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Potassium- and Calcium-Induced Alterations in Lipid Interactions of Isolated Plasma Membranes from *Blastocladiella emersonii*. Evidence for an Adenosine 5′-Triphosphate Requirement†

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ABSTRACT: The physical–chemical properties of the isolated plasma membranes from zoospores of the chytridiomycete *Blastocladiella emersonii* were investigated, with electron spin resonance (ESR) spectroscopy, using the spin-label 5-nitroxystearate (5-NS). Both isolated plasma membranes and aqueous dispersions of the lipids extracted from the plasma membranes were spin-labeled and analyzed. Plots of the hyperfine splitting parameter ($2T_{\parallel}$) vs. temperature indicated that the middle break point, T_M, initially observed in experiments with spin-labeled zoospores in vivo [Leonards, K. S., & Haug, A. (1980) *Biochim. Biophys. Acta 600*, 805–816], was the result of a lipid–lipid interaction (glycolipid–glycolipid or glycolipid–neutral lipid) rather than a lipid–protein interaction. This interaction was markedly affected by Ca^{2+} ions, which interacted directly with the lipid components, increasing T_M from 11 ± 1 (Ca^{2+} removed by EDTA) to 21 ± 1 °C (10 mM Ca^{2+}) in the lipid dispersions and from 12 ± 1 to 23 ± 1 °C in the plasma membrane preparations. The initial ESR studies on spin-labeled zoospores in vivo had also demonstrated that the addition of K^+ ions could reverse the Ca^{2+} ion effect, downshifting T_M from 22 ± 1 to 10 ± 1 °C. The addition of K^+ ions to the isolated plasma membrane had no affect on T_M, indicating that K^+ ions do not simply replace Ca^{2+} ions but exert their effect indirectly on the membrane. However, after the inclusion of ATP, K^+ ions could reverse the Ca^{2+} ion effect. It was determined that the ATP generated an "energized membrane" state which permitted the K^+ ions to reverse the Ca^{2+} effect. Since K^+ ions have been shown to depolarize the membrane potential in both zoospores and isolated zoospore plasma membrane preparations (generated by ATP), we suggest that the K^+ ion induced reversal of the Ca^{2+} ion effect, and therefore the change in the lipid–lipid interactions responsible for T_M, is a consequence of the K^+ ion induced depolarization of the membrane potential.

Zoospores of the chytridiomycete *Blastocladiella emersonii* have proved to be an excellent model system for studying the role(s) of the plasma membrane in eukaryotic cell differentiation and development. The plasma membrane is intimately involved in the differentiation process. Although zoospore encystment does not seem to require either protein or RNA[1] synthesis (Soll & Sonneborn, 1971; Lovett, 1975), it does involve extensive membrane alterations. The first detectable changes which occur during encystment include the induction of cell adhesiveness (Cantino et al., 1968), alterations in cell surface monitored by FITC-concanavalin A (Jen & Haug, 1979), and the fusion of vesicles derived from the γ particles with the plasma membranes (Truesdell & Cantino, 1970; Myers & Cantino, 1974). In addition, the differentiation process is markedly affected by zoospore's ionic environment, with Ca^{2+} ions inhibiting and K^+ ions inducing encystment (Cantino et al., 1968; Soll & Sonneborn, 1969, 1972).

We have previously demonstrated a correlation between cation- and temperature-induced changes in the physical–chemical properties of the zoospore plasma membrane, in vivo, and the effects of temperature and cations on zoospore differentiation and viability (Leonards & Haug 1979a, 1980a). These studies indicated the presence of three break points (T_L, T_M, and T_H), as observed with ESR spectroscopy, in plots of $2T_{\parallel}$, S, and f vs. temperature. Of the three, T_L and T_H were observed in zoospore total lipid extracts. Further studies on the isolated lipid components of the zoospore indicated that it was the zoospore glycolipids rather than the phospholipids which gave rise to T_L and T_H (Leonards & Haug, 1979b, 1980b).

However, it was the middle break point, T_M, which was affected by the external cation concentration. The cation-induced shifts in T_M were closely correlated with the temperature dependence and physiological effects of cations on zoospore differentiation, suggesting that the physical–chemical properties of the plasma membrane were involved in regulating the initial changes during zoospore encystment (Leonards & Haug, 1979a, 1980a).

The absence of the middle break point in both the total lipid extracts and in the zoospore phospholipid and glycolipid dis-

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[1] Abbreviations used: Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; AMP-PNP, adenylyl imidodiphosphate; 5-NS or 5-nitroxystearate, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy; TLC, thin-layer chromatography; RNA, ribonucleic acid; ATP, adenosine 5′-triphosphate; cAMP, adenosine cyclic 3′,5′-phosphate.

