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Translocation of lipocortin (annexin) 1 to the membrane of U937 cells induced by phorbol ester, but not by dexamethasone

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1 Induction of lipocortin 1 secretion by dexamethasone has been demonstrated, although the secretory mechanism is still unknown. We have studied the effects of 12-tetradecanoyl phorbol 13-acetate (TPA) and/or dexamethasone on the expression, translocation, and secretion of lipocortin 1 in U937 cells.

2 The expression of lipocortin 1 and its mRNA increased during TPA-induced differentiation of U937 cells to a maximum of 1.9 fold and 8.2 fold, respectively, after 48 h. Both the protein and the mRNA levels decreased after 48 h.

3 TPA caused the translocation of lipocortin 1 from the cytosol to the membrane of U937 cells in a time-dependent manner, as determined by Western blot analysis. The translocation was concurrent with the differentiation of the cells. After 48 h of TPA treatment, $82.6 \pm 6.5\%$ of lipocortin 1 was present in the membrane fraction compared to $41.6 \pm 1.7\%$ in untreated cells.

4 The amount of lipocortin 1 that was externally bound (associated) with the membrane increased to 3.2 fold as the cytosol to membrane translocation of lipocortin 1 increased.

5 Dexamethasone decreased the externally bound lipocortin 1, but had no effect on the cytosol to membrane translocation.

6 This offers a model system with which the function and the secretion mechanism of lipocortin 1 can be studied. Our data is consistent with the hypothesis that the secretory mechanism is through an unknown pathway, involving the translocation of lipocortin 1 from the cytosol to the internal membranes, and then, its secretion to the external membrane.

Keywords: Lipocortin-1; membrane translocation; 12-tetradecanoyl phorbol 13-acetate; dexamethasone; U937 cell

Introduction

Lipocortins (also called annexins) are structurally related, calcium-dependent phospholipid-binding proteins (Glenny *et al.*, 1987; Ando *et al.*, 1989; Crompton *et al.*, 1988; Flower, 1988). Human lipocortin 1 has been shown to be a substrate for epidermal growth factor receptor kinase (Pepinski & Sinclair, 1986; Haigler *et al.*, 1987; Futter *et al.*, 1993) and protein kinase C (Varticovski *et al.*, 1988; Schlaepfer & Haigler, 1988; Oudinet *et al.*, 1993). Lipocortin 1 has also been implicated in the regulation of signal transduction pathways involving phospholipase A_2 inhibition (Kim *et al.*, 1994).

Recently, lipocortin 1 has been proposed to act as a mediator of some of the actions of glucocorticoids *in vivo* (Goulding & Guyre, 1993; Perretti & Flower, 1993; Duncan *et al.*, 1993; Peers *et al.*, 1993). A favorable hypothesis is that lipocortin 1 is secreted upon glucocorticoid treatment, and then binds to the putative receptor, which generates autocrine signals (Perretti & Flower, 1993; Duncan *et al.*, 1993). Since lipocortin 1 is a cytosolic protein, and lacks a hydrophobic signal peptide (Frey *et al.*, 1991), its secretion from the cell is likely to involve an unconventional mechanism.

The effects of dexamethasone on the expression and secretion of lipocortin 1 have been controversial in studies with various cells, including macrophages, neutrophils, fibroblasts, and lymphocytes (Blackwell, 1983; Errasfa *et al.*, 1985; Fradin *et al.*, 1988; Bronnegard *et al.*, 1988). The U937 cell line, an immature human monocytic line that can be induced to differentiate into macrophage-like cells by treatment with 12-tetradecanoyl phorbol 13-acetate (TPA), provides a model system to study the function of lipocortin 1 (Hass *et al.*, 1989; Solito *et al.*, 1991). Studies by Solito and coworkers have indicated that dexamethasone induces mRNA expression and release of lipocortin 1 in differentiated, but not in undifferentiated U937 cells (Solito *et al.*, 1991). These authors have also shown, by flow cytometry, that membrane-bound lipocortin 1 is increased by dexamethasone (Solito *et al.*, 1994). In contrast, other studies have indicated that both the mRNA and the protein levels of lipocortin 1 in U937 cells increase during TPA-induced differentiation, but dexamethasone has no effect on these parameters (Isacke *et al.*, 1989). Despite the controversy, these studies agree on the point that lipocortin 1 is a secreted protein.

