

# Regulation of endothelial cell migration by amphiphiles—are changes in cell membrane physical properties involved?

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**Abstract** Endothelial cell (EC) migration is an integral part of angiogenesis and a prerequisite for malignant tumor growth. Recent studies suggest that amphiphilic compounds can regulate migration of bovine aortic ECs by altering the physical properties of the cell membrane lipid bilayers. A number of structurally different amphiphiles thus regulate the migration in quantitative correlation with their effects on the plasma membrane microviscosity. Many amphiphiles that affect EC migration and angiogenesis alter the physical properties of lipid bilayers, suggesting that such a regulatory mechanism may be of general importance. To investigate this notion, we studied the effects of lysophospholipids that inhibit migration of bovine aortic ECs and decrease cell membrane microviscosity, and of other amphiphiles that decrease membrane microviscosity (Triton X-100, octyl- $\beta$ -glucoside, arachidonic acid, docosahexaenoic acid, ETYA, capsaicin) on the migration of porcine aortic ECs. We further studied whether the enzyme secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>) would affect migration in accordance with the changes in membrane microviscosity induced by its hydrolysis products lysophospholipids and polyunsaturated fatty acids. Arachidonic acid, at low concentrations, promoted cell migration by a mechanism involving metabolic products of this compound. Apart from this effect, all the amphiphiles, as well as sPLA<sub>2</sub>, inhibited cell migration. A semi-quantitative analysis found a similar correlation between the effects on

migration and on lipid bilayer stiffness measured using gramicidin channels as molecular force transducers. These results suggest that changes in cell membrane physical properties may generally contribute to the effects of amphiphiles on EC migration.

**Keywords** Endothelial cell migration · Microviscosity · Lipid bilayer stiffness · Fluidity · Capsazepine

## Introduction

Endothelial cell (EC) migration is an integral part of angiogenesis and a prerequisite for malignant tumor growth [1]. Several growth factors and signaling pathways regulate EC migration in a specific manner [2]. Recent investigations suggest that EC migration can also be regulated by changes in the plasma membrane physical properties [3–5]. A number of structurally different amphiphilic compounds thus regulate the migration of bovine aortic endothelial cells (BAECs) in quantitative correlation with their effects on the plasma membrane microviscosity, as evaluated from the fluorescence anisotropy of membrane-embedded 1,6-diphenyl-1,3,5-hexatriene (DPH) [5].<sup>1</sup> There is a biphasic relation between the effects on migration and on membrane microviscosity. Three different lysophospholipids that decrease microviscosity inhibit migration in linear correlation with the effects on microviscosity. A small increase in microviscosity,

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<sup>1</sup> Characterizing changes in the physical properties of a lipid membrane by such measurements of microviscosity is not unproblematic, as will be described below. However, for the present a change in microviscosity will be taken to reflect that the membrane physical properties are altered.

induced by cholesterol,  $\alpha$ -tocopherol or other compounds, promotes migration with a similar relation between the effects on microviscosity and on migration. A further increase in microviscosity, induced by higher concentrations of cholesterol or  $\alpha$ -tocopherol, inhibits migration [5]. Many amphiphiles that regulate EC migration and have been implicated in angiogenesis and tumor growth, at similar concentrations, alter the physical properties of lipids bilayers (e.g., polyunsaturated fatty acids (PUFAs) [6–13], lysophospholipids (LPLs) [5, 14, 15], cholesterol [5, 16, 17], capsaicin [18–21], or genistein [22, 23]). A mechanism whereby EC migration is regulated by changes in bilayer physics thus may be of general importance.

Enzymes of the phospholipase A<sub>2</sub> (PLA<sub>2</sub>) superfamily catalyze hydrolysis of the *sn*-2 fatty acyl ester bond of cellular phospholipids to LPLs and free fatty acids [24]. Usually a polyunsaturated fatty acid (PUFA) is found at the *sn*-2 position, so primarily PUFAs, such as arachidonic acid (AA), are released [25]. Secretory PLA<sub>2</sub> (sPLA<sub>2</sub>) can activate BAEC migration by binding to plasma membrane receptors [26]. However, both LPLs and PUFAs decrease membrane microviscosity [5, 11, 12, 27]. Thus, sPLA<sub>2</sub> might affect cell migration by altering the physical properties of the cellular membranes. Whereas LPLs regulate BAEC migration in correlation with their effects on the plasma membrane microviscosity [5], AA and other PUFAs can promote [7, 8, 28] or inhibit [28, 29] cell migration. It is unknown whether changes in membrane physics are involved in the effects of PUFAs.

Capsaicin, which decreases cell membrane microviscosity [20], inhibits EC and cancer cell migration, as well as angiogenesis and tumor growth [18, 19]. Capsaicin is a specific agonist at the TRPV1 receptor, but interactions with this receptor do not seem to be involved, and the underlying mechanisms are poorly understood.

In order to further investigate the relation between the effects of amphiphiles on EC migration and on cell membrane physical properties, we have studied the effects of a number of structurally different amphiphiles, known to decrease cell membrane microviscosity (LPLs, Triton X-100, octyl- $\beta$ -glucoside, AA, docosahexaenoic acid, ETYA, capsaicin), on the migration of porcine aortic ECs (PAECs). We further studied whether the effects of the enzyme secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>) from *Naja Mossambica* would conform to those expected from a decrease in membrane microviscosity induced by LPLs and PUFAs. We finally studied the relation between

the amphiphile-induced effects on migration and on lipid bilayer stiffness, measured using gramicidin channels as molecular force transducers.

## Materials and methods

### Cell culture

Porcine aortic endothelial cells stably expressing the human VEGF receptor 2 were a gift from Y. Cao, Karolinska Institute, Stockholm, Sweden. The cells were grown at the bottom of plastic wells (diameter 16 mm, Falcon, Franklin Lakes, NJ, USA) coated with 25  $\mu$ g/ml collagen-IV (Fluka, Switzerland). The growth medium was: D-MEM:F12 (1:1) + GlutaMAX medium (Gibco, USA), supplemented with 10 % heat inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (all from Gibco, USA). The cells were passaged by gentle trypsinization. Cells in passage from 5 to 50 were seeded at 25,000–100,000 cells/cm<sup>2</sup>, and grown until confluence at 37°C in an ambient atmosphere with 5% CO<sub>2</sub>.

