Temperature-dependent changes in plasma-membrane lipid order and the phagocytotic activity of the amoeba *Acanthamoeba castellanii* are closely correlated

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The relationship between temperature-dependent changes in phagocytotic activity of *Acanthamoeba castellanii* and the fatty acid composition and physical properties of plasma membrane-enriched fractions were determined in cultures acclimated to 30 °C and 15 °C. Chilling (from 30 °C to 15 °C) had a very pronounced short-term inhibitory effect on phagocytosis only in stationary-phase cultures, which displayed a low degree of fatty acid unsaturation. A subsequent increase in phagocytosis by these cells was correlated with a low-temperature-induced increase in fatty acid unsaturation [shown previously [Jones, Lloyd and Harwood (1993) *Biochem. J.* 296, 183–188] to be due to *n*-6 desaturase induction]. Plasma membrane-enriched fractions from 15 °C-acclimated cells also showed a marked increase in the relative proportion of polyunsaturated fatty acids. Steady-state fluorescence depolarization studies, using the membrane probe diphenylhexatriene, revealed increases in plasma membrane order with decreasing assay temperature. Over the upper assay-temperature range (25–40 °C), fluorescence anisotropy values were higher in membranes from 30 °C-acclimated cells; a 3.3 °C relative displacement of plots indicated that temperature-induced

changes in membrane lipid composition compensated for approx. 22% of the ordering effect of low temperature. Changes in the temperature-dependence of fluorescence anisotropy, possibly corresponding to lateral phase separations or alterations in other bulk physical properties of membranes, occurred between 20 and 25 °C in membranes from 30 °C-acclimated cells and between 15 and 20 °C in membranes from 15 °C-acclimated cells. Fluorescence anisotropy plots were superimposed at assay temperatures between 5 and 15 °C. Short-term phagocytotic rates in whole cells decreased with assay temperature. Arrhenius discontinuities in rates of phagocytosis occurred at approx. 25.0 °C and 17.5 °C in 30 °C- and 15 °C-acclimated cells respectively, and in each case were thus within the temperature ranges of slope-change in the corresponding fluorescence anisotropy plots. The results show a direct correlation between plasma membrane fatty acid unsaturation, membrane physical properties and phagocytotic activity in *A. castellanii*. Therefore, a specific integrated physiological process has been correlated with fatty acid desaturase induction for the first time.

INTRODUCTION

One of the most important mechanisms employed by organisms to enable adaptation to low temperature involves maintenance of membrane order through appropriate manipulation of membrane composition [1,2]. The rigidifying effect of low temperature on membranes can be counteracted in a number of ways, the most common being an increased relative synthesis and incorporation of unsaturated fatty acids compared with saturated fatty acids [1,3]. Thus, membrane adaptation may involve a lowering of the melting points of constituent lipids to enhance the fluid-like properties of chilled membranes. It is now generally considered that a prerequisite for physiological activity and growth in many organisms is that the majority of membrane lipids exist in a liquid-crystalline state at ambient or body temperatures [4–7]. Thus, a lowering of the membrane gel-to-liquid-crystalline phase transition temperature, to a value below the new incubation temperature, is commonly observed during low-temperature adaptation [7,8]. Furthermore, approximate phase-transition temperatures of high- and low-temperature-acclimated membranes, estimated by independent physical tech-

niques, have been found to correlate with breaks in Arrhenius plots of certain membrane-bound enzyme (e.g. transport-ATPase) activities in a range of organisms (see [1,9,10]). Despite these findings, there is some disagreement as to the role of the lipid environment in regulating certain membrane-mediated functions; alternative explanations for low-temperature-induced alterations in membrane-bound protein activity have included changes in the viscosity of the external aqueous phase [11], changes in protein–protein interactions, or in the conformation of the proteins themselves [12,13]. Moreover, a clear relationship between an integrated functional physiological process and membrane order has yet to be demonstrated.

In order to establish a relationship between physiological function and the physical properties determined by membrane lipid composition, it is desirable to select a model organism that is susceptible to low-temperature-induced (or other) modifications of lipid metabolism. Recent studies have revealed that the soil amoeba *Acanthamoeba castellanii* displays rapid and marked increases in its overall degree of membrane lipid unsaturation during early-exponential growth in batch culture [14] and following chilling of late-exponential- or stationary-

Abbreviation used: DPH, 1,6-diphenyl-1,3,5-hexatriene.

