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Relation between membrane fluidity and signal transduction in the human megakaryoblastic cell line MEG-01

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Abstract

The fluidity of the plasma membrane is thought to affect the responsiveness of blood platelets. We measured membrane fluidity in a single cell by Fluorescence Recovery after Photobleaching (FRAP) of the lipophilic probe $DiIC_{14}$. Since platelets are too small for this technique, we used the human megakaryoblastic cell-line MEG-01, which shares many properties with platelets. MEG-01 cells were cultured for 44 h with simvastatin or mevalonate to change the cholesterol content, enabling analysis of signal processing at cholesterol/phospholipid ratios (C/P) between 0.20 and 0.31. The diffusion of $DiIC_{14}$ correlated inversely with the C/P ratio with lateral diffusion coefficients (D) of $3.28 \cdot 10^{-9}$ cm^2/s at a low C/P decreasing to $2.55 \cdot 10^{-9}$ cm^2/s at a high C/P ratio. The mobile fraction was 65% and constant at the different C/P ratios. The relation between lipid diffusion and signal processing was measured following stimulation with 10 U/ml thrombin at 22° C. There were only little differences in phosphatidylinositol metabolism, Ca^{2+} influx or mobilization and prostaglandin I_2-induced formation of cyclic AMP. At 37° C, cells with a high C/P ratio showed increased phosphatidylinositol metabolism, but these differences had no major effect on the Ca^{2+} responses. These data demonstrate that in megakaryoblasts the lateral diffusion of lipids is inversely correlated with the C/P ratio, but within the range of 0.20–0.31 the influence on signal processing is minor.

Keywords: Membrane; Fluidity; Signal processing; Megakaryoblast; (Human)

1. Introduction

Human platelets from patients with hyperlipoproteinemia are enriched in cholesterol and respond to platelet activating agents with increased aggregation and secretion [1]. Also when normal platelets are incubated with cholesterol-rich liposomes their sensitivity to agonists increases [2]. Above the phase transition temperature cholesterol is known to decrease membrane fluidity and to raise the degree of order of the lipid bilayer and the packing density in the membrane [3]. It also interferes with the van der Waals forces between lipids by decreasing the number of cis double bonds and sterically blocking large motions of fatty acyl chains.

Kramer et al. [4] proposed that the fluidity of the plasma membrane affects stimulus-induced changes in membrane lipids. In studies with cholesterol-enriched platelets, a more rigid plasma membrane was accompanied by increased expression of thrombin receptors [5], a higher phospholipase (PLA_2) activity and more liberation of arachidonic acid [4], and a greater rise in cytosolic Ca^{2+}, $[Ca^{2+}]_i$ [6]. Tandon et al. [7] compared the expression of thrombin receptors and stimulus-response mechanisms in platelets with different cholesterol content. Incubation with cholesterol-rich liposomes increased the expression of high affinity receptors thought to initiate aggregation but the number of occupied receptors required for half-maximal aggregation did not change. Apparently, post-receptor signal processing was undisturbed and the hyperaggregability caused by increased numbers of thrombin receptors. In contrast, platelets from hypercholesterolaemic patients had normal receptor numbers but aggregated better at the same

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receptor occupancy, suggesting that post-receptor events were enhanced. Sorisky et al. [6] stimulated cholesterol-enriched platelets with AlF_4^-, an activator of heterotrimeric G proteins, and found increased PLA_2-activity and Ca^{2+}-mobilization also indicating that processes distant from surface receptors are affected by the fluidity of the plasma membrane.

