in medium containing different Ca^{2+} and Mg^{2+} concentrations (results not shown). Finally, neither total PKC activity nor the ratio of active/inactive PKCs was significantly affected by repeated washing of isolated cell membranes or by preincubating these membranes at 27°C for 10 min before the assay, indicating that both active and stimulatable PKCs associated with the native membranes were stable pools. Thus, many, if not all, cells appeared to maintain a substantial pool of stimulatable or activatable PKCs in their membranes.

The physiological significance of an activatable pool of membrane PKCs was studied in more detail with IL-2 and lymphoid cells. The role of PKCs in IL-2 signalling has been controversial in that some studies [3,34], but not others [10,35,36], have shown that IL-2 causes a rapid redistribution of cytosolic PKCs to

Figure 2 Effects of IL-2 on (a) membrane PKC activity, (b, c) the subcellular distribution of PKCs and (d) the phosphorylation of endogenous 85 kDa protein

(a) CTLL-2 cells, WEHI-231 B-lymphoma cells, and S49 T-lymphoma cells were washed and maintained in serum-free RPMI medium (25×10^6 cells/2 ml) for 4 h and then left untreated ($\square$), or treated with 80 units/ml IL-2 ($\blacksquare$) or $1 \mu\text{M}$ PMA ($\hatchedbox$) for 10 min at 37°C . Cells were then fractionated and membrane PKC activity was measured by the direct method and cytosolic PKC levels by the conventional method (see the Materials and methods section). Double-headed arrows (a) indicate the total PKC activity (active + stimutable) in membranes from untreated cells ($\square$) as determined by measuring enzyme activity in the presence of $1 \mu\text{M}$ PMA in the reaction mixture (see Table 1). The difference between total (dotted horizontal lines) and active membrane PKC ($\square$) represents the amount of stimutable PKCs in membranes of unstimulated cells. Data represent means $\pm$ S.D. of 3 experiments performed in triplicate. (b) Solubilized membrane (10 μg) (lanes a–c) and 20 μg of cytosol (lanes d–f) from untreated (lanes a and d), IL-2-treated (lanes b and e), and PMA-treated (lanes c and f) WEHI-231 cells were subjected to immunoblotting with pan-PKC antibody as described in the Materials and methods section. (c) Membrane (lanes a and b) and cytosolic (lanes c and d) fractions from untreated (lanes a and c) and IL-2-treated (lanes b and d) WEHI cells were probed with either a mixture of the α -, β -, γ -, δ -, ϵ - and ζ -isoform-specific PKC antibodies (M) or with α -, β -, δ - and ζ -antibodies individually. (d) WEHI-231 cells were prelabelled with $^{32}\text{P}_i$ as described in the Materials and methods section. The labelled cells ($10 \times 10^6/\text{ml}$) were then either left untreated (lane a) or treated with 80 units of IL-2 (lane b) or $1 \mu\text{M}$ PMA (lane c) for 10 min. The cells were then rapidly sedimented, solubilized, and 85 kDa protein phosphorylation was visualized by autoradiography.

membranes. Clearly, the activation of an IL-2 responsive pool of membrane PKCs which could not be detected by the conventional activity assay might at least partially explain these contradictions. Approx. 40–50 % of the PKCs in isolated native membranes of unstimulated CTLL-2 T-lymphoma cells, S49T-lymphoma cells and WEHI B-lymphoma cells was PMA-stimulatable (Table 3, Figure 2a). A 10 min exposure of these cells to IL-2 triggered a 1.7–2.0-fold increase in membrane PKC activity without causing a detectable reduction in cytosolic PKC activity (Figure 2a), indicating that this cytokine stimulated membrane PKC activity without translocating cytosolic enzymes. By contrast, exposing intact cells to PMA rapidly increased membrane-associated, and decreased cytosolic, PKC activity (Figure 2a), a characteristic feature of enzyme translocation. Therefore, the observed 2.5-fold increase in membrane PKC activity in PMA-treated WEHI cells (Figure 2a) arose from the combined result of the stimulation of resident membrane PKCs and the translocation of cytosolic enzymes. These observations (i.e. distribution of PKCs) were confirmed by immunoblot analysis using a pan-PKC antibody which revealed that, whereas PMA caused a dramatic redistribution of immunodetectable cytosolic PKCs to membranes in WEHI cells, IL-2 treatment did not (Figure 2b). WEHI cells

express at least four PKC isoforms, α -, β -, δ - and ζ - (Figure 3). It is possible that the IL-2-induced increase in membrane PKC activity might have been due to the selective redistribution of a particular PKC isoform(s) which was not detected by the pan-PKC antibody. However, there was no detectable redistribution of any of these PKC isoforms when cytosol and membranes from untreated and IL-2-treated WEHI cells were probed with either a mixture of α -, β -, γ -, δ -, ϵ - and ζ -isoform-specific antibodies or individual α -, β -, δ - or ζ -specific antibodies (Figure 2c). Thus, both activity measurements and immunoblot analyses indicated that neither a bulk nor a selective translocation of cytosolic PKCs was responsible for the increased PKC activity observed in native membranes from IL-2-treated WEHI cells.

