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Transmembrane signaling: tumor promoter distribution

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Diacylglycerol plays a critical role in transmembrane signaling by activating protein kinase C (PKC). The tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA) mimics that action, and in the human erythrocyte, TPA-activated PKC phosphorylates membrane proteins. Although molecular aspects of this process have been investigated, details of the interaction of TPA with plasma membranes remain elusive. Because TPA is hydrophobic, it has been assumed that it associates with the lipid bilayer. However, there is no direct evidence for its transbilayer distribution. Because knowledge of its location would limit molecular models proposed to explain its mode of action, we have used membrane-splitting techniques, based on freeze-fracture of planar cell monolayers, to quantify transmembrane partitioning of [^{3}H]TPA. Under conditions where PKC-mediated phosphorylation was stimulated by [^{3}H]TPA and where more than 90% of the [^{3}H]TPA was associated with the human red cell plasma membrane, two-thirds of the TPA partitioned with the cytoplasmic leaflet after bilayer splitting. This represents the first direct topographic localization of TPA in a biological membrane and supports the hypothesis that the mechanism of TPA activation requires its association with the cytoplasmic leaflet of the bilayer.

Introduction

In a common form of transmembrane signaling, cells interact with their environment via plasma membrane receptors that activate cellular processes [1]. Two major signaling pathways have been proposed. In one, extracellular interactions of ligands with membrane receptors modulate cytoplasmic levels of a second messenger, cyclic AMP. In the other, a signaling event activates a phospholipase that cleaves phosphatidylinositol bisphosphate releasing two second messengers, inositol trisphosphate and diacylglycerol (DAG) [2]. DAG activates protein kinase C (PCK) [3], which in turn phosphorylates key intermediates such as membrane-associated coupling factors [4] and transporters [5]. The pathway has special relevance to developmental and cancer biology, since tumor-promoting phorbol diesters, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA), mimic DAG in activating PKC [3].

Because TPA is lipophilic, it has been thought to partition into and exert its effect at the membrane bilayer [6]. It has also been proposed that cytosolic PKC diffuses to the membrane to form an active quaternary complex with DAG (or TPA), Ca^{2+}, and phosphatidylserine [3]. In support of this hypothesis, inactive PKC can be isolated from the cytosolic fraction and active PKC from the membrane fraction [7–11]. However, a fluorescent derivative of TPA has been shown to label the cytoplasm [12], suggesting that the derivative passes through the membrane to bind to cytosolic PKC. Although it is generally assumed that unmodified TPA must partition to or through the cytoplasmic leaflet, there is no direct evidence about the transmembrane distribution of unmodified TPA. Knowledge of the distribution of TPA across the plane of the membrane of the intact cell is of biological relevance since that knowledge would help evaluate models proposed to explain its mechanism at the molecular level.

Planar cell and membrane monolayer methods that employ membrane splitting by freeze-fracture can be

Abbreviations: *A*, absorbance; DAG, diacylglycerol; DBLAMS, double-labeled membrane splitting; Me_2SO, dimethylsulfoxide; *F*, fluorescence; FITC-Con A, fluorescein isothiocyanate conjugated concanavalin A; HBS, Hepes-buffered saline; LSC, liquid-scintillation counting; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PLG, polylysine-treated glass; RBC, human red blood cell; SDS, sodium dodecyl sulfate; SMM, single membrane monolayer; TLC, thin-layer chromatography; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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used to examine the transmembrane distribution of proteins and lipids [13–17]. The location of amphiphilic or lipophilic (hydrophobic) molecules, physically stabilized by freezing, can be quantified after fracturing. Investigations of the human red blood cell (RBC) have shown that it contains PKC and that TPA can induce the phosphorylation of specific membrane proteins [12,18–21]. In this paper we report on the transmembrane partitioning pattern of [^{3}H]TPA in the human RBC membrane. Using planar splitting methods [22–25] we show that at low but physiologically relevant concentrations the transmembrane distribution of [^{3}H]TPA is asymmetric: two-thirds of the TPA partitions with the cytoplasmic leaflet of the membrane.

Materials and Methods

Erythrocyte labeling. Human RBCs (A^+) were washed [23] and labeled with [^{3}H]TPA [11] at 37°C for 10 min at 20% hematocrit in 12 to 900 nM [^{3}H]TPA (Dupont New England Nuclear, Boston, MA, or Amersham Searle, Arlington Heights, IL) in 145 mM NaCl, 5 mM KCl, 10 mM Hepes (Sigma Chemical Co., St. Louis, MO), 0.1 mM sodium phosphate (monobasic), and 20 units/ml penicillin and streptomycin (pH 7.4) (Buffer A or TPA-buffer). TPA-labeled cells were washed once with TPA-buffer then labeled with fluorescein isothiocyanate conjugated concanavalin A (FITC-Con A), purified on an agarose column, and concentrated by centrifugation [23].