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phase cultures [15,16]. Rapid induction of the microsomal ω 6-desaturase (within 10 min) results in large increases in the relative proportion of the polyunsaturated fatty acid linoleate and appears to be primarily responsible for increases in the overall unsaturation index (average number of double bonds per fatty acid) of *A. castellanii* [16,17]. However, detailed investigations examining the relationships between these compositional changes and membrane physical properties and physiological functions of *A. castellanii* have yet to be undertaken. Avery et al. [16] recently attempted to relate membrane composition to physiological function in *A. castellanii* and found that whereas a low-temperature-induced temporary cessation in cell division and change in the rate of oxygen uptake were independent of lipid composition, there appeared to be a correlation between changes in phagocytotic rates at low temperature and the degree of membrane lipid unsaturation.

The purpose of the present study was to assess how changes in plasma membrane fatty acid composition at low temperature are related to the temperature-dependencies of membrane order and phagocytotic activity. The results indicate a clear association of changes in the temperature-dependence of phagocytosis with plasma membrane physical properties in *A. castellanii*, and represent the first example of a macroscopic functional physiological process being directly dependent on desaturase induction and altered membrane composition at low temperature.

MATERIALS AND METHODS

Organism, medium and culture conditions

Acanthamoeba castellanii was grown in P.G.Y. medium, containing (% w/v): Difco Proteose Peptone, 0.75; Oxoid yeast extract, 0.75; and glucose, 1.5. Cultures (200 ml in 500-ml Erlenmeyer flasks) were inoculated with cells from 48 h cultures, to give an initial cell density of approx. $2 \times 10^5 \text{ ml}^{-1}$, and were incubated at 30 °C with rotary aeration at 200 rev. min^{-1} . For rapid shift to low temperature, flasks containing 48 h cultures were placed in an ice slurry and agitated using a magnetic stirrer. After approx. 4.5 min, when the temperature had decreased to approx. 15 °C, the flasks were transferred to a shaking incubator (200 rev. min^{-1}) at 15 °C. Cells acclimated to 15 °C were used for experimental purposes after 12 h incubation at this temperature, when low-temperature-induced changes in fatty acid composition were largely complete [15,16].

Phagocytosis assay

Undiluted samples (0.9 ml) from *A. castellanii* cultures were transferred to 1.5-ml microcentrifuge tubes, either with or without 12.5 mM sodium azide. These were immersed for a short time (0–60 s, depending on temperature shift) in a water bath at 0–2 °C and, after rapid attainment of the required assay temperature (10–30 °C), tubes were incubated with rotary aeration (200 rev. min^{-1}) at the desired incubation temperature. After 5 min pre-incubation, phagocytosis was initiated by the addition of 100 μl of fluorescently labelled yellow-green latex microbeads, diameter 1.0 μm (Polysciences; Warrington, PA, U.S.A.) to a final density of approx. $5 \times 10^7 \text{ beads} \cdot \text{ml}^{-1}$. After 10 min incubation with latex beads, phagocytosis was stopped by dilution of the cell-plus-bead suspension to 10 ml with buffer (100 mM mannitol, 15 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, pH 7.2, adjusted with 2 M NaOH) at 4 °C. Cells were separated from free beads by centrifugation for 1 min at 750 g (after which > 95% of beads remained in suspension) and the bead-containing supernatant was discarded. The cell pellet was suspended in 1 ml of buffer and maintained at 4 °C until required for analysis. All samples were

analysed within 24 h of isolation; control experiments confirmed that no change in total numbers of phagocytosed or cell-surface-bound beads occurred during storage of washed cells at 4 °C for up to 1 week. Uptake of latex beads by cells was determined using a Skatron Argus flow cytometer (Skatron; Tromsø, Norway), as described previously [18]. Analysis of cells incubated in the presence of sodium azide gave average numbers of beads bound to the cell surface and these values were subtracted from those obtained for cells incubated in the absence of sodium azide to give the average number of internalized (phagocytosed) beads. Phagocytosis by *A. castellanii* is linear for approx. 30 min under the present conditions [18], and calculated numbers of beads phagocytosed after 10 min were converted into initial phagocytotic rates.

Plasma membrane isolation

Isolation of plasma membrane-enriched fractions was performed by differential centrifugation of disrupted silica-bead-coated cells, as described previously [15].

Lipid extraction and analysis

Lipids were extracted from pellets of either whole cells or plasma membrane fractions using the method of Bligh and Dyer [19] as modified by Griffiths and Harwood [20].