In the present study we investigated the relation between membrane fluidity and signal processing in more detail by comparing the mobility of lipids with signal generation via the phospholipase C and adenylyl cyclase pathways. Lipid mobility was measured by fluorescence recovery after photobleaching (FRAP) of the lipophylic probe 1,1'-ditetradecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate ($DiIC_{14}$), which is a sensitive means to evaluate the lateral diffusion of lipids in the plasma membrane of a single cell [8]. Since platelets are too small for this technique we used the megakaryoblastic cell MEG-01. MEG-01 cells resemble the early maturation stage of normal megakaryocytopoiesis and share many properties with megakaryocytes and platelets. The membranes have the same phospholipid composition [9]; the cells express the glycoprotein IIb/IIIa complex [10] and prostaglandin/thromboxane receptors [11] but lack the demarcating membrane system and α-granules [10]. MEG-01 cells respond to ADP, thrombin and epinephrine with an increase in $[Ca^{2+}]_i$ [12], Ca^{2+} oscillations [13] and activation of protein kinase C [14], suggesting that the initial steps in the activation cascade are intact. Changes in lipid mobility were introduced by interfering with the cell's cholesterol synthesis during culture. The cholesterol content was lowered with simvastatin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyzes the conversion of HMG-CoA to mevalonate, the committed substrate for cholesterol production. An increased cholesterol content was induced by culturing the cells in the presence of mevalonate. Thus, changes in cholesterol/phospholipid content, lateral diffusion of lipids and signal generation could be compared.

2. Materials and methods

2.1. Chemicals

RPMI 1640, fetal calf serum (FCS), Ultroser G, L-glutamine, penicillin, and streptomycin were purchased from Gibco, Grand Island, NY, USA. Bovine serum albumin (BSA), fatty acid free BSA, α-thrombin, agarose and mevalonate were from Sigma, St. Louis, MO, USA. ADP, and Fura-2-acetoxymethyl ester (Fura-2-AM) were from Boehringer Mannheim, Mannheim, Germany. 1,1'-Ditetradecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate ($DiIC_{14}$), and 2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1*H*-benzimidazole trihydrochloride (Hoechst 33342) were from Molecular Probes, Junction City, OR, USA. Prostacyclin (PGI_2) came from Cayman Chemicals, Ann Arbor, MI, USA. Culture flasks were purchased from Nunc, Roskilde, Denmark. Ionomycin was bought from Calbiochem Behring, La Jolla, CA, USA. The cyclic AMP radioimmunoassay kit was from Amersham International, Amersham, Bucks, UK. $[^{32}P]P_i$ (specific radioactivity 314 TBq/mmol) was purchased from New England Nuclear, Boston, MA, USA. Simvastatin was a kind gift of Merck Sharpe and Dohme, Rahway, NJ, USA.

2.2. MEG-01 cultures

MEG-01 cells were cultured as described [10]. In short, the cells were grown in plastic tissue culture flasks in RPMI 1640 supplemented with 20% FCS and 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin, at 37° C in 5% CO_2 and a fully humidified atmosphere.

2.3. Modulation of cholesterol content

44 h before the start of the experiments MEG-01 cells were centrifuged ($200 \times g$, 5 min, 22° C) and resuspended in culture medium with the low density lipoprotein-free serum substitute Ultroser G to prevent the uptake of cholesterol. The medium contained RPMI, 2% Ultroser G, 100 U/ml penicillin and 100 U/ml streptomycin at $1 \cdot 10^6$ cells/ml. The cells were grown for 44 h in plastic tissue culture flasks at 37° C in 5% CO_2 and a fully humidified atmosphere in the absence of modulators of cholesterol synthesis or in the presence of 5 μM simvastatin to inhibit cholesterol synthesis or 5 and 10 mM mevalonate to increase cholesterol synthesis.

2.4. Fluorescence recovery after photobleaching

The lateral diffusion of lipids in the plasma membrane of a single MEG-01 was measured by Fluorescence Recovery After Photobleaching (FRAP), using $DiIC_{14}$ as a lipid probe as described in detail in earlier publications [15,16]. In short, a 514 nm laser beam (Argon laser, Coherent CR-4) in the Gaussian mode, focussed with a Zeiss 50 × water-immersion objective (N.A. = 1.00) to a spot with an estimated $1/e^2$ radius of 1.23 μm, was used to exitate the fluorescent lipid probe and to bleach the fluorescence during 20–30 ms by a 1000-fold increase in light intensity. Fluorescence intensity was measured by a single photon counting system equipped with a photomultiplier (EMI 9863/100, EMI, Hayes, Middlesex, UK) and an Ortec 9302 amplifier discriminator (ORTEC, Oak Ridge, TN, USA). For calculation of the lateral diffusion coefficients (D), half-times of recovery were used using the mathematical method developed by Van Zoelen et al. [17].