TPA-mediated phosphorylation. Pelleted, washed RBCs (400 μl) were incubated with 400 μCi [^{32}P]orthophosphate in 2 ml TPA-buffer [11] at 37°C for 4 h. Cells were washed with Hepes-buffered saline (HBS: 10 mM Hepes, 143 mM NaCl, 5 mM Mg^{2+}), treated with nonradioactive TPA as described above, and washed with HBS. Intact cells and isolated cell membranes [26] were solubilized and analyzed by SDS-polyacrylamide gel electrophoresis [27]. Gels were either silver-stained [28] or dried and analyzed by autoradiography and scintillation counting.

Effects of TPA solvents. Washed RBCs (200 μl) were suspended in 1 ml TPA-buffer at 37°C and treated with 0%, 0.1%, 0.2%, 0.5% or 1.0% (v/v) dimethylsulfoxide (Me_2SO) or similar concentrations of methanol for 10 min. Cells were pelleted, supernatants decanted, and equal volumes of resuspended cells and supernatants were added to SDS-borate buffer for measurement of hemoglobin (Hb) absorbance [23].

Cell and membrane monolayer formation. To prepare cell monolayers, labeled, washed, RBCs (50 μl of 2 parts pellet to 1 part buffer) were applied to one side of 11 × 22 mm coverglasses treated with poly(L-lysine) (PLG), and unattached cells were washed free. To prepare ghost monolayers, RBCs were lysed in hypotonic phosphate buffer [26] and washed thrice prior to being applied to PLG. To prepare single membrane monolayers (SMM), cell or ghost monolayers were submerged in HBS, and unattached membrane was removed by shearing with a forceful stream of buffer delivered by a syringe rapidly sweeping over the coverglass 1 to 2 mm above and perpendicular to its surface (25-ml syringe, 22 gauge needle, 40 s to deliver 30 ml HBS, 0–4°C). For splitting, monolayers of labeled cells were sandwiched against a second glass, frozen, and fractured [23].

Cell fragility. RBCs were incubated with TPA, concentrations as indicated, at 37°C for 10 min, washed, and labeled with FITC-Con A as described above. FITC fluorescence (F) and Hb absorbance (A) of cells in suspension or after attachment to PLG were measured after cells were solubilized in SDS-borate buffer [23], and F/A ratios calculated.

Cellular distribution of [^{3}H]TPA. For each measurement, two cell monolayers of [^{3}H]TPA-labeled RBCs were prepared (1 μCi/400 μl cells, 37°C, 10 min). To form ghost monolayers, one of the two cell monolayers was transferred to 40 ml of rapidly stirring 7.3 mM phosphate-buffered saline (pH 7.4) [26] at 0–4°C for 1 min then washed with a wash bottle for 10 s. Intact cell and ghost monolayers were transferred to 600 μl SDS-borate buffer [23] in scintillation vials, sonicated for 5 s, suspended in 10 ml Aquasol II and radioactivity was measured by LSC.

Extraction of membrane-bound TPA. Single membrane monolayers prepared from fresh RBCs labeled with ^{125}I by lactoperoxidase-catalyzed radioiodination [29] or with [^{3}H]TPA were extracted by transfer to 100 ml vigorously stirring 0.1 M NaOH, 1.0 M NaCl, or 0.1% Nonidet P-40 at 4°C. Extractions were terminated by vigorous 10-s rinses with HBS. The back surfaces of the glasses were wiped dry and glasses transferred to scintillation vials for LSC as described above. Control and extracted samples were also solubilized and analyzed by SDS-PAGE [27] followed by silver-staining [28] or fluorography. For acetone extractions [^{3}H]TPA-labeled RBCs were washed thrice in TPA-buffer then twice in HBS-buffer; cell monolayers were prepared and single glasses plunged into stirring acetone (50 ml) at −78°C for 10 s to 30 min [30]. Extraction was terminated by transfer to LSC vials as previously described. The percentage of radioactivity extracted was calculated by comparing the radioactivity remaining after extraction to that of unextracted control cell monolayers processed in stirred ice cold buffer under identical conditions.

TPA integrity. Stock [^{3}H]TPA and [^{3}H]TPA extracted [31] from RBCs were analyzed by thin-layer chromatography (TLC) on silica gel G plates (Adsorbosil Plus Prekotes, Alltech Assoc., Inc.). Plates were developed with methylene chloride/acetone (3:1, v/v), lanes divided into 2-mm strips which were scraped into

10 ml Aquasol 11 in LSC vials, and radioactivity measured.

TPA-stimulated phosphorylation. Human RBCs (200 μl pellet) were incubated with 400 μCi [^{32}P]orthophosphate in 1 ml TPA-buffer (20% hematocrit) for 4 h at 37°C, treated with 0.5 μCi [^{3}H]TPA (25 nM) for 10 min at 37°C, washed thrice with HBS (0–4°C), once with PBS, and lysed and washed thrice with hypotonic phosphate buffer [26]. Approx. 1 μg of total membrane protein containing 8000 cpm ^{32}P was applied to each lane of duplicate gels. One gel was silver-stained [28] and its companion left unstained, dried, and autoradiographed for 30 h at −100°C.