Perhaps the most compelling evidence that the observed increases in PKC activity in native membranes isolated from IL-2-treated cells accurately reflected the changes in membrane PKC activity that actually occurred in intact cells comes from the effect IL-2 had on the phosphorylation of endogenous PKC-selective substrates. As shown in Figure 2(d), IL-2 rapidly stimulated the phosphorylation of a PKC-specific substrate, the 80–85 kDa MARCKS protein [28,29] in intact WEHI cells, although the cytokine did not cause the translocation of cytosolic

Figure 3 Immunoblot analysis of subcellular distribution of PKCs in WEHI-231 cells

Membrane and cytosolic fractions from untreated WEHI-231 cells were prepared as described in the Materials and methods section. Proteins (equivalent to 3×10^6 cells) in cytosolic (C) and membrane (M) fractions were separated by SDS/PAGE and subjected to immunoblot analysis using pan-specific as well as isoform-specific PKC antibodies as described in the Materials and methods section. Crude rat-brain extract (B) was used as a standard to detect immunoreactivity of isoform-specific PKC antibodies. Brain samples were also probed with antibodies that were pre-incubated with the corresponding isozyme-specific peptides against which they were raised to determine antibody specificity towards PKC (B + P).

PKCs. Therefore the measurements of PKC activities by the direct assay truly mirrored the changes in membrane-associated PKC activities occurring in intact cells. A 1.7–2.0-fold increase in membrane PKC activity induced by IL-2 represented the stimulation of a significant fraction of WEHI cells' complement of PKCs, because, as with 3T3-L1 cells, a large fraction ($\geq 50\%$) of the cellular PKCs was associated with native membranes from WEHI cells (Table 2). This distribution based on activity measurements was confirmed by immunoblot analyses using pan- and isoform-specific PKC antibodies, which revealed that the amount of PKCs in the native membrane fraction was higher than that in the cytosol fraction (Figure 3).

The question as to how growth factors might activate membrane PKCs independently from translocation of cytosolic PKCs was then addressed. The translocation of PKCs appears to be caused by the combined actions of physiologically small amounts of DAGs and the Ca^{2+} -mobilizing *myo*-inositol 1,4,5-trisphosphate (IP_3) generated by the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) [37–44]. However, neither EGF nor IL-2 stimulated intracellular Ca^{2+} surges in 3T3-L1 or WEHI cells preloaded with the Ca^{2+} -specific fluoroprobe Fura-2 (results not shown), which agrees with previous reports that these agonists may not generate Ca^{2+} -mobilizing IP_3 from polyphosphoinositides in 3T3 fibroblasts and lymphoma cells [45–49]. Thus, the hydrolysis of phospholipids other than PIP_2 , such as phosphatidylcholine, may produce enough DAGs to activate membrane-associated PKCs but not the IP_3 -induced Ca^{2+} surge needed to translocate cytosolic enzymes to the membrane. Indeed, exposing WEHI cells to low levels of OAG (5–10 μM) for 10 min activated virtually all of their activatable membrane PKCs without triggering a detectable translocation of cytosolic enzyme to membranes (Figure 1a). These low concentrations of OAG, like IL-2, stimulated the phosphorylation of the 85 kDa PKC-specific substrate in intact cells (Figure 4d), indicating that the stimulation of the activatable pool of membrane PKCs could

trigger downstream events. OAG could stimulate the translocation of cytosolic PKCs, but only at much higher concentrations (Figure 4a). The effect of PMA on WEHI cells was similar to that of OAG in that low concentrations ($< 10 \text{ nM}$) stimulated membrane PKCs without a detectable translocation of cytosolic enzymes but higher concentrations were needed to stimulate translocation of cytosolic PKCs (Figure 4b). Finally, exogenous phospholipase C (PLC) has been used to generate DAGs in various cells [50–52], and treating WEHI cells with lower concentrations of a phosphatidylcholine-specific PLC selectively stimulated PKCs already resident in membranes without translocating cytosolic enzymes, whereas higher concentrations of PLC stimulated translocation (Figure 4c). Thus DAGs produced from the breakdown of phospholipids other than PIP_2 can stimulate membrane-associated PKCs without the translocation of cytosolic enzymes. Collectively, these results strongly support the possibility that PKCs may remain associated with the membranes in an inactive but 'primed' state and that this membrane-associated PKC pool can be activated by agonists that generate PKC-activating second messengers such as DAGs [18,53].