Calculations:

D was calculated from the equation:

$$D = \left(\omega^2/4 \cdot \tau_{1/2}\right) \cdot \gamma_d$$

in which ω, $\tau_{1/2}$, and γ_d stand for the $1/e^2$ radius of the spot, half times of recovery and bleaching parameter, respectively.

Mobile fractions were calculated from the equation:

$$Mf = (F_\infty - F_0)/(F - F_0)$$

in which F, F_0, and F_∞ are the fluorescence intensities of the spot before, immediately after and long after bleaching, respectively.

2.5. Selection of MEG-01 cells for FRAP measurements

Approximately 3 h before the start of the FRAP experiments 1 μg/ml (final concentration) of the fluorescent benzimidazole dye Hoechst 33342 was added. Hoechst 33342 binds reversibly to DNA of living cells without interfering with cell function [18]. It has an excitation maximum at 350 nm and an emission maximum at 510 nm. After labelling, the cells were excitated with a UV-lamp making the nuclei of the cells visible by light microscopy. After selection of a cell with a multilobulated nucleus (average cell size 20 ± 1 μm), FRAP measurements were performed on the same cell by switching to 514 nm laser excitation. Each measurement of unstimulated MEG-01's was performed on a separate cell.

2.6. Determination of $[Ca^{2+}]_i$

MEG-01 cells ($1 \cdot 10^6$/ml) were incubated at 37° C for 45 min with 1 μM Fura-2-AM in culture medium. Thereafter, the cells were pelleted ($200 \times g$, 5 min, 22° C), resuspended in Ca^{2+}-free Hepes-Tyrode buffer (pH 6.5) and stored at room temperature. 5 min before the start of each measurement, the suspension was diluted to 200.000 cells/ml in Hepes-Tyrode (pH 7.2) containing 0.4 mM extracellular Ca^{2+}, $[Ca^{2+}]_o$. The suspension was mildly stirred (about 50 rev/min) at 22 or 37° C, as indicated in Section 3. $[Ca^{2+}]_i$ was calculated from the equation $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where K_d is the dissociation constant of the Fura-2-Ca^{2+} complex, F is the relative fluorescence, F_{min} is the F value of free Fura, and F_{max} the value for Fura saturated with Ca^{2+} [19]. F_{max} and F_{min} values were obtained after addition of 1% Triton X-100 and 10 mM EGTA (final conc.) respectively. Fluorescence measurements were carried out on a Hitachi F-3000 fluorescence spectrophotometer at respective excitation and emission wavelengths of 340 and 495 nm.

2.7. Determination of cholesterol / phospholipid ratio

MEG-01 cells were cultured for 44 h in lipoprotein-free medium without and with simvastatin (5 μM, final concentration) or mevalonate (5 and 10 mM, final concentrations). Thereafter, the lipids of $15 \cdot 10^6$ cells were extracted according to Rose and Oklander [20]. The lipid phosphorus content was determined as described by Fiske-SubbaRow [21] and total cholesterol content was determined by a colorimetric assay [22].

