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Transbilayer transport of a propyltrimethylammonium derivative of diphenylhexatriene (TMAP-DPH) in bovine blood platelets and adrenal chromaffin cells

Shuji Kitagawa, Eiichi Tachikawa and Takashi Kashimoto

Abstract

The membrane fluorescent probe *N*-((4-(6-phenyl-1,3,5-hexatrienyl)phenyl)propyl)trimethylammonium (TMAP-DPH) has an additional three-carbon spacer between the fluorophore and the trimethylammonium substituent of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH). As a basic study to clarify the transport mechanism of amphiphilic quaternary ammoniums, we observed the characteristics of the transbilayer transport of TMAP-DPH in bovine blood platelets and bovine adrenal chromaffin cells using the albumin extraction method. We compared these inward transport rates with those of TMA-DPH. TMAP-DPH crossed into the cytoplasmic layers of the membranes more slowly than TMA-DPH after rapid binding to the outer halves of the plasma membranes. The transport rate markedly depended on temperature. Time to reach the half-maximal incorporated amount of TMAP-DPH increased threefold accompanied by an increase in the concentration from 0.2 to 1.5 μM. The transport was stimulated significantly by various types of membrane perturbations such as modification of sulfhydryl-groups by *N*-ethylmaleimide and benzyl alcohol-induced increase in the fluidity of the lipid bilayer. The saturation phenomenon suggested the presence of the regulatory process in the transbilayer transport of TMAP-DPH.

Introduction

Diphenylhexatriene (DPH) and its derivatives such as the trimethylammonium derivative of diphenylhexatriene (TMA-DPH) have been used widely to monitor the fluidity of the membrane lipid bilayer by measuring their fluorescence polarization or fluorescence anisotropy. The fluidity at different depths in the lipid bilayer has been reported to depend on the location of the fluorophores (Trotter & Storch 1989; Kaiser & London 1998). These fluorophores differ also in their transbilayer and lateral localization in the membrane depending on their alkyl chain lengths and electronic charge (Kitagawa et al 1991, 1995; Beck et al 1993). Another cationic derivative of diphenylhexatriene, propyltrimethylammonium derivative of diphenylhexatriene (TMAP-DPH) (Figure 1), preferably partitions into the fluid phase of the lipid bilayer (Beck et al 1993). However, its transbilayer permeability in biological cell membranes is still unknown.

Amphiphilic quaternary ammonium fluorescence probes such as TMA-DPH first bind to the outer leaflet of the plasma membrane and then move to the cytoplasmic leaflet by a flip process (Bevers et al 1990; Kitagawa et al 1991, 1998). In a state of equilibrium they bind mainly to the inner membrane leaflet, which has a more negative surface charge. However, the mechanisms of the transbilayer transport of these probes as well as other amphiphilic quaternary ammoniums are unknown. Therefore, in this study we have observed the transbilayer transport of TMAP-DPH by the albumin extraction method as a basic study to clarify the mechanism of the transbilayer transport of amphiphilic quaternary ammoniums. For this purpose, we used bovine platelets and bovine adrenal chromaffin cells, because much is known about their membrane structures. We compared the inward transport rates of this probe with those of TMA-DPH to demonstrate the relationship between the structures of TMA-DPH analogues and their transbilayer transport rates. Overall fluidity in the membranes

Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, Kamishin'ei-cho 5-13-2, Niigata 950-2081, Japan

Shuji Kitagawa

Department of Pharmacology, School of Medicine, Iwate Medical University, Uchimaru 19-1, Morioka 020-8505, Japan

Eiichi Tachikawa, Takashi Kashimoto

Correspondence: S. Kitagawa, Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, Kamishin'ei-cho 5-13-2, Niigata 950-2081, Japan. E-mail: kitagawa@niigata-pharm.ac.jp

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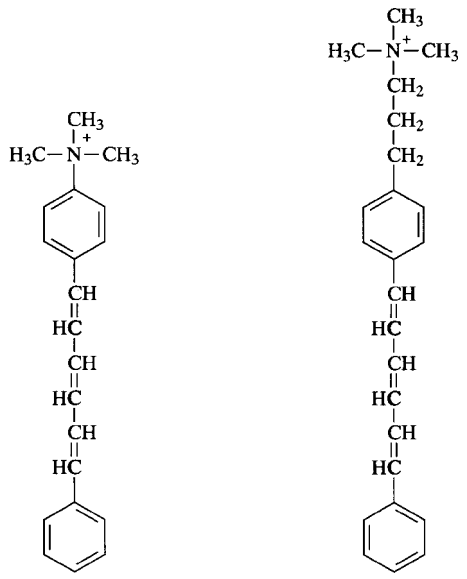

TMA-DPH TMAP-DPH

Figure 1 Chemical structure of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) and *N*-((4-(6-phenyl-1,3,5-hexatrienyl)phenyl)propyl)trimethylammonium (TMAP-DPH).

of these cells and possible coexisting domain structure of their lipid phase, which might affect both partitioning and permeability of the probes, will be changed by the temperature. Therefore, the transport rates were examined at 25°C and at 37°C.