In an attempt to understand better the function of lipocortin 1 and the mechanism of its secretion, we have examined the cytosol to membrane translocation of lipocortin 1 in U937 cells. We were interested in determining: (1) whether the cytosol to membrane translocation occurs before the secretion of lipocortin 1, and (2) the effects of TPA and dexamethasone on the translocation.

Methods

Culture of U937 cells

U937 cells were cultured in RPMI 1640 medium (Gibco BRL, U.S.A.) containing 10% (v/v) foetal bovine serum (FBS) (Gibco BRL). To induce differentiation, U937 cells were plated at a density of 4×10^5 ml^{-1} in medium containing 25 ng ml^{-1} TPA (Sigma Chemical Co., St. Louis, MO, U.S.A.). Differentiation status was monitored by morphological changes and division rates. Dexamethasone (Sigma) was dissolved in dimethyl sulphoxide (Sigma) and was added to the culture media when needed.

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Non-ionic detergent solubilization of U937 cells

The cells were washed once with ice-cold phosphate-buffered saline (PBS: NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 8 mM, KH_2PO_4 1.5 mM) and were collected either by centrifugation at 1,500 *g* for 5 min at 4°C or with a rubber policeman. The cells were resuspended in Triton X-100 lysis buffer (NaCl 100 mM, Tris 50 mM, pH 7.5, 0.5% Triton X-100) containing 1 mM phenylmethylsulphonyl fluoride (PMSF, Sigma). The suspension was incubated for 30 min at 4°C. The nuclei were removed by centrifugation at 6,500 *g* for 15 min at 4°C.

Preparation of the cytosolic and membrane fractions

The collected cells were washed with PBS, resuspended in 'fractionation buffer' (10 mM Tris-HCl, pH 7.6, 10% glycerol, 5 mM 2-mecaptoethanol, 0.1 mM PMSF), and disrupted by sonication. The cellular organelles were removed by centrifugation at 6,500 *g* for 20 min at 4°C. The cytosolic and the membrane fractions were separated by centrifugation at 100,000 *g* for 1 h in a Beckman TL100 ultracentrifuge at 20°C. The pelleted membrane fraction was resuspended in the 'fractionation buffer'.

To extract the lipocortin 1 that was externally bound (associated) to the plasma membrane, PBS-washed cells were suspended in 'fractionation buffer' containing 10 mM EDTA, and were then incubated for 30 min at 4°C. The cells were removed by centrifugation at 1,500 *g* for 5 min at 4°C.

Western blot analysis

Procedures for SDS–PAGE (polyacrylamide gel electrophoresis) and Western blotting were essentially as described (Laemmli, 1970), and according to the instructions from Pierce (Rockford, IL, U.S.A.). The protein concentration was determined using a protein assay kit (Bio-Rad Labs., Richmond, CA, U.S.A.). For SDS–PAGE, 30 μg of protein lysate was loaded in each lane. The rabbit anti-human lipocortin 1 polyclonal antibody was prepared using recombinant lipocortin 1 produced in *E. coli* (Huh *et al.*, 1990; Weng *et al.*, 1993). Immunodetection was performed using the anti-human lipocortin 1 antibody (1 : 1000 dilution) and mouse anti-rabbit immunoglobulin (1 : 10000 dilution, Pierce) conjugated to alkaline phosphatase with 5-bromo-4-chloro-3′-indolylphosphate-toluidine salt and nitro-blue tetrazolium chloride as substrates (Pierce). Band areas on the Western blot were evaluated with an image analyzer (Quantimet 570 Image Processing and Analysis System, Cambridge Instruments Ltd., Cambridge, U.K.).

