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Thromboplastin (tissue factor) in plasma membranes of human monocytes

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The synthesis of thromboplastin, a potent trigger of blood coagulation, can be induced in human peripheral blood monocytes. Indirect evidence suggests that newly synthesized thromboplastin becomes in part available on the cell surface. We have attempted to study the localization and availability of thromboplastin more directly by isolating plasma membranes from isolated human peripheral blood monocytes. The specific activities of the plasma membrane markers increased 16–22-fold in these preparations with a recovery of about 15%. The contamination by mitochondria, lysosomes, nuclei and endoplasmic reticulum was low as estimated by marker enzymes and electron microscopy. In both unstimulated and stimulated monocytes thromboplastin was largely recovered in this plasma membrane fraction, providing direct evidence for its membrane localization. Phospholipase C (E.C. 3.1.4.3) is a potent inactivator of thromboplastin through its hydrolysis of the phospholipids necessary for thromboplastin activity [Otnæss, Prydz, Bjørklid & Berre (1972) *Eur. J. Biochem.* **27**, 238–243]. About 70% of the total membrane thromboplastin activity was inactivated when whole cells were treated with phospholipase C and the membranes subsequently isolated. Following stimulation to induce thromboplastin synthesis, the plasma membranes showed a shift in their relative content of phosphatidylcholine and phosphatidylethanolamine consistent with a transmethylation process.

Human monocytes respond to various stimuli with increased synthesis of a procoagulant activity which both functionally and immunologically shares the features of thromboplastin (tissue factor, Factor III) (Prydz & Allison, 1978). Thromboplastin is an integral membrane glycoprotein (apoprotein III) of M_r about 52000, the activity of which is dependent upon surrounding phospholipids (Bjørklid *et al.*, 1973; Bjørklid & Storm, 1977). The apoprotein alone has no procoagulant activity, but in complex with the appropriate phospholipids it is the most potent trigger of blood coagulation yet isolated.

Monocytes are the only cells in peripheral blood able to produce thromboplastin (Rivers *et al.*, 1975). Normally they contain low or even no thromboplastin activity, but substantial increases due to synthesis *de novo* of apoprotein III occur following stimulation by agents such as endotoxins (Rivers *et al.*, 1975; Prydz & Allison, 1978), lectins (Lyberg & Prydz, 1980), immune complexes (Rothberger *et al.*, 1977; Prydz *et al.*, 1979), complement factor C3b (Prydz *et al.*, 1977) and phorbol esters (Lyberg & Prydz, 1981). The criteria for identifying this procoagulant activity as thromboplastin include neutralization by antibodies against apoprotein III, phospholipase C sensitivity and lack of procoagulant activity in factor VII-deficient plasma (Prydz & Allison, 1978).

Previous studies have suggested that a major portion of the thromboplastin synthesized upon stimulation in monocytes and endothelial cells appears on the cell surface anchored in the plasma membrane (Lyberg *et al.*, 1983; Østerud & Bjørklid, 1982). The present study was carried out to investigate this more directly.

Abbreviation used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

Materials and methods

Chemicals

Chemicals were obtained as follows: RPMI 1640 (low endotoxin) medium was from Gibco Biocult, Paisley, Scotland, U.K.; MEM-S medium

and fetal calf serum from Flow Laboratories, Irvine, Scotland, U.K.; Lymphoprep from Nyegaard, Oslo, Norway; Dextran T-500 from Pharmacia, Uppsala, Sweden; endotoxin (*Escherichia coli* 055:B5) from Difco Laboratories, Detroit, MI, U.S.A.; anti-(human albumin) (rabbit) from Dakopatts, Copenhagen, Denmark; phytohaemagglutinin HA 16 from Wellcome Research Laboratories, Beckenham, Kent, U.K.; TPA from P-L Biochemicals, Milwaukee, WI, U.S.A.; human serum albumin and standard phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and sphingomyelin) from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were of pro analysis quality.

Isolation of monocytes

Mononuclear cells were isolated as described by Lyberg & Prydz (1980) from healthy donors by use of a cell separator (Haemonetics Model 30 Blood Processor; Haemonetics, Natick, MA, U.S.A.) or from buffy coats from individual donors, essentially by the method of Bøyum (1968).