### Razor wound assay

Cell migration was studied using the razor wound technique, in which a sterile razor blade is gently pressed through a confluent cell monolayer and swept approximately 4 mm laterally to remove cells on one side of the “demarcation” line [30]. The cells were washed twice using serum-free growth medium. Subsequently 1 ml migration medium (serum-free growth medium, supplemented with 10 mg/ml collagen-IV) was added to each well. The compound under investigation was added to the migration medium from a stock solution, as described below. The cells migrated for 24 hours at 37 °C in ambient atmosphere with 5% CO<sub>2</sub>, and were subsequently fixated using absolute methanol, and stained with modified Giemsa-Wright stain (Gibco, USA). In each experiment a given experimental situation was studied in 4–6 wells, each with two razor wounds. The number of cells that had migrated across the demarcation line (number of migrated cells, NMC), was determined in 8–12 randomly chosen microscope visual fields, using computer-assisted counting by the image analysis program ImagePro 5.1 plus (MediaCybernetics, Silver Springs, MD, USA). Independent of the NMC, the result obtained using this method corresponded to a fixed ratio of the NMC, determined by manual counting (slope of linear regression  $\pm$  SE equal to  $0.85 \pm 0.01$ ,  $r = 0.96$ ). In each experiment the average NMC obtained under a

given experimental condition was normalized by the average NMC observed under control conditions. Unless otherwise noted, the results given represent the geometric average of the normalized NMC obtained in at least three independent experiments.

## Chemicals

The following compounds were dissolved in migration medium from 2–200 mM stock solutions in ethanol: Arachidonic acid (AA), docosahexaenoic acid (DHA), 1-palmitoyl-*sn*-glycero-3-phospho-L-choline (LPC), 1-palmitoyl-*sn*-glycero-3-phospho-L-serine (LPS), 5,8,11,14-Eicosatetraynoic acid (ETYA), 17-octadecynoic acid (all from Sigma, USA), 1-palmitoyl-*sn*-glycero-3-phospho-L-inositol (LPI) from Avanti Polar Lipids (Alabaster, AL, USA), capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) (ICN Biomedicals, Irvine, CA, USA), capsazepine (*N*-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide) (Tocris Cookson, Ellisville, MI, USA), 5-bromo-2[4-fluorophenyl]-3-[4-methylsulfonylphenyl]-thiophene (DuP-697) and nordihydroguaiaretic acid (NDGA) (both from Calbiochem, USA). The ethanol concentration never exceeded 0.4%, which did not affect cell migration or viability. Secretory phospholipase A<sub>2</sub> from *Naja mossambica mossambica* (Sigma, USA) was dissolved from a stock solution in PBS. Triton X-100 (Sigma) and octyl- $\beta$ -glucoside (Calbiochem, USA) were dissolved and diluted directly into the migration medium.

## Viability assay

Cell death was detected using the propidium iodide uptake method [31]. 20  $\mu$ l propidium iodide solution (1 mg/ml) (Gibco, USA) was added to 1 ml migration medium bathing cells in a confluent monolayer. After incubation for 30 min, cellular uptake of propidium iodide, visualized using a fluorescence microscope, was considered indicative of cell death.

## Lipolysis assay

Cells in confluent monolayers were incubated for 24 h in serum-free growth medium containing 0.5  $\mu$ Ci [<sup>3</sup>H] arachidonic acid (Perkin–Elmer, USA) and subsequently washed 3 times in serum-free growth medium, containing 3 mg/ml fatty acid free bovine serum albumin (BSA) (Sigma). The cells were incubated for 2 h in migration medium, containing sPLA<sub>2</sub> and BSA

(3 mg/ml). The supernatant was recovered and centrifuged at 15,000 *g* for 10 min. The resulting supernatant was transferred to Ultima Gold scintillation fluid (Perkin–Elmer) and the activity was determined using a beta counter.

## Inhibition of sPLA<sub>2</sub> by para-bromophenacyl bromide

The enzymatic activity of sPLA<sub>2</sub> was inhibited using para-bromophenacyl bromide (p-BPB) (Fluka, Switzerland), following the method described in [32]. sPLA<sub>2</sub> (1 mg/ml) +2 mM p-BPB dissolved in PBS was incubated at room temperature for 14–20 h. The resulting solution was centrifuged for 10 min at 20,000 *g* and the supernatant was dialyzed twice for 8–16 h against PBS using protein dialysis cells with a cutoff of 5 kDa.

## Statistical analysis

Unless otherwise noted, the number of migrated cells is given as the geometric average  $\pm$  SE of three independent experiments, as described above.

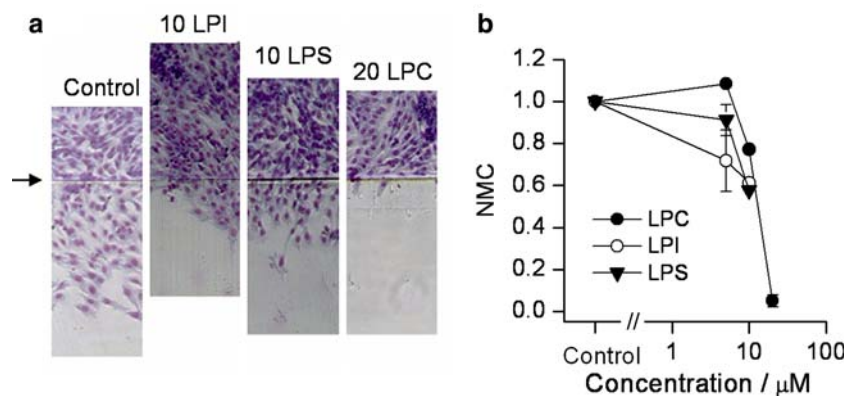
## Results

### Migration assay

PAEC migration was studied using the razor wound technique [30]. Cellular migration over 24 h was quantified by determining the number of cells that had traversed the demarcation line (number of migrated cells, NMC) using computer-assisted automatic counting (see Methods).

### Effects of lysophospholipids on PAEC migration

We initially studied whether the inhibitory effects of LPLs on BAEC migration [5] could be reproduced using PAECs. As shown in Fig. 1, lysophosphatidylinositol (LPI), lysophosphatidylserine (LPS), or lysophosphatidylcholine (LPC) inhibited PAEC migration from a concentration of 10  $\mu$ M (Fig. 1). Fig. 1a shows images from experiments using 10  $\mu$ M LPI, 10  $\mu$ M LPS, or 20  $\mu$ M LPC. As evaluated from the cellular morphology, there were no signs of cell damage using these concentrations of the LPLs (Fig. 1a). Fig 1b shows the concentration-dependent decrease in the NMC.



