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Membrane potential in liposomes measured by the transmembrane distribution of $^{86}Rb^+$, tetraphenylphosphonium or triphenylmethylphosphonium: Effect of cholesterol in the lipid bilayer

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Valinomycin-induced potassium diffusion potential ($\Delta\psi$, inside negative) in the liposomes made of phosphatidylcholine and various amounts of cholesterol was measured by uptake of $^{86}Rb^+$, tetraphenylphosphonium (TPP^+) or triphenylmethylphosphonium ($TPMP^+$). In any liposome, the values of membrane potential obtained by $^{86}Rb^+$ uptake ($\Delta\psi_{Rb}$) agreed well with those calculated from the imposed potassium concentration gradient using the Nernst equation, and were not affected by the presence of cholesterol. However, both $\Delta\psi_{TPP}$ and $\Delta\psi_{TPMP}$ showed smaller values than $\Delta\psi_{Rb}$ when the cholesterol content in liposomes increased. $\Delta\psi_{TPMP}$ at a stationary state was much smaller than $\Delta\psi_{TPP}$. The orientational order parameter of the lipids' bilayer with various cholesterol content was estimated from fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene. The results indicated that the permeation of TPP^+ or $TPMP^+$ into liposomes containing a large amount of cholesterol is strongly restricted by the high ordering of phosphatidylcholine acyl chains.

Introduction

The membrane potential ($\Delta\psi$) of cells or organelles is often determined from the transmembrane distribution of lipophilic cation probes between the interior and the exterior space. Among such membrane-permeable probes, tetraphenylphosphonium (TPP^+) and triphenylmethylphosphonium ($TPMP^+$) have frequently been used [1–7]. Schuldiner and Kaback [2] measured the membrane potential in the vesicles of *Escherichia coli* by the uptake of $TPMP^+$ and $^{86}Rb^+$ (+ valinomycin), and the values obtained with both probes were comparable. There have been, however, a number of articles reporting that different distribution probes gave inconsistent results for the estimation of $\Delta\psi$ [5–7]. For example, Bakker [5] reported that the estimated $\Delta\psi$ in *E. coli* K-12 cells depended on the kinds of probe used. Demura et al. [7] showed that a large amount of lipophilic cations, such as TPP^+ and $TPMP^+$, bound to the membrane surface of *Halobacterium*

Abbreviations: TPP^+, tetraphenylphosphonium; $TPMP^+$, triphenylmethylphosphonium; TPB^-, tetraphenylboron; $\Delta\psi$, membrane potential; $\Delta\psi_{TPP}$, $\Delta\psi_{TPMP}$ and $\Delta\psi_{Rb}$, membrane potentials from transmembrane distribution of TPP^+, $TPMP^+$ and Rb^+, respectively; PC, phosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 9-AA, 9-aminoacridine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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halobium vesicles and this binding resulted in an overestimation of $\Delta\psi$.

The use of protein-free liposomes is significant for the fundamental understanding of the $\Delta\psi$-dependent distribution behavior of the probes. In this study, the valinomycin-induced K^+ diffusion potential (inside negative) was measured by the uptake of distribution probes TPP^+, $TPMP^+$ and $^{86}Rb^+$ in large unilamellar liposomes. Then the effects of cholesterol in liposomes and the physical state of liposome membrane on the $\Delta\psi$-dependent uptake of these probes were analyzed. The $\Delta\psi$ values obtained from the distribution of TPP^+ or $TPMP^+$ were smaller than those obtained from $^{86}Rb^+$ distribution and were dependent on the cholesterol content in liposomes. The results are discussed in terms of the restricted permeation of the lipophilic cation probes into liposome membrane due to an increase in acyl-chain ordering of PCs.