Double-labeled membrane splitting (DBLAMS). Monolayers of intact RBCs labeled with [^{3}H]TPA and FITC-Con A were frozen and fractured, and split membrane fractions were analyzed [23]. Absorbance was measured with a diode array spectrophotometer (Model 8451A, Hewlett Packard, Palo Alto, Ca), fluorescence with a home-built fluorometer, and radioactivity with a scintillation counter (Model LS-7000, Beckman Instruments, Irvine, CA). Data were corrected for background, enhancement, and quench, and percentage distribution was calculated [23].

Light and electron microscopy. For light microscopy, sheared intact monolayers of RBC ghosts were fixed with 1% glutaraldehyde in 20 imosM phosphate buffer (pH 7.4), for 1 h at 20°C, rinsed twice with buffer, 5 min each, dehydrated with a six-step ethanol concentration series, 1 min each step, rinsed twice in acetone, 1 min each, at 20°C, and dried with a nitrogen gun. Dry samples were examined and photographed with a Zeiss photomicroscope using phase optics and Kodak Technical Pan Film 2415. To achieve optimal contrast, film was developed in undiluted Kodak D-19 for 4 min at 20°C. For electron microscopy sheared RBC monolayers were frozen in Freon-22, freeze-dried, and shadowed with Pt-C. Replicas were stripped from the glass by floating onto HF, washed, transferred to grids and examined with a Siemens 101 electron microscope.

Results

As background to the membrane-splitting experiments we examined several features of the TPA–RBC membrane interaction: the effect of TPA solvents on cell lysis, the distribution of TPA between cytoplasmic and membrane fractions and between peripheral and integral membrane proteins, the properties of TPA after labeling, and the degree of TPA-induced phosphorylation of membrane proteins. Several different preparations were used in these experiments (Fig. 1): cells in suspension, single cell monolayers analyzed by

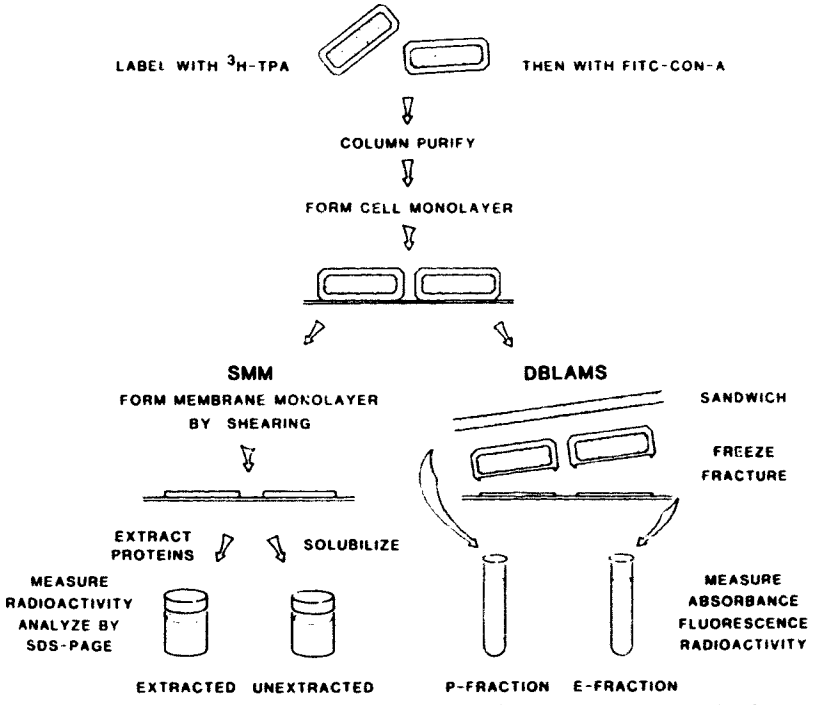

Fig. 1. Scheme for the preparation of single membrane monolayers (SMM) and single cell monolayers for double-labeled membrane splitting (DBLAMS). SMM were used for membrane protein analysis, and DBLAMS was used to study the transmembrane distribution of [^{3}H]TPA.

DBLAMS. ghost monolayers, and single membrane monolayers (SMM).

Effect of TPA carrier solvents on RBC fragility

We measured hemoglobin release from intact RBCs after incubation at 37°C for 10 min in two carrier solvents: dimethyl sulfoxide and methanol. In 0.1% Me_2SO, 0.9% of the hemoglobin was released; in 0.2% Me_2SO, 1.1% was released; in 0.5% Me_2SO, 2.1% was released; in 1.0% Me_2SO, 3.1% was released; and in 0.1%, 0.2%, 0.5%, and 1.0% methanol, 0.5% or less of the hemoglobin was released. Thus cells were routinely labeled by incubation in 0.1 to 1.0% (v/v) methanol in isotonic buffers.