The final mononuclear cell suspension was plated at $(5–8) \times 10^7$ cells/10cm culture dish (Costar; Oxnard, CA, U.S.A.), and incubated in an atmosphere of CO_2/air (1:19) (95% relative humidity) at 37°C for 2h. Non-adherent cells were then removed by four washings with RPMI 1640 containing 5% (two washes) and 20% (two washes) fetal calf serum. The final preparation contained more than 95% monocytes as judged by nonspecific esterase staining (Yam *et al.*, 1971) and May–Grunwald–Giemsa staining. The final cell cultures were incubated for 4–18h in RPMI 1640 containing 20% foetal calf serum with or without stimulant as indicated.

Stimulation of cells

The following stimulants were used: endotoxin (10–25 μg/ml for 4–5h), phytohaemagglutinin (20 μg/ml for 4–10h), TPA (1–10ng/ml for 15–18h) and immune complexes (10 μg of antigen/ml for 14–18h). Immune complexes were formed by incubation of human serum albumin (Sigma) and corresponding antiserum (Dakopatts, Copenhagen, Denmark) for 90min at room temperature at antigen:antibody equivalence (Lyberg & Prydz, 1982).

Preparation of monocyte plasma membranes

The procedure for plasma membrane isolation is modified from the methods of Wang *et al.* (1976), Segel *et al.* (1979) and DiPersio *et al.* (1979). All operations were performed at 0–4°C.

After incubation/stimulation the cells were scraped off with a rubber policeman, washed in barbital-buffered saline (Hjort, 1957), resuspended [$(1–3) \times 10^7$ cells/ml)] in 10ml of lysis medium (1mM-$NaHCO_3$, pH7.4) and homogenized by 15–20 strokes in a tight-fitting Dounce homogenizer. The homogenate was diluted to 50ml with lysis medium and centrifuged at 500*g* for 20min. The pellet was rehomogenized in lysis medium and centrifuged again as above. The combined 500*g* supernatants were then centrifuged at 15000*g* for 20min. The pellet obtained was carefully suspended in 3.5ml of lysis medium and added to 10.5ml of sucrose (60%, w/v, in lysis medium) to give a crude membrane suspension in 45% sucrose. An equal volume of 35% (w/v) sucrose in lysis medium was carefully layered over the crude membrane suspension in two 13ml tubes (Beckman Ultraclear) and centrifuged at 96000*g* for 2h in a SW 41 Ti rotor. The interface band was carefully harvested and diluted with barbital-buffered saline to 60ml. The plasma membrane fraction was sedimented at 100000*g* for 1h. The resulting pellet was then treated as indicated for further analysis or electron microscopy.

Assay of marker enzymes

The following enzymes were assayed: lactate dehydrogenase (EC 1.1.1.27), alkaline phosphatase (EC 3.1.3.1) and γ-glutamyltransferase (EC 2.3.2.13) according to the Scandinavian Committee on Enzymes (1974, 1976); phosphodiesterase I (EC 3.1.4.1) as described by Touster *et al.* (1970); NADH dehydrogenase (EC 1.6.99.3) according to Kamat & Wallach (1965); succinate dehydrogenase (EC 1.3.99.1) according to Pennington (1961), cytochrome *c* oxidase (EC 1.9.3.1) according to de Duve *et al.* (1955) and acid phosphatase (EC 3.1.3.2) as described by Kachmar (1970).

Treatment of monocytes with phospholipase C

Phospholipase C was purified to homogeneity from *Bacillus cereus* culture supernatants (Hetland & Prydz, 1982). About $(1–2) \times 10^7$ adherent cells were incubated in 10cm dishes for 30min at 37°C with phospholipase C at a final concentration of 10 μg/ml. The incubations were terminated by addition of EDTA to a final concentration of 10mM and cooling. The cells were washed twice in barbital-buffered saline containing 10mM-EDTA, then twice with barbital-buffered saline only. Cell viability after treatment with phospholipase C was monitored by the uptake of 0.05% Trypan Blue and the release of lactate dehydrogenase.