Materials and Methods

Large unilameller liposomes of egg yolk PC containing various amounts of cholesterol were prepared by the reverse-phase evaporation method, as described previously [8]. The liposomal content of PC and cholesterol were determined with Phospholipid-test Wako kit and Cholesterol B-test Wako kit (Wako Pure Chemical Industries, Japan), respectively. The cholesterol content in liposomes ranged from 10 to 50 mol%. The potassium concentration gradient was established as follows: liposomes prepared in a KCl buffer (150 mM KCl/20 mM Hepes at pH 7.4) were passed through a Sephadex G-50 column preequilibrated with the isoosmotic elution buffer ([KCl] + [NaCl] = 150 mM, 20 mM Hepes at pH 7.4).

A flow dialysis cell was made of acryl blocks. The upper and lower chambers were separated by cellulose acetate membrane (6000–8000 molecular weight cutoff; Union Carbide Co.). The dialysis buffer was arranged to flow continuously at 0.7 ml/min through the lower chamber (0.8 ml). A liposome suspension (3.0 ml) was placed in the upper chamber, then $^{86}Rb^+$ or radiolabeled probe ([^{14}C]TPP^+ or [^{14}C]$TPMP^+$) was added to the suspension. The final concentrations of lipophilic cations were 6 μM and that of $^{86}Rb^+$ was no greater than 2.5 μM. K^+ diffusion potential was generated by the addition of valinomycin (final concn. 7 μM) to the liposomes suspension (1–5 μmol PC). 2.0 ml of each dialysis buffer were collected, and then 1.0 ml aliquots were used for the radioactivity measurement with a liquid scintillation counter (Aloka LSC-700).

$\Delta\psi_{TPP}$ was also measured with a laboratory-made TPP^+-selective electrode according to the method reported previously [9]. A liposome suspension (2 ml, 1–5 μmol PC) was placed in a cuvette, and then an adequate amount of TPP^+ stock solution (1 mM in water) was added stepwise (final concn. 6 μM) to record a calibration curve for the electrode response. After the equilibrium was attained, valinomycin (final concn. 0.3 μM) was added, and then the $\Delta\psi$-dependent uptake of TPP^+ was recorded.

The valinomycin-induced membrane potential, $\Delta\psi$, was calculated from the Nernst equation:

$$\Delta\psi(\mathrm{mV}) = -59 \log\left(\frac{C_i}{C_o}\right)$$

Here, C_i and C_o are the intravesicular and external concentrations of TPP^+, $TPMP^+$, $^{86}Rb^+$ or K^+. Encapsulation (%) of liposomes for each preparation was determined by the calcein cobalt method [10], prior to gel filtration. The encapsulation (%) of liposomes after gel filtration treatment was determined by comparing the turbidity of the liposome suspension at 600 nm before and after the gel filtration. Encapsulation (%) of the liposomes used in the present experiments was in the range of 1–5%. If not stated otherwise, all the $\Delta\psi$ data were an average of the data taken from at least three independent experiments. The deviation from the average did not exceed ± 10 mV.

The steady-state fluorescence polarization of 1.6-diphenyl-1,3,5-hexatriene (DPH) in liposomes was measured with a Hitachi 650-60 fluorescence spectrophotometer equipped with a polarization accessory. The excitation wavelength and monitored fluorescence wavelength were 355 and 435 nm, respectively. The steady-state fluorescence anisotropy (r_s) was calculated from

$$r_s = 2P/(3-P)$$

Here, P is the degree of fluorescence polarization

of DPH embedded in liposomes. In the case of DPH in liposomal membranes, r_s can be resolved into a static part (r_∞) and a dynamic part (r_f): $r_s = r_f + r_\infty$. Here, r_∞ is the limiting fluorescence anisotropy, which reflects the structural order of membrane [11–13]. To obtain r_∞ directly, a time-resolved fluorescence anisotropy measurement is necessary. However, Van Blitterswijk et al. [14] reported an empirical relation between r_s and r_∞ on DPH-labeled artificial membrane. Then, the orientational order parameter, S, can be calculated from following equations

$$r_\infty = \tfrac{4}{3} r_s - 0.1 (0.13 < r_s < 0.28),$$

$$S^2 = r_\infty / r_0.$$

Here, r_0 is the maximal fluorescence anisotropy value in the absence of any rotational motion of the fluorophore and is taken to be 0.4.