**Fig. 1 (a)** Regulation of cell migration by lysophospholipids. The arrow indicates the position of the demarcation line. **(a)** Effects of 10  $\mu\text{M}$  lysophosphatidylinositol (LPI), 10  $\mu\text{M}$  lyso-

phosphatidylserine (LPS), or 20  $\mu\text{M}$  lysophosphatidylcholine (LPC) on the NMC. **(b)** Concentration-dependent effects of the LPLs. Geometric average  $\pm$  SE,  $n \geq 3$

### Effects of synthetic amphiphiles on PAEC migration

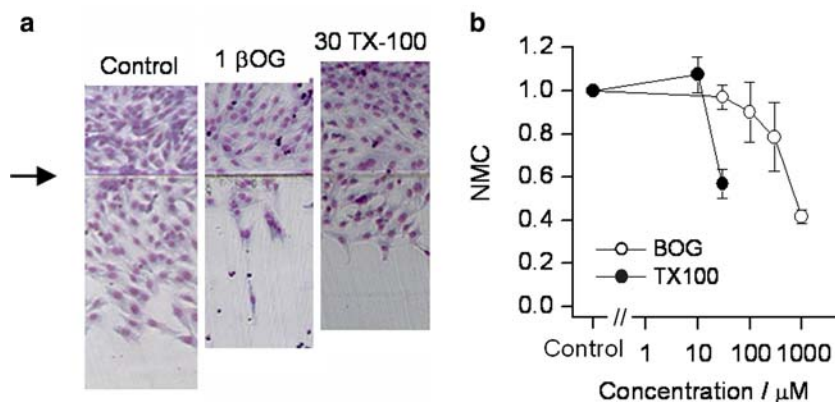
As a further test of whether amphiphiles that decrease membrane microviscosity would inhibit PAEC migration, we also studied the effects of Triton X-100 and octyl- $\beta$ -glucoside. These synthetic amphiphiles are structurally unrelated to LPLs, but also decrease membrane microviscosity [33]. As may be seen from Fig. 2a–b, Triton X-100 or octyl- $\beta$ -glucoside inhibited migration from concentrations of 30  $\mu\text{M}$  or 300  $\mu\text{M}$ , respectively. We note that these compounds, due to their ability to dissolve lipid bilayers, are commonly used in the reconstitution of membrane proteins. However, Triton X-100 in such studies is typically used at concentrations of  $\sim 2\%$  v/v ( $\sim 30$  mM) (e.g., [34]), which is 1000-fold higher than the lowest concentrations affecting migration. Further at the concentrations used in the present study, Triton X-100 and octyl- $\beta$ -glucoside reversibly modulate the function of a number of membrane proteins (e.g., N-type calcium channels [16]; voltage dependent sodium channels [17], and

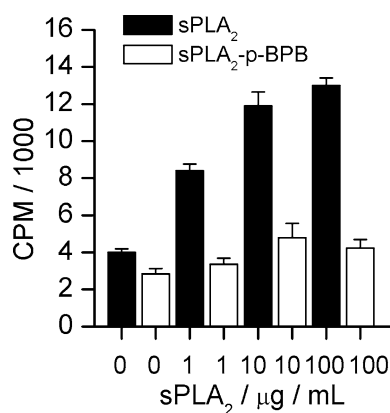
GABA<sub>A</sub> receptors [35]) without affecting the integrity or the plasma membrane conductance of the cells investigated. In the present experiments neither the cellular morphology (Fig. 2a), nor test of cell viability using propidium uptake (see below), indicated that the cells were damaged at the concentrations used.

### Lipolytic activity of sPLA<sub>2</sub>

In order to study the regulation of PAEC migration by sPLA<sub>2</sub> from *Naja Mossambica Mossambica*, we initially investigated whether the enzyme would hydrolyze the phospholipids in these cells. Cells loaded with radioactively labeled arachidonic acid ( $[^3\text{H}]\text{AA}$ ) were exposed to varying concentrations of sPLA<sub>2</sub>. As shown in Fig. 3, sPLA<sub>2</sub> caused a concentration-dependent release of  $[^3\text{H}]\text{AA}$ . When the enzymatic activity of sPLA<sub>2</sub> was blocked by para-bromophenacyl bromide (p-BPB) [32], the release of  $[^3\text{H}]\text{AA}$  was not increased above the background level (Fig. 3). sPLA<sub>2</sub> thus hydrolyzes the cellular phospholipids in a manner that can be blocked by p-BPB.

**Fig. 2** Regulation of migration by Triton X-100 and octyl- $\beta$ -glucoside ( $\beta\text{OG}$ ). The arrow indicates the position of the demarcation line. **(a)** Effects of 30  $\mu\text{M}$  Triton X-100 or 1 mM octyl- $\beta$ -glucoside. **(b)** Concentration-dependent effects of the two compounds. Geometric average  $\pm$  SE,  $n \geq 3$



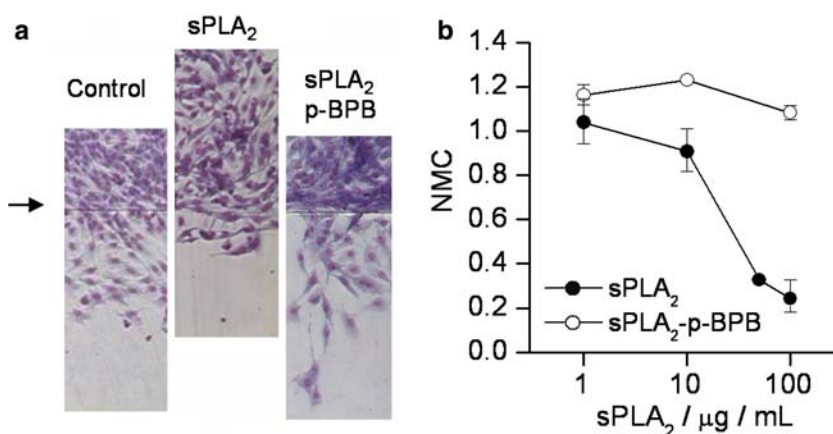


**Fig. 3** Effects of sPLA<sub>2</sub> on [<sup>3</sup>H]AA-release from porcine aortic endothelial cells. sPLA<sub>2</sub> causes concentration-dependent increase in the counts per minute (CPM) detected in the supernatant. Results from three independent experiments, each comprising 2–6 wells. Mean ± SE