Phospholipid and cholesterol determinations

Lipids were extracted from homogenized cells with chloroform/methanol (2:1, v/v) according to Bligh & Dyer (1959). The final residue was

dissolved in a small volume of chloroform/methanol (9:1, v/v) and analysed for lipids.

Individual phospholipids were separated by t.l.c. (Skipski & Barclay, 1969). The spots were identified by standards run in parallel, scraped off and analysed for phospholipid phosphorus by the method of Bartlett (1959) as modifed by Dittmer & Wells (1969). Cholesterol was analysed by g.l.c.

Thromboplastin determination

Total thromboplastin activity was determined in triplicate after freezing–thawing of the cells or membranes followed by homogenization. The clotting system (Hvatum & Prydz, 1966) consisted of 0.1 ml of cell or membrane homogenate in barbital-buffered saline, 0.1 ml of citrated, pooled human plasma (or factor VII-deficient plasma when appropriate) and 0.1 ml of $CaCl_2$ (0.03 M). Reference curves were prepared by dilution of a standard human brain thromboplastin preparation (Hjort, 1957) arbitrarily chosen to contain 100 units/ml.

Electron microscopy

Samples of plasma membrane material pelleted at 100000 *g* for 1 h were fixed in 2% glutaraldehyde in 0.1 M-cacodylate buffer/0.1 M-sucrose, pH 7.3, and postfixed in 1% OsO_4 in the same buffer. The specimens were dehydrated in graded ethanol and propylene oxide and embedded in Epon 812. Ultrathin sections were cut from top, bottom and middle regions of each specimen, stained with uranyl acetate and lead citrate and examined in a Jeol 100B electron microscope.

Monoclonal and polyclonal antibodies

A monoclonal antibody (1D5, IgG_1) against a plasma membrane antigen of human blood monocytes (Kaplan & Gaudernack, 1982) was purified from ascites fluid by using a protein A–Sepharose column (Pharmacia). Purified 1D5 was radioiodinated (^{125}I) by the Iodogen method (Markwell & Fox, 1978) to a specific activity of 8.5 mCi/mg of protein.

Adherent cells in culture dishes were incubated with the ^{125}I-labelled antibody (0.17 μCi/ml) for 2 h at 4°C. The cells were harvested, washed and their plasma membranes isolated. Radioactivity was measured in a Packard Model 578 gamma scintillation spectrometer.

The polyclonal neutralizing antiserum to thromboplastin (apoprotein III) was raised in goats and the Ig fraction was isolated as described by Bjørklid & Storm (1977).

Protein determination

Protein was determined by the method of Markwell *et al.* (1978) using bovine serum as standard.

Results

Characterization of the plasma membrane preparation

Plasma membranes were prepared from unstimulated cells and from cells stimulated with endotoxin, phytohaemagglutinin or TPA. When 10 ng of TPA/ml was used the recovery of monocytes was markedly reduced and only a small amount of plasma membrane material was obtained. When 1 ng of TPA/ml was used, however, plasma membranes of high purity were successfully isolated (Table 1). The plasma membranes of cells stimulated with immune complexes behaved very differently during the fractionation, probably due to the massive binding of antigen–antibody complexes. This resulted in a yield of only about 1% and an anomalously high protein content of the fraction.

Electron microscopy of sections from various parts of the membrane pellets showed some stratification. The upper and middle parts of the pellet contained almost exclusively vesicles with the appearance of plasma membrane material (Figs. 1*a* and 1*b*). Sections from the bottom part contained similar vesicles and in addition some swollen mitochondria and amorphous material (Fig. 1*c*). These contaminating structures were only seen in a small part of the pellets, consistent with the low activity or absence of marker enzymes for other organelles.