The fluorescence-decay constant was determined by analyzing the decay curve measured by the single-photon counting method using an Ortec system with the Norland multichannel analyzer. The excitation wavelength was selected with a 340 nm interference filter (half-width of the exiting light pulse was 2 ns) and the emitted fluorescence was detected through a 440 nm cut-off filter. All the measurements above were carried out at 20 °C.

The zeta potential of liposomes was measured as follows. The electrophoretic mobility (U) of liposomes in the medium containing 150 mM NaCl, 20 mM Hepes (pH 7.4) was measured at 25 °C with Lazer Zee Meter Model 500 (Pen-Ken). The measured mobility was converted to zeta potential (ζ) by using the Helmholtz-Smoluchowski equation, $\zeta = 4\pi\eta U/\epsilon$. Here, π and ϵ are the viscosity and dielectric constants of the medium, respectively.

The $\Delta\psi$-driven proton influx experiments with 9-aminoacridine (9-AA) fluorescence was carrier out as follows. 3 ml of 150 mM KCl-loaded liposome suspension (30 nmol PC in a buffer comprising 20 mM Hepes/149.8 mM NaCl/0.15 mM KCl (pH 7.4)) were placed in a cuvette, and 9-AA was added (final concn. 10 μM) to the liposome suspension. Valinomycin was added (final concn. 0.2 μM) in order to generate a K^+ diffusion potential. The protonophore, FCCP, was then added (final concn. 1 nM) in order to induce $\Delta\psi$-driven, FCCP-mediated H^+ influx into liposomes. The change in 9-AA fluorescence intensity was monitored at 430 nm (excitation at 400 nm).

Materials used were as follows: egg yolk PC (type III-F, purity 99%), gramicidin from Sigma, ^{86}RbCl (1 mCi/mg), $H^{36}Cl$ (0.1 mCi/g), ^{22}NaCl (carrier-free), [^{14}C]TPP-bromide (20 mCi/mmol), [^{14}C]TPMP-iodide (20 mCi/mmol) from New England Nuclear, TPP-chloride from Dojin, valinomycin from P-L Biochemicals, TPB-Na, cholesterol and 9-aminoacridine hydrochloride, and other chemical reagents were purchased from Wako.

Results and Discussion

Comparison of $\Delta\psi_{TPP}$ and $\Delta\psi_{TPMF}$ with $\Delta\psi_{Rb}$ in liposomes containing various amount of cholesterol

The time-course of extravesicular concentration change of $^{86}Rb^+$ and radioactive lipophilic cations was followed by the flow dialysis. Fig. 1 shows a typical result for the liposomes containing 38 mol%

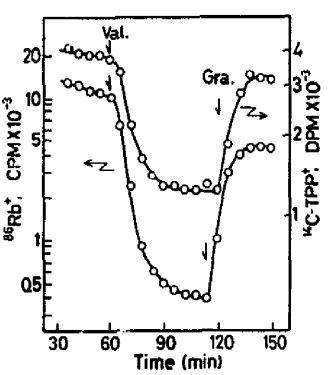

Fig. 1. The typical time-course of extravesicular concentration change of $^{86}Rb^+$ or [^{14}C]TPP$^+$ followed by flow dialysis. Upper curve is for the uptake of [^{14}C]TPP$^+$ and lower curve is for the uptake of $^{86}Rb^+$. Large unilameller PC/cholesterol liposomes containing 38 mol% cholesterol were used. Liposomes loaded with 150 mM KCl were suspended in external buffer containing 0.15 mM KCl and 149.8 mM NaCl. The concentrations of [^{14}C]TPP$^+$, $^{86}Rb^+$, valinomycin and gramycidin were 6, 2.5, 7 and 3 μM, respectively. The encapsulation % of liposomes to total suspension volume (3.0 ml) was 2.5%. Arrows show the time when valinomycin (Val.) and gramicidin (Gra.) were added. See Materials and Methods for further details.