For fatty acid analysis, methyl esters were generated by acid-catalysed methanolysis [2.5% (v/v) H_2SO_4 in methanol] at 70 °C for 2 h. After extraction with light petroleum ether (b.p. 60–80 °C), aliquots of methyl esters were analysed by gas-liquid chromatography. Pentadecanoate was used as an internal standard. Separations were routinely achieved using 10% SP-2330 on 100/120 Supelcoport (Supelco) packed into a glass column [1.5 m $\times$ 4 mm (internal diam.)]. Fatty acids were identified by comparison with authentic standards and had been fully identified previously [15]. Fatty acids were quantified using the method of Carroll [21] or by using integrating software (Rachel, supplied by LabLogic, Sheffield, U.K.).

Steady-state fluorescence depolarization

Plasma membrane fractions were suspended in buffer (20 mM Tes, 0.3 M sucrose, pH 6.9, adjusted using 2 M NaOH) to a final density of approx. 50 μg of membrane (dry wt.) $\cdot \text{ml}^{-1}$. 1,6-Diphenyl-1,3,5-hexatriene (DPH, 5 μl), from a stock solution in tetrahydrofuran, was added to 3 ml of membrane suspension to attain a final concentration of 3 μM DPH. The suspension was vortexed and left to stand for at least 20 min prior to analysis. Suspensions were analysed in a 10-mm-pathlength quartz cuvette, using a Perkin Elmer LS5 fluorescence spectrometer with polarization accessory. A thermostatically controlled water bath and circulator were used to control precisely the cuvette temperature at assay temperatures in 5 min steps from 40 °C to 5 °C (after pre-warming the samples). DPH was excited with vertically polarized light at 365 nm. The vertical and horizontal vectors of the emitted light were measured at 430 nm (band pass 10 nm). Fluorescence anisotropy (r) was calculated according to the equation: $r = (I_{VV} - I_{VH}) / (I_{VV} + 2I_{VH})$, where I_{VV} refers to the intensity of the vertical component of emitted light (from the vertically polarized excitation source) and I_{VH} to the intensity of the horizontal component of depolarized light. Light scattering was determined by using identical membrane samples in the absence of DPH. This was found to account for less than 4% of the total emitted light and therefore no corrections were employed [22–24].

RESULTS

Effect of chilling on fatty acid unsaturation and phagocytotic activity of *A. castellanii*

Previous studies have shown that whereas a marked increase in the degree of fatty acid unsaturation of *A. castellanii* results from chilling of late-exponential- and stationary-phase cultures [15,16], such changes are not apparent following chilling of mid-exponential-phase cultures where membrane lipids already display a high degree of unsaturation [16]. Table 1 shows changes in the unsaturation index (average number of double bonds per fatty acid) of whole-cell fatty acids from mid-exponential- (24 h) and early-stationary-phase (48 h) cultures of *A. castellanii* prior to, shortly after, and 12 h after, chilling from 30 °C to 15 °C. Chilling of 24 h cultures had no discernible effect on the fatty acid unsaturation index, which remained constant at 2.15 throughout the 12 h incubation. In contrast, while chilling to 15 °C had no immediate (within 10 min) effect on the unsaturation index of 48 h cultures, after 12 h of incubation at 15 °C the unsaturation index of these cells had risen from 1.76 to 2.18.

These changes in unsaturation index were correlated with alterations in phagocytotic rates of *A. castellanii*. The rate of phagocytosis in 24 h cultures was reduced by approx. 45% on chilling from 30 °C to 15 °C. However, as with the unsaturation index of these cultures, a further 12 h incubation at 15 °C resulted in no further change in the rate of phagocytosis (measured at 15 °C) (Table 1). Chilling had a much more pronounced effect on phagocytosis of 48 h cultures, which displayed a lower initial degree of fatty acid unsaturation (Table 1). Within 10 min of chilling, the rate of phagocytosis of these cultures was reduced to only approx. 2% of that observed at 30 °C. However, in contrast to chilled mid-exponential-phase cultures, a 12-fold increase in the rate of phagocytosis was found after incubation at 15 °C for 12 h, and was correlated with an increase in the unsaturation index of these cells. Thus after 12 h incubation at 15 °C, by which time low-temperature-induced changes in fatty acid composition are largely complete [15,16], unsaturation indices and phagocytotic rates in cultures of both ages were similar (Table 1).