Effects of sPLA<sub>2</sub> and polyunsaturated fatty acids on PAEC migration

sPLA<sub>2</sub> inhibited PAEC migration in a concentration-dependent manner. Figure 4a shows images from an experiment using 100 μg/ml sPLA<sub>2</sub>, which decreased the NMC by 80% (Fig. 4b). Neither the cellular morphology (cf. Fig. 4a), nor the test of cell viability using propidium iodide uptake, (see below), indicated that the cells were damaged by sPLA<sub>2</sub>. sPLA<sub>2</sub> blocked by p-BPB did not affect the NMC (Fig 4a–b). We note that the experiment shown in Fig. 4a indicates that the cellular migration pattern is altered by p-BPB. Due to the general variation in the results, the experiments did not allow for a conclusion of whether this is a systematic effect. If this were the case, it would imply that sPLA<sub>2</sub> affects the migration pattern also by a mechanism not dependent on enzymatic activity.

**Fig. 4** Regulation of cell migration by sPLA<sub>2</sub>. (a) Results from experiments using 100 μg/ml sPLA<sub>2</sub> or 100 μg/ml sPLA<sub>2</sub> blocked by para-bromophenacyl bromide (p-BPB). The arrow indicates the position of the demarcation line. (b) Relation between sPLA<sub>2</sub> concentration and NMC. Geometric average ± SE, n ≥ 3

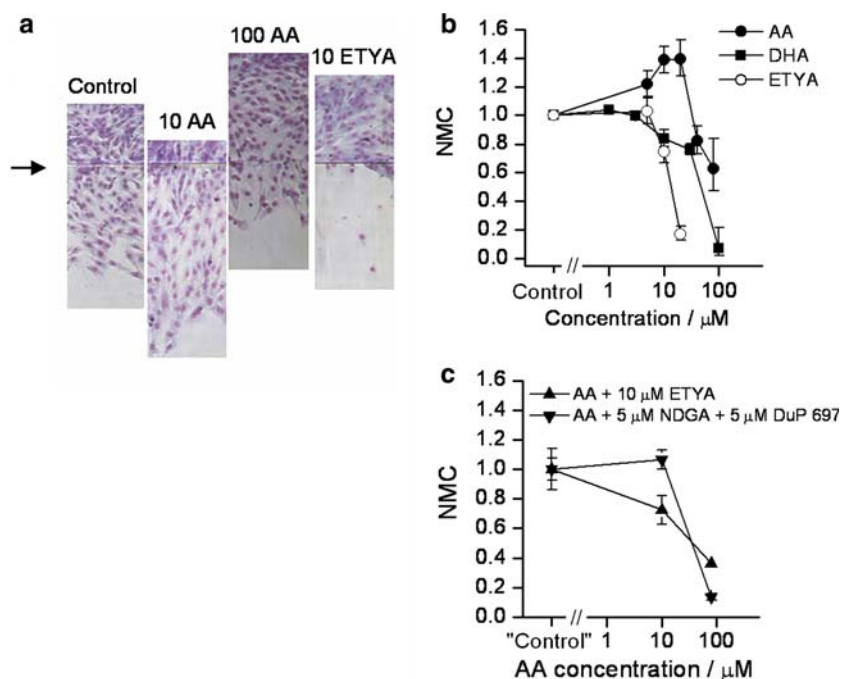


We further studied the regulation of PAEC migration by PUFAs. AA has previously been shown to promote EC and cancer cell migration at concentrations of 0.5–50 μM [8, 28], and inhibit smooth muscle cell migration at 30 μM [29]. In the present study AA had biphasic effects; concentrations of 5–40 μM increased the NMC by up to 40%, whereas higher concentrations *decreased* the NMC by up to 40% (Fig. 5a–b). Eicosatetraynoic acid (ETYA), a non-hydrolyzable AA analogue, which as AA decreases cell membrane microviscosity [27], in contrast, inhibited migration in a monotonic manner from a concentration of 10 μM (Fig. 5a–b). Docosahexaenoic acid (DHA), another PUFA, which previously has been shown to inhibit cancer and smooth muscle cell migration (at concentrations of 10–30 μM [28, 29]), similarly inhibited migration from a concentration of 10 μM (Fig. 5b).

Biphasic effects of arachidonic acid on migration—role of metabolic pathways

The fact that low concentrations of AA increased the NMC, whereas ETYA and DHA did not have this effect, suggested that metabolic products of AA were involved in the promotion of migration. This notion was investigated by exposing the cells to AA in the presence of ETYA, which blocks the major metabolic pathways for AA [36]. As shown in Fig. 5c, AA in the presence of 10 μM ETYA decreased the NMC in a concentration-dependent monotonic manner (the NMC shown in Fig. 5c is normalized by the value in the presence of 10 μM ETYA). Further, 10 μM AA, which in the absence of ETYA increased the NMC by 40%, actually *decreased* the NMC compared to the situation where only 10 μM ETYA was present. Blocking AA metabolism thus prevented the AA-induced promotion

**Fig. 5** (a) Effects of 10  $\mu\text{M}$  AA, 100  $\mu\text{M}$  AA or 10  $\mu\text{M}$  ETYA on migration. The arrow indicates the position of the demarcation line. (b) Concentration-dependent effects of AA, ETYA or DHA on the NMC. (c) Effects of AA in the presence of 10  $\mu\text{M}$  ETYA or 5  $\mu\text{M}$  Dup-697 + 5  $\mu\text{M}$  NDGA. Values normalized by the NMC in the presence of 10  $\mu\text{M}$  ETYA or 5  $\mu\text{M}$  Dup-697 + 5  $\mu\text{M}$  NDGA, respectively. Geometric average  $\pm$  SE,  $n \geq 3$



of migration at low concentrations, but did not affect the inhibition of migration.