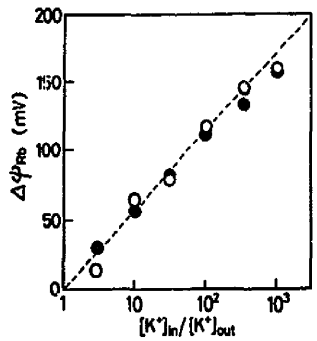

Fig. 2. The values of $\Delta\psi_{Rb}$ in PC liposomes containing 10 and 38 mol% cholesterol obtained at various K^+ gradients. Liposomes loaded with 50 mM KCl was suspended in isoosmotic external buffer containing KCl and NaCl in the desired proportions. The line represents the theoretical values of $\Delta\psi$ calculated from imposed K^+ gradients using the Nernst equation. (○), liposomes with 10 mol% cholesterol; (●), liposomes with 38 mol% cholesterol. Further details are described in Materials and Methods.

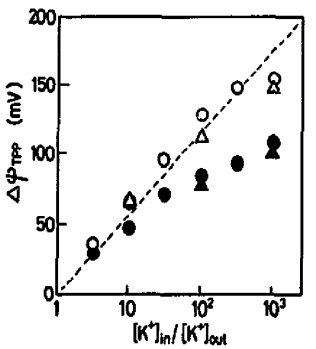

Fig. 3. The values of $\Delta\psi_{TPP}$ in PC liposomes containing 10 and 38 mol% cholesterol obtained at various K^+ gradients. Liposomes loaded with 150 mM KCl was suspended in isoosmotic external buffer containing KCl and NaCl in the desired proportions. The line represents the theoretical values of $\Delta\psi$ calculated from imposed K^+ gradients using the Nernst equation. Open symbols are the data for 10 mol% cholesterol liposomes obtained by flow dialysis (○) and obtained with a TPP^+-selective electrode (△). Closed symbols are the data for 38 mol% cholesterol liposomes obtained by flow dialysis (●) and obtained with a TPP^+-selective electrode (▲). Further details are described in Materials and Methods.

cholesterol at an imposed K^+ gradient of $[K^+]_{in}/[K^+]_{out} = 10^3$. The probe uptake by liposomes occurred after the addition of valinomycin and a stationary state was attained within 30 min. These probes were released from the liposomes by a subsequent addition of gramicidin in response to the collapse of $\Delta\psi$. Therefore, the accumulation of these probes into liposomes was $\Delta\psi$-dependent. Fig. 2 shows the observed $\Delta\psi_{Rb}$ in the liposomes containing 10 or 38 mol% cholesterol at various K^+ gradients; it was in good agreement with $\Delta\psi$ calculated from the initial K^+ gradient across the liposome membranes using the Nernst equation. In the 10 mol% cholesterol liposomes, $\Delta\psi_{TPP}$ was very close to the $\Delta\psi_{Rb}$, as shown in Fig. 3. However, $\Delta\psi_{TPP}$ in the 38 mol% cholesterol liposomes was considerably smaller than the $\Delta\psi_{Rb}$. The $\Delta\psi_{TPP}$ obtained with the TPP^+-selective electrode agreed well with the $\Delta\psi_{TPP}$ obtained by the flow dialysis, as shown in Fig. 3. Fig. 4 shows $\Delta\psi_{Rb}$, $\Delta\psi_{TPP}$ and $\Delta\psi_{TPMP}$ observed at the imposed K^+ gradient of $[K^+]_{in}/[K^+]_{out} = 10^3$ at various values of cholesterol content. For the liposomes containing cholesterol lower than 21 mol%, the values of $\Delta\psi_{Rb}$ and $\Delta\psi_{TPP}$ were comparable, while in the liposomes containing more than 30 mol% cholesterol $\Delta\psi_{TPP}$ decreased as the cholesterol content increased. When $TPMP^+$, which has been considered to be less membrane-permeable than TPP^+, was used as a probe, $\Delta\psi_{TPMP}$ obtained was smaller than $\Delta\psi_{TPP}$ in the liposomes containing cholesterol > 21 mol%.