Statistical analysis of the data by Student's *t*-test confirmed that there was no significant difference between unsaturation indices of unchilled and chilled 24 h cultures, and of 48 h cultures chilled for 12 h. However, unsaturation indices of 48 h cultures maintained at 30 °C or chilled for 10 min were very highly significantly different ($P < 0.1\%$) from the former set. The *t*-test analysis also confirmed that there was no significant difference between phagocytotic rates of 12 h cultures chilled for 10 min and of 12 h and 48 h cultures chilled for 12 h.

Table 2 Fatty acid composition of plasma membrane-enriched fractions from 30 °C- and 15 °C-acclimated *A. castellanii*

Fatty acids are abbreviated with the first figure showing the number of carbon atoms and the second indicating the number of double bonds. The positions of double bonds were determined [15] and are given below. Average values for percentage fatty acid composition from two independent experiments, each with three replicate determinations, are shown $\pm$ S.E.M. ($n = 6$).

Fatty acid	Acclimation temperature ...	Composition (%)	
		30 °C	15 °C
14:0		9.6 $\pm$ 0.6	4.4 $\pm$ 0.1
16:0		5.4 $\pm$ 0.7	4.2 $\pm$ 0.1
16:1 ($n-7$)		1.0 $\pm$ 0.8	1.9 $\pm$ trace
18:0		3.6 $\pm$ 1.5	6.0 $\pm$ 0.2
18:1 ($n-9$)		32.0 $\pm$ 0.6	9.2 $\pm$ 0.2
18:2 ($n-6$)		6.6 $\pm$ 2.5	27.5 $\pm$ 1.5
18:3 ($n-6$)		1.0 $\pm$ 0.1	0.8 $\pm$ 0.1
20:2 ($n-6$)		18.9 $\pm$ 0.2	10.9 $\pm$ 1.2
20:3 ($n-6$)		8.4 $\pm$ 0.7	13.3 $\pm$ 1.2
20:4 ($n-6$)		13.9 $\pm$ 0.3	22.0 $\pm$ 0.6

Effect of chilling on fatty acid composition of plasma membrane-enriched fractions from *A. castellanii*

Because phagocytosis is primarily a plasma membrane-mediated activity, we sought to determine whether changes in the fatty acid unsaturation index (and phagocytosis) of whole cells, following chilling of 48 h cultures, were reflected by changes in the fatty acid composition of plasma membrane-enriched fractions. Differential centrifugation of homogenates prepared from disrupted silica-bead-coated cells yielded a fraction enriched in plasma membranes. Marker enzyme assays confirmed a satisfactory degree of enrichment of the plasma membrane fraction, similar to that described previously [15]. For example, the activity of this fraction accounted for approx. 2% and 7% of total homogenate activity of endoplasmic reticulum (choline phosphotransferase) and mitochondrial (succinate dehydrogenase) marker enzymes respectively, but approx. 27% of homogenate activity of 5' nucleotidase, a marker for the plasma membrane of *A. castellanii*. Plasma membrane-enriched fractions obtained from 30 °C- and 15 °C-acclimated cells displayed markedly different fatty acid compositions (Table 2). The most notable differences occurred in the relative proportions of unsaturated fatty acids. For example, the linoleate (18:2) to oleate (18:1) ratio was 14-fold higher in membranes from 15 °C-acclimated cells than in those from 30 °C-acclimated cells. Furthermore,

Table 1 Effect of chilling on unsaturation index and phagocytosis in *A. castellanii*

Mid-exponential- (24 h) and stationary-phase (48 h) cultures were either maintained at 30 °C or chilled to 15 °C. Unsaturation index refers to the average number of double bonds per fatty acid. Values shown are means from three independent experiments $\pm$ S.E.M.

Age of culture (h)	Time after chilling (h)	Unsaturation index	Rate of phagocytosis [beads $\cdot$ (100 cells) $^{-1} \cdot$ min $^{-1}$]
24	0	2.15 $\pm$ 0.02	7.3 $\pm$ 0.1
24	0.17	2.15 $\pm$ 0.01	4.1 $\pm$ 0.8
24	12	2.15 $\pm$ 0.01	4.9 $\pm$ 0.4
48	0	1.76 $\pm$ 0.01	17.5 $\pm$ 0.2
48	0.17	1.76 $\pm$ 0.03	0.4 $\pm$ 0.1
48	12	2.18 $\pm$ 0.02	4.6 $\pm$ 0.2