2.8. Measurement of [^{32}P]phosphoinositol derivatives

Changes in ^{32}P-labelled phosphoinositol and derivatives were measured according to a modified procedure described by Jolles et al. [23] for rat brain cells. In short, MEG-01 cells were labelled with 3.7 MBq [^{32}P]-orthophosphate (60 min, 37° C) and isolated by centrifugation ($200 \times g$, 5 min, 22° C). The cells were stimulated with 10 U/ml thrombin at 22 or 37° C. The reaction was terminated after 5 min by addition of 2 ml chloroform/methanol/13 M HCl (100:50:1, by volume; 0° C). Phospholipids were separated by high-performance thin-layer chromatography on HPTLC silica 60 gel plates (Merck) (22° C) and the radioactivity was determined by autoradiography on Kodak Royal X-Omat film. The spots were scraped off and counted for radioactivity by liquid scintillation counting. The ^{32}P radioactivity of phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylinositol monophosphate (PIP) and phosphatidylinositol bisphosphate (PIP_2) was expressed as a percentage of that of unstimulated cells.

2.9. Measurement of cyclic AMP concentration

MEG-01 cells ($1 \cdot 10^6$/ml) were cultured as described and incubated with 30 ng/ml PGI_2 (final concentration). Cyclic AMP concentrations were measured at 0, 5, and 10 min, at 22 or 37° C, after addition of PGI_2 using a radioimmunoassay, performed according to the manufacturer's instructions.

2.10. Statistics

The data were expressed as mean $\pm$ standard error of the mean (S.E.M.); statistical significances were calculated by Student's t-test for paired data and show the comparison between control cells and cells grown in the presence of agents that interfered with cholesterol synthesis.

3. Results

3.1. Modulation of the cholesterol / phospholipid ratio in MEG-01 cells

MEG-01 cells grown for 44 h in lipoprotein-free medium (control cells) had a C/P ratio of 0.23. In the presence of simvastatin the C/P ratio decreased to 0.20, which was significantly lower than in the control cells ($P = 0.03$). Incubation with 5 and 10 mM mevalonate led to C/P ratios of 0.29 ($P = 0.047$) and 0.31 ($P = 0.014$), respectively. Thus, the different incubation conditions resulted in

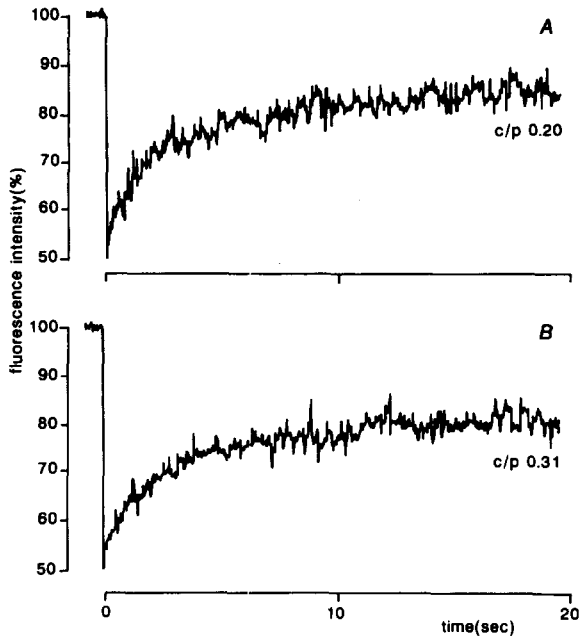

Fig. 1. FRAP curves of the lateral mobility of $DiIC_{14}$ in the plasma membrane of two MEG-01 cells with different cholesterol/phospholipid ratios. Tracings are examples of recovery patterns seen after photobleaching (20 ms) unstimulated cells with C/P ratios of 0.20 (*A*) and 0.31 (*B*).

