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EFFECT OF TEMPERATURE ON MEMBRANE FLUIDITY AND CALCIUM CONDUCTANCE OF THE EXCITABLE CILIARY MEMBRANE FROM *PARAMECIUM*

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Fluorescence anisotropy and average fluorescence lifetime of diphenylhexatriene were measured in artificial lipid membrane vesicles. Within the temperature range investigated (15–52°C) both parameters correlate and can be used interchangeably to measure membrane fluidity. Fluorescence anisotropy of DPH in membrane vesicles of cilia from the protozoan *Paramecium tetraurelia* decreased slightly from 5 to 37°C, yet, no phase transition was observed. An estimated flow activation energy of approx. 2 kcal/mol indicated that the ciliary membrane is very rigid and not readily susceptible to environmental stimuli. The ciliary membrane contains two domains of different membrane fluidity as indicated by two distinct fluorescence lifetimes of diphenylhexatriene of 7.9 and 12.4 ns, respectively. Ca^{2+} flux into ciliary membrane vesicles of *Paramecium* as measured with the Ca^{2+} indicator dye arsenazo III showed a nonlinear temperature dependency from 5 to 35°C with a minimum around 15°C and increasing flux rates at higher and lower temperatures. The fraction of vesicles permeable for Ca^{2+} remained unaffected by temperature. The differences in temperature dependency of Ca^{2+} conductance and membrane fluidity indicate that the Ca^{2+} permeability of the ciliary membrane is a membrane property which is not directly affected by the fluidity of its lipid environment.

Introduction

Higher organisms maintain ion gradients across their cellular membrane and utilize ion fluxes along these gradients and concomitant potential changes for signal transduction. In case of an action potential, a small voltage change (receptor potential) temporarily opens voltage-sensitive ion-specific channels in the membrane to allow transient passive ion fluxes. For most channels the mechanism of ion permeation and the process of termination of these fluxes is poorly understood and needs detailed studies of isolated channels and of membrane properties.

In *Paramecium* a Ca^{2+}/K^+ action potential exists, the voltage-sensitive Ca^{2+}-channels are localized in the ciliary membrane [1,2]. Ca^{2+} entry depolarizes the cell and reverses the beating direction of the cilia (avoiding reaction, [3]). K^+ outward currents through voltage- and Ca^{2+}-operated K^+ channels repolarize the cell and restore normal forward swimming [3]. Interference with this reaction sequence changes the behavioral pattern of *Paramecium*. For example, Kung and coworkers have isolated several types of mutants with defective Ca^{2+} conductance, called 'pawns' [4]. The mutant cells are unable to show an avoiding reaction since not sufficient Ca^{2+} can enter the cilia to trigger ciliary reversal. Electrophysiological studies of these behavioral mutants yielded valuable infor-

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Abbreviations: DMPC, dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, dipalmitoyl-*sn*-glycero-3-phosphocholine; Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

mation about the ionic currents involved in sensory transduction [5].

In biochemical studies of this pathway we demonstrated Ca^{2+} influx into ciliary membrane vesicles of *Paramecium* [6]. This Ca^{2+} flux was considerably reduced in vesicles from pawn mutants, i.e. in wild-type vesicles it occurred at least in part through the voltage-sensitive Ca^{2+} channels [6]. In this paper we examined the temperature dependence of the Ca^{2+} flux and correlate it to membrane fluidity as measured by fluorescence depolarization and lifetime of diphenylhexatriene.

Materials and Methods

Axenic mass cultivation of *Paramecium tetraurelia* wild-type 51s, preparation of cilia and vesicles loaded with arsenazo III were described previously [6–8]. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were from Fluka, ionophore A 23187 from Calbiochem, all-*trans*-1,6-diphenyl-1,3,5-hexatriene from Sigma. Ca^{2+} flux into ciliary membrane vesicles was determined spectrophotometrically as change in absorbance of the enclosed Ca^{2+} indicator dye arsenazo III at 652 nm [6]. Measurements of the initial velocity were made with a thermostated Aminco Morrow Stopped flow cell adapted to a Zeiss PMQ3 unit [9]. Ca^{2+} flux after 90 s was measured in a Zeiss PM 6 photometer. The signal is expressed as the fraction of enclosed dye which reacted with Ca^{2+}. After the experiment the amount of enclosed arsenazo III was determined by addition of the Ca^{2+} ionophore A 23187 (10 μM), which rendered all vesicles permeable to Ca^{2+}, or by lysis of vesicles with 0.1% Triton X-100 and subsequent determination of the released dye [6]. Both methods yielded identical results. Saturation of arsenazo III by Ca^{2+} was almost instantaneous using vesicles preincubated with the Ca^{2+} ionophore. This proved that the formation of the Ca^{2+}-arsenazo III complex within vesicles was not rate limiting but rather Ca^{2+} entry through specific channels.