Northern blot analysis

Total RNA was prepared from 5 to 10 million cells according to the methods described by Chomczynski and Sacchi (1987). Briefly, cells were lysed in 0.5 ml of guanidium thiocyanate (GT) buffer (guanidium thiocyanate 4 M, sodium citrate 25 mM (pH 7.0) and 0.72% (v/v) β-mercaptoethanol). To this lysate 50 μl of 2 M sodium acetate (pH 5.7), 0.5 ml of phenol, and 0.1 ml of chloroform-isoamyl alcohol (24 : 1) were serially added. The phases were mixed by vortexing and separated by centrifugation. The upper aqueous phase was transferred to a new tube and RNA was precipitated with equal volume of isopropanol. The pellet was resuspended in 0.15 ml of GT buffer and RNA was precipitated with equal volume of isopropanol. The pellet was washed with 100% ethanol, dried, and resuspended in RNase free water.

Aliquots of total RNA (30 μg) were denatured by heating, separated on a 1% formaldehyde/agarose gel, and then blotted onto a GeneScreen Plus nylon membrane (NEN Research Products, Boston, MA, U.S.A.). Details of Northern blot hybridization were according to the described methods (Sambrook *et al.*, 1989). The 1.4 kb BamHI fragment of the human lipocortin 1 cDNA (Huh *et al.*, 1990) and the EcoRI fragment of the mouse β-actin gene (from ATCC, MD, U.S.A.) were used as probes. Autoradiographs were evaluated with an image analyzer as described for the Western blot analysis and the data were normalized according to the β-actin signals. The Western and Northern blot data are presented as mean ± s.e. mean and analyzed by one-way ANOVA with Bonferroni post-test using a statistical program of StatView IV (StatView IV, Abacus Concepts, Inc., Berkely, CA, U.S.A.).

Results

Modulation of lipocortin 1 expression during U937 cell differentiation

To determine the change in lipocortin 1 expression during differentiation, U937 cells were cultured in the presence of 25 ng ml^{-1} of TPA for up to 72 h. Treatment with TPA caused a time-dependent morphological change in U937 cells. The cells became adherent to the substratum after about 24 h of treatment, and obtained a macrophage-like morphology after 48 h. The cells were harvested at 2, 6, 12, 24, 48 and 72 h, and then lipocortin 1 and its mRNA levels were determined by Western blot (Figure 1a) and Northern blot (Figure 1b) analyses, respectively. The lanes in Figure 1a and b were traced with an image analyzer, and the relative gray densities as compared to the control (0 h) are plotted in Figure 1c. Both lipocortin 1 protein and mRNA levels increased until 48 h, after which they decreased. The amounts of lipocortin 1 compared to the control at 2, 6, 12, 24, 48 and 72 h were 0.86 ± 0.04, 1.18 ± 0.04, 1.25 ± 0.03, 1.49 ± 0.02, 1.93 ± 0.27, and 1.27 ± 0.03 fold, respectively (Figure 1c). The levels of lipocortin 1 mRNA compared to the control at the corresponding time points were 1.0 ± 0.11, 1.24 ± 0.04, 1.69 ± 0.01, 2.49 ± 0.41, 8.20 ± 1.10 and 2.34 ± 0.96 fold, respectively (Figure 1c). These changes were significantly different from 0 h control ($P < 0.05$) except the mRNA value at 2 h point.

Lipocortin 1 translocates to the cell membrane upon TPA treatment

U937 cells were cultured in the absence or presence of TPA (25 ng ml^{-1}), and were harvested at 0, 0.25, 1, 6 and 24 h. The cells were sonicated in the 'fractionation buffer', and the cytosolic and the membrane fractions were separated by centrifugation, as described in the Methods. The amount of lipocortin 1 in each fraction was analyzed by SDS–PAGE and Western blot.