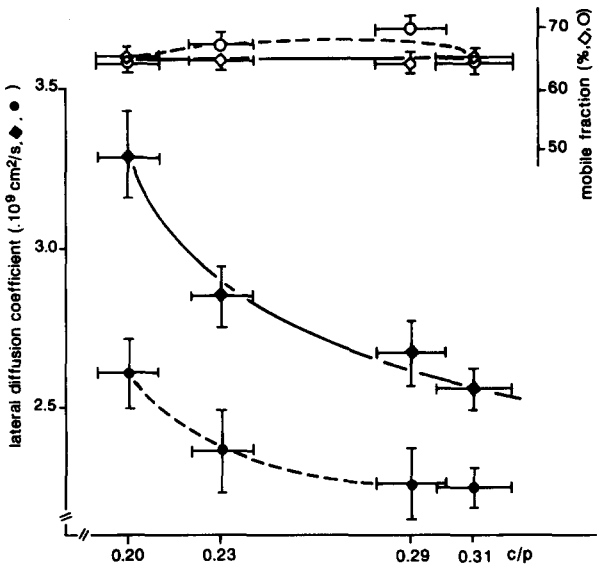

Fig. 2. Lateral diffusion of $DiIC_{14}$ in MEG-01 cells with different cholesterol/phospholipid ratios. The solid curves show the lateral diffusion coefficients and mobile fractions of unstimulated $DiIC_{14}$-labelled MEG-01 cells grown in the presence of 5 μM simvastatin (C/P ratio 0.20), no supplementation (C/P ratio 0.23), 5 mM mevalonate (C/P ratio 0.29) and 10 mM mevalonate (C/P ratio 0.31). The dotted lines show the lateral diffusion coefficients and mobile fractions of these cells at 2–5 min after stimulation with 10 U/ml thrombin.

MEG-01 cells with C/P ratios ranging between 0.20 and 0.31.

3.2. Lateral diffusion of $DiIC_{14}$ in MEG-01 cells with different cholesterol / phospholipid ratios

Fig. 1 illustrates representative tracings of the recovery of $DiIC_{14}$ fluorescence following a 20 ms bleaching to about 50% of the initial fluorescence. The upper curve shows a FRAP tracing of a MEG-01 cell with a C/P ratio of 0.20 with a $\tau_{1/2}$ of 1347 ms. A recovery to 82% of the initial fluorescence was calculated. These findings led to a lateral diffusion coefficient D of $3.29 \cdot 10^{-9}$ cm^2/s and a mobile fraction Mf of 66%. The lower curve shows the recovery of a cell with a C/P ratio of 0.31, leading to a $\tau_{1/2}$ of 1927 ms and a recovery to 81% of the initial fluorescence. This resulted in a D of $2.22 \cdot 10^{-9}$ cm^2/s and an Mf of 65%. These findings illustrate that the lateral diffusion of the lipophilic probe $DiIC_{14}$ correlated inversely with the C/P ratio, whereas the mobile fraction remained constant.

Fig. 2 shows the relation between $DiIC_{14}$ diffusion and C/P ratio in more detail. Simvastatin treated cells, with a C/P ratio of 0.20 had a lateral diffusion coefficient D of about $3.28 \cdot 10^{-9}$ cm^2/s. A C/P ratio of 0.23 found in the control medium correlated with a D of $2.84 \cdot 10^{-9}$ cm^2/s ($P = 0.01$), whereas the C/P ratios of 0.29 (cells incubated with 5 mM mevalonate) and 0.31 (10 mM mevalonate) correlated with respective D values of $2.66 \cdot 10^{-9}$

Table 1
Cholesterol/phospholipid ratio and lateral diffusion of $DiIC_{14}$ in the plasma membrane of MEG-01 cells after modulation of the cholesterol synthesis

	5 μM simvastatin	control	5 mM mevalonate	10 mM mevalonate
C/P	0.20 ± 0.01 * (5)	0.23 ± 0.01 (5)	0.29 ± 0.02 * (5)	0.31 ± 0.02 * (5)
D ($\cdot 10^9$ cm^2/s)				
unstimulated	3.28 ± 0.13 * (52)	2.84 ± 0.10 (44)	2.66 ± 0.10 (44)	2.55 ± 0.07 * (61)
thrombin (10 U/ml)	2.60 ± 0.11 [a] (35)	2.36 ± 0.13 [a] (27)	2.25 ± 0.11 [a] (19)	2.24 ± 0.06 [a] (39)
Mf (%)				
unstimulated	65 ± 1 (52)	64 ± 2 (44)	64 ± 2 (44)	65 ± 1 (61)
thrombin (10 U/ml)	65 ± 3 (35)	67 ± 2 (27)	69 ± 2 (19)	64 ± 2 (39)

Cholesterol/phospholipid ratio (C/P), lateral diffusion coefficient (D), and mobile fraction (Mf) are expressed as means ± SEM. Data of cells stimulated with 10 U/ml thrombin are derived from multiple measurements between 2 and 5 min after stimulation. The number of determinations is given in brackets.