Judged by the specific activities of the subcellular marker enzymes, the plasma membrane preparations appeared to be 16–22-fold purified (Table 1). Similar results were found with membranes from unstimulated and TPA-stimulated cells. The variations in the specific activities of alkaline phosphatase and succinic dehydrogenase in different experiments (Table 1) are probably due to differences among individual batches of monocytes. The relative specific activity of plasma membranes:cell homogenate remained essentially unaltered. The monoclonal antibody (1D5) was bound to its antigen on the surface of whole unstimulated cells with high avidity and there was no detectable release of soluble ^{125}I-labelled antibody at any stage, i.e. dissociation of the antigen–antibody binding during the plasma membrane purification was negligible. This is in accordance with the very high binding constant for this antibody (4×10^{12} litre/mol; G. Gaudernack, unpublished work). Purification and yield of plasma membranes were thus conveniently monitored by this surface-located antigen–^{125}I-antibody complex. The yield of ^{125}I in the final plasma

Table 1. *Activity of subcellular markers in human monocyte plasma membrane preparations*
Enzyme activities are in nkat/mg of protein. Values are means of triplicate analyses from two representative experiments, one with and one without TPA stimulation. Surface antigen was measured by binding (c.p.m./mg of protein) of monoclonal antibody 1D5 labelled with ^{125}I (see the Materials and methods section). Thromboplastin activity (units/mg of protein) is the mean ± S.E.M. for seven and one experiment(s), respectively.

Enzyme	TPA added (1 ng/ml)	Cell homogenate	Plasma membranes	Relative specific activity	Recovery (%)
Phosphodiesterase I	−	11.5×10^{-3}	24.0×10^{-2}	20.9	15.0
	+	11.9×10^{-3}	20.2×10^{-2}	17.0	15.7
Alkaline phosphatase	−	26.2×10^{-3}	50.0×10^{-2}	19.1	13.5
	+	4.7×10^{-3}	10.0×10^{-2}	21.6	20.0
γ-Glutamyl transferase	−	29.3×10^{-3}	47.3×10^{-2}	16.2	13.1
Acid phosphatase	−	2.50	2.73	1.1	1.2
NADH dehydrogenase	−	6.76	3.37	0.5	0.7
Succinic dehydrogenase	−	94.5×10^{-3}	$< 6.6 \times 10^{-3}$	0.07	< 0.1
	+	131×10^{-3}	25.1×10^{-3}	0.2	0.2
Surface antigen	−	58×10^{3}	1057×10^{3}	18.6	15.3
Thromboplastin	−	0.75 ± 0.14	17.9 ± 1.2	23.8	12.0
	+	16.0	323	20.2	20.5

membrane preparations from unstimulated cells was 15–16% and the specific activity increased about 19-fold (Table 1). Membranes isolated from cells incubated for 15–18 h with 1 ng of TPA/ml gave, however, very low antibody binding. Membrane marker enzymes showed a recovery of about 13–15% in the plasma membrane fraction from unstimulated cells and 15–20% in that from TPA-stimulated cells. The recovery of ^{125}I-1D5 antibody in the latter fraction was below 1% of that in the former. These data are consistent with a rapid disappearance of the 1D5 antigen from the surface of TPA-stimulated cells. In preliminary experiments $t_{\frac{1}{2}}$ 4 h has been observed (G. Gaudernack, unpublished work).

Contamination by other subcellular structures was monitored by measurement of succinic dehydrogenase and cytochrome *c* oxidase (mitochondrial), acid phosphomonoesterase (lysosomes) and NADH dehydrogenase (endoplasmic reticulum). No or very low activity of succinic dehydrogenase and cytochrome *c* oxidase was found, indicating a very low amount of mitochondria in the plasma membrane preparation (Table 1). The presence of a lysosomal marker enzyme at unchanged specific activity compared with that of whole cell homogenates indicates a low lysosomal contamination. The specific activity of NADH dehydrogenase was about halved in the final preparations compared with the cell homogenates. The recovery of protein in the plasma membrane preparations was 1.0 ± 0.2% ($n = 7$).

The phospholipid and cholesterol composition data (Table 2) show a 2-fold higher cholesterol: phospholipid ratio in the plasma membranes compared with whole cells. The phospholipid: protein and the cholesterol: protein ratios were 4.3- and 8.2-fold higher in the membrane preparations than in whole cells, respectively. The plasma membrane preparations from unstimulated cells contained a higher proportion of sphingomyelin (22.4%) than did whole cells (14.7%) with a corresponding reduction in phosphatidylcholine (42.6% in whole cells, 35.5% in the membranes). The other phospholipid fractions (phosphatidylethanolamine and phosphatidylserine/phosphatidylinositol) were essentially identical (Table 2).