The absorbance of the arsenazo III-Ca^{2+} complex is highly sensitive to pH changes [10]. Therefore, the pH of the buffer, in which the vesicles were suspended prior to measurement (10 mM Mops, pH 7.20/50 mM sucrose/50 μM EGTA/20 mM KCl), was adjusted such that at the experimental temperature the pH was 7.20. Fluorescence depolarization was measured in a setup consisting of two Zeiss MM12 double monochromators for selection of excitation and emission light, a Xenon lamp (Osram XB450W), and Glan-Thompson prisms for polarization of the excitation light and for analyzing the emitted light. An RCA 7265-photomultiplier in connection with an Ortec 454 amplifier and an Ortec single-photon counter was used to measure fluorescence depolarization [11]. Polarization due to the monochromators was corrected according to Ref. 12. Fluorescence lifetimes were determined with time-correlated photon counting in a setup consisting of an Ortec flashlamp (bandwidth 2 ns), a Spex double monochromator, for selection of emitted light, and a cooled photomultiplier (RCA C31034). All electronic equipment as amplifiers, discriminators, and a time to pulse height converter with a multi-channel analyzer were from Ortec. The data were analyzed according to Refs. 13 and 14 with a multiexponential least-square fit. The detailed procedure will be published elsewhere. Artificial lipid vesicles were prepared by dissolving the respective phospholipid in carbontetrachloride (20 mg/ml) in a glass tube under nitrogen atmosphere. Evaporation of solvent with a stream of nitrogen left a dry lipid film on the glass surface. Vortexing with 0.1 M NaCl/10 mM Mops, pH 7.1 at 4°C and subsequent sonication for 2 min (Branson Sonifier B12, Microtip) yielded a translucent vesicle suspension (0.4 mg lipid/ml).

Vesicles were incubated for 8–14 hours in the presence of diphenylhexatriene (1 μM) prior to the measurement to allow equilibration of the fluorophore with the membrane. A ratio of lipid : diphenylhexatriene of 500–1000 was maintained throughout.

Results

Calcium flux into ciliary membrane vesicles at various temperatures

In preliminary experiments we found that the Ca^{2+} sensor arsenazo III leaked out of ciliary membrane vesicles at elevated temperatures. We

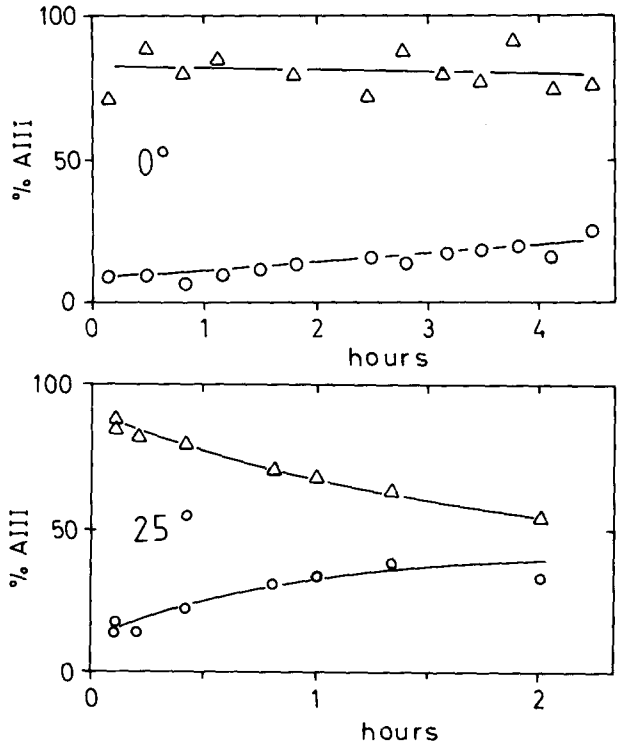

Fig. 1. Stability of ciliary membrane vesicles loaded with 15 mM arsenazo III (AIII) as a function of temperature. The vesicle suspension was stored at 0°C (top) and 25°C (bottom) and centrifuged at appropriate time intervals. Arsenazo III was then determined in the pellet (△——△) and in the supernatant (○——○).