* $P < 0.05$, significantly different from control cells.

[a] $P < 0.05$, significantly different from unstimulated cells that had undergone the same treatment.

cm^2/s (ns) and $2.55 \cdot 10^{-9}$ cm^2/s ($P = 0.016$, Table 1). The mobile fraction (Mf) of cells with the control C/P ratio was about 64%, and not significantly different at the different incubation conditions (Table 1).

Stimulation with 10 U/ml thrombin led in all groups to a rapid fall in diffusion velocity, in accordance with earlier observations [15,16]. The fall was steeper in cells with a low C/P ratio, where the decrease in D was $0.68 \cdot 10^{-9}$ cm^2/s, compared with cells with a high C/P ratio, where D fell with $0.31 \cdot 10^{-9}$ cm^2/s ($P = 0.006$, Table 1). At the different C/P ratios the Mf remained unchanged after stimulation (Fig. 2, Table 1).

3.3. Signal transduction at different cholesterol / phospholipid ratios

To investigate the effect of lipid diffusion on signal processing through the plasma membrane, thrombin-induced changes in the metabolism of PIP_2 and derivatives and in $[Ca^{2+}]_i$ were measured in MEG-01 cells with different C/P ratios incubated in the presence of extracellular Ca^{2+} ions to enable Ca^{2+} influx as well as mobilization. The $[Ca^{2+}]_i$ values were virtually similar in resting MEG-01 cells cultured with or without modulators of cholesterol synthesis (about 140 nM). Stimulation with thrombin carried out at 22° C to make comparisons with the FRAP data possible induced only minor changes in the PI metabolites (Table 2). Cells with a lowered C/P ratio contained less PIP and PIP_2 than the controls or cells with an increased C/P ratio but the differences were small. In contrast, a 30 to 60% increase in $[Ca^{2+}]_i$ was found, which was virtually the same at the different C/P ratios. Addition of EGTA immediately before stimulation to chelate extracellular Ca^{2+} ions did not change $[Ca^{2+}]_i$ of unstimulated cells (about 130 nM) and showed that 90% of the increase was due to mobilization from intracellular stores. These data were not different between cells cultured in the presence of simvastatin or mevalonate.

Table 2
Signal processing at different cholesterol/phospholipid ratios in MEG-01 cells

	C/P	0.20	0.23	0.29	0.31
22° C	PA	94 ± 6	106 ± 3	109 ± 5	109 ± 4
	PIP	83 ± 8 [a]	111 ± 4	96 ± 8	80 ± 5 [a]
	PIP_2	81 ± 6 [a]	103 ± 7	100 ± 5	88 ± 3
	Ca^{2+} total	131 ± 5 [a]	165 ± 8	148 ± 5	145 ± 17
	Ca^{2+} mobil.	126 ± 4	125 ± 4	135 ± 6	134 ± 7
37° C	PA	175 ± 4	185 ± 5	136 ± 4 [a]	112 ± 4 [a]
	PIP	78 ± 3	76 ± 1	99 ± 4 [a]	100 ± 4 [a]
	PIP_2	96 ± 2	97 ± 3	110 ± 3 [a]	112 ± 3 [a]
	Ca^{2+} total	220 ± 12	198 ± 11	225 ± 14	221 ± 13
	Ca^{2+} mobil.	152 ± 7	146 ± 4	145 ± 7	177 ± 12 [a]