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persions suggested that T_M either is the result of lipid–protein interaction or is due to a lipid–lipid interaction which requires a specific organization or lipid composition of the zoospore plasma membrane.

The purpose of this paper is to specifically study the middle break point, T_M, in isolated zoospore plasma membrane preparations in order to determine (a) whether T_M is the result of a lipid–protein or lipid–lipid interaction, (b) whether calcium ions are involved in this interaction, (c) under what conditions potassium ions can reverse the calcium ion effect, as observed in zoospores in vivo, and (d) how the properties of T_M observed in the isolated plasma membrane preparations compare to those obtained for zoospores in vivo?

Materials and Methods

Organism, Growth, and Membrane Isolation. Cultures of *Blastocladiella emersonii* were routinely grown on PYG agar at 22 °C as previously described (Cantino & Hyatt, 1953). Zoospores were harvested from first generation plants with a 5 mM 3-(*N*-morpholino)propanesulfonic acid (Mops) plus 10 mM $CaCl_2$ solution (pH 6.7). The resulting zoospore suspension was then collected and filtered to remove germlings. Zoospore plasma membranes were isolated according to the method of Leonards & Haug (1980c). Because of the zoospore's ultrastructure, it is possible to selectively rupture the cell osmotically and remove the nuclear cap, nucleus, mitochondrion, and side-body complex (microbody) as a single unit (there is no endoplasmic reticulum in the zoospore). The plasma membranes are then separated from these and the other cytoplasmic organelle, the γ particles, by filtration and differential centrifugation.

The plasma membranes were resuspended in a minimal volume of supernatant (250–300 μL) which contained 5 mM Mops plus 10 mM $CaCl_2$ (pH 6.7). The supernatant was from the last step of the membrane isolation procedure and was essentially a wash solution. Protein concentration varied between 3 and 4 mg/mL. These preparations were used immediately for the electron spin resonance studies on isolated membranes.

Chemicals. All organic solvents were either redistilled or spectra grade distilled in glass (Burdick and Jackson Laboratories, Inc.). ATP (both disodium salt, grade II, 95–98%, and disodium salt from equine muscle, vanadium free, 99–100%), EDTA (tetrasodium salt, 99%), 3-(*N*-morpholino)propanesulfonic acid (Mops), adenylyl imidodiphosphate (AMP-PNP, 90–95%), and Sephadex G-25 were from Sigma Chemical Co. 5-Nitroxystearate (5-NS) was from Syva Research Chemicals.

Lipid Isolation and Analytical Determinations. Plasma membrane lipids were extracted and isolated (Leonards & Haug, 1980c). Nonlipid contaminants were removed from the preparations by chromatography on Sephadex G-25 according to the method of Radin (1969). Protein removal was verified by protein determination before and after Sephadex chromatography. Protein was measured by using the modified Lowry method of Wang & Smith (1975), with bovine serum albumin as the standard.

The lipid composition of the plasma membrane preparations was monitored by thin-layer chromatography (TLC) on silica gel G and H using chloroform/methanol/water (65:25:4 v/v/v) and petroleum ether/diethyl ether/acetic acid (80:20:1 v/v/v) as solvents. Individual lipid components were identified by cochromatography with standards (Supelco), extracted zoospore total, phospho-, glyco-, and neutral lipid samples, and comparisons to previously obtained results (Mills & Cantino, 1974; Leonards & Haug, 1979b, 1980b).

Spin-Labeling Procedure. Isolated membrane preparations were spin-labeled as follows: (a) The membrane preparation was sonicated in a bath sonicator for 10 min at room temperature to break up the membrane sheets and generate vesicles of a relatively uniform size. (b) 5-Nitroxystearate (5-NS) was added as an ethanolic solution (usually 1–2 μL) to give a final spin-label/protein concentration $\leq$0.2% wt/wt. (c) The sample was again sonicated for 5 min to incorporate the spin-label. (d) The sample was transferred into the ESR cuvette, and spectra were obtained over the temperature range 0–30 $\pm$ 2 °C. (e) The sample was transferred back to the test tube. (f) KCl (final concentration 50 mM) and/or EDTA (final concentration 10 mM) were added and the suspension mixed and again transferred to the ESR cuvette for the next run.

This basic procedure was modified for specific experiments. In some cases, KCl and/or EDTA were added between steps c and d as a control. Alternatively, the sample was extracted with chloroform/methanol (2:1 v/v) at step e for TLC. The effects of ATP and AMP-PNP on T_M in isolated membrane preparations were measured by using freshly prepared (kept on ice) solutions in 5 mM Mops (pH 6.7) which were added to the sample immediately before step a.