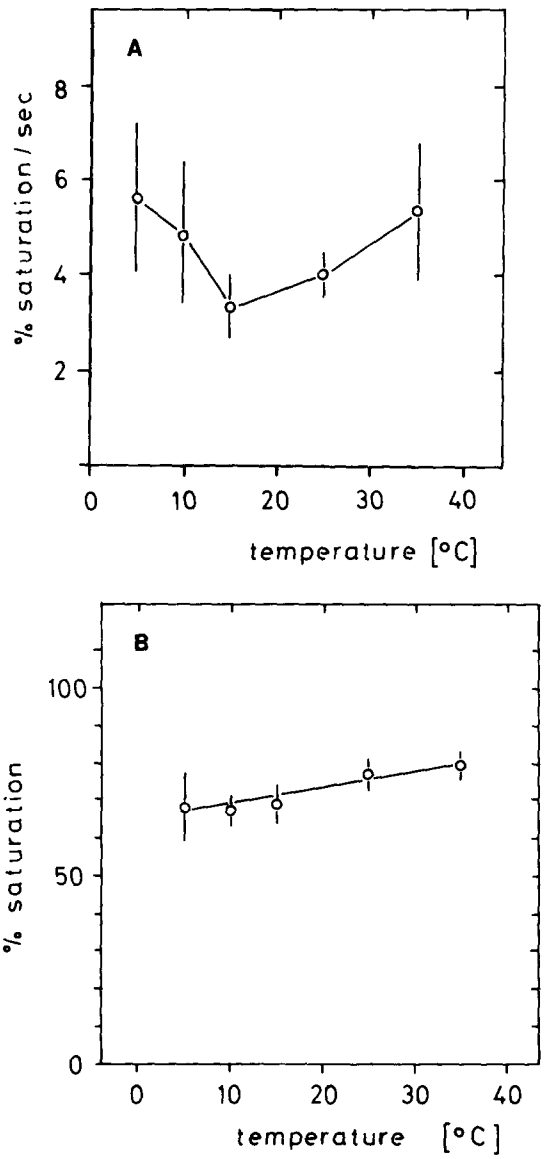

Fig. 2. Temperature dependence of Ca^{2+} flux into ciliary membrane vesicles of *Paramecium*. (A) Initial Ca^{2+} flux rates as determined from stopped-flow absorbance traces (60–600 ms). (B) Vesicle volume accessible to Ca^{2+} as determined 90 s after onset of Ca flux. The final Ca^{2+} concentration in the experiments was 50 μM. $N = 3$–10, each point is $\bar{x} \pm$ S.D. The differences are statistically not significant.

determined the stability of arsenazo III-loaded vesicles at 0 and 25°C (Fig. 1). Vesicles kept at 25°C lost half of the arsenazo III within 1.5 h. At 0°C the release of dye was much slower, $t_{1/2}$ was 10 h. Therefore, arsenazo III-loaded vesicles were kept on ice and equilibrated at the experimental temperature for 3 min before measuring Ca^{2+} flux. Even at 45°C there was no measurable loss of arsenazo III from the vesicles during the experiment (about 5 min).

Fig. 2 shows the Ca^{2+} flux as a function of temperature. The initial Ca^{2+} flux rates were calculated from the linear part of the kinetics (60–600 ms [9]). A very slight increase in arsenazo III saturation after completion of the fast Ca^{2+} influx indicated further Ca^{2+} entry into vesicles which did not contain Ca^{2+} specific entry sites. This unspecific Ca^{2+} leakage was also observed in preparations from the Ca^{2+}-channel deficient mutant pawn A/pawn B and contributed less than 10% to the total initial Ca^{2+} flux at all temperatures. The initial velocity changed nonuni-

formly with temperature up to 40°C (Fig. 2A). Around 15°C the flux was minimal and increased towards higher and lower temperatures. At all temperatures the fast Ca^{2+} flux was completed after 90 s at which timepoint the fraction of Ca^{2+} permeable vesicles was determined (Fig. 2B). A slight and steady increase with temperature was observed suggesting that unspecific Ca^{2+} leakage of vesicles was related to increased lipid mobility.