Thromboplastin activity in the plasma membrane preparations

Monocytes not deliberately stimulated to synthesize thromboplastin have a low and variable procoagulant activity (0.75 ± 0.14 units/mg of protein) (Fig. 2 and Tables 1 and 4). The mean increase in the specific procoagulant activity (17.9 ± 1.2 units/mg of protein) in the plasma membrane preparations was about 24-fold above that in cell homogenates with a recovery of 12%. Essentially all of this activity was due to thromboplastin, as evidenced by inactivation by phospholipase C and neutralization with antiserum against apoprotein III (Table 4).

When adherent, unstimulated cells in culture were subjected to phospholipase C and their plasma membranes subsequently isolated, the specific activity of thromboplastin was reduced by about 70% (Table 3). Of the total plasma membrane phospholipid (0.65 μmol/mg of protein), about 0.16 μmol/mg (sphingomyelin and phosphatidylinositol) is not hydrolysable by phospholipase C from *B. cereus* under the present conditions. Of

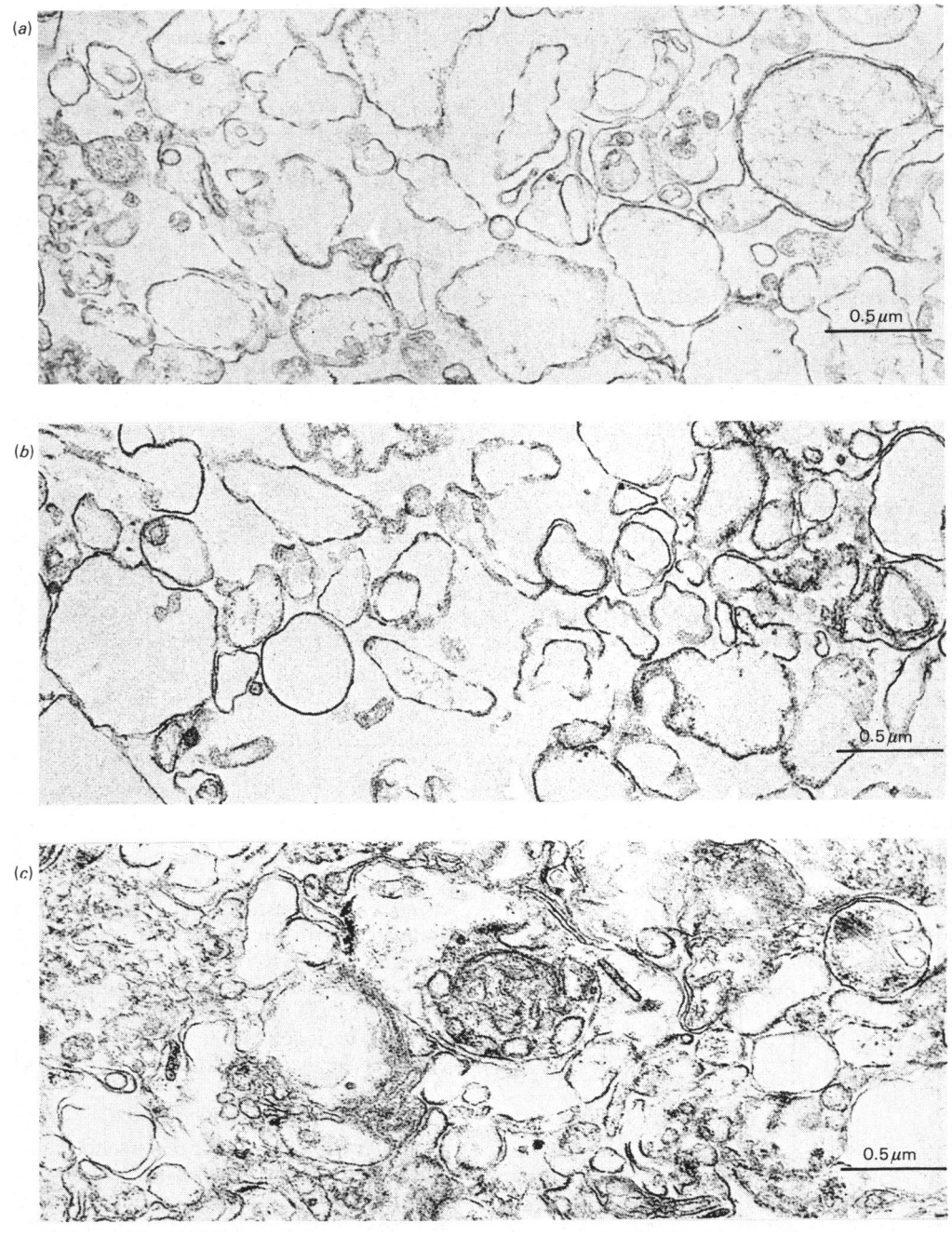

Fig. 1. *Electron micrographs of monocyte membrane fraction*
(*a*), Section from top of pellet; (*b*), section from middle of pellet; (*c*), section from bottom of pellet. Magnification ×30000.

the hydrolysable fraction (0.49 μmol/mg), about 73% (0.36 μmol/mg) was broken down by phospholipase C. Cell viability was unaltered, as evidenced by Trypan Blue exclusion and no leakage of lactate dehydrogenase.