We examined the effects of *N*-ethylmaleimide and benzyl alcohol on the transbilayer transport of TMAP-DPH, because these compounds have been reported to modify the transbilayer passage of aminophospholipids (Basse et al 1992; Haest et al 1997). The membrane-permeable sulfhydryl-reactive reagent *N*-ethylmaleimide is known to inhibit the transbilayer movement of aminophospholipids, which are transported by ATP-dependent aminophospholipid translocase (Ding et al 2000), similarly to other sulfhydryl reagents (Zachowski et al 1987; Shroit 1994). On the other hand, benzyl alcohol, which has been suggested to modify membrane transport possibly due to its effects on the fluidity of membrane lipid bilayer (Friedlander et al 1987; Kitagawa et al 1995), is known to stimulate the transbilayer flip-flop of aminophospholipids (Basse et al 1992). To compare the effects of these compounds on the transbilayer transport of TMAP-DPH with those on the transport of aminophospholipids, we examined their effects on transbilayer transport of the cationic probe in chromaffin cells.

Materials and Methods

Materials

p-Toluenesulfonate salts of *N*-((4-(6-phenyl-1,3,5-hexatrienylphenyl)propyl)trimethylammonium (TMAP-DPH) and 1-(4-trimethylammoniumphenyl)-6- phenyl-1,3,5-hexatriene (TMA-DPH) were purchased from Molecular Probes (Junction City, OR). 5-Doxylmethylstearate and bovine serum albumin (essentially fatty acid-free) were obtained from Sigma-Aldrich Japan Co. (Tokyo, Japan). Other reagents were all from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Preparation of suspensions of platelets and adrenal chromaffin cells

Platelet suspension was prepared as described by Kitagawa et al (1982). Platelet-rich plasma was obtained from fresh bovine (Holstein) blood with 10% by volume ACD anticoagulant solution (74.8 mM sodium citrate, 38.1 mM citric acid and 122 mM dextrose). The platelet-rich plasma was centrifuged at 1000 *g* for 10 min and the platelets were suspended in Na,K-Tris medium (in mM: 137 NaCl, 5.4 KCl, 11 dextrose, 25 Tris-HCl adjusted to pH 7.4). To prevent spontaneous platelet aggregation during preservation 129 mM citrate (adjusted to pH 7.4) was added to the suspension at a volume ratio of 1:9. The final platelet concentration was approximately 7×10^9 mL^{-1}. Adrenal chromaffin cells were isolated by collagen digestion using the method described by Tachikawa et al (1989). Cells were suspended in oxygenated Krebs-Ringer-HEPES (KRH) buffer (in mM: 125 NaCl, 4.8 KCl, 2.6 $CaCl_2$, 1.2 $MgSO_4$, 25 HEPES-NaOH, 5.6 glucose, pH 7.4) at a concentration of 3×10^6 cells mL^{-1}.

Measurement of binding and transbilayer transport of TMAP-DPH

Binding of TMAP-DPH to the platelets or chromaffin cells was quantified by measuring fluorescence intensity in the platelets or cells after centrifugation (Kitagawa et al 1998). After incubation of the platelet or cell suspension with 1.5 μM (or other concentration) TMAP-DPH at 37°C, 0.5-mL samples of the suspension were collected periodically and centrifuged at 1800 *g* for 2 min at 20°C. The precipitated platelets or cells were solubilized with 1.5% sodium dodecyl sulfate. The fluorescence intensity of TMAP-DPH in the solubilized sample was then measured using excitation and emission wavelengths of 363 nm and 432 nm, respectively, in an F-2500 spectrofluorometer (Hitachi Seisakusho, Tokyo, Japan) and the amounts of the bound probe were calculated.