Fluorescence depolarization of diphenylhexatriene in the ciliary membrane

For a further interpretation of these data information is necessary about biophysical properties of the membrane in the temperature range investigated. Membrane fluidity was determined by fluorescence depolarization of diphenylhexatriene incorporated into intact cilia or membrane vesicles. The anisotropy of fluorescence decreased only marginally from 5°C to 37°C. No phase transition was observed (Fig. 3) nor any hysteresis.

Addition of Ca^{2+} (500 μM) to the external medium raised the fluorescence anisotropy of diphenylhexatriene marginally (Fig. 3). Upon 8-fold dilution the anisotropy of the cilia increased by 10%, while with membrane vesicles the changes in anisotropy upon dilution were less than 1%. Thus, the anisotropy difference between intact cilia and ciliary membrane vesicles was most likely due to multiple depolarization of the incident light by repeated scattering in the turbid suspension. An Arrhenius diagram from the anisotropy data approximated according to Shinitzki and Barenholz [15] yielded a flow activation energy of 1.7 kcal/mol (data not shown). This extremely small value shows that the ciliary membrane is susceptible to temperature dependent fluidity changes only to a very limited extent.

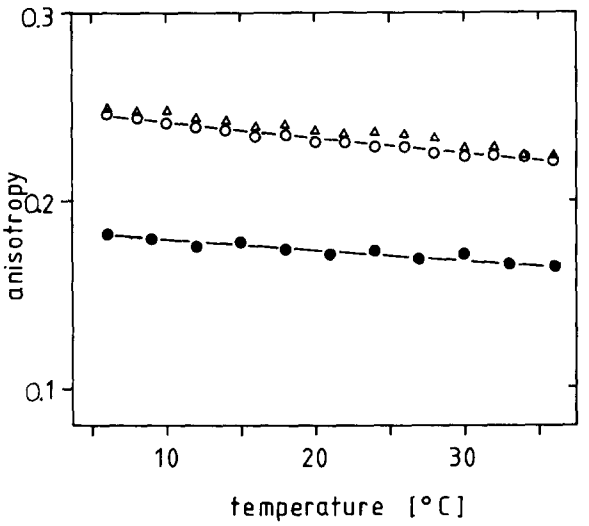

Fig. 3. Fluorescence anisotropy of diphenylhexatriene incorporated into cilia (●———●) and ciliary membrane vesicles (○———○) from *Paramecium*. Note that addition of 500 μM Ca to ciliary membrane vesicles (△———△) caused only a marginal change in membrane fluidity.

Fluorescence decay of diphenylhexatriene in artificial and in ciliary membrane vesicles

In an attempt to further analyze the properties of the ciliary membrane we used the fluorescence lifetime of diphenylhexatriene as sensor for changes in the microenvironment of the fluorophore. Previous measurements [16] indicated that the lifetime of diphenylhexatriene incorporated into artificial lipid vesicles was temperature dependent. Comparing fluorescence anisotropy and fluorescence decay of diphenylhexatriene in DMPC and DPPC vesicles we found an excellent correlation of both measures (Fig. 4). Analogous to the anisotropy, changes of fluorescence lifetime were greatest near the transition temperature of the membrane. From the derivatives of these curves identical transition temperatures of 23.8 $\pm$ 0.2°C for DMPC and 40.9 $\pm$ 0.2°C for DPPC, respectively, were obtained. A plot of anisotropy versus lifetime (Fig. 4C) shows the correlation between both parameters. Apparently it is identical for both types of vesicles indicating that the average fluorescence lifetime and fluorescence anisotropy of diphenylhexatriene can be used interchangeably irrespective of the lipid used.

The fluorescence of diphenylhexatriene is longest in the crystalline state of the membrane and decreases when the membrane becomes less ordered. This may indicate that not only the interaction with lipid but also access of water [15] and/or oxygen, both potent quenchers of diphenylhexatriene fluorescence, determine the lifetime of the excited fluorophore. The decay of diphenylhexatriene fluorescence in vesicles of homogeneous composition was approximated by a monoexponential function, i.e. the obtained lifetime values