In stimulated cells the specific activity of thromboplastin was 6–38-fold higher than in unstimulated cells, and a similar relative increase in the plasma membrane thromboplastin activity was found (Fig. 2). Plasma membranes isolated

Table 2. *Cholesterol and phospholipid in whole human monocytes and isolated plasma membranes*
Values are means ± S.E.M. for three to nine experiments. The cells were unstimulated controls (A), stimulated with immune complexes (B) and with TPA (C).

		Whole cells	Plasma membranes	Recovery
Cholesterol (μmol/mg of protein)		0.038±0.003	0.31±0.04	8.7±0.4
Total phospholipid (μmol/mg of protein)		0.15±0.01	0.65±0.06	5.0±1.2
Phospholipid composition (mol%)				
Phosphatidylcholine	A	42.6±0.3	35.6±0.5	
	B	47.8±0.2	39.1±0.3	
	C	47.9±1.3	45.3±1.8	
Phosphatidylethanolamine	A	30.8±0.3	30.7±0.5	
	B	29.8±2.1	29.2±0.6	
	C	25.9±0.6	26.4±0.2	
Sphingomyelin	A	14.7±0.4	22.4±0.6	
	B	13.0±0.4	20.9±0.5	
	C	15.9±0.5	18.9±0.4	
Phosphatidylserine + phosphatidylinositol	A	11.8±0.4	11.2±0.3	
	B	9.4±2.6	10.7±0.4	
	C	10.2±0.8	9.4±0.8	
Cholesterol:phospholipid (molar ratio)		0.25	0.48	

Table 3. *Thromboplastin and phospholipids in plasma membranes from adherent human monocytes treated with phospholipase C*
Cells were incubated with phospholipase C (10 μg/ml for 30 min at 37°C) while still adherent to the culture dishes prior to plasma membrane isolation. Values are the means of duplicates from two experiments.

	Plasma membranes		Whole cells	
Phospholipase C ...	−	+	−	+
Thromboplastin (units/mg)	24.0	7.1	1.45	0.41
Phospholipid (μmol/mg)	0.65	0.29	0.15	0.09