Finally, we attempted a preliminary characterization of the association of the activatable pool of PKCs with the cell membranes. There appears to be two kinds of association of PKCs with membranes [15,54–56]: peripherally bound PKCs, which are readily stripped off the membranes by Ca^{2+} -chelating agents such as EGTA; and 'membrane-inserted PKCs', which are tightly bound to the membrane and cannot be removed by EGTA. *In vitro* studies using phospholipid vesicles suggest that membrane-inserted PKCs are constitutively active [15,54–56]. However, the physiological significance of peripherally bound membrane PKC is unknown because it is normally removed from the membranes during lysis and membrane preparation by the EDTA and EGTA used in the conventional PKC assay [20,21]. Thus the conventional assay only measures membrane-inserted PKC. By contrast, the direct PKC method [22] does not use chelating agents and, therefore, gives a measure of the total active PKC in both the tightly and loosely bound states. Because the direct method also readily detects activatable PKCs associated with either of these pools (by adding PMA), it can be used to determine the ratio of active/inactive (i.e. PMA-stimulatable) PKCs in both the peripherally and tightly bound states. In unstimulated WEHI cells, $\sim 50\%$ of the cellular PKCs is membrane-associated (Table 2, Figure 3), and up to 50% of this membrane enzyme is inactive (PMA-stimulatable) (Figure 2a, Table 3). As seen in Table 6, treating membranes from unstimulated WEHI cells with EGTA reduced the basal PKC activity by 70–80%, indicating that only ~ 20 –30% of the total active membrane PKCs was membrane-inserted whereas most of the active enzyme was peripherally bound. The residual PKCs activity in EGTA-treated membranes was not increased further by adding 1 μM PMA to the reaction mixture (Table 6), indicating that membrane-inserted PKC was fully active, and that the PMA-activatable PKCs in control membranes must have been EGTA-extractable, and hence peripherally bound. Therefore, membrane-inserted PKC is constitutively active in cellular membranes, as previously indicated by results of experiments using phospholipid vesicles [15,54–56]. The present results indicate that EGTA-sensitive, peripherally bound PKC occurs in both active and inactive (PMA-stimulatable) forms on some cell membranes. Thus PKCs appear to exist in three states in WEHI and perhaps other cell membranes: (a) active, membrane-inserted; (b) active, peripherally bound; and (c) inactive, peripherally bound. The increase in membrane PKC activity induced by EGF and FGF in 3T3-L1 cells (Table 1), and by IL-2 in WEHI cells (Figure 2a),

Figure 4 Low levels of OAG, PMA and PLC stimulate membrane PKCs without translocation in intact cells

WEHI-231 cells ($25 \times 10^6/2$ ml) were exposed to the indicated concentrations of (a) OAG, (b) PMA or (c) phosphatidylcholine-specific PLC for 10 min at 37 °C. The cells were then fractionated and membrane-PKC activity (●) was measured by the direct assay and cytosol PKC (○) by the conventional assay. Membrane PKC activity in untreated cells was measured in the presence (total PKC activity, shown by horizontal dotted line) and the absence (active PKC) of PMA in the reaction mixture. The difference between total and active PKCs, denoted by the double-headed arrow, gives a measure of stimutable PKCs in the membranes of untreated cells. Data represent means $\pm$ S.D. of 2–3 independent experiments performed in triplicate. (d) The effect of (a) no additions, (b) 5 μ M OAG, (c) 10 μ M OAG and (d) 1 μ M PMA on the phosphorylation of endogenous 85 kDa protein, measured as described in the legend to Figure 2(c).

Table 6 Effect of EGTA on active and stimutable PKCs in native membranes from WEHI cells

Membranes from unstimulated WEHI cells were suspended in assay buffer (control membranes) or the same buffer containing 5 mM EGTA and 0.5 mM EDTA (EGTA membranes), and incubated on ice for 3 min with intermittent vortexing. Membranes were pelleted by centrifugation at 100 000 *g* for 10 min, and washed three times in assay buffer. PKC activity in the washed membranes was measured by the direct method in the presence and absence of 1 μ M PMA in the reaction mixture.

Treatment	PKC activity (pmol/min per mg of protein)	
	Control membranes	EGTA membranes
No PMA	689.2 $\pm$ 60.1	200.4 $\pm$ 12.3
PMA	1169.1 $\pm$ 81.8	218.0 $\pm$ 13.6

most probably resulted from the stimulation of these inactive, EGTA-sensitive, peripherally bound PKCs.

In conclusion, the results of this study indicate the presence of an activatable pool of PKCs anchored in an EGTA-sensitive manner to the membranes of some cells. These results suggest that a small increase in DAGs from the receptor-induced hydrolysis of membrane phospholipids can activate these PKCs without necessarily triggering the translocation of more PKCs from the cytosol to the membrane, and that these DAG-activated

membrane PKCs can then stimulate downstream events such as the phosphorylation of target proteins. Although activation of resident inactive membrane PKCs has been proposed to be the probable mechanism by which PMA (or DAGs) and physiological agents stimulate membrane PKC activity in the absence of translocation, we do not rule out other possible mechanisms such as an increase in intrinsic specific activity of resident membrane PKCs resulting from an as yet unidentified modification of enzymes in response to receptor stimulation.

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