Aqueous dispersions of the isolated plasma membrane lipids were made by drying the lipids first under N_2 and then under vacuum. The sample was resuspended on a vortex stirrer into 200–300 μL of 5 mM Mops (pH 6.7) with a glass bead. The lipid concentration was between 3 and 5 mg/mL. The suspension was then sonicated in a sonic water bath, followed by steps b–f as above. The spin-label concentration was $\leq$0.2% wt/wt. $CaCl_2$ (final concentration 10 mM) was added just before step d. ATP and/or KCl were added just before step d or at step f. EDTA was added at step f or in control experiments just before step d.

Spin-Label Measurements and Analysis. ESR measurements were made with a Varian X-band spectrometer (Model E-112). The temperature was regulated with a Varian variable temperature controller and monitored inside the cuvette with a calibrated thermocouple connected to a digital readout meter (Omega Model 250). All spectra were recorded at power and modulation amplitude settings which were previously determined to be below those causing saturation or line width broadening. The spectra were analyzed with a Varian 620/L-100 computer. The experimental plots of the hyperfine splitting parameter ($2T_{\parallel}$) vs. temperature were analyzed in terms of linear components by fitting regression lines to appropriate sections using the method of least squares. This empirical method of analysis has been previously used in the interpretation of results obtained from ESR experiments on synthetic lipid dispersions (Shimshick & McConnell, 1973) and prokaryotes (Weller & Haug, 1977; Yang & Haug, 1979), as well as *B. emersonii* (Leonards & Haug, 1980a,b). The temperatures at which break points are observed, as indicated by the intersections of straight lines, are in good agreement with those obtained by other physical–chemical techniques (Shimshick & McConnell, 1973; Lee, 1975). The break points obtained with this form of analysis were confirmed with a second analytical method, an iterative least-squares program (Brunder et al., 1980) using normalized β splines (Dierckx, 1975).

Results

The middle break point, T_M, originally observed with electron spin resonance spectroscopy for zoospores in vivo, spin-labeled with 5-nitroxystearate (5-NS) or 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo) was markedly affected by

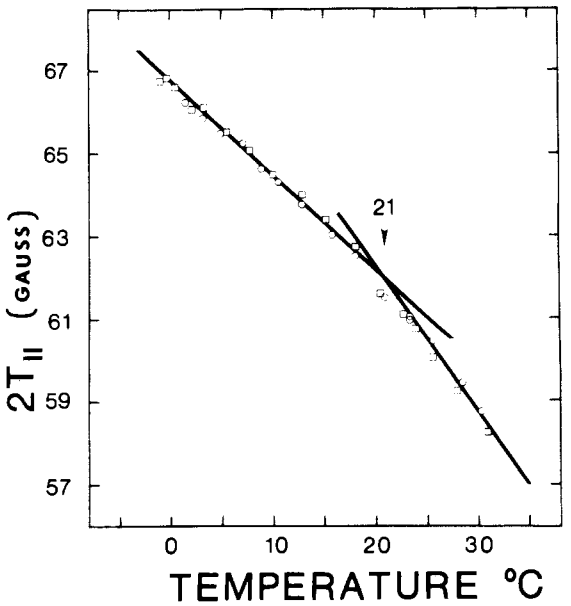

FIGURE 1: Plots of $2T_{||}$ vs. temperature for zoospore plasma membrane preparations (□) and aqueous dispersions of the plasma membrane lipids (○) spin-labeled with 5-nitroxystearate (5-NS) and measured in the presence of 10 mM $CaCl_2$ (pH 6.7).

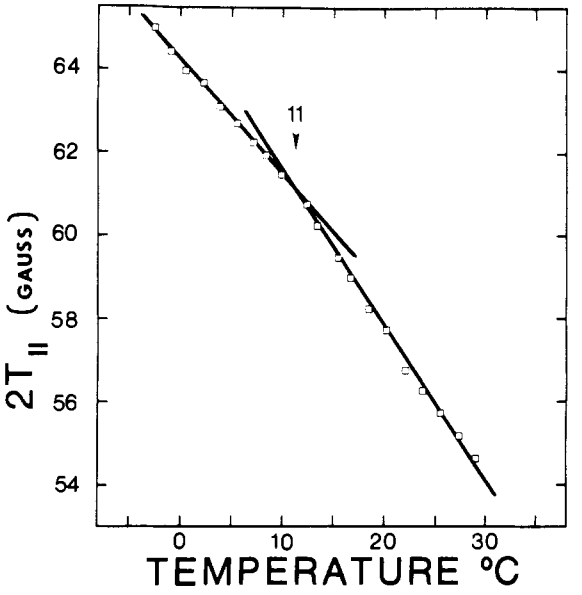

FIGURE 2: Plot of $2T_{||}$ vs. temperature for aqueous dispersions of plasma membrane lipids (□) spin-labeled with 5-nitroxystearate (5-NS) and measured in the presence of 10 mM $CaCl_2$ plus 10 mM EDTA (pH 6.7), demonstrating the reversal of the Ca^{2+} effect of EDTA.