Similar results were obtained when the metabolism of AA by the LOX and COX2 pathways were blocked by exposing the cells to nordihydroguaiaretic acid (NDGA) and 5-bromo-2[4-fluorophenyl]-3-[4-methylsulfonylphenyl]-thiophene (DuP-697). When AA was added in the presence of 5  $\mu\text{M}$  NDGA + 5  $\mu\text{M}$  DuP-697, the promotion of cell migration was prevented, whereas the inhibitory effect of AA was maintained. Figure 5c shows the effects of AA on the NMC normalized by the value in the presence of 5  $\mu\text{M}$  NDGA + 5  $\mu\text{M}$  DuP-697. In contrast, when the CYP450 metabolic pathway was blocked by exposing the cells to 10  $\mu\text{M}$  17-octadecynoic acid, neither the activating nor the inhibiting effects of AA were affected (results not shown).

In summary, when the metabolism of AA is blocked, the effects of this compound are similar to those of two structurally different PUFAs; migration is inhibited in a concentration-dependent monotonic manner.

#### Effects of capsaicin and capsazepine on PAEC migration

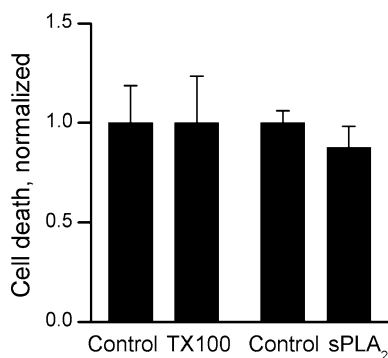
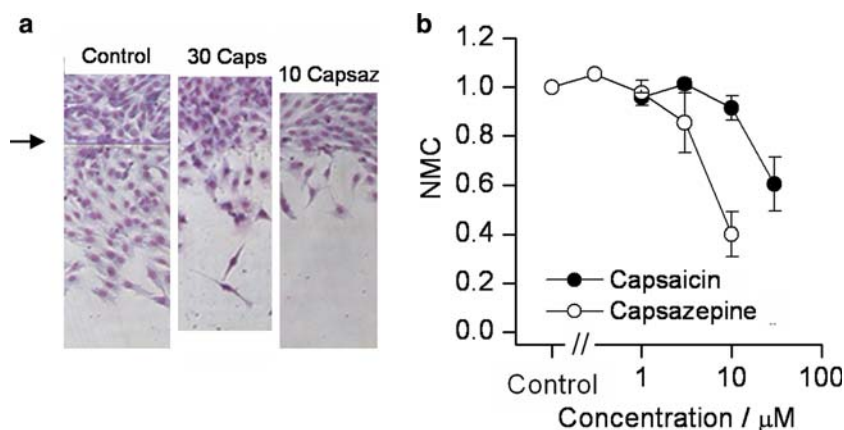
We finally studied the effects of capsaicin on PAEC migration. In previous investigations capsaicin (at concentrations of 5–20  $\mu\text{M}$ ) has been shown to inhibit EC and cancer cell migration as well as angiogenesis [18, 19]. In the present study capsaicin decreased the NMC from a concentration of 10  $\mu\text{M}$

(Fig. 6). To investigate whether capsaicin inhibited migration by a mechanism involving interactions with the TRPV1 receptor, we also studied the effects of capsazepine, which is an antagonist at the TRPV1 receptor, but as capsaicin decreases lipid bilayer stiffness measured using gramicidin channels as molecular force transducers [37] (see below). Capsazepine inhibited migration from a concentration of 3  $\mu\text{M}$  (Fig. 6).

#### Cell viability

As evaluated from the cellular morphology, none of the compounds used in the study caused cell damage at the concentrations used. Moreover, the cellular density in the part of the confluent monolayer not damaged by razor blade scraping was unaltered (see Figs. 1–2 and 4–6). To further investigate whether cell damage could be involved in the effects on migration, the cellular viability after exposure to test compound for 24 h was determined using the propidium iodide uptake method (see Methods). Cellular uptake of propidium iodide, following incubation for 30 min, was considered indicative of cell death. The average number of dead cells in three representative monolayer areas of  $60 \times 150 \mu\text{m}$  was determined using a fluorescence microscope. Figure 7 shows results from experiments studying the effects of 30  $\mu\text{M}$  Triton X-100 or 100  $\mu\text{g}/\text{ml}$  sPLA<sub>2</sub>. Neither of these procedures increased the number of dead cells relative to timed control experiments. Similarly none of the other amphiphiles

**Fig. 6** (a) Effects of 30  $\mu\text{M}$  capsaicin or 10  $\mu\text{M}$  capsazepine on migration. The arrow indicates the position of the demarcation line. (b) Concentration-dependent effects of the two amphiphiles. Geometric average  $\pm$  SE,  $n \geq 3$



**Fig. 7** Effects of 30  $\mu\text{M}$  Triton X-100 or 100  $\mu\text{g/ml}$  sPLA<sub>2</sub> on cell death evaluated using the propidium uptake method. The number of cells showing propidium uptake, in a confluent monolayer of 60  $\times$  150  $\mu\text{m}$ , was normalized by the corresponding number in timed control experiments. Geometric average  $\pm$  SE,  $n \geq 3$

affected the cellular viability at the concentrations described above (results not shown).

**Discussion**

The present study shows that the inhibition of BAEC migration by LPLs can be reproduced using PAECs. The inhibitory effects of a number of other amphiphiles known to decrease membrane viscosity (Triton X-100, octyl- $\beta$ -glucoside, AA, DHA, ETYA and capsaicin), extend the correlation between amphiphile-induced changes in EC migration and cell membrane physical properties beyond that previously described.

Effects of arachidonic acid, lysophospholipids and sPLA<sub>2</sub>

Low concentrations of AA promote migration, while higher concentrations inhibit migration. When

DuP-697 and NDGA is used to block AA metabolism by the COX2 and LOX pathways, the promotion of migration is prevented, whereas the inhibition is maintained. When AA is applied in the presence of ETYA the same effects are observed. Moreover, in the presence of ETYA, AA at a low concentration (10  $\mu\text{M}$ ) inhibits rather than promotes migration. The fact that the promotion of migration can be inhibited by two different procedures that block AA metabolism, lead us to conclude that metabolic products of AA are involved in this effect. Further, when the metabolism of AA is blocked, the inhibitory effects of this PUFA conform to the general correlation with the changes in membrane microviscosity.