The cholesterol in liposomes influenced not only $\Delta\psi_{TPP}$ ($\Delta\psi_{TPMP}$) at steady state but also the rate of uptake of lipophilic cations. The time-course of TPP^+ uptake by liposomes monitored

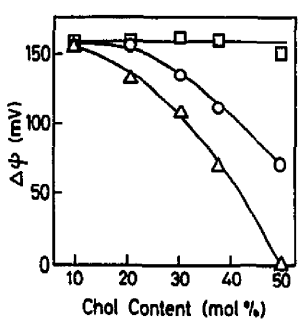

Fig. 4. The values of $\Delta\psi_{TPP}$ (○), $\Delta\psi_{TPMP}$ (△) and $\Delta\psi_{Rb}$ (□) in liposomes with various cholesterol content. All values of $\Delta\psi$ were obtained under gradient $[K]_{in}/[K]_{out} = 10^3$, $[K^+]_{in} = 150$ mM. Conditions were the same as in Fig. 1. The concentration of $TPMP^+$ was 6 μM. Each point represents a single experiment.

with a TPP^+ electrode showed that the TPP^+ uptake by liposomes containing 10 mol% cholesterol reached a stationary state within 1 min, whereas the maximum accumulation of TPP^+ in liposomes containing 38 mol% cholesterol took over 8 min under the same conditions. The initial rate of TPP^+ uptake by the former liposomes was about 3-times greater than that by the later liposomes. The influx of $TPMP^+$ into 38 mol% cholesterol liposomes occurred more slowly than that of TPP^+. On the other hand, after the addition of valinomycin, the fluorescence quenching of 3,3'-dipropylthiodicarbocyanine, a membrane-potential-sensitive fluorescent dye, occurred rapidly within 1 min in both liposomes (data not shown). This showed that $\Delta\psi$ reached a steady state within this time range. Thus, a large amount of cholesterol clearly retards the uptake of lipophilic cations, but does not affect the rate of $\Delta\psi$ generation in each of the liposomes. This retardation was more significant in $TPMP^+$ uptake than in TPP^+ uptake.

The effect of TPP^+ on $^{86}Rb^+$ uptake was examined in 38 mol% cholesterol liposomes, in order to clarify whether or not lipophilic cations would cause depolarization of the membrane potential. $\Delta\psi_{Rb}$ in the presence of nonradioactive TPP^+ (6–20 μM) agreed well with $\Delta\psi_{Rb}$ without TPP^+. Thus, the accumulation of lipophilic cations in the liposomes does not diminish the $\Delta\psi$ generated under these experimental conditions.

Movement of Cl^-, Na^+ and H^+

As one of the possible causes for the unexpectedly small $\Delta\psi_{TPP}$ and $\Delta\psi_{TPMP}$, one may point out that the presence of these probes increases the efflux of Cl^- from the liposomes or the influx of Na^+ or H^+ into the liposomes. First, we examined the efflux of Cl^- from $^{36}Cl^-$-loaded liposomes containing 38 mol% cholesterol. At the imposed K^+ gradient of $[K^+]_{in}/[K^+]_{out} = 10^3$, the efflux of $^{36}Cl^-$ was small and was not affected in the presence of 6 μM TPP^+ (data not shown). Further, we examined whether the values of $\Delta\psi_{TPP}$ in 38 mol% cholesterol liposomes would depend on anion species or not. Here, as counter anions for K^+ and Na^+, SO_4^{2-}, F^- and gluconate$^-$ were used, which are generally considered to be less permeable than Cl^-. The values of $\Delta\psi_{TPP}$, obtained at the imposed K^+ gradient of $[K^+]_{in}/[K^+]_{out} = 10^3$, were independent of these anion species; 110 mV for Cl^-, 119 mV for F^-, 104 mV for SO_4^{2-} and 114 mV for gluconate$^-$. Thus, it was concluded that the efflux of counter anions accompanying with TPP^+ uptake is not the cause of the unexpectedly small value of $\Delta\psi_{TPP}$.