the external Ca^{2+} ion concentration. Specifically, Ca^{2+} ions increased the value of T_M from 12 ± 1 (no Ca^{2+} added) to 22 ± 1 °C (10 mM $CaCl_2$ present), which was the growth temperature (Leonards & Haug, 1980a).

To ascertain if T_M was the result of a cation-modulated lipid–lipid or lipid–protein interaction, we isolated the zoospore plasma membrane, spin-labeled it with 5-NS, and measured the hyperfine splitting parameter, $2T_{||}$, as a function of temperature (Figure 1). A break point was observed at 21 ± 1 °C for the membrane preparation, which was isolated and suspended in a buffered 10 mM $CaCl_2$ solution, pH 6.7. The lipids were then extracted from the preparation, nonlipid contaminants were removed (including more than 99% of the proteins), and aqueous dispersions of the membrane lipids were reexamined, in the presence of 10 mM $CaCl_2$ (pH 6.7), as a function of temperature. The results obtained (Figure 1) were the same as those found for the plasma membrane preparations, indicating that the break point observed at 21 ± 1 °C in the presence of 10 mM $CaCl_2$ was a lipid–lipid interaction. Control experiments, using lipids extracted from membrane preparations which had not been previously used for ESR experiments, gave the same result.

Although the Ca^{2+} ion effect involved lipid–lipid interactions, the possibility existed that the T_M value obtained in the absence of Ca^{2+} ions was due to a lipid–protein interaction, which dissociated in the presence of Ca^{2+} ions. This possibility was examined by making spin-labeled aqueous dispersions of the plasma membrane lipids and measuring $2T_{||}$ as a function of temperature in the presence of 10 mM $CaCl_2$ plus 10 mM EDTA (pH 6.7). A break point was observed at 11 ± 1 °C (Figure 2), indicating that the middle break point obtained in the absence of free Ca^{2+} ions was also due to a lipid–lipid interaction and that the Ca^{2+} ion effect could be reversed by EDTA.

The initial ESR experiments with spin-labeled zoospores indicated that K^+ ions could reverse the effects of Ca^{2+} ions, downshifting T_M from 22 ± 1 to 10 ± 1 C (Leonards & Haug, 1979a, 1980a). The ability of K^+ ions to reverse the effects of Ca^{2+} ions in the isolated plasma membrane preparations was tested by first measuring the hyperfine splitting parameter, $2T_{||}$, of a spin-labeled membrane sample in the presence of a

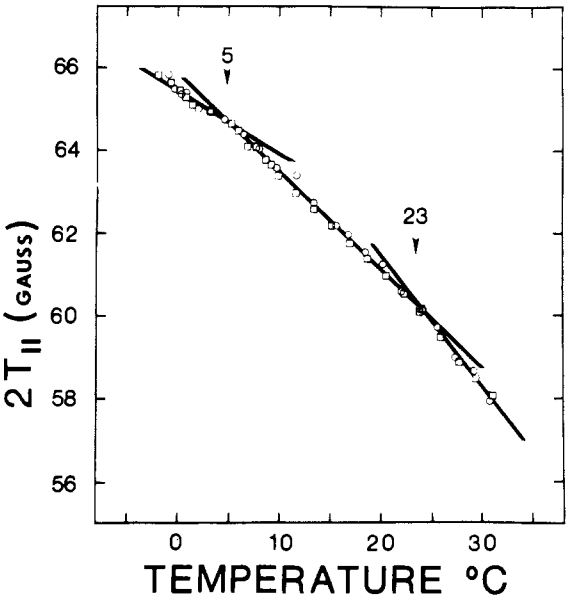

FIGURE 3: Plots of $2T_{||}$ vs. temperature for zoospore plasma membrane preparations spin-labeled with 5-nitroxystearate (5-NS), indicating the inability of K^+ ions to reverse the Ca^{2+} ion effect in isolated membranes. Plasma membranes in the presence of 10 mM $CaCl_2$ (pH 6.7) (○). Plasma membrane sample after KCl (final concentration 50 mM) was added to the preparation containing 10 mM $CaCl_2$ to reverse the Ca^{2+} effect (□).