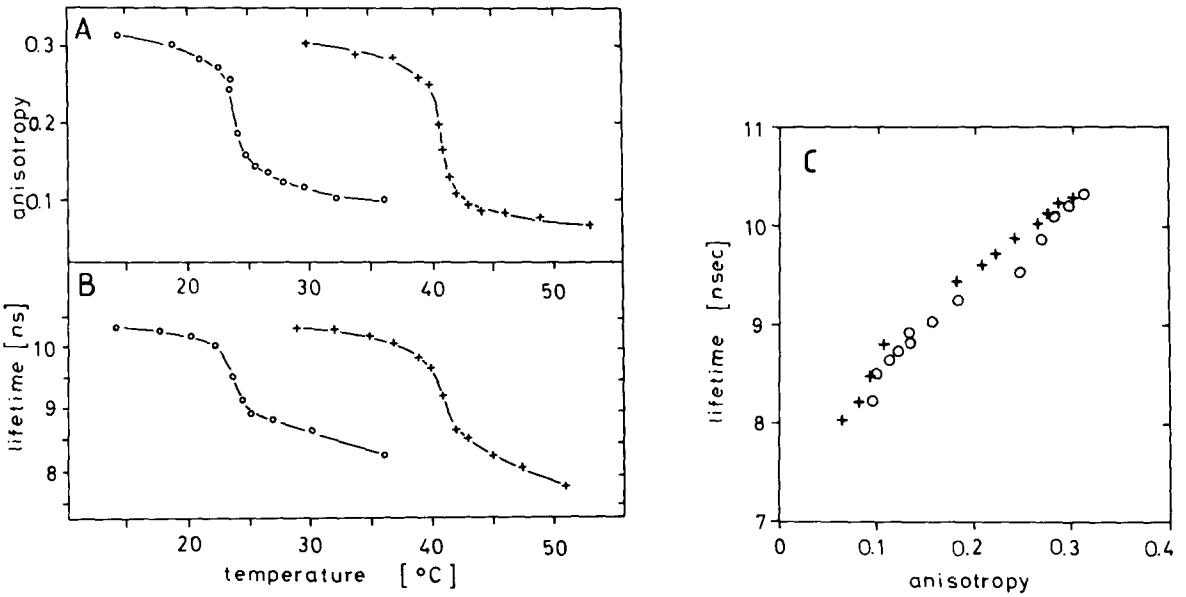

Fig. 4. Fluorescence of diphenylhexatriene incorporated into dimyristoylphosphatidylcholine (O——O) and dipalmitoylphosphatidylcholine (+——+) vesicles as function of temperature. (A) Fluorescence anisotropy. (B) Average lifetime of fluorescence decay. (C) Correlation of fluorescence anisotropy and lifetime. For details see text.

represent weight averages over all existing subpopulations [15].

To test to what extent we could resolve distinct lifetimes we measured diphenylhexatriene fluorescence in mixtures of separately prepared DMPC- and DPPC-vesicles at 32°C. At this temperature DMPC formed fluid membranes whereas DPPC was in the crystalline state (Fig. 4). Multiexponential analysis of diphenylhexatriene fluorescence decay yielded optimal approximation of the data with two exponentials. We found two distinct lifetimes for all mixtures (Table I). Even in

TABLE I

DETERMINATION OF THE FRACTION OF DIMYRISTOYLPHOSPHATIDYLCHOLINE AND DIPALMITOYLPHOSPHATIDYLCHOLINE VESICLES BY DIPHENYLHEXATRIENE FLUORESCENCE DECAY MEASUREMENTS AT 32°C

Vesicles were prepared separately and mixed as indicated. The composition was calculated from the amplitudes of a biexponential fit assuming that both vesicle populations contribute to the fluorescence decay according to their respective amplitudes found in homogeneous suspension.

Experimental composition DMPC : DPPC	Decay of diphenylhexatriene fluorescence			Calculated composition DMPC : DPPC
	Lifetimes (ns)		Ratio of amplitudes	
100 : 0	7.8	11.1	89 : 11	–
90 : 10	7.7	11.3	80 : 20	87 : 13
80 : 20	8.0	11.2	73 : 27	77 : 23
50 : 50	7.6	11.3	55 : 45	51 : 49
20 : 80	8.0	11.1	32 : 68	19 : 81
10 : 90	7.6	11.2	23 : 77	7 : 93
0 : 100	7.8	11.1	19 : 81	–

TABLE II

FLUORESCENCE DECAY OF DIPHENYLHEXATRIENE INCORPORATED INTO CILIARY MEMBRANE VESICLES OF *PARAMECIUM*

	Temperature (°C)	Short lifetime		Long lifetime	
		ns	% amplitude	ns	% amplitude
+ 50 μM EGTA	10	8.0	43.9	12.4	56.1
	20	7.9	45.1	12.4	54.9
	30	7.9	45.3	12.4	54.2
+ 500 μM Ca^{2+}	10	8.1	33.6	12.3	66.4
	20	7.8	41.3	12.4	59.7
	30	8.2	38.2	12.3	62.8

homogeneous vesicle preparations a small contribution of a second lifetime was observed as reported earlier [17]. Assuming that both vesicle types contribute to the amplitudes as they do in homogeneous suspension, the fraction of each vesicle species of the mixture was determined (Table I). The calculated composition of the mixtures agreed well with the theoretical values thus proving that the method is capable of resolving differences in lifetimes.