We find that sPLA<sub>2</sub> promotes phospholipid hydrolysis and inhibits PAEC migration. The effects on AA release are observed from a concentration of 1  $\mu\text{g/ml}$ , while the effects on migration occur from a concentration of 10  $\mu\text{g/ml}$ . At low concentrations of AA the effects on migration are opposite to those of LPLs (promoting and inhibiting migration, respectively), while at higher concentrations both AA and LPLs inhibit migration. sPLA<sub>2</sub> thus regulates migration in accordance with the combined effects of LPLs and AA - and with the general correlation with the changes in membrane microviscosity.

Mechanism underlying the regulation of migration

The amphiphile-induced regulation of cellular migration may involve specific molecular interactions and/or more or less “non-specific” effects of their adsorption to hydrophobic surfaces. Specific mechanisms may contribute to the effects observed. However, the quantitative correlation between the effects of a number of structurally different amphiphiles on membrane microviscosity and cellular migration, observed by Ghosh et al [5], and the further qualitative correlation

observed in the present study, suggests that changes in the physical properties of cell membrane lipid bilayers are involved—be it in absence or presence of additional specific mechanisms.

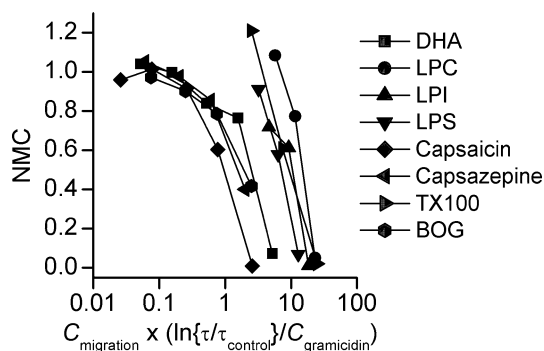
Changes in the physical properties of cell membrane lipid bilayers may affect cellular migration by several mechanisms, such as; by changing the energetic cost of the bilayer deformations involved in cellular movement [38]; by changing the energetic cost of vesicular release/adsorption from/to the cell membrane lipid bilayers [38]; or by changing the energetic cost of the bilayer deformations associated with membrane protein conformational changes [37]. It is beyond the scope of the present study to evaluate the possible role of these mechanisms in the observed effects. However, it should be noted that several of the amphiphiles (LPLs, Triton X-100, octyl- $\beta$ -glucoside, capsaicin and capsazepine), at concentrations similar to those used in the present study, can regulate the function of lipid bilayer-embedded proteins by altering the bilayer physical properties [15–17, 21, 37].

How could the adsorption of amphiphiles to a lipid bilayer affect the energetic cost of a bilayer deformation? Amphiphile-induced changes in the physical properties of lipid bilayers have been described using a number of different descriptors (cf. [37]). The basis for the present investigation was the previously observed quantitative correlation between the effects on EC migration and on membrane microviscosity, as evaluated from changes in the fluorescence anisotropy of membrane-embedded DPH [5]. The present findings extend this correlation. The advantage of evaluating changes in the physical properties of cellular membranes by measuring the fluorescence anisotropy of membrane-embedded DPH is that such measurements can be done in the membranes of living cells. However, the interpretation of the results of such measurements is problematic. Strictly, the microviscosity (1/fluidity) of a lipid bilayer refers to the rate of molecular motion in the bilayer. Nevertheless, the fluorescence anisotropy of DPH is determined by both the rate and extent of bilayer molecular motion [39], as well as by a number of other factors [39–41]. Moreover, the energetic cost of a lipid bilayer deformation cannot be altered by a change in microviscosity, per se, and the possible causal relation between membrane microviscosity and cell function has never been clear [39]. Thus, while the effects of the amphiphiles on cellular migration correlate with the changes in the fluorescence anisotropy of DPH, this correlation does not provide information about the changes in the bilayer material properties that determine the energetics of lipid bilayer deformations.

If not membrane microviscosity, what then? The energetic cost of a lipid bilayer deformation may be altered by changes in monolayer spontaneous curvature (the curvature adopted by the monolayers in the absence of the hydrophobic interactions between the monolayers, cf. [37]), and the effects of amphiphiles on cellular function have often been ascribed to changes in monolayer spontaneous curvature [42, 43]. However, LPLs and Triton X-100 promote positive monolayer curvature and octyl- $\beta$ -glucoside is likely to do the same [21], while capsaicin and PUFAs tend to promote negative monolayer curvature [21, 44]. The effects on PAEC migration thus do not correlate with the expected changes in monolayer spontaneous curvature.

Amphiphile-induced changes in the energetic cost of a lipid bilayer deformation may be characterized using gramicidin channels as molecular force transducers. Briefly, gramicidin channel formation in a lipid bilayer where the unperturbed thickness of the hydrophobic core (hydrophobic thickness) exceeds the length of the channel hydrophobic exterior (hydrophobic length) involves a bilayer deformation, where the bilayer hydrophobic thickness locally adjusts to match the channel hydrophobic length. As the bilayer deformation energy contributes to the energetic cost of channel formation, amphiphile-induced changes in the bilayer elastic properties are reflected in the channel appearance rate and lifetime. A change in the bilayer elasticity, which increases both the channel appearance rate and lifetime, is defined as decrease in bilayer stiffness and vice versa ([17, 21], for a recent review, see also [37]). LPLs, Triton X-100, octyl- $\beta$ -glucoside, PUFAs, capsaicin, and capsazepine all decrease lipid bilayer stiffness. Given that specific interaction between an amphiphile and the gramicidin channel can be excluded (which is the case for the LPLs used in the present study, as well as for Triton X-100, octyl- $\beta$ -glucoside, capsaicin and capsazepine [37]), the amphiphile-induced change in the energetic cost of the bilayer deformation associated with channel dissociation ( $\Delta\Delta G_{\text{def}}$ ) is equal to:  $\ln\{\tau_{\text{amph}}/\tau_{\text{control}}\} \cdot RT$ , where  $\tau_{\text{amph}}$  and  $\tau_{\text{control}}$  denote the channel lifetime in the presence and absence of amphiphile, respectively, and  $R$  and  $T$  are the gas constant and the temperature in Kelvin [37]. The effects of low concentrations of Triton X-100, octyl- $\beta$ -glucoside and capsaicin, on the function of voltage dependent sodium channels, are an approximately linear function of  $\ln\{\tau_{\text{amph}}/\tau_{\text{control}}\}$  [17, 21, 37]. We may perform a similar comparison for the amphiphile-induced effects on PAEC migration. Figure 8 shows the NMC as the function of  $C_{\text{migration}} \cdot (\ln\{\tau_{\text{amph}}/\tau_{\text{control}}\}/C_{\text{gA}})$ , where  $C_{\text{migration}}$  is the nominal amphiphile concentration in the migration experiments, and