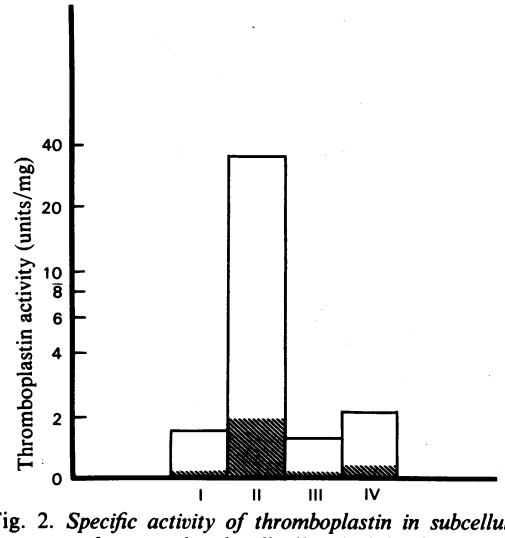

Fig. 2. *Specific activity of thromboplastin in subcellular fractions of unstimulated cells (hatched bars) and cells stimulated with PHA (open bars)*
Plasma membranes were isolated as described in the Materials and methods section. I, Cell homogenate; II, sucrose gradient interface fraction (plasma membranes); III, 15000*g* supernatant; IV, sucrose gradient bottom fraction.

from TPA, endotoxin- and phytohaemagglutinin-stimulated cells demonstrated a 20–25-fold increase in the specific activity of thromboplastin compared with the corresponding cell homogenate, with a mean recovery of 21% (±1.8) ($n = 4$). In cells stimulated with immune complexes, about 60–70% of the thromboplastin activity was susceptible to inactivation by phospholipase C as evidence for its localization on the cell surface.

Identity of the procoagulant activity

The identity of the procoagulant activity in stimulated cells was established (Prydz & Allison, 1978) by its lack of activity in factor VII-deficient plasma, by its sensitivity to phospholipase and by its inhibition by antibodies against apoprotein III. The procoagulant activity of homogenates of stimulated cells and their plasma membranes was greatly reduced by all these treatments, showing that this procoagulant activity indeed is thromboplastin (Table 4).

Shedding of TPA-stimulated thromboplastin activity

Thromboplastin newly synthesized in cells in culture remains normally cell-bound. In cells

Table 4. *Characteristics of stimulated and unstimulated monocyte thromboplastin activity of whole cell homogenate and of isolated plasma membranes*

Values are means ± S.E.M. of two to four experiments with unstimulated and stimulated cultures as indicated. Cultures were stimulated with phytohaemagglutinin (20 μg/ml), endotoxin (10 μg/ml) or TPA (1 ng/ml). Neutralization with anti-(apoprotein III) took place at 37°C for 30 min followed by 2 h at 0–4°C. Phospholipase C was used at 10 μg/ml for 10 min at 37°C.

	Thromboplastin (units/mg)			
	Whole cell homogenate		Plasma membranes	
Sample Stimulation ...	−	+	−	+
Activity in normal plasma	0.25 ± 0.1	7.7 ± 3.1	4.9 ± 2.8	144 ± 67
Activity in Factor VII-deficient plasma	<0.01	0.3	0.4 ± 2.8	2.5 ± 1.0
After incubation with anti-(apoprotein III)	<0.01	<0.01	<0.01	0.3 ± 0.2
After incubation with phospholipase C	<0.01	<0.01	<0.01	<0.01

stimulated with phytohaemagglutinin, immune complexes or endotoxin, regularly less than 5% of the total procoagulant activity was found in the medium. In cells stimulated with TPA (1–10 ng/ml), however, 15–27% of the total activity (21.4 ± 3.6%, mean ± S.E.M.) appeared in the medium as an activity sedimentable at 100000 *g* for 1 h but not at 5000 *g* for 20 min. The specific activity of this material was regularly about 20-fold higher than that of the corresponding cell homogenates, except when TPA at 10 ng/ml was used, when a 7-fold increase was observed. The procoagulant activity was fully sensitive to phospholipase C and required factor VII (results not shown). All these properties suggest that this procoagulant activity in the medium represents thromboplastin in plasma membrane material released by shedding.

Changes in phospholipids induced by stimulation

In whole cells as well as plasma membranes, the phospholipid:protein ratio increased by stimulation (in whole cells from 0.15 to 0.22 μmol/mg, in plasma membranes from 0.65 to 0.85 μmol/mg). This was accompanied by a change in the proportions of the individual phospholipids (Table 2). An increase in phosphatidylcholine and a decrease in phosphatidylethanolamine were observed in both stimulated whole cells and in their isolated plasma membranes.