The analysis of fluorescence lifetimes of diphenylhexatriene incorporated into ciliary membrane vesicles from *Paramecium* yielded two distinct values of 8.0 + 0.3 and 12.4 + 0.2 ns, respectively, with about equal amplitudes (Table II).

Fluorescence lifetimes were constant within the measured temperature range. This strongly indicates the presence of two major hydrophobic domains of different fluidity within the ciliary membrane of *Paramecium*. The fraction of each was found to be constant from 10 to 30°C.

Ca^{2+} had no effect on the fluorescence lifetimes of diphenylhexatriene in either hydrophobic domain, however, it increased noticeably the proportion of rigid domains.

Discussion

In biological membranes proteins such as ionic channels are embedded in a lipid matrix and changes in lipid fluidity can affect the function of membrane proteins [18]. The rotational mobility of diphenylhexatriene as measured by its fluorescence depolarization is an accepted measure for membrane fluidity [15]. Using artificial lipid vesicles we correlated fluorescence depolarization and the average fluorescence lifetime of diphenylhexatriene and showed that both parameters are interchangeable, i.e. the average fluorescence lifetime can also be used to monitor membrane fluidity.

Due to their protein and sterol content biological membranes show less pronounced fluidity changes than artificial lipid bilayers [18]. The ciliary membrane of *Paramecium* seems rigid in that almost no fluidity changes were observed at physiologically relevant temperatures. This may be caused by the very high content of proteins and sterols in the ciliary membrane, both rendering the membrane less susceptible to short term changes of their environment [19,20]. Apparently the ciliary membrane of *Paramecium* can provide a rather constant environment for its protein constituents in spite of frequent environmental stimuli.

Ciliates adapt the lipid composition of their membranes to long term changes of their environment [21] indicating that the interaction between lipids and other membrane components is carefully regulated *. An example is a Ba-shy *Para-*

* Noteworthy in this context are the surface antigens of *Paramecium*, prominent membrane proteins, which are anchored in the membrane and protrude into the surrounding aqueous medium. Their appearance on the cell surface is regulated by growth temperature. It will be interesting to investigate whether these proteins to some extent take part in adaptation to temperature by influencing membrane properties.

mecium mutant, d4-592, which has an altered membrane lipid composition and concomitantly decreased Ca^{2+} and K^+ conductances. Growth in presence of stigmasterol can cure both the biochemical and the electrophysiological defects of this mutant [22]. *Tetrahymena,* a related ciliate, responds to a decrease of the growth temperature by replacing saturated by unsaturated fatty acids in position one of its phospholipids thus counteracting the decrease of membrane fluidity [21].

If Ca^{2+} conductances were strongly dependent on lipid fluidity one would expect small changes with temperature considering the properties of the ciliary membrane. We found a moderate influence of temperature on Ca^{2+} fluxes and the changes did not correlate with alterations in membrane fluidity. Thus the Ca^{2+} conducting structure seems to be rather independent of its lipid environment. The biphasic curve may indicate that actually two counteracting processes are involved one of which might be an interaction of the channel structure with its lipid environment.

Little is known about the molecular nature of the membrane constituent generally termed 'Ca^{2+} channel' [23]. In principle ion channels or a large concentration of ion carrier molecules can account for Ca^{2+} fluxes. We had hoped to find a clear phase transition of the ciliary membrane. The simultaneous fluidity change would alter the mobility of a carrier and therefore its transport rate. With the experimental evidence, however, we cannot exclude a carrier model at present.

We have indications that Ca^{2+} passes through the membrane via a protein. Freezing of cilia or incubation of cilia for 3 min at pH 2 caused a significant reduction of Ca^{2+} conductance. Also, the observed temperature dependency of the Ca^{2+} conductance may be taken as preliminary evidence for the protein nature of the Ca^{2+}-conducting complex.