Figure 1 Temperature-dependence of fluorescence depolarization in *A. castellanii* plasma membrane fractions

Plasma membrane-enriched fractions were obtained from 30 °C- (○) and 15 °C- (●) acclimated cells and labelled with DPH (3 μM). Fluorescence anisotropy (r) of samples, excited with vertically polarized light at 365 nm, was calculated according to the Materials and methods section. The vertical axis is plotted on a logarithmic scale. Means $\pm$ S.E.M. ($n = 3$) are shown where the latter values exceeded the dimensions of the symbols.

whereas the relative proportion of eicosadienoate (20:2) was lower in plasma membrane fractions of cells chilled to 15 °C, proportions of eicosatrienoate (20:3) and arachidonate (20:4) were approx. 58% higher than in plasma membranes from unchilled cells. These changes in $n-6$ fatty acids reflected the induction of $\omega 6$ -desaturase activity caused by low temperature [17]. The proportion of myristate (14:0) was also greater in plasma membrane fractions from unchilled cells. However, t -test analysis revealed that there were no significant differences in the relative compositions of palmitate (16:0), palmitoleate (16:1), stearate (18:0) and linolenate (18:3) between membranes from unchilled and chilled cells. (Significant, to very highly significant, changes in the compositions of all the other membrane fatty acids resulted from chilling.) Unsaturation indices (calculated from data provided in Table 2) for plasma membrane fractions from unchilled and chilled cells were found to be 1.68 and 2.18 respectively, and were similar to unsaturation indices of whole cells incubated under the same conditions (see Table 1).

Steady-state fluorescence depolarization of DPH-labelled plasma membrane fractions from *A. castellanii*

Steady-state fluorescence depolarization studies of intact plasma membrane fractions, over an assay temperature range of between 5 °C and 40 °C, employed DPH as a probe of lipid order. Fluorescence anisotropy was calculated from measured values of the vertical and horizontal vectors of light emitted from samples which were excited with vertically polarized light (see the Materials and methods section). In all cases, fluorescence anisotropy was found to increase with decreasing assay temperature, suggesting reduced freedom of motion of the probe in membrane samples (Figure 1). However, fluorescence anisotropy plots displayed reproducible changes in the slopes of temperature-dependence. The temperature range over which the slope change occurred (approx. 20–25 °C) was higher in plasma membranes from 30 °C-acclimated cells than that in 15 °C-acclimated cells, where the change occurred between approx. 15 and 20 °C. Thus, the increase in plasma-membrane fatty acid unsaturation (Table

Figure 2 Arrhenius plots of phagocytotic activity in *A. castellanii*

Phagocytosis was initiated by the addition of 1.0-μm-diam. fluorescently labelled latex beads to cell suspensions. Short-term (10 min) rates of phagocytosis were determined in 30 °C- (○) and 15 °C- (●) acclimated cells at various assay temperatures (10–30 °C). Means $\pm$ S.E.M. ($n = 3$) are shown where the latter values exceeded the dimensions of the symbols.

2), after a 15 °C shift in the incubation temperature (from 30 °C to 15 °C) of *A. castellanii*, correlated with a shift in the temperature range over which the temperature-dependence of fluorescence anisotropy was altered.

Slopes of plots for the two membrane samples were similar above 25 °C (linear regression analysis gave gradients of 0.183 and 0.184) and below 15 °C (linear regression analysis gave gradients of 0.116 and 0.127), and were superimposed in the latter case (Figure 1). However, over the upper assay-temperature range (25–40 °C), anisotropy values were always higher at any given temperature in plasma membranes from 30 °C-acclimated cells than in those from 15 °C-acclimated cells. The difference in assay temperature required to give approximately equal anisotropy values in the different membrane samples over this upper temperature range (i.e. the relative displacement of plots) was approx. 3.3 °C (Figure 1).