As shown in Figure 2, translocation of lipocortin 1 from the cytosolic fraction to the membrane fraction was not observed until 24 h of TPA treatment. The translocation of lipocortin 1 was concurrent with the time when the cells became adherent and started the morphological change to macrophages. A longer incubation with TPA (over 48 h) caused no additional change in the translocation of lipocortin 1 (see Figure 3, lanes 3 vs 5) ($P > 0.1$).

Effects of dexamethasone on the translocation of lipocortin 1

The effects of dexamethasone on the translocation of lipocortin 1 to the cell membrane were examined in three types of U937 cells, undifferentiated, differentiating, and fully differentiated. The undifferentiated U937 cells (5×10^5 ml^{-1}), and the cells previously treated with 25 ng ml^{-1} TPA for 24 h (differentiating) or 48 h (fully differentiated) were incubated for an additional 24 h in the presence or absence of 1 μM dexamethasone.

The results of quantitation of the 37 kDa (lipocortin 1) band by densitometric tracing are shown in Figure 3. Similar to the results shown in Figure 2, the cytosol to membrane translocation of lipocortin 1 was observed in TPA-treated cells (lanes 3–6). The fractions of lipocortin 1 in the membrane and

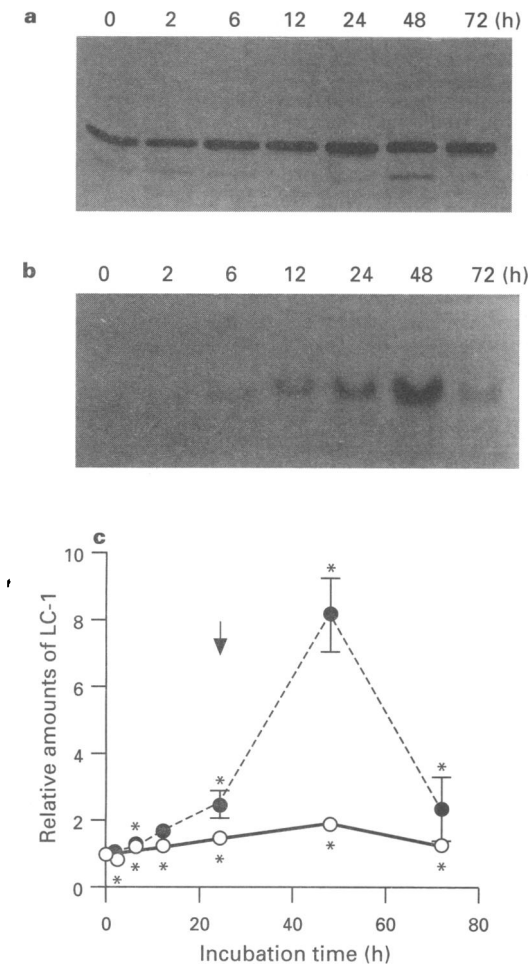

Figure 1 Effects of TPA-induced differentiation on lipocortin 1 expression in U937 cells. U937 cells (4×10^5 ml^{-1}) were cultured in RPMI 1640 containing 10% FBS and 25 ng ml^{-1} TPA. The cells were harvested at 0, 2, 6, 12, 24, 48 and 72 h, and non-ionic protein lysates and total RNAs were prepared. The lipocortin 1 protein and mRNA levels were determined by Western blot (a) and Northern blot (b) analyses, and were then quantitated with an image analyzer (c). (a) The same amount of protein lysate (30 μg) was loaded in each lane. The Western blot was probed with antisera to lipocortin 1 (LC-1). (b) Total RNA (30 μg per lane) was probed with lipocortin 1 cDNA. The intensity was normalized using β-actin as an internal control. (c) The data in (a) and (b) were quantitated with an image analyzer and plotted. (●) mRNA; (○) lipocortin 1 protein. The arrow shows the time point when most of the cells started to adhere to the substratum. The data shown are the average of three experiments. The values are mean ± s.e. mean where $*P<0.05$ vs value at 0 h by one-way ANOVA with Bonferroni post test.