Effect of TPA treatment on RBC fragility

To examine cell fragility after TPA treatment, we used DBLAMS techniques (Fig. 1) to monitor *F/A* ratios of TPA-labeled versus nonlabeled cells before and after cell monolayer formation. Both treated and control cells were washed and incubated at 37°C prior to monolayer formation. These experiments consistently showed a slight increase in the ratio of fluorescence to absorbance (*F/A*) of untreated cells attached to PLG versus cells in suspension. Light microscopy revealed that this increase correlated with the presence of RBC ghosts at the perimeter of the cell monolayer. Approximately 6% of the total 11 × 22 mm PLG area contained RBC ghosts as quantified by planimetry of photographically enlarged monolayers. However, treatment with TPA concentrations in the range of 0 to 100 nM (Table I), as well as higher concentrations, e.g. 1 μM (data not shown), did not significantly alter the *F/A* ratios. For TPA concentrations of 12 to 900 nM, 37 ± 4% (mean ± S.D., $n = 3$) of the [^{3}H]TPA partitioned into the RBC fraction independent of TPA concentration.

[^{3}H]TPA distribution between membrane and cytosol

To quantify TPA distribution beteen cytosol and membrane fractions at the time of membrane splitting, monolayers of intact cells labeled with [^{3}H]TPA were prepared. Cells were lysed by immersing the cell monolayers in rapidly stirring hypotonic phosphate buffer [26] forming ghost monolayers (Fig. 2a). Table II shows data for five pairs of RBC monolayers, one monolayer of each pair was lysed to form a ghost monolayer (cell plasma membrane). Monolayers were solubilized in SDS, scintillation fluid added, and radioactivity measured. After lysis 93 ± 1% (mean ± S.D., $n = 5$) of the radioactivity remained with the membrane fraction.

Extraction of [^{3}H]TPA associated with the membrane

To determine what fraction of the [^{3}H]TPA was associated with integral membrane proteins and/or the lipid bilayer, and what fraction with peripheral membrane proteins, single membrane monolayers (Fig. 2c,d) of [^{3}H]TPA-labeled cells and ^{125}I-lactoperoxidase radioiodinated cells were extracted at 0–4°C with 0.1 M NaOH, 1.0 M NaCl, or 0.1% Nonidet P-40 [32]. Table III shows the results of salt, alkaline, and detergent extraction of SMM. Because ^{125}I-labeled predominantly band 3 and glycophorin A (both integral membrane proteins), extraction data indicates the effect of treatment on the removal of total membrane from the PLG surface. Treatment with 1 M NaCl or 0.1 M NaOH for 10 min removed 0 to 4% of the total membrane. However, these treatments removed approx. 15% of the [^{3}H]TPA, concomitant to the loss of peripheral membrane proteins as monitored by SDS gel electrophoresis. At 30 min a net loss of about 20% [^{3}H]TPA (28% minus 7%) was observed. Although the detergent Nonidet P-40 extracted 100% of the [^{3}H]TPA activity it also removed about half of the integral membrane protein. Cell mono-

TABLE I

Fragility of TPA-treated RBCs as indicated by F/A ratios

TPA concentration (nM)	*F/A* ratios	
	cells in suspension	cells attached to PLG
0	0.24	0.26
10	0.25	0.27
50	0.20	0.24
100	0.27	0.30

TABLE II

Cell-membrane distribution of TPA

No. Pair	[^{3}H]TPA (cpm)	
	intact RBC monolayer	lysed RBC ghost monolayer [a]
1	2249	2100 (93%)
2	2368	2231 (94%)
3	1955	1793 (92%)
4	2046	1898 (93%)
5	1855	1707 (92%)

[a] Mean ± S.D. = 93 ± 1%.

TABLE III

Extraction of radiolabeled single membrane monolayers

Treatment	% radioactivity extracted	
	[^{3}H]TPA	^{125}I
NaCl (1.0 M, 10 min)	14	0
NaOH (0.1 M, 5 min)	15	–
NaOH (0.1 M, 10 min)	16	4
NaOH (0.1 M, 15 min)	20	–
NaOH (0.1 M, 30 min)	28	7
NP-40 (0.1%, 10 min)	100	49