Temperature-dependence of phagocytosis in *A. castellanii*

In order to relate membrane physical properties to phagocytosis in *A. castellanii*, short-term phagocytotic rates were determined over a range of assay temperatures (10–30 °C); low rates of phagocytosis precluded their accurate determination at temperatures below 10 °C, while phagocytosis became inhibited at temperatures greater than 30 °C. Rates of phagocytosis were found to increase with increasing assay temperature. Arrhenius plots of logarithmic values of phagocytotic rates against reciprocal assay temperature (K^{-1}) revealed discontinuities in the temperature dependence of phagocytosis (Figure 2). The temperature at which the discontinuity of phagocytosis occurred was higher in 30 °C- than in 15 °C-acclimated cells; discontinuities in these cells were evident at approx. 25.0 °C and 17.5 °C respectively. These discontinuity temperatures for whole-cell phagocytosis each occurred within the temperature range over which changes in the temperature-dependence of fluorescence anisotropy were evident in the corresponding membranes (Figure 1). Activation energies for phagocytosis were calculated from the slopes of Arrhenius plots using linear regression and found to be approx. 97 ($r^2 = 0.9919$) and 195 ($r^2 = 0.9991$) $\text{kJ} \cdot \text{mol}^{-1}$ in 30 °C-acclimated cells and approx. 58 ($r^2 = 0.9974$) and 153 ($r^2 =$

0.9801) $\text{kJ} \cdot \text{mol}^{-1}$ in 15 °C-acclimated cells, above and below the discontinuities respectively.

DISCUSSION

The results presented in this study indicate that phagocytotic activity of *A. castellanii* is markedly influenced by the degree of plasma membrane fatty acid unsaturation and lipid order. Differences in the fatty acid unsaturation indices of whole cells, and changes in these values after chilling to 15 °C, correlated well with low-temperature-induced changes in the rates of phagocytosis of mid-exponential- and stationary-phase cultures of *A. castellanii*. These results showed that phagocytosis of cells displaying a low initial degree of fatty acid unsaturation is highly susceptible to low-temperature inhibition. In addition, changes in whole-cell fatty acid unsaturation after temperature shift were found to be similar to those of plasma membrane-enriched fractions; these changes were largely attributable to an increased relative proportion of polyunsaturated fatty acids, particularly linoleate. Previous studies have also indicated similar fatty acid compositions of different membrane fractions and whole cells of *A. castellanii* [14,15].

While there is no simple linear relationship between fatty acid unsaturation and membrane 'fluidity' [25], it is generally accepted that increases in the relative degree of fatty acid unsaturation, such as those observed in *A. castellanii* plasma membranes after chilling, result in a lowering of the melting temperatures of constituent lipids and increased membrane disorder [1,2]. We attempted to relate further temperature-dependent changes in phagocytotic activity with fatty acid unsaturation by investigating the physical properties of plasma membrane-enriched fractions using steady-state fluorescence depolarization, with DPH. While this technique has some limitations, particularly for prokaryotic membranes [6], the relative independence of DPH fluorescence lifetime on membrane fluidity and the fairly homogeneous distribution of DPH in heterogeneous membranes dictate that the probe is highly suitable for reporting on the average bulk-phase lipid order [26–29]. Furthermore, the rate component of probe motion, which can be determined by time-resolved analysis [30,31], is small and measurements are dominated by the steady-state order (angular amplitude of motion) component [30,32]. Thus, steady-state fluorescence anisotropy reflects membrane 'order' almost as accurately as if order itself were actually measured [30].

In the present study, fluorescence anisotropy in plasma-membrane fractions was never constant with changing temperature but increased with decreasing temperature, indicating a gradual decrease in amplitude of lipid rotational motion. The 3.3 °C difference in assay temperature required to give equal fluorescence anisotropy values in 15 °C- and 30 °C-acclimated membranes, over the assay-temperature range 25–40 °C, indicated that temperature-induced changes in membrane lipid composition compensated for approx. 22 % of the ordering effect of low temperature. This value is within the range of 'homeoviscous efficacies' [6,32] commonly observed for bacterial membranes [6], but compares unfavourably with those estimated for fish membranes (30–75 %) [32] and those for evolutionary adaptations where values may approach 100 % [1] (it should be noted that the term 'homeoviscous efficacy' strictly refers to a rate process and therefore cannot be truly described by the steady-state measurements employed here). Thus, low-temperature-induced adaptations in fatty acid composition of the *A. castellanii* plasma membrane appear to only partially compensate for the rigidifying effect of a lower incubation temperature.