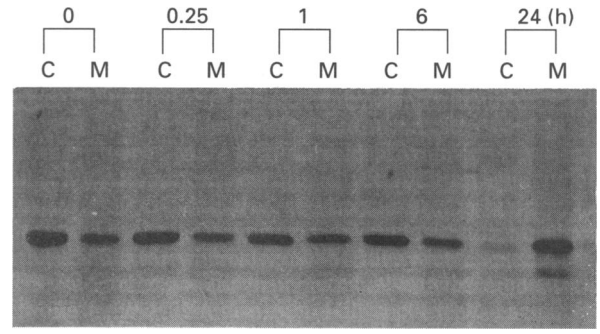

Figure 2 Translocation of lipocortin 1 during TPA-induced differentiation. Cells were cultured in the presence of 25 ng ml^{-1} TPA and were harvested at 0, 0.25, 1, 6 and 24 h. The membrane and the cytosolic fractions were prepared as described in the Methods. The cells were disrupted in the 'fractionation buffer' (10 mM Tris-HCl, pH 7.6, 10% glycerol, 5 mM 2-mercaptoethanol, 0.1 mM PMSF). The presence of lipocortin 1 in each sample was determined by Western blot as in Figure 1. The letters C and M represent the cytosolic and membrane fractions, respectively. The figure shows a representative blot from one of three experiments.

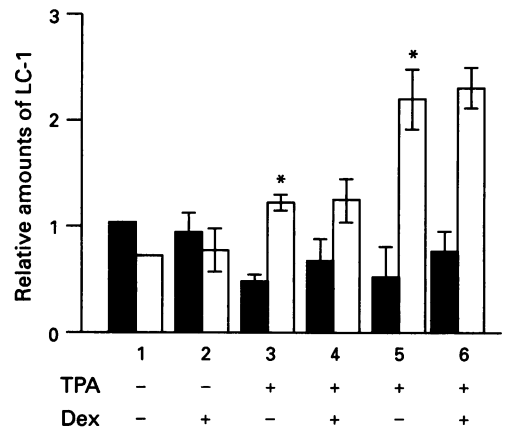

Figure 3 Effect of dexamethasone on the translocation of lipocortin 1 (LC-1). The undifferentiated U937 cells (5×10^5 ml^{-1}) (lanes 1, 2), and the cells previously treated with 25 ng ml^{-1} TPA for 24 h (differentiating, lanes 3, 4) or 48 h (fully differentiated, lanes 5, 6) were incubated for 24 h in the presence (lanes 2, 4, 6) or absence (lanes 1, 3, 5) of 1 μM dexamethasone (Dex). Details for lipocortin 1 analysis are as in Figures 1 and 2. The gray densities on the Western blot were quantitated with an image analyzer and relative values to the value of cytosolic fraction in TPA(−) Dex(−) cells were plotted. Open columns, membrane fractons; solid columns, cytosolic fractions. The data show an average of three analyses and represent mean ± s.e. mean where $*P<0.05$ vs value of TPA(−) by one-way ANOVA with Bonferroni post-test. The error bar in the membrane fraction of lane 1 does not show on the scale.

cytosol were calculated using the values shown in Figure 3. Lipocortin 1 in the membrane fraction increased from 41.6 ± 1.7% of the total in the undifferentiated (lane 1) to 70.6 ± 8.7% in differentiating (lane 3) and 82.6 ± 6.5% in fully differentiated (lane 5) cells ($P<0.05$). Dexamethasone had no effect on the translocation of lipocortin 1 in all three types of cells (open columns in lanes 1 vs 2, 3 vs 4, 5 vs 6) ($P>0.05$).