In analogy to the Na^+ channel one generally considers an ion pore to be responsible for Ca^{2+} entry [5]. This assumption is based mainly on an estimation of net Ca^{2+} fluxes as measured electrophysiologically [5]. The model proposes a finite number of Ca^{2+} pores in the ciliary membrane with a single-channel conductance of similar magnitude to other ion channels. The assumption that cilia or vesicles contain only a limited number of channels is supported by the observation that conditions that reduce initial Ca^{2+} flux rates into membrane vesicles always cause a proportional decrease in the vesicle volume accessible to Ca^{2+} [9]. With many channels per vesicle one would expect that at least some channels remain functional so that the accessible volume would be unaltered. The Ca^{2+} influx per vesicle (about 10000 calcium ions per s) which may be calculated from our flux data (see also Ref. 9) is smaller than the Na^+ channel conductance [24] but probably too large for a carrier-mediated Ca^{2+} flux, thus favoring a channel.

At present we do not know how these presumed Ca^{2+} channels interact with the hydrophobic domains of the ciliary membrane. The two fluorescence lifetimes of diphenylhexatriene indicate that rigid hydrophobic domains can coexist with more fluid domains. This result corroborates the notion of a heterogeneous ciliary membrane which is indicated by the finding of at least two distinct vesicle subpopulations which carry differential enzyme patterns [8,25]. A comparison of biophysical membrane properties with the Ca^{2+} conductancies of these vesicle fractions should allow to determine whether the Ca^{2+} channel can be assigned to a distinct membrane domain.

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References

1 Dunlap, K. (1977) J. Physiol. (London) 127, 119–133
2 Ogura, A. and Takahashi, K. (1976) Nature 264, 170–172
3 Oertel, D., Schein, S.J. and Kung, C. (1977) Nature 268, 120–124
4 Kung, C. (1971) Z. Vergl. Physiol. 71, 142–164
5 Eckert, R. and Brehm, P. (1979) Annu. Rev. Biophys. Bioeng. 8, 353–383
6 Thiele, J. and Schultz, J.E. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 3688–3691
7 Thiele, J., Honer-Schmid, O., Wahl, J., Kleefeld, G. and Schultz, J.E. (1980) J. Protozool. 27, 118–121
8 Thiele, J., Klumpp, S., Schultz, J.E. and Bardele, C.F. (1982) Eur. J. Cell Biol. 28, 3–11
9 Thiele, J., Otto, M.K., Deitmer, J.W. and Schultz, J.E.

(1983) J. Membrane Biol. 76, 253–260
10 Chiu, V.C.K. and Haynes, D. (1980) Membrane Biochem. 3, 169–183
11 Krabichler, G. (1981) Diplomarbeit, Universität Tübingen
12 Azumi, T. and Glynn, S.P. (1962) J. Chem. Phys. 37, 2413–2420
13 O'Connor, D.V., Ware, W.R. and André, J. (1979) J. Phys. Chem. 83, 1333–1343
14 Kirchhoff, W.H. (1972) J. Mol. Spectrosc. 41, 333–380
15 Shinitzky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367–394
16 Prendergast, F.G., Haugland, R.P. and Callahan, P.J. (1981) Biochemistry 20, 733–738
17 Chen, L.A., Dale, R.E., Roth, S. and Brand, L. (1977) J. Biol. Chem. 252, 2163–2169
18 Melchior, D.L. and Stein, J.M. (1976) Annu. Rev. Biophys. Bioeng. 5, 205–238
19 Brugerolle, G., Andrivon, G. and Bohatier, J. (1980) Biol. Cell. 37, 251–260
20 Adoutte, A., Ramanathan, R., Lewis, R.W., Dute, R.R., Lin, K.-Y., Kung, C. and Nelson, D.L. (1980) J. Cell Biol. 84, 711–738
21 Watanabe, T., Fukushima, H., Kasai, R. and Nozawa, Y. (1981) Biochim. Biophys. Acta 665, 55–73
22 Forte, M., Satow, Y., Nelson, D. and Kung, C. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 7195–7199
23 Hagiwara, S. and Byerly, L. (1981) Annu. Rev. Neurosci. 4, 69–125
24 Neher, E. and Sakmann, B. (1976) Nature 260, 799–801
25 Schultz, J.E. and Klumpp, S. (1983) FEBS Lett. 154, 347–350