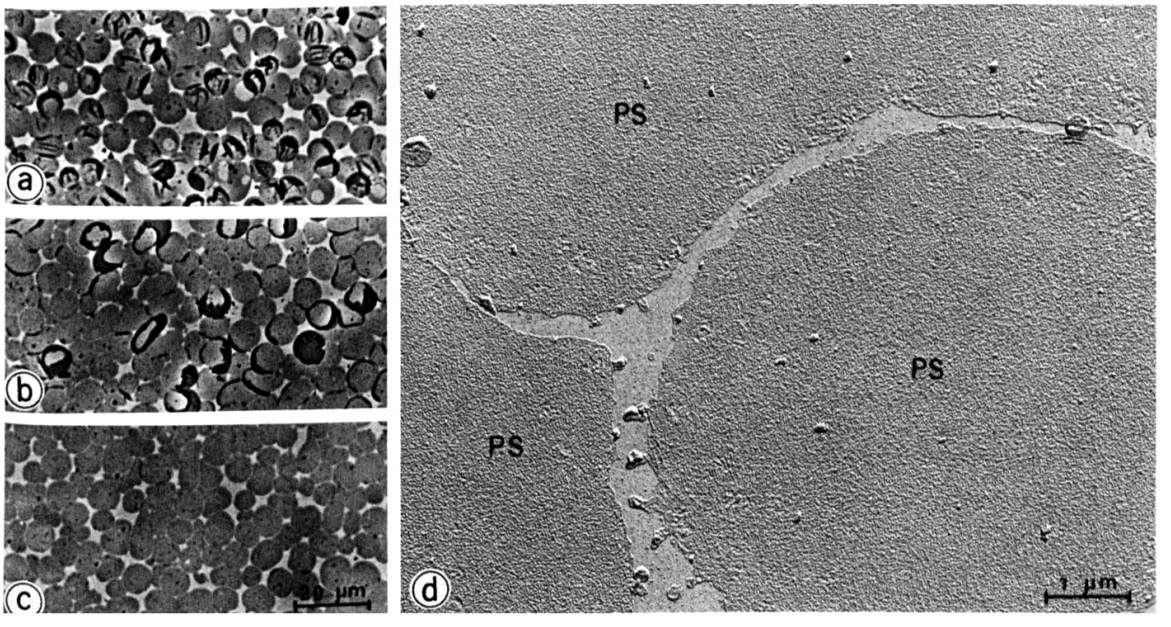

Fig. 2. Light (a–c) and electron (d) micrographs of planar RBC ghost and sheared membrane monolayers on polylysine-treated glass. (a) RBC ghost monolayer before shearing. (b) Partially sheared RBC ghost monolayer. Most ghosts have been sheared to single membranes, but some have burst to form 'slippers'. Peripheral crescent-shaped areas represent overlapped double membrane. (c) Sheared RBC ghost monolayer. Shearing removes all overlapped or free membrane. Flattened membranes remain attached to the glass. a–c, ×550. (d) Electron micrograph of a Pt-C shadowed, freeze-dried sheared RBC monolayer. Flattened, non-overlapped, single membranes are attached to the glass surface with cytoplasmic surfaces (PS) exposed. ×12600.

layers were also extracted at −78°C with 100% acetone [30]. Relative to controls, more than half of the [^{3}H]TPA was extracted from intact cell monolayers in less than 1 min. At 5 min and 30 min 68% of the activity was extracted from the monolayer.

[^{3}H]TPA integrity

Possible metabolic modification of [^{3}H]TPA during short term incubation with intact red cells was monitored by TLC and LSC (Fig. 3). [^{3}H]TPA had essentially the same relative mobility on silica gel G plates after incubation with cells as it did prior to incubation.

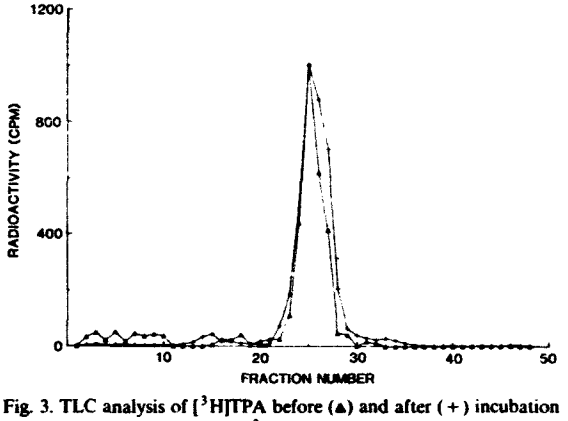

Fig. 3. TLC analysis of [^{3}H]TPA before (▲) and after (+) incubation with intact RBCs. Extracted [^{3}H]TPA was spotted on TLC plates, developed, 2 mm wide strips scraped into scintillation fluid and radioactivity measured by LSC.

[^{3}H]TPA-stimulated phosphorylation

To determine if [^{3}H]TPA promoted phosphorylation of membrane proteins, we incubated intact cells with [^{32}P]orthophosphate for 4 h, then with 25 nM [^{3}H]TPA for 10 min [11]. Cells were washed, plasma membranes isolated, and membrane proteins examined by SDS-PAGE and fluorography [29]. Fig. 4 shows that [^{3}H]TPA stimulated the phosphorylation of five protein bands as previously reported [11,18–21].

Transmembrane partitioning of TPA

The transmembrane partitioning pattern of TPA was quantified using double-labeled membrane splitting, DBLAMS [23]. Table IV shows data from three experiments at two concentrations of TPA. For each experiment two split fractions were examined: one enriched in extracellular leaflets, ES (the side attached to the glass), and one enriched in cytoplasmic leaflets, PS (the unattached side). Data are given as the means ± S.D. where n = the number of pairs of glasses analyzed. For these three experiments 33 ± 11% (mean ± S.D., n = 39) of the radioactivity partitioned with the extracellular leaflet, 67% with the cytoplasmic leaflet. At higher TPA

TABLE IV

Transmembrane distribution of [^{3}H]TPA

Experiment	TPA (nM)	Fraction	Absorbance (404 nm)	Fluorescence (525 nm)	Radioactivity (cpm)	Fraction in extracellular leaflet
1 (n = 14)	12	ES	0.057 ± 0.029	0.089 ± 0.017	54 ± 17	39 ± 10%
		PS	0.237 ± 0.035	0.089 ± 0.017	142 ± 26	
2 (n = 11)	90	ES	0.076 ± 0.025	0.069 ± 0.009	32 ± 8	27 ± 10%
		PS	0.214 ± 0.030	0.068 ± 0.009	66 ± 12	
3 (n = 14)	90	ES	0.078 ± 0.033	0.089 ± 0.026	124 ± 45	31 ± 9%
		PS	0.245 ± 0.030	0.116 ± 0.022	289 ± 48	

concentrations, e.g. 900 nM, TPA was more randomly distributed across the membrane: 50 ± 14% (mean ± S.D., n = 10) in the extracellular leaflet.