Since the permeability of choline$^+$ in lipid bilayer is lower than that of Na^+, choline chloride was substituted for NaCl, but we obtained the same $\Delta\psi_{TPP}$ value as in the system using NaCl. Hence, Na^+ influx to the liposomes is also not the cause for the small value of $\Delta\psi_{TPP}$. When $\Delta\psi$ of 177 mV was generated, the $\Delta\psi$-driven proton influx into the 38 mol% cholesterol liposomes was monitored by the quenching of 9-AA fluorescence. The fluorescence intensity of 9-AA did not change after the addition of valinomycin, indicating that H^+ influx into liposomes was negligibly small, even when large $\Delta\psi$ was generated. However, the subsequent addition of the protonophore, FCCP, caused a significant decrease in the fluorescence intensity. This indicates that the acidification of intravesicular medium caused by $\Delta\psi$-driven H^+ influx into liposomes occurs only when protonophore is present. A similar result was obtained in the presence of 6 μM TPP^+. Thus, transmembrane H^+ permeability in the 38 mol% cholesterol liposomes was extremely low under large $\Delta\psi$, as was observed in the liposomes of PC/cholesterol = 9 : 1 (mole ratio) [8].

Effect of surface charge of liposomes on $\Delta\psi_{TPP}$

Binding of lipophilic cations to the membrane constituents of cells has been reported [4,7,15–17]. If this is the case with the liposomes used here, $\Delta\psi_{TPP}$ and $\Delta\psi_{TPMP}$ must be larger than $\Delta\psi_{Rb}$. In order to clarify the effect of probe binding to liposome membranes on $\Delta\psi$ estimation, we examined whether or not the surface charge of liposome membrane would affect the values of $\Delta\psi_{TPP}$. Here, PC/cholesterol/dicetylphosphate liposomes and PC/cholesterol/stearylamine liposomes were prepared, and their zeta potentials were measured as an index of surface charge. The former had more negative surface charge than the 10 mol% cholesterol liposomes and the latter had positive surface charge. Both the zeta potential and $\Delta\psi_{TPP}$ obtained are summarized in Table I.

TABLE I

THE EFFECTS OF CHOLESTEROL ON $\Delta\psi_{TPP}$, $\Delta\psi_{TPMP}$, THE STEADY-STATE FLUORESCENCE ANISOTROPY OF DPH (r_s), THE ORIENTATIONAL ORDER PARAMETER (S) OF LIPID BILAYER AND ZETA POTENTIAL OF LIPOSOMES

$\Delta\psi_{TPP}$ and $\Delta\psi_{TPMP}$ are the values of $\Delta\psi$ obtained by TPP^+- and $TPMP^+$-uptake under imposed K^+ gradient of $[K^+]_{in}/[K^+]_{out} = 10^3$ ($[K^+]_{in} = 150$ mM), respectively. Details are the same as in Fig. 1. The values in brackets are $\Delta\psi$ obtained after the addition of 60 nM TPB. All values are an average of triplicate experiments. The deviation of data values from the average did not exceed ±3 mV. DCP and SA are dicetyl phosphate and stearylamine, respectively.