Effects of dexamethasone on the secretion of lipocortin 1

The membrane-bound lipocortin 1 in Figures 2 and 3 defines the total amount of lipocortin 1 bound to the internal membranes (endoplasmic reticulum, Golgi apparatus, and other vesicles) as well as to the internal and external surfaces of the plasma membrane. Experiments were designed to separate the lipocortin 1 bound to the external side of the plasma membrane from the internally bound lipocortin 1. The externally bound lipocortin 1 was analyzed using the same groups of cells as in Figure 3. Proteins externally bound to the plasma membrane were extracted from whole cells with the 'fractionation buffer' containing 10 mM EDTA, and were analyzed for lipocortin 1 by Western blot, as described in the Methods. Figure 4 shows the results of the densitometric analysis. The plasma membrane-bound lipocortin 1 increased markedly in TPA-induced U937 cells. The relative intensities of the lipocortin 1 bands compared to the control (undifferentiated cells, lane 1) in differentiating cells (lane 3) and differentiated cells (lane 5) were 2.53 ± 0.02 and 3.17 ± 0.10, respectively ($P<0.05$). Treatment with dexamethasone decreased the plasma membrane bound lipocortin 1 in undifferentiated (lanes 1 vs 2) cells, as well as in differentiating (lanes 3 vs 4)

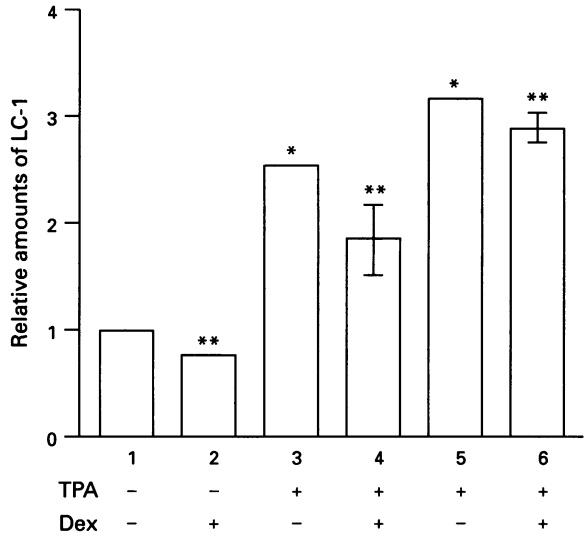

Figure 4 Effects of dexamethasone and TPA on the plasma membrane-bound lipocortin 1 (LC-1). Proteins bound externally on the plasma membrane were extracted with the 'fractionation buffer' containing 10 mM EDTA, and were analyzed for lipocortin 1 by Western blot. For SDS–PAGE, 30 μg of the protein extract was loaded on each lane. The same groups of cells as in Figure 3 were used in this experiment. Lanes 1 to 6 correspond to lanes 1 to 6 in Figure 3. Other details are as in Figures 2 and 3. $^{*}P<0.05$ vs value of TPA(−), and $^{**}P<0.05$ vs value of Dex(−) by one-way ANOVA with Bonferroni post-test. The error bars in lanes 2, 3, and 5 do not show on the scale.

and in fully differentiated (lanes 5 vs 6) cells by 21.5±0.7%, 27.3±13.0%, and 8.99±6.02%, respectively ($P<0.05$).

Discussion

The current hypothesis of lipocortin 1 mediation of the anti-inflammatory action of glucocorticoids involves the secretion of lipocortin 1 from the cell and the binding of the protein to the putative receptor (Perretti & Flower, 1993; Peers *et al.*, 1993). The secretion of lipocortin 1 by glucocorticoids is still a controversial issue.

In the present study we have used U937 cells to address the issue of the cytosol to membrane translocation and the secretion of lipocortin 1 by a glucocorticoid and/or TPA. The treatment of U937 cells with TPA resulted in: (1) a time dependent change in the lipocortin 1 protein and mRNA levels; (2) time dependent translocation of lipocortin 1 from the cytosol to the membrane; and (3) release of lipocortin 1 into the culture medium.

It has been shown that a pool of lipocortin 1 is bound to the external side of the plasma membrane in a dication-dependent manner (Browning *et al.*, 1990). In all experiments presented here, cells were washed and fractionated in buffers without calcium ions. When the experiments were performed using buffers containing calcium ions, similar results were obtained except that the portion of the N-terminally cleaved band (the smaller band in Figure 2, 24 h) was increased (data not shown). Therefore the trace amount of calcium ion originated from the culture medium is enough to hold the membrane bound lipocortin 1.