Discussion

We have used planar membrane splitting techniques to measure the transmembrane distribution of the lipophilic tumor promoter TPA. Several features of the lipophile–cell interaction were investigated, and a sequence of experiments was developed to address the following questions.

1. Does the carrier solvent perturb the bilayer? We assume that an ideal solvent would not disrupt the bilayer, and that gross bilayer perturbation would lead to cell fragility or lysis. Evaluation of cell intactness is also necessary for DBLAMS methods since hemoglobin is used as a quantitative marker for the cytoplasmic leaflet. Cells treated with increasing concentrations of solvents were centrifuged, and the absorbance of the supernatant was measured. Methanol produced less lysis than dimethylsulfoxide and thus was used as the solvent for TPA.

2. Do the lipophilic molecules perturb the bilayer? Incubation of red cells at 37°C for extended times with lipophilic molecules such as TPA or cholesterol can make cells more susceptible to lysis during formation of cell monolayers. We monitor cell fragility by comparing the F/A ratios of cells in suspension to the F/A of cell monolayers [23]; cell lysis, loss of hemoglobin, leads to an increase in the F/A ratio. We interpret the observation that TPA treatment produced monolayer F/A ratios identical to those for untreated monolayers as evidence for minimal lytic perturbation of the bilayer.

3. What proportion of the molecules associates with the membrane? It is generally assumed that lipophilic molecules tend to partition into the lipid bilayer of biomembranes. Cell monolayers provide a convenient and controllable tool both for exposure of the plasma membrane to labeling reagents and for separation of the plasma membrane from the cytosol; i.e., for quantifying the proportion of molecules associated with the membrane. For the TPA experiments 93 ± 1% of the [^{3}H]TPA remained with the membrane. Because a small percentage of membrane is lost during ghosting, this 93% represented the minimum amount of [^{3}H]TPA that was associated with the membrane under our labeling conditions.

4. What proportion associates with membrane lipids and with integral and peripheral proteins? Quantification

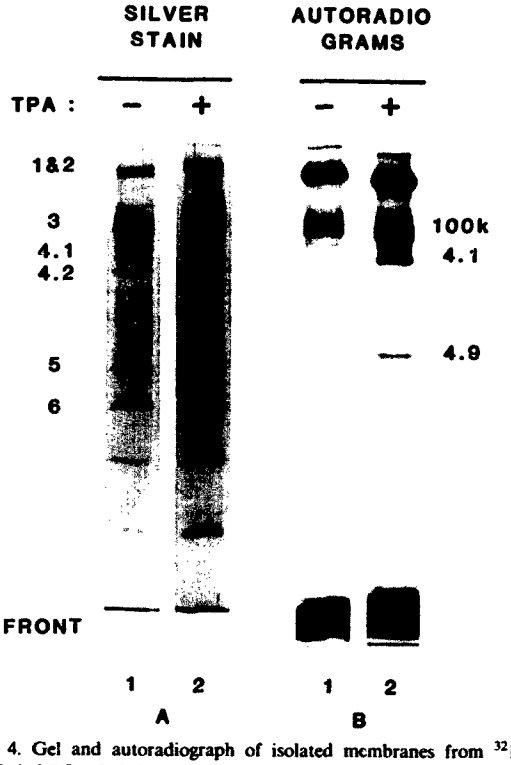

Fig. 4. Gel and autoradiograph of isolated membranes from ^{32}P-labeled RBCs. Labeled cells were either treated with methanolic [^{3}H]TPA (+) or methanol only (−), washed, membranes isolated, solubilized, and applied to duplicate gels for simultaneous electrophoresis. One gel was silver-stained (A), and the other dried and autoradiographed (B). TPA treatment stimulated ^{32}P association with 5 bands: two 100 kDa bands, and bands 4.1a, 4.1b, and 4.9.

of the distribution of isotope between membrane lipid and protein is essential for accurate interpretation of molecular topographies from splitting data. Monolayer preparations again are convenient for such analyses. Cell, ghost, and single membrane monolayers can be extracted [31] and analysed by TLC [22], and peripheral and integral proteins by SDS gel electrophoresis, silver staining, and fluorography [29].