Changes in [^{32}P]phosphatidic acid (PA), -phosphatidylinositol monophosphate (PIP) and -bisphosphate (PIP_2) following 5 min stimulation with 10 U/ml thrombin at 22° C and 37° C. Also shown is the increase in cytosolic Ca^{2+} content in the presence of 1 mM extracellular Ca^{2+} (added 5 min before stimulation, Ca^{2+} total) and in the presence of 1 mM EGTA (added 30 s before stimulation, Ca^{2+} mobilization). Data (means ± S.E.M., $n = 7$) are expressed as % of unstimulated cells; $[Ca^{2+}]_i$ in resting cells was 139 ± 16 nM and 127 ± 10 nM, respectively, without and with EGTA at 22° C, and 314 ± 31 and 118 ± 19 nM at 37° C.
[a] Significant difference with control cells (C/P 0.23).

Table 2 also shows the metabolism of PIP_2 in MEG-01 cells at 37° C. Unfortunately, at this temperature the FRAP measurements were disturbed by endocytosis of the lipid probe, limiting the comparisons to the relation between C/P ratio and PI metabolites. Stimulation with thrombin induced a 75% increase in [^{32}P]PA in cells with a low C/P ratio, which was not different from the accumulation in cells cultured in the absence of simvastatin. In contrast, cells with an increased C/P ratio had accumulated less [^{32}P]PA after stimulation. [^{32}P]PI radioactivity was about 20% higher than in unstimulated cells and not significantly affected by the C/P ratio (not shown). The difference between cholesterol-depleted and cholesterol-enriched cells was also reflected by a 10% increase in PIP_2 and a 20% increase in PIP at the high C/P ratios. Together, these data reflected a faster turnover through the phospholipase C pathway in MEG-01 cells with an increased C/P ratio compared with cells cultured in the absence of mevalonate. However, the differences were small indicating that also at 37° C alterations in C/P ratio in the range investigated in this study induced only minor differences. Although the increase in $[Ca^{2+}]_i$ triggered by thrombin at 37° C was slightly higher than at 22° C, there were no differences between the different MEG suspensions. Again the resting levels were similar (about 300 nM) and also the mobilization from intracellular stores (about 40% of total) was the same. Taken together, these data indicate that within the range between 0.20 and 0.31 the C/P ratio had only a minor effect on the signal transducing pathways leading to PIP_2 metabolism and Ca^{2+} mobilization/influx.

A possible effect of a changed C/P ratio on the pathway that leads to the synthesis of cyclic AMP was investigated by measuring cyclic AMP concentrations in MEG-01 cells treated with 30 ng/ml PGI_2. Unstimulated cells (22° C) with a C/P ratio of 0.20 had a slight but significantly lower cyclic AMP concentration than cells with a normal or increased C/P ratio (1.3 ± 0.2 compared with 2.6 ± 0.3 pmol/10^6 cells). Stimulation with PGI_2 for 5 min had little effect at this temperature, but at 37° C this value increased in cells with a C/P ratio of 0.20 to 4.3 ± 0.4 pmol/10^6 cells ($P = 0.005$). With a normal or higher C/P ratio, cyclic AMP increased from about 2.6 pmol/10^6 cells to 7.7 ± 1.0 pmol/10^6 cells ($P < 0.001$). However, the relative increase was similar at the different C/P ratios (250–300%, $P > 0.05$).

4. Discussion

In the present study we have demonstrated that lipids in the plasma membrane of MEG-01 cells have a slower

diffusion velocity as the C/P is increased. In contrast, the number of lipid molecules that takes part in this process is not affected by a changed cholesterol/phospholipid ratio. This finding is in concert with observations in artificial bilayers of phosphatidylcholine and cholesterol where the lateral diffusion of the probe nitrobenzoxadiazol-phosphatidylethanolamine (NBD-PE) decreased from about $3 \cdot 10^{-8}$ to $1 \cdot 10^{-8}$ cm^2/s when the cholesterol content was increased [24]. Also diffusion studies in the plasma membrane of carcinoma cells showed an inverse correlation between the lateral diffusion of $DiIC_{16}$ and the cholesterol content [25]. Unfortunately, size limitations oppose a similar FRAP study on platelets and it remains therefore uncertain whether the present diffusion characteristics apply to this cell type. However, studies based on 1,6-diphenyl-1,3,,5-hexatriene (DPH) anisotropy reveal an increase in membrane viscosity when platelets are artificially enriched with cholesterol supporting the concept that a high cholesterol content makes the plasma membrane more rigid [7,26].