The increased expression of lipocortin 1 and its mRNA by U937 cells during differentiation with TPA treatment (Figure 1) is consistent with previous observations (Isacke *et al.*, 1989; Solito *et al.*, 1991). Interestingly, both the protein and the mRNA levels decreased after 48 h, when the differentiation was nearly completed. This suggests that the expression of lipocortin 1 is related to the differentiation process of U937 cells, rather than to the TPA treatment itself. The change in the mRNA level was several times higher than that of the protein level. It is possible that this is due to the non-linear response of the Northern blot and Western blot signals to the actual amounts of mRNA and protein.

Comparison of Figures 3 and 4 reveals that: (1) in the cells without dexamethasone treatment, the amount of plasma membrane-bound lipocortin 1 (Figure 4, lanes 1, 3, 5) correlates to the amount of the total lipocortin 1 that is associated with the membrane (Figure 3, lanes 1, 3, 5); and (2) dexamethasone decreased the plasma membrane bound lipocortin 1 (Figure 4, lanes 2, 4, 6), but had no effect on the cytosol to membrane translocation of lipocortin 1 (Figure 3, lanes 2, 4, 6).

The decrease in the externally bound lipocortin 1 by dexamethasone (Figure 4) is in apparent contrast with recent studies (Solito *et al.*, 1994), where flow cytometry shows a biphasic increase by dexamethasone in the membrane-bound lipocortin 1. The discrepancy may come from the use of different methods. The flow cytometry determined the externally bound proteins using a monoclonal antibody, whereas our methods analyzed the externally bound proteins that were EDTA extractable.

The mechanism by which dexamethasone decreases the externally bound lipocortin 1 is unclear at this point. Since dexamethasone has no effect on the cytosol to membrane translocation of lipocortin 1, it is tempting to speculate that dexamethasone increases the release of lipocortin 1 from the plasma membrane. This point needs to be clarified in future studies.

Recently, it has been proposed that cytosolic proteins without a signal sequence, like lipocortin 1 and interleukin 1, are likely to be secreted through as yet unknown pathways that do not involve the endoplasmic reticulum and the Golgi apparatus (Muesch *et al.*, 1990; Rubartelli *et al.*, 1990; Solito *et al.*, 1991). A role for dexamethasone as an activator of this unknown pathway has been proposed (Solito *et al.*, 1991).

It is tempting to speculate that the secretory mechanism is through an unknown pathway, involving the translocation of lipocortin 1 from the cytosol to the internal membranes, and then, its secretion to the external membrane. Binding of lipocortin 1 to the putative receptor on the plasma membrane would occur after secretion, as previously postulated (Goulding & Guyre, 1993). The status of the lipocortin 1 bound to the internal membranes is unclear at this point. Studies on the subcellular localization of lipocortin 1 are in progress in our laboratory.

The results in Figure 2 show that translocation of lipocortin 1 to the membrane occurred at 24 h, but not at 6 h of TPA treatment. Phosphorylation of lipocortin 1 by protein kinase C (PKC) occurs within 3 h after the activation of PKC by phorbol 12-myristate 13-acetate (PMA) (Strulovici *et al.*, 1989), suggesting that PKC-dependent phosphorylation of lipocortin 1 may not be important for its translocation.

Phospholipase A_2 translocates to the cell membrane upon treatment of the cells with PMA, resulting in the activation of the enzyme and the release of arachidonate (Rehfeldt *et al.*, 1991). In view of the hypothesis that lipocortin 1 may act as a regulator of the cytosolic form of phospholipase A_2 (Perretti & Flower, 1993; Kim *et al.*, 1994), it can be postulated that the translocation of lipocortin 1 may be related to the regulation of phospholipase A_2. Studies are in progress in this laboratory to test this hypothesis.

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