**Fig. 8** Relation between the effects of the amphiphiles on the NMC and on lipid bilayer stiffness measured using gramicidin channels. The NMC is expressed as a function of  $C_{\text{migration}} \cdot (\ln\{\tau_{\text{amph}}/\tau_{\text{control}}\}/C_{\text{gA}})$ , where  $C_{\text{migration}}$  is the concentration in the migration experiments, and  $\ln\{\tau_{\text{amph}}/\tau_{\text{control}}\}$  represents the change in gramicidin channel lifetime induced by a low amphiphile concentration ( $C_{\text{gA}}$ ) in previous studies. Previously determined values of  $\ln\{\tau_{\text{amph}}/\tau_{\text{control}}\}$ : 0.747 (3  $\mu\text{M}$  Triton X-100) and 0.750 (300  $\mu\text{M}$  octyl- $\beta$ -glucoside) from [17]; 0.259 (10  $\mu\text{M}$  capsaicin) from [21]; 0.155 (3  $\mu\text{M}$  DHA), from [13] and Michael Bruno and Olaf S. Andersen, personal communication. The values of  $\ln\{\tau_{\text{amph}}/\tau_{\text{control}}\}$  represent the lowest amphiphile concentrations studied. The NMC is given as geometric average  $\pm$  SE,  $n \geq 3$

$\ln\{\tau_{\text{amph}}/\tau_{\text{control}}\}$  represents the change in  $\tau$  induced by a small nominal amphiphile concentration ( $C_{\text{gA}}$ ) in gramicidin channel experiments [13, 16, 17, 21].<sup>2</sup>

As may be seen from Fig. 8, the curves describing the effects of the different amphiphiles are similarly shaped, but shifted over a little more than a decade on the x-axis. The different positions of the curves indicates some specificity in the observed effects. However, the curves describing the effects of the three LPLs are similar—and similar to that for Triton X-100—a structurally different amphiphile. Further, the curves for DHA, capsaicin and capsazepine and octyl- $\beta$ -glucoside, four structurally different amphiphiles, are similar. If the amphiphiles acted only by specific mechanisms such findings would be very unlikely. Moreover, the fact that the curves for capsaicin and capsazepine, as well as for two structurally unrelated amphiphiles, are similar strongly suggest that the

<sup>2</sup> Triton X-100 and octyl- $\beta$ -glucoside decrease lipid bilayer stiffness measured using gramicidin channels in living cells [35], but amphiphile-induced changes in  $\tau$  have not been quantitatively studied in cells. The comparison of the effects of amphiphiles on cell migration and on  $\tau$  (shown in Fig. 8) therefore is based on gramicidin channel experiments done in dioleoylphosphatidylcholine/*n*-decane lipid bilayers. This becomes relevant, first, because of the problems involved in evaluating changes in membrane physics on the basis of the microviscosity, second because no other measures of such changes - even qualitatively - correlate with the effects on migration.

effects of capsaicin are not due to interactions with the TRPV1 receptor.

In conclusion, we have further investigated the correlation between the effects of amphiphiles on EC migration and on the physical properties of lipid bilayers. We find that the effects of a number of structurally different amphiphiles extend the correlation beyond that previously observed. Thus the present findings support the notion that changes in bilayer physical properties contribute to the effects on migration. Such changes further may contribute to the effects of capsaicin [18, 19] and sPLA<sub>2</sub> [24] on tumor growth and angiogenesis.

## Reference

- Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1:27–31
- Hoeben A, Landuyt B, Higley MS, Wildiers H, Van Oostrom AT, De Bruijn EE (2004) Vascular endothelial growth factors and angiogenesis. *Pharmacol Rev* 56:549–580
- Gojova A, Barakat AI (2005) Vascular endothelial wound closure under shear stress: role of membrane fluidity and flow-sensitive ion channels. *J Appl Physiol* 98:2355–2362
- Van Aalst JA, Burmeister W, Fox PL, Graham LM (2004) Alfa-tocopherol preserves endothelial cell migration in the presence of cell-oxidized lipoprotein by inhibiting changes in cell membrane fluidity. *J Vasc Surg* 39:229–237
- Ghosh PK, Vasanji A, Murugesan G, Eppell SJ, Graham LM, Fox PL (2002) Membrane microviscosity regulates endothelial cell motility. *Nat Cell Biol* 11:894–900
- Antoniotti S, Fiorio Pla A, Pregnolato S, Mottola A, Lovisolo D, Munaron L (2003) Control of endothelial cell proliferation by calcium influx and arachidonic acid metabolism: A pharmacological approach. *J Cellular Physiol* 197:370–378
- Kanayasu-Toyoda T, Morita I, Murota S (1996) Docosapentaenoic acid (22:5, n-3), an elongation metabolite of eicosapentaenoic acid (20:5, n-3) is a potent stimulator of endothelial cell migration on pretreatment in vitro. *Prostaglandin Leukotr Essent Fatty Acids* 54:319–325
- Sa G, Fox PL (1994) Basic fibroblast growth factor-stimulated endothelial cell movement is mediated by a pertussis toxin-sensitive pathway regulating phospholipase A2 activity. *J Biol Chem* 269:3219–3225
- Bogatcheva NV, Sergeeva MG, Dudek SM, Verin AD (2005) Arachidonic acid cascade in endothelial cell pathobiology. *Microvasc Res* 69:107–127
- Larsson SC, Kumlin M, Ingelman-Sundberg M, Wolk A (2004) Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms. *Am J Clin Nutr* 79:935–945
- Beck R, Bertolino S, Abbot SE, Aaronson PI, Smirnov SV (1998) Modulation of arachidonic acid release and membrane fluidity by albumin in vascular smooth muscle and endothelial cells. *Circ Res* 83:923–931
- Stillwell W, Wassall SR (2003) Docosahexaenoic acid: membrane properties of a unique fatty acid. *Chem Phys Lipids* 126:1–27
- Bruno MJ, Koeppe RE II, Andersen OS (2005) Modification of gramicidin channel function by PUFAs depends on double-bond structure. *Biophysical J* 88:575A