Discussion

The plasma membrane preparations isolated in the present experiments contained about 1% of total cell protein, 9% of total cholesterol and 5% of total phospholipid. Recovery of the various plasma membrane markers in these experiments was 13–20%, slightly higher (15–20%) when stimulated rather than unstimulated (13–16%) cells were used. The rise in specific activity of these markers was 16–22-fold, which corresponds well with that observed by others (Johnsen *et al.*, 1974; Wang *et al.*, 1976; Segel *et al.*, 1979; DiPersio *et al.*, 1979; Sauvage *et al.*, 1981; Record *et al.*, 1982; Rawyler *et al.*, 1983) and suggests that our membrane preparations are of high purity.

Results obtained with the monoclonal antibody 1D5 to a surface antigen and the markers for other cellular organelles give further supporting evidence to this end. Anti-(plasma membrane) IgG (polyclonal) was also used by Sauvage *et al.* (1981) as a plasma membrane marker in rat HTC cells. They reported essentially superimposable distribution patterns for bound antibody and alkaline phosphodiesterase I with an increased specific activity and a protein recovery very similar to that found in our experiments. Finally, the electron micrographs indicate a high degree of purity. We conclude therefore that our membrane preparations are of a high degree of purity, although the presence of endo- or exocytotic (recirculating) vesicles in these plasma membrane preparations cannot be excluded.

Based on the recovery and specific activity of thromboplastin in such preparations we conclude that in stimulated cells, as well as in not deliberately stimulated cells, by far the largest part of thromboplastin is found in the plasma membrane or connected with it. This does not mean that all of it necessarily is available on the cell surface. Earlier studies (Lyberg *et al.*, 1983) have suggested that 50–70% of the total thromboplastin activity in whole cells is susceptible to trypsin or antibody neutralization from the outside. Following phospholipase C treatment of whole cells, the specific thromboplastin activity of plasma membranes subsequently isolated was decreased by about 70%, as was the total activity recovered. Thus, this is in good accordance with earlier data. There is also a very good correlation between this loss of thromboplastin activity and the amount of relevant phospholipid hydrolysed. Sphingomyelin and

phosphatidylinositol are only very poor or not at all substrates for phospholipase C (Hetland & Prydz, 1982), and they are also unable to reconstitute the thromboplastin activity of purified apoprotein III (Bjørklid & Storm, 1977). The total amount of phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine hydrolysed is 70–75%, corresponding to the 70% loss of procoagulant activity.

The resistance of about 30% of the thromboplastin activity to phospholipase C inactivation may be due to its location in the inner membrane leaflet or in intracellular (transport) vesicles which may copurify with the plasma membranes. This result is in good accordance with the data of Lopez-Saura *et al.* (1978) demonstrating that about 30% of alkaline phosphodiesterase I is 'latent' in homogenates of HTC cells. A similar figure was found for the surface area of endocytotic vesicles as a fraction of total plasma membrane area (Leroy-Houyet *et al.*, 1978). One must, however, bear in mind that phospholipase C only indirectly inactivates thromboplastin via hydrolysis of the necessary phospholipids. This phospholipase C-induced deficiency of phospholipids necessary for thromboplastin activity can be counteracted by synthesis and/or insertion and lateral membrane mobility of phospholipids, resulting in some thromboplastin activity apparently being phospholipase C-resistant.

The mechanism behind the observed changes of phosphatidylcholine and phosphatidylethanolamine in the plasma membrane following monocyte stimulation is not known, but transmethylation is an obvious possibility, although it represents a minor pathway for phosphatidylcholine synthesis. Whether these changes are related to afferent signal transduction leading to induction of thromboplastin synthesis or to efferent expression of thromboplastin procoagulant activity is a field for further study. It is interesting to note that an (admittedly unspecific) inhibitor of transmethylation (3-deaza-adenosine) (Galdal *et al.*, 1984; Hetland *et al.*, 1985) inhibits the thromboplastin response in human peripheral blood monocytes as well as in unbilical cord endothelial cells.

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