buffered 10 mM $CaCl_2$ solution (pH 6.7) as a function of temperature and then rerunning the same sample after adding KCl (final concentration 50 mM) to the suspension. The results are illustrated in Figure 3. Two break points were observed in the presence of 10 mM $CaCl_2$ over the temperature range of 3–32 °C. The first occurred at 5 ± 1 °C and the second at 23 ± 1 °C. These values are the same as the values obtained for T_L and T_M with 5-NS spin-labeled zoospores under the same conditions (5 ± 1 and 22 ± 1 °C, respectively) (Leonards & Haug, 1980a). Addition of K^+ ions had no effect on T_L or T_M; the results obtained being indistinguishable from those observed with Ca^{2+} ions only (Figure 3). The absence of any change in T_L, as a consequence of cation addition, is in accord with previous results obtained for spin-labeled

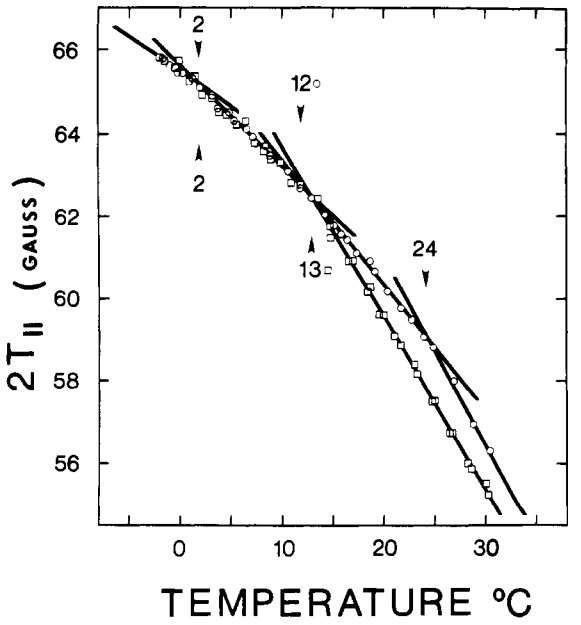

FIGURE 4: Plots of $2T_{\|}$ vs. temperature for zoospore plasma membrane preparations spin-labeled with 5-nitroxystearate (5-NS) in the presence of 10 mM $CaCl_2$, 50 mM KCl, and 5 mM ATP (pH 6.7) (○) and 10 mM $CaCl_2$, 50 mM KCl, 5 mM ATP, and 10 mM EDTA (pH 6.7) (□), indicating that K^+ ions can reverse the Ca^{2+} ion effect in plasma membrane samples if ATP is included. The sample was reexamined after EDTA addition to confirm the effect on T_M of Ca^{2+} removal.

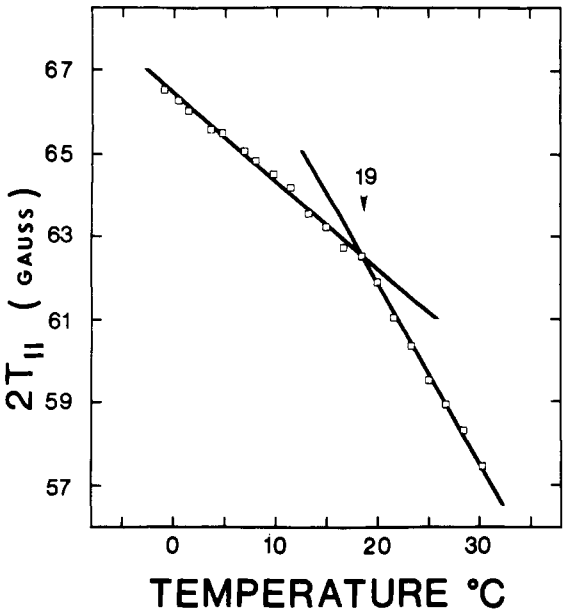

FIGURE 5: Plot of $2T_{\|}$ vs. temperature for zoospore plasma membrane preparation spin-labeled with 5-nitroxystearate (5-NS) in the presence of 10 mM $CaCl_2$, 50 mM KCl, and 5 mM AMP-PNP (pH 6.7) (□), indicating that AMP-PNP, a structural analogue of ATP, cannot substitute for ATP.

zoospores (Leonards & Haug, 1979a, 1980a) and with the isolated lipid components from zoospores (Leonards & Haug, 1979b, 1980b). In contrast, the inability of K^+ ions to downshift T_M was inconsistent with the results of spin-labeled zoospore experiments, under seemingly similar conditions.