Extraction data suggest a predominant association of [^{3}H]TPA with integral membrane proteins, lipids, and possibly non-extractable, tightly bound peripheral membrane proteins. NaOH and NaCl treatments of single membrane monolayers labeled with [^{3}H]TPA extracted less than 15% of the radioactivity even though such treatments removed essentially all peripheral membrane protein. It is likely that TPA is associated with membrane-bound PKC [3], a hypothesis supported by low temperature acetone extraction data. This method [30] removed 68% of the activity from the red cell membrane. Thus, as much as 32% of the activity may be receptor/PKC associated. In addition, it is known that PKC can be extracted with nonionic detergents, but not with NaCl or NaOH [11]. Although our data show total extraction of [^{3}H]TPA with Nonidet P-40, it is also known that this detergent extracts membrane lipids [32]. To summarize, these data suggest the association of TPA with membrane proteins, possibly PKC, as well as with membrane lipids in the cytoplasmic leaflet.

4. Are membrane-associated molecules functionally active? Modification of membrane properties can be used as evidence of lipophile incorporation into the bilayer. For example, TPA is known to stimulate PKC-mediated phosphorylation of cytosolic membrane proteins in the red cell. We found that [^{3}H]TPA stimulated the phosphorylation of protein bands as previously reported and attributed to stimulation of PKC [11,18–21]. Thus membrane-associated [^{3}H]TPA was functionally active at the nanomolar concentrations used in the membrane splitting experiments to determine transbilayer topographies.

6. Are the molecules metabolically altered? The DBLAMS assay of [^{3}H]TPA measures radioactivity only. To equate tritium activity with the TPA molecule required the evaluation of TPA after incubation with intact cells, since it is known that TPA can be enzymatically and chemically modified. TLC showed that [^{3}H]TPA had the same relative mobility before and after incubation with intact red cells. Although we realize that TLC cannot resolve small differences among TPA metabolites [6], our serum-free labeling conditions would not be expected to promote TPA hydrolysis. It is thus likely that [^{3}H]TPA was not metabolically altered during our labeling procedure.

7. What fraction of the molecules partitions with each split leaflet? Previous studies have shown that DBLAMS can be used to quantify split membrane fractions [23,24].

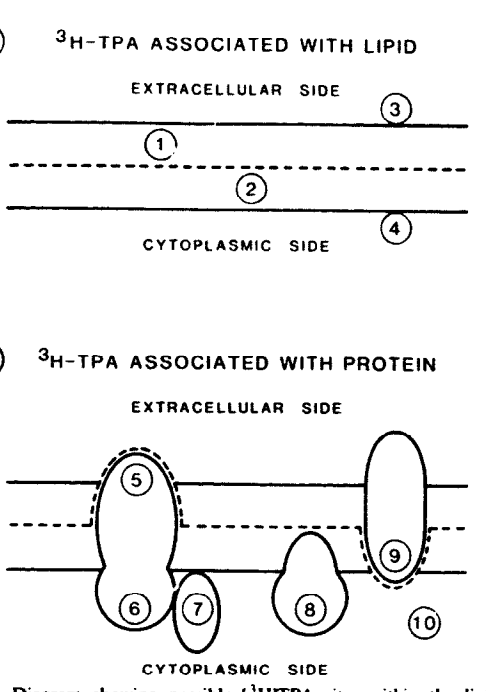

Fig. 5. Diagram showing possible [^{3}H]TPA sites within the lipid bilayer (a) or associated with membrane proteins and/or cytosol (b). The plane of fracture (------) for human red cell membranes deviates around integral transmembrane proteins without cleaving covalent bonds. Labeling, extraction, and splitting data suggest the most probable sites for [^{3}H]TPA are 2 and 8 (possibly 6).

At TPA concentrations of 90 nM or less, about one third of the label (mean = 33%) was associated with the extracellular leaflet and two-thirds with the cytoplasmic leaflet. At higher TPA concentrations the distribution of label was more random, about 50% in each leaflet. Importantly, [^{3}H]TPA was associated with the cytoplasmic leaflet at all concentrations tested. Student's *t*-distribution evaluation of pooled data of splitting experiments of membranes labeled at low TPA concentration versus high concentration revealed that the means, 33% versus 50%, were significantly different at the 99.9% confidence level. The random distribution of TPA at the higher concentrations presumably represents nonspecific association of the hydrophobic molecule with both leaflets of the bilayer.

8. How is the partitioning pattern interpreted? As shown in Fig. 5 there are several membrane sites that a lipophile such as TPA can potentially occupy. When attempting to equate transbilayer topography with the measured partitioning of the molecule, lipophile–membrane interactions and the effect of freeze-fracture on membrane protein distribution must be considered. Because of its lipophilicity, TPA can be assumed to partition into the extracellular leaflet of the lipid bilayer and perhaps readily equilibrate with the cytoplasmic leaflet.

Results of other studies also suggest an interaction with cytoplasmic leaflet lipids. A phorbol ester photoaffinity probe has been shown to label phosphatidylserine and phosphatidylethanolamine [33], and these phospholipids are enriched in the cytoplasmic leaflet of the human RBC plasma membrane [34]. If unmodified TPA acts like the photoaffinity probe, then one would expect an asymmetric distribution of TPA in the human erythrocyte, more partitioning to the cytoplasmic leaflet. The present study supports the model of TPA enrichment in the cytoplasmic leaflet resulting from amino phospholipid interaction and asymmetric distribution.