Chol/(PC+Chol) (mol%)	$\Delta\psi_{TPP}$ (mV)	$\Delta\psi_{TPMP}$ (mV)	r_s [a]	S [b]	zeta potential [c] (mV)
10	153 (153)	156	0.119	0.380	−28
21	155	131	0.147	0.490	−22
30	135	108	0.178	0.585	−20
38	110 (128)	70	0.224	0.705	−19
50	71 (135)	≒0	0.258	0.781	
(mol ratio)					
PC/DCP/Chol = 5.0/0.50/1	141		0.158	0.527	−38
PC/SA/Chol = 4.3/0.55/1	150		0.148	0.494	31

[a] The steady-state fluorescence anisotropy of DPH in liposomes at 20°C. Each r_s value is an average from two independent measurements. The average error is within ±2.5%.

[b] The orientational order parameter of lipid bilayer obtained with DPH. The values of S were estimated by using equations described in Materials and Methods.

[c] The values of zeta potential were obtained under following conditions: 150 mM NaCl, 20 mM Hepes, (pH 7.4) 25°C.

The $\Delta\psi_{TPP}$ is little affected by the surface charge, indicating that the TPP^+ binding to the liposome membrane is not so significant as to affect the $\Delta\psi_{TPP}$.

Dependence of $\Delta\psi_{TPP}$ or $\Delta\psi_{TPMP}$ on PC acyl chain ordering in liposomes

These observations that the $\Delta\psi$ obtained with lipophilic cation probes depended both on the liposomal cholesterol content and on the inherent permeability of probes must be explained in terms of other factors than those discussed above. It is well known that the hydrocarbon chain ordering in phospholipid bilayers increases as the cholesterol content in the membrane increases. In order to examine the effect of hydrocarbon chain ordering on the probe distribution properties, we measured the fluorescence polarization of DPH in the liposomes and evaluated the orientational order parameter (S) of the lipid bilayer. The polarity of the environment of DPH was little changed by the presence of cholesterol, because the decay constants of DPH fluorescence were independent of the amount of cholesterol in liposomes (data not shown). TPP^+ (6 μM) did not affect the anisotropy and the decay constant of DPH fluorescence, indicating that the effects of TPP^+ on the physical state of the liposome membrane were negligibly small.

The value of S increased as the cholesterol content in liposomes increased, as shown in Table I. $\Delta\psi_{TPP}$ and $\Delta\psi_{TPMP}$ decreased with an increase in the S value. Thus, the suppressed permeation of TPP^+ and $TPMP^+$ into liposomes containing a large amount of cholesterol is attributable to the decrease of acyl chain mobility of PC in the liposomes. This idea is further supported by the results as of the cholesterol effect on the rate of probe uptake (see above).

Effect of TPB^- or TPP^+ or $TPMP^+$ or $TPMP^+$ uptake of liposomes

The above results indicate there is a possibility that an equilibrium distribution of TPP^+ or $TPMP^+$ will not be attained in liposomes of high cholesterol content, though the distribution of the probe appears to reach a stationary level from the profile of $\Delta\psi$-dependent uptake of TPP^+ (Fig. 1). To check this, the effect of tetraphenylboron (TPB^-) on the lipophilic cation uptake was ex-

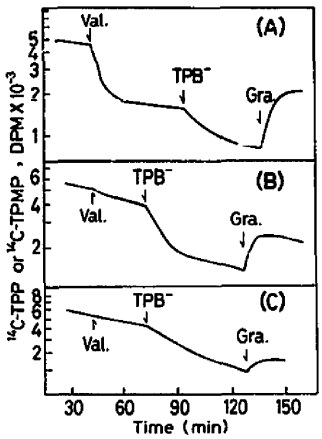

Fig. 5. The effect of TPB^- on $\Delta\psi$-dependent uptake of TPP^+ or $TPMP^+$ by liposomes with a large amount of cholesterol. Curves (A) and (B) are TPP^+-uptake profiles for liposomes with 38 and 50 mol% cholesterol, respectively. Curve (C) is a $TPMP^+$-uptake profile for liposomes with 50 mol% cholesterol. Arrows show the time when 7 μM valinomycin (Val.), 60 nM TPB^- and 3 μM gramycidin (Gra.) were added. Conditions were the same as in Fig. 1; $[K^+]_{in}/[K^+]_{out} = 10^3$, $[K^+]_{in} = 150$ mM.

amined, because TPB^- is known to enhance the value of $\Delta\psi$ when lipophilic cation is used as a $\Delta\psi$ probe [18].