A major difference between the experiments with the spin-labeled zoospores and with the isolated plasma membranes was the absence of an energy source in the latter. Metabolically, the zoospores are quite active, expending energy derived from endogenous reserves to maintain an osmotic balance with the medium, since the zoospore lacks a cell wall, and to provide energy for motility (Suberkropp & Cantino, 1972, 1973). In addition, living cells possess an electrochemical gradient across their plasma membranes, a condition which did not hold for the isolated plasma membrane preparations. The importance of such a gradient is supported by experiments which indicate that K^+ ions change the membrane potential of *B. emersonii* zoospores and that these changes may be involved in the initial events of zoospore encystment (Jen & Haug, 1981). Previous studies have also demonstrated that a transmembrane electrical potential ($\Delta\Psi$) can be generated by *Neurospora* plasma membrane vesicles in the presence of added ATP (Stroobant & Scarborough, 1979).

In order to determine if the K^+ ion induced reversal of the Ca^{2+} ion effect was related to an energy-dependent process, we added freshly prepared ATP to the isolated plasma membrane preparation immediately before the initial sonication step (step a, Materials and Methods). If the reversal process required the presence of an energized membrane and this requirement could be fulfilled by ATP, a break point should be observed at ~10 °C. The results obtained for membrane samples in the presence of 5 mM ATP, 10 mM $CaCl_2$, and 50 mM KCl (pH 6.7), measured as a function of temperature, are illustrated in Figure 4. Three break points were observed, the lowest one (2 ± 1 °C) being similar to the value previously noted for T_L. The second and third break points occurred at 12 ± 1 and 24 ± 1 °C. The 24 ± 1 °C break is the same as that observed in Figure 3. The 12 ± 1 °C break is virtually the same as that hypothesized above for K^+ ion reversal of the Ca^{2+} ion effect. The presence of both breaks is what one would expect for a membrane preparation composed of a mixture of right side out and inverted vesicles, and/or a mixture of sealed and leaky vesicles. Both types of ATP used gave the same results.

To further investigate the possibility that the membrane preparation was composed of such a mixed population of vesicles, we added buffered EDTA to the preparation (final concentration 10 mM) and remeasured the hyperfine splitting value, $2T_{\|}$, as a function of temperature. These results are also shown in Figure 4. A break point was observed at 13 ± 1 °C, but the break point previously observed at 24 ± 1 °C was absent. The $2T_{\|}$ values obtained up to 13 °C were also indistinguishable from those observed before EDTA addition, confirming the break point at 12 ± 1 °C.

To eliminate the possibility that the downshift in T_M observed in the presence of ATP was the result of an ATP chelation effect, we examined control experiments with aqueous dispersions of the membrane lipid extracts (proteins removed) as a function of temperature in the presence of buffered 5 mM ATP, 10 mM $CaCl_2$, and 50 mM KCl (pH 6.7). The results obtained were the same as those previously observed for aqueous dispersions of the membrane lipid extracts in the presence of 10 mM $CaCl_2$ (Figure 1), indicating that the downshift in T_M by ATP was not due to a chelation effect.

To obtain further information concerning the "energized membrane" requirement, and as another control experiment, we replaced ATP with its structural analogue AMP-PNP in the membrane preparations. Although AMP-PNP is structurally similar to ATP, it cannot be used as an energy source by most organisms. The results obtained for membrane samples in the presence of 5 mM AMP-PNP, 10 mM $CaCl_2$, and 50 mM KCl (pH 6.7) are illustrated in Figure 5. Only one break point was observed, at 19 ± 1 °C, indicating that AMP-PNP could not substitute for ATP. Why the break point was downshifted from 24 ± 1 to 19 ± 1 °C, however, is not known at this time.

Discussion

Analysis of the hyperfine splitting parameter, $2T_{\parallel}$, obtained for spin-labeled plasma membrane preparations and aqueous dispersions of the plasma membrane lipid extracts indicates that the middle break point, T_M, found in the zoospore plasma membrane samples is the result of a lipid–lipid interaction rather than a lipid–protein interaction. The values obtained for T_M in the plasma membrane preparations are also the same as those observed in the ESR studies of spin-labeled zoospores in vivo (Leonards & Haug, 1979a, 1980a), indicating that the lipid–lipid interaction found in the plasma membrane preparations is also responsible for the middle break point found in the zoospores in vivo.