The potential interaction of TPA with integral red cell membrane proteins is also important to consider, since different classes of integral proteins are known to partition differently during membrane splitting [17,35, 36]. Glycophorin A, for example, preferentially partitions to the extracellular leaflet. Since our data show an asymmetric association of TPA with the cytosolic leaflet, we can tentatively conclude that TPA does not preferentially associate with glycophorin A and thus co-partition to the extracellular leaflet during membrane splitting.

In summary, our data show an asymmetric partitioning of TPA across the red blood cell plasma membrane and support a model (Fig. 5) that depicts the interaction of TPA with lipids and protein(s) associated with the cytoplasmic leaflet of the membrane (Fig. 5, sites 2 and 8 and possibly 6).

Acknowledgments

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References

1 Cohen, P. and Houslay, M.D. (eds.) (1985) Molecular Mechanisms of Transmembrane Signalling, 487 pp., Elsevier, New York.
2 Downes, C.P. and Michell, R.H. (1985) in Molecular Mechanisms of Transmembrane Signalling (Cohen, P. and Houslay, M.D., eds.), pp. 3–56, Elsevier, New York.
3 Nishizuka, Y. (1984) Nature 308, 693–698.
4 Sibley, D.R., Jeffs, R.A., Daniel, K., Nambi, P. and Lefkowitz, R.J. (1986) Arch. Biochem. Biophys. 244, 373–381.
5 Witters, L.A., Vatler, C.A. and Lienhard, G.E. (1985) Nature 315, 777–778.
6 Lee, L.-S. and Weinstein, I.B. (1978) J. Environ. Pathol. Toxicol. 1, 627–639.
7 Farrar, W.L. and Anderson, W.B. (1985) Nature 315, 233–235.
8 Hirota, K., Hirota, T., Aguilera, G. and Catt, K.J. (1985) J. Biol. Chem. 260, 3243–3246.
9 Kraft, A.S. and Anderson, W.B. (1983) Nature 301, 621–623.
10 Nel, A.E., Wooten, M.W., Landreth, G.E., Goldschmidt-Clermont, P.J., Stevenson, H.C., Miller, P.J. and Galbraith, R.M. (1986) Biochem. J. 233, 145–149.
11 Palfrey, H.C. and Waseem, A. (1985) J. Biol. Chem. 260, 15021–16029.
12 Liskamp, R.M.J., Browthman, A.R., Arcoled, J.P., Miller, O.J. and Weinstein, I.B. (1985) Biochem. Biophys. Res. Commun. 131, 920–927.
13 Fisher, K.A. (1980) Annu. Rev. Physiol. 42, 261–273.
14 Fisher, K.A. (1987) in Advances in Cell Biology (Miller, K.R., ed.), Vol. 1, pp. 1–29, JAI Press, Greenwich.
15 Nermut, M.V. (1982) Eur. J. Cell Biol. 28, 160–172.
16 Nermut, M.V. (1983) Trends Biochem. Sci. 8, 303–306.
17 Edwards, H.H., Mueller, T.J. and Morrison, M. (1979) Science 203, 1343–1345.
18 Cohen, C. and Foley, S.F. (1986) J. Biol. Chem. 261, 7701–7709.
19 Ling, E. and Sapirstein, V. (1984) Biochem. Biophys. Res. Commun. 120, 291–298.
20 Raval, P.J. and Allan, D. (1985) Biochem. J. 232, 43–47.
21 Fisher, K.A. and Yanagimoto, K.C. (1988) Biochim. Biophys. Acta 970, 39–50.
22 Fisher, K.A. (1976) Proc. Natl. Acad. Sci. USA 73, 173–177.
23 Fisher, K.A. (1982) J. Cell Biol. 92, 44–52
24 Fisher, K.A. (1982) J. Cell Biol. 93, 155–163.
25 Fisher, K.A. (1982) Meth. Enzymol. 88, 230–235.
26 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130.
27 Laemmli, U.K. (1970) Nature 227, 680–685
28 Poehling, H.M. and Neuhoff, V. (1981) Electrophoresis 2, 141–147.
29 Fisher, K.A. and Yanagimoto, K.C. (1986) J. Cell Biol. 102, 551–559.
30 Ashendel, C.L. and Boutwell, R.K. (1981) Biochem. Biophys. Res. Commun. 99, 543–549.
31 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
32 Yu, J., Fischman, D.A. and Steck, T.L. (1973) J. Supramol. Struct. 1, 233–249.
33 Delclos, K.B., Yeh, E. and Blumberg, P.M. (1983) Proc. Natl. Acad. Sci. USA 80, 3054–3058.
34 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71.
35 Fisher, K.A. (1989) in Freeze-Fracture Studies of Membrane Structure (Hui, S., ed.), (in press), CRC Press, Boca Raton.
36 Fisher, K.A. and Yanagimoto, K.C. (1985) J. Cell Biol. 101, 307a.