Cholesterol-enriched platelets and platelets of hypercholesterolaemic patients show increased aggregation and secretion [1,2]. This finding led to the hypothesis that a more rigid plasma membrane facilitates signal transducing pathways in the lipid bilayer either through increased expression of receptors for activating agents or better post-receptor signal processing. In general, cholesterol enrichment requires prolonged shaking with lipid vesicles, which impairs the platelets' responsiveness to stimulation. The use of MEG-01 cells enabled us to modulate the C/P ratio by interfering with the synthesis of cholesterol. This approach is not possible in platelets since these cells lack significant cholesterol synthesis [9]. A possible disadvantage of the use of simvastatin is a shortage of other products of the mevalonate pathway e.g. farnesyl pyrophosphate which is an intermediate in the synthesis of Ras proteins [27]. However, when the mevalonate concentration is limited, preferentially products of the non-sterol pathways are produced, because their enzymes have higher affinities for mevalonate-derived substrates than those of the sterol pathway [27].

Our data show that interfering with cholesterol synthesis changes the C/P ratio in MEG-01 cells between 0.20 and 0.31. This range is smaller than after cholesterol enrichment of platelets where the C/P ratio ranged between 0.31 and 0.45 [28] and between 0.50 and 0.85 [7]. Megakaryocytes are known to have a lower C/P ratio than platelets. For instance, in guinea pigs the C/P ratio of these cells is 0.35 and in platelets 0.55 [29]. The mature megakaryocyte is rich in intracellular membranes, which probably have a lower cholesterol content than the plasma membrane. In general, the C/P ratio of the whole cell is between 0.28 and 0.42, while that of the plasma membrane is between 0.52 and 0.70. In fibroblasts 90% of total cholesterol is localized in the plasma membrane [30].

Stimulation with thrombin induced a fall in the lateral diffusion coefficient at all C/P ratios tested. Also MEG-01 cells cultured under normal conditions and megakaryocytes show such a decrease in D after stimulation with thrombin, ADP and Ca^{2+}-ionophore, whereas the mobile fraction remains constant [16]. The importance of the decrease in fluidity is uncertain. Since a more rigid membrane prior to stimulation enhances platelet aggregation and secretion [1,2], one may speculate that the fall in D seen after stimulation contributes to cell activation. The fall was steep in cells with a high diffusion coefficient before stimulation and gradually became smaller as the initial D was lower. Apparently, there is a limit to which lipid diffusion can decrease after cell activation.

A comparison between lipid diffusion and signal processing through the phospholipase C and adenyl cyclase pathways shows that the metabolism of (poly)phosphatidylinositol is affected by an increase in C/P ratio. At 37° C the data indicate a higher turnover of PI cycle at high C/P ratio, but apparently this does not result in major changes in Ca^{2+} homeostasis. At 22° C the differences are small and again Ca^{2+} influx and mobilization are similar in the different MEG suspensions. Apart from a slight difference in the cyclic AMP concentration, similar changes were induced in the different MEG suspensions after stimulation with PGI_2. Thus, under our experimental conditions there is little influence of the C/P ratio on thrombin- and PGI_2-induced signal processing within the range between 0.20 and 0.31. It is possible that the rigidity of the plasma membrane of MEG-01 cells [16] and megakaryocytes [15] makes signal processing in these cells relatively independent of fluidity changes in the membranes. On the other hand, the present attempt to change membrane fluidity by interfering with cholesterol synthesis differs markedly with the liposome enrichment during prolonged incubation of platelets used in most studies and provides a more physiological means to alter the C/P ratio.

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