Relatively sharp (within a 5 °C range) changes in the slope of temperature-dependence of fluorescence anisotropy, such as those described here, are surprising considering the heterogeneous mixture of lipid species prevalent in the *A. castellanii* plasma membrane [15]. In several studies, discontinuities in fluorescence anisotropy plots derived from membrane samples have been interpreted as the mid-points of gel-to-liquid-crystalline phase transitions [5,9,33]. However, as steady-state fluorescence depolarization predominantly reports on orientational order, rather than dynamic events, the temperature-dependence of derived data would not be expected to follow an Arrhenius-Law type of behaviour (although it should be noted that the present results do not discount the possibility of associated changes in motional rates). Nevertheless, the results do clearly indicate a change in slope of temperature-dependence over a limited temperature range, which is presumably related to a change in the physical environment of DPH. This could be interpreted in general terms as a steep change in the overall lipid chain motion of the membrane over a limited temperature range. A more specific explanation, which satisfies the phase rule [1,34], is that a proportion of the plasma-membrane lipids are undergoing a lateral separation at 20–25 °C or 15–20 °C (depending on temperature of acclimation). The generally broad nature of lateral-phase separations [1], as well as the high sterol content of the *A. castellanii* plasma membrane (approx. 10 % of total macromolecular composition) [35], suggest that such an event would occur over a wider temperature range than observed here. There does not seem to be an obvious reason why the present apparent phase separations occur within a 5 °C temperature range and why changes in fluorescence anisotropy are linear between 5 and 15 °C and between 25 and 40 °C. It is possible that the observed discrete events only reflect separations of a small fraction of membrane lipids and that further, more extensive, separations occur outside the physiological temperature range.

The change in temperature-dependence of DPH-depolarization occurred over a lower temperature range in plasma membrane fractions from 15 °C-acclimated cells compared with membranes from 30 °C-acclimated cells. This observation was consistent with the increased proportion of polyunsaturated fatty acids in the former. Moreover, temperature-dependent changes and Arrhenius discontinuities in short-term rates of phagocytosis by *A. castellanii*, acclimated to 30 °C and 15 °C, showed a good correlation with fluorescence anisotropy measurements. Earlier studies on protozoal phagocytosis have also described a decline in phagocytosis with temperature [36–38]. However, while possible discontinuities in temperature-dependence have been hinted at [36,37], in the absence of data relating to the lipid composition and physical properties of the plasma membrane, these observations were attributed to energetic considerations [37]. Reductions in cellular metabolic rates at low temperature will certainly contribute (possibly in conjunction with continued increases in membrane lipid order, see Figure 2) towards further reductions in phagocytotic activity below the main discontinuity temperature. However, the occurrence of discontinuities in phagocytosis within the temperature range of apparent changes in the physical properties of the plasma membrane, derived by fluorescence depolarization, strongly implies a role for plasma membrane order as a control on phagocytosis.

Sudden changes in the temperature-dependence of membrane-dependent activities (Arrhenius discontinuities) are commonly interpreted as an effect of the thermotropic phase behaviour of membrane lipids [13]. Such relationships are particularly clear where the discontinuity temperature decreases with increasing membrane fatty acid unsaturation [1], or where discontinuities

can be correlated with temperature-dependent changes in membrane properties determined by independent physical methods [13]. Both of these criteria were fulfilled in the present study. The correlation between plasma membrane lipid composition or membrane order and phagocytotic activity is particularly strong as phagocytosis, a macroscopic process which involves the rapid expansion, invagination and fusion of plasma membrane, involves relatively extensive membrane areas and will thus quite accurately reflect (or rely on) bulk membrane properties. In contrast, difficulties can be associated with assigning temperature effects on an isolated membrane-protein activity to the local lipid environment, particularly when possible direct effects on the protein itself have not been ascertained [12,13]. Moreover, these correlations generally give no clear indication of any functional advantages which may result from low-temperature adaptation [32]. It should be noted that the shift in phase-transition temperature was indeed effective in maintaining high phagocytotic rates in *A. castellanii* at low temperature. For example, while rates of phagocytosis in 30 °C-acclimated cells dropped rapidly below 25 °C, the rate at 17.5 °C in 15 °C-acclimated cells was still approximately equal to that at 25 °C in 30 °C-acclimated cells.

To our knowledge, this is the first demonstration that a specific physiological process (i.e. phagocytosis) is directly correlated with plasma membrane order, which in turn has been altered by low-temperature-induced fatty acid desaturase induction. Such an environmental adaptation is clearly necessary for *A. castellanii* to continue to feed and grow in its natural soil habitat.

The financial support of the Natural Environment Research Council (GR 3/8456) is gratefully acknowledged. We also thank Professor P. J. Quinn (King's College, London) for helpful discussions. Silica beads were a kind gift from Ms. Kelly L. Auer (University of Massachusetts at Amherst).

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