The possibility that the middle break point, T_M, represents the gel to liquid-crystalline phase transition of a bulk lipid phase is unlikely. The lower, T_L, and upper, T_H, break points have been observed in experiments with spin-labeled zoospores in vivo (Leonards & Haug, 1979a, 1980a), zoospore plasma membrane preparations (unpublished data), aqueous dispersions of the total lipids extracted from zoospores (Leonards & Haug, 1979a, 1980b), and aqueous dispersions of the glycolipid fraction isolated from zoospores (Leonards & Haug, 1979b, 1980b). However, the middle break point, T_M, has not been detected either in aqueous dispersions of the total lipids extracted from zoospores or in aqueous dispersions of any of the isolated lipid components.

The presence of Ca^{2+} ions markedly affected the middle break point observed in the plasma membrane lipid extract, T_M being shifted from 21 ± 1 (10 mM $CaCl_2$) down to 11 ± 1 °C (Ca^{2+} ions removed by EDTA). This change in T_M is identical with that obtained in ESR studies on spin-labeled zoospores in vivo, where very different spin-label probes 5-NS and Tempo (2,2,6,6-tetramethylpiperidinyl-1-oxy) demonstrated the same Ca^{2+} ion specific effect on T_M (Leonards & Haug, 1980a). During the initial studies with spin-labeled zoospores in vivo control experiments also indicated that T_M was independent of pH, with a maximum shift of 2 °C over the pH range 6.0–7.5. Chemical analysis of the lipid components isolated from the zoospore plasma membrane has shown that this membrane is almost entirely composed of diglucosyldiglycerides (45% by wt) and neutral lipids (43% by wt, no triglycerides) rather than phospholipids (12% by wt). Of the phospholipids present ~90% are phosphatidylcholine and phosphatidylethanolamine (Leonards & Haug, 1980c). In addition, experiments with aqueous dispersions of the lipid components isolated from the zoospores have shown that the zoospore's glycolipids interact strongly with Ca^{2+} ions, whereas Ca^{2+} ions have no detectable effect on the zoospore's phospholipid fraction (Leonards & Haug, 1979b, 1980b). The lipid–lipid interactions observed therefore cannot be attributed to the properties of the phospholipids. Since the middle break point, T_M, is the result of a lipid–lipid interaction which can be influenced by Ca^{2+} ions but cannot be attributed to either a lipid phase transition or the zoospore's phospholipid fraction, the most plausible explanation is a glycolipid–glycolipid or glycolipid–neutral lipid interaction.

The specific molecular nature of a Ca^{2+} ion–glycolipid interaction is still unknown but certainly involves the lipid head group. Because neither the glycolipids nor neutral lipids (excluding free fatty acids) are charged molecules, an interaction analogous to that induced by Ca^{2+} ions in phosphatidylserine or phosphatidic acid containing mixtures is unlikely. However, the interaction of Ca^{2+} ions with glycolipids has been shown to increase the hydration capacity of diglucosyldiglyceride head groups (Wieslander et al., 1978), possibly by altering the orientation of the glycolipid head group. Hydration-related head group orientation changes have also been reported for thin films of phospholipid membranes (Jendrasiak & Mendible, 1976). A Ca^{2+} ion induced glycolipid head group change is also consistent with the results obtained with aqueous dispersions of zoospore glycolipids (Leonards & Haug, 1979b, 1980b).

The addition of K^+ ions to the isolated plasma membrane preparations, in the presence of Ca^{2+} ions, had no effect on the middle break point, T_M, when ATP was omitted. The absence of any change in the plots of $2T_{\parallel}$ vs. temperature for these preparations, after K^+ ion addition, indicates that K^+ ions do not reverse the Ca^{2+} ion effect simply by replacing the Ca^{2+} ions. That is, K^+ ions and Ca^{2+} ions do not have the same interaction site. This conclusion is supported by the results obtained for aqueous dispersions of the zoospore glycolipids, which demonstrated that an increase in $2T_{\parallel}$ observed as a consequence of Ca^{2+} ion inclusion could be neither prevented nor reversed by the addition of K^+ ions (Leonards & Haug, 1980b).