14. Sutphen R, Krischer JP (2004) Lysophospholipids as potential biomarkers in ovarian cancer. *Cancer Epidemiol Biomarkers Prev* 13:1185–1191
15. Lundbæk JA, Andersen OS (1994) Lysophospholipids modulate channel function by altering the mechanical properties of lipid bilayers. *J Gen Physiol* 104:645–673
16. Lundbæk JA, Birn P, Girshman J, Hansen AJ, Andersen OS (1996) Membrane stiffness and channel function. *Biochemistry* 35:3825–3830
17. Lundbæk JA, Birn P, Hansen AJ, Sogaard R, Nielsen C, Girshman J, Bruno MJ, Tape SE, Egebjerg J, Greathouse DV, Mattice GL, Koeppe RE 2nd, Andersen OS (2004) Regulation of sodium channel function by bilayer elasticity: the importance of hydrophobic coupling. Effects of micelle-forming amphiphiles and cholesterol. *J Gen Physiol* 123:599–621
18. Min JK, Han KY, Kim EC, Kim YM, Lee SW, Kim OH, Kim KW, Gho YS, Kwon YG (2004) Capsaicin inhibits in vitro and in vivo angiogenesis. *Cancer Res* 64:644–651
19. Mori A, Lehmann S, O'Kelly J, Kumagai T, Desmond JC, Pervan M, McBride WH, Kizaki M, Koeffler H (2006) Capsaicin, a component of red peppers, inhibits the growth of androgen-independent, p53 mutant prostate cancer cells. *Cancer Res* 66:3222–3229
20. Meddings JB, Hogaboam CM, Tran K, Reynolds JD, Wallace JL (1991) Capsaicin effects on non-neuronal plasma membranes. *Biochim Biophys Acta* 1070:43–50
21. Lundbæk JA, Birn P, Tape SE, Toombes GES, Sogaard R, Koeppe RE 2nd, Gruner SM, Hansen AJ, Andersen OS (2005) Capsaicin regulates voltage-dependent sodium channels by altering lipid bilayer elasticity. *Mol Pharmacol* 68:680–689
22. Su SJ, Yeh TM, Chuang WJ, Ho CL, Chang KL, Cheng HL, Liu HS, Cheng HL, Hsu PY, Chow NH (2005) The novel targets for anti-angiogenesis of genistein on human cancer cells. *Biochem Pharmacol* 69:307–301
23. Hwang TC, Koeppe RE 2nd, Andersen OS (2003) Genistein can modulate channel function by a phosphorylation-independent mechanism: Importance of hydrophobic mismatch and bilayer mechanics. *Biochemistry* 42:13646–13658
24. Murakami M, Kudo I (2002) Phospholipase A2. *J Biochem* 3:285–292
25. Bonventre JV (1992) Phospholipase A2 and signal transduction. *J Am Soc Nephrol* 3:128–150
26. Rizzo MT, Nguyen E, Aldo-Benson M, Lambeau G (2000) Secreted phospholipase A(2) induces vascular endothelial cell migration. *Blood* 96:3809–3815
27. Brown M., Anderson KM, Patel H, Hopfinger J, Harris JE (1992) Eicosatetraenoic acid and arachidonic acid-induced changes in cell membrane fluidity consonant with differences in computer aided design-structures. *Biochim Biophys Acta* 1105:285–290
28. Brown MD, Hart CA, Gazi E, Bagley S, Clarke NW (2006) Promotion of prostatic metastatic migration towards human bone marrow stroma by Omega 6 and its inhibition by Omega 3 PUFAs. *Brit J Cancer* 94:842–853
29. Mizunati M, Asano M, Roy S, Okuda Y (1997) Omega 3 polyunsaturated fatty acids inhibit migration of human vascular smooth muscle cells in vitro. *Life Sciences* 61:269–274
30. Burk RR (1973) A factor from a transformed cell line that affects cell migration. *PNAS* 2:369–372
31. Dengler WA, Fiebig HH (1995) Development of a propidium iodide fluorescence assay for proliferation and cytotoxicity assays. *Anticancer Drugs* 6:522–532
32. Roberts MF, Deems RA, Dennis EA (1977) Chemical modification of the histidine residue in phospholipase A2 (Naja Naja Naja). A case of half site reactivity. *J Biol Chem* 252:2405–2411
33. Rinken A, Harro J, Engstrom L, Orelund L (1998) Role of fluidity of membranes on the guanyl nucleotide-dependent binding of cholecystokinin-8S to rat brain cortical membranes. *Biochem Pharmacol* 55:423–431
34. Jones MN (1999) Surfactants in membrane solubilisation. *Int J Pharm* 177:137–159
35. Sogaard R, Werge TM, Bertelsen C, Lundbye C, Madsen KL, Nielsen CH, Andersen MB, Lundbæk JA (2006) GABA<sub>A</sub> receptor function is regulated by lipid bilayer elasticity. *Biochemistry* 45:13118–13129
36. McGiff JC (1991) *Annu Rev Pharmacol Toxicol*. Cytochrome P450 metabolism of arachidonic acid 31:339–369
37. Lundbæk JA (2006) Regulation of membrane protein function by the elasticity of the host lipid bilayer. A single molecule technology to measure the bilayer properties experienced by an embedded protein. *J Phys Cond Matt* 18:S1305–S1344
38. Navarro A, Andand-Apte B, Parat M (2004) A role for caveolae in cell migration. *FASEB J* 18:1801–1811
39. Lee AG (1991) Lipids and their effects on membrane proteins: evidence against a role for fluidity. *Prog Lipid Res* 30:323–348
40. Lentz BR (1993) Use of fluorescent probes to monitor molecular order and motions within liposome bilayers. *Chem Phys Lipids* 64:99–116
41. Muller JM, van Faassen EE, van Ginkel G (1994) Experimental support for a novel compound motion model for the time resolved fluorescence anisotropy decay of TMA-DPH in lipid vesicle bilayers. *Chem Phys* 185:393–404
42. McMahon HT, Gallop JL (2005) Membrane curvature and mechanisms of dynamic cell membrane remodelling. *Nature* 438:590–596
43. Zimmerberg J, Kozlov MM (2006) How proteins produce cell membrane curvature. *Nat Rev Mol Cell Biol* 7:9–19
44. Seddon JM (1990) Structure of the inverted hexagonal (HII) phase, and non-lamellar phase transitions of lipids. *Biochim Biophys Acta* 1031:1–69