An addition of a small amount of TPB^- (60 nM) caused further uptake of TPP^+ to a new stationary level in the 38 mol% cholesterol liposomes, as shown in Fig. 5A. In the 50 mol% cholesterol liposomes, the TPP^+ uptake after the addition of valinomycin was very small, but again a subsequent addition of TPB^- caused further significant uptake of TPP^+ to a new stationary level (see Fig. 5B). When $TPMP^+$ was used, we could not detect any uptake of the probe in the 50 mol% cholesterol liposomes until TPB^- was added to the system, as shown in Fig. 5C. In the 10 mol% cholesterol liposomes, such TPB^--induced enhancement of TPP^+ uptake could not be observed (data not shown). TPB^- (60 nM) did not alter the physical state of liposome membranes, since the fluorescence anisotropy and the decay constant of DPH were not affected by TPB^-. The above results indicate that the nonequilibrium distribution of lipophilic cations is caused by their restricted permeation across the highly ordered membrane.

The TPB^--induced additional uptake of TPP^+ (or $TPMP^+$) can be explained in terms of two different populations of the liposomes. TPP^+ and $TPMP^+$ permeate into the liposomes with low cholesterol content, but they permeate into those with very high cholesterol content only when TPB^- is added. The latter fraction becomes large with an increase in the amount of cholesterol present at the stage of liposome preparation. The different behavior of TPP^+ and $TPMP^+$ is ascribable to the difference in their membrane permeability or lipophilicity. Contrary to the behavior of lipophilic cations, the value of $\Delta\psi$ obtained by uptake of $^{86}Rb^+$, which was mediated by a cation carrier, valinomycin, was not affected by cholesterol. Since the TPB^--enhanced uptake of lipophilic cation was independent of the amount of TPB^- (60–600 nM), and even a small amount of TPB^- enhanced the uptake of TPP^+ or $TPMP^+$, the effect of TPB^- was due not to its charge but to its action as a carrier of TPP^+ or $TPMP^+$, as reported previously [17,19].

Concluding remarks

This study reveals that the abnormal distribution of the lipophilic cation probes for $\Delta\psi$ measurement is caused mainly by the extremely lowered permeability of probes due to highly ordered state of lipid membrane caused by the presence of a large amount of cholesterol. So far, it is generally accepted that the erroneous estimation of $\Delta\psi$ in cells may result from the binding of lipophilic cation probes to the membrane and/or to the intracellular constituents. However, our results with regard to the effect of liposomal surface charge on the uptake of TPP^+ or $TPMP^+$ show that the probe binding to the liposomes is negligibly small. The present study also denies the possibility that the smaller value of $\Delta\psi_{TPP}$ or $\Delta\psi_{TPMP}$ relative to that of $\Delta\psi_{Rb}$ is due to the enhanced permeabilities of Cl^-, H^+ and Na^+ in the presence of lipophilic cation probes. The fact that $\Delta\psi_{TPMP}$ was more significantly influenced than $\Delta\psi_{TPP}$ by the presence of cholesterol at the same K^+ gradient (see Fig. 4) supports the idea that the probe permeability depends on acyl chain ordering of phospholipids, as well as on the probe's inherent hydrophobicity (or permeability). Thus, the erro-

neous estimation of $\Delta\psi$ is caused by an inhibition of the probe permeation across the membrane due to its physicochemical structural change, that is, the increase in phospholipid acyl chain ordering. Since cell membranes from prokaryotes or eukaryotes are composed of a variety of lipids and contain a large amount of cholesterols, proteins and sugars, which obviously change the physicochemical states of their lipid membranes, special care must be taken in the measurement of $\Delta\psi$ in cells by using lipophilic cations.

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