A comparison of the results illustrated in Figure 4 to those in Figure 2 indicates that the temperature to which T_M is shifted by K^+ ions plus ATP and EDTA in the isolated plasma membrane preparations is the same as that found for the aqueous dispersions of the plasma membrane lipid extracts in the presence of EDTA (12 ± 1 and 11 ± 1 °C, respectively). This value for T_M is also similar to the T_M values obtained for the K^+ ion reversal of the Ca^{2+} ion effect in spin-labeled zoospores in vivo, 10 ± 1 °C (Leonards & Haug, 1979a, 1980a). These results indicate that the Ca^{2+} ion effect could be reversed in at least a portion of the vesicle preparation by K^+ ions, after adding ATP. This conclusion was confirmed by the data obtained after EDTA addition, which demonstrated that the removal of Ca^{2+} ions resulted in the downshifting of T_M to 13 ± 1 °C and eliminated the break point at 24 ± 1 °C (Figure 4). These results suggest that the membrane proteins do not significantly affect the observed interaction. However, the mechanism of ATP action was not the same as that observed for EDTA, since the control experiments indicated that ATP was not downshifting T_M by a chelation effect and AMP-PNP could not substitute for ATP.

The analysis of the results obtained before and after the inclusion of ATP in the plasma membrane preparations suggests that the ATP is being utilized to generate an energized membrane. This suggestion was directly confirmed by experiments in our laboratory which demonstrated that a membrane potential was generated in zoospore plasma membrane preparations when ATP was added to the sample. Without the addition of ATP no such membrane potential existed. In addition, cAMP could not substitute for ATP (Jen & Haug, 1981). Such an interpretation is also consistent with the studies on *Neurospora* plasma membrane vesicles which indicated that a transmembrane potential ($\Delta\Psi$) could be generated in the presence of ATP (Stroobant & Scarborough, 1979).

Given that ATP can be used to generate a membrane potential in zoospore plasma membrane preparations and that once an energized membrane state is achieved, K^+ ions can reverse the Ca^{2+} ion effect causing a downshift in T_M, what is the connection between this phenomenon and T_M? Specifically, how is a change in a lipid–lipid interaction, which can be accomplished by EDTA and Ca^{2+} ions in lipid extracts and involves the lipid head group, related to the generation of an energized membrane by ATP, which requires the presence of membrane proteins?

Since K^+ ions do not simply replace Ca^{2+} ions, the reversal of the Ca^{2+} ion effect must involve other membrane parameters. Besides the downshift in T_M, the addition of K^+ ions has been shown to result in the immediate release of $^{45}Ca^{2+}$ from preloaded zoospores (Soll & Sonneborn, 1972). K^+ ion addition also causes a depolarization of the membrane potential, both in zoospores in vivo (Brunt & Harold, 1980) and in plasma membrane preparations after a membrane potential was first generated by ATP addition (Jen & Haug, 1980). In addition, experiments with lipid membrane vesicles have demonstrated that a transmembrane potential interacts strongly with phosphatidylserine and phosphatidylcholine head groups, alterations in the orientation of the surface dipoles of the lipid molecules being caused by different membrane potentials (Lelkes, 1979). Together, these results suggest that the reversal of the Ca^{2+} ion effect, and thus the downshift in T_M, is a consequence of the K^+ ion induced depolarization of the membrane potential. This hypothesis is consistent with the effects of K^+ ions on the membrane potential, which explains the requirement for an energized membrane, and at the same time indicates how K^+ ions could reverse the Ca^{2+} ion effect, altering a lipid–lipid interaction. It should be noted that this hypothesis does not exclude other consequences of either K^+ ion addition or the depolarization of the membrane potential on cellular functions. Nor does this hypothesis indicate a specific molecular mechanism by which K^+ ions cause a depolarization of the membrane potential.

The physiological significance of the middle break point is that it is closely correlated with the ability of zoospores to differentiate (Leonards & Haug, 1980a). The lipid–lipid interactions observed, as T_M, in the plasma membrane seem to be intimately involved with the temperature dependence of zoospore encystment. The encystment process entails the fusion of vesicles derived from the zoospores γ particles, which contain the last enzyme of the biochemical pathway for chitin synthesis (chitin synthase EC 2.4.1.16), with the plasma membrane (Myers & Cantino, 1974). Given that glycolipids comprise the major lipid fraction of both the plasma membrane (Leonards & Haug, 1980c) and γ particles (Mills & Cantino, 1978) and are also thought to be the intermediate for chitin synthesis in this organism (Mills & Cantino, 1980), changes in the physicochemical properties involving glycolipids at the cell surface may be important in regulating the encystment process.

Ca^{2+} ions seem to alter the temperature dependence of T_M by interacting directly with the lipid molecules involved. In contrast, K^+ ions exert their influence on T_M indirectly. If the hypothesis that K^+ ions reverse the Ca^{2+} effect, downshifting T_M via a depolarization of the membrane potential, is correct, it indicates that lipid–lipid interactions in a plasma membrane can be altered by changes in a transmembrane electrical potential. The regulation of zoospore differentiation may, therefore, involve both electrical and structural properties of the zoospore plasma membrane.

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