The inward transbilayer transport of TMAP-DPH in platelets and chromaffin cells was quantified by measuring fluorescence intensity remaining after albumin extraction. The albumin extraction method has been used previously (Schrier et al 1992; Schwichtenhövel et al 1992) to evaluate the amounts of amphiphilic drugs and phospholipid analogues incorporated in the inner membrane leaflets. After incubation of the platelet or chromaffin cell suspension with 1.5 μM (or other concentration) TMAP-DPH at 37°C, 0.5-mL samples were collected periodically and transferred to 1 mL 3% albumin solution at 20°C (Kitagawa et al 1998). The samples were centrifuged as

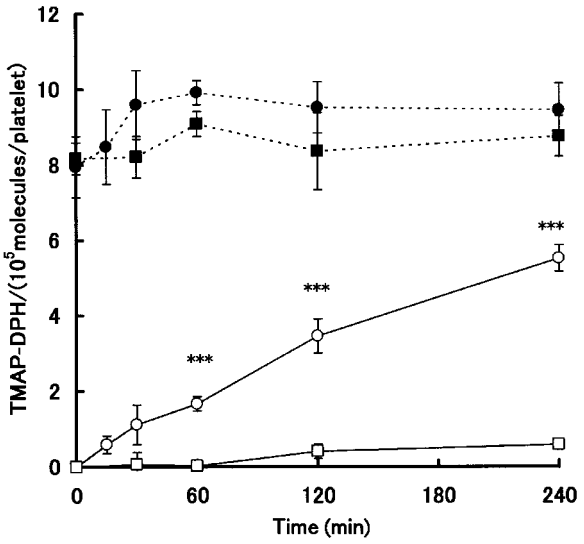

Figure 2 Time courses of total binding (closed symbols) and amount of TMAP-DPH incorporated into the cytoplasmic side (open symbols) of bovine blood platelets at 37°C (●, ○) and 25°C (■, □). Platelets were incubated with 1.5 μM TMAP-DPH. Data present are means±s.d. of three experiments. ***$P < 0.01$ compared with the value at 25 °C.

described above, and the precipitated platelets or cells were washed once more with 1% albumin at 20°C and then solubilized with 1.5% sodium dodecyl sulfate. Fluorescence intensity of TMAP-DPH in the solubilized samples was measured as described above and the amounts of the probes incorporated were calculated. Transbilayer transport of TMA-DPH was similarly observed. Time to reach the half-maximal amount, $t_{\frac{1}{2}max}$, of the probes incorporated into the cytoplasmic side was obtained as a measure of inward transbilayer transport rates. It was determined from time courses of changes in the amount of cationic probes incorporated into the cytoplasmic side (Figure 2).

N-Ethylmaleimide-treated chromaffin cells were prepared by incubating the cell suspension with 1 mM *N*-ethylmaleimide at 37°C for 10 min. The chromaffin cells were washed with KRH twice and suspended in KRH at the original concentration (3×10^6 cells mL^{-1}).

Measurement of apparent rotational correlation time of spin labelled chromaffin cells

Spin labelling of chromaffin cells with 5-doxylmethylstearate was carried out as described by Kitagawa et al (1990). Chromaffin cells treated with 1 mM *N*-ethylmaleimide as described above or those to which benzyl alcohol was added at various concentrations were incubated with 25 μM 5-doxylmethylstearate at 37°C for 2 min. The ESR spectra were measured at 37°C with TE-200 (X-band) spectrometer (JEOL, Tokyo, Japan) with 100 kHz field modulation frequency and 0.2 mT modulation amplitude at an output power of 8 mW. The apparent rotational correlation time was obtained from the relatively isotropic spectra by measuring the peak width of the central signal and peak lengths of the signals at central and lower magnetic fields (Kitagawa et al 1990).

Statistical analysis

The Mann–Whitney U-test was used to analyse the difference between sets of data. The level of significance was adjusted by Bonferroni's method. A *P*-value less than 0.05 was considered significant.

Results

Binding and transbilayer transport of TMAP-DPH in platelets and adrenal chromaffin cells

We examined binding and inward transbilayer transport of TMAP-DPH in platelets and chromaffin cells during incubation with the probe and compared them with those of TMA-DPH. The cationic probe bound to the platelets immediately after addition (Figure 2). However, the inner leaflet incorporated TMAP-DPH gradually increased at 37°C, and the increase continued over 240 min at 1.5 μM. A similar gradual inward transbilayer transport of TMAP-DPH was observed in chromaffin cells, although the transfer was slightly faster than that in the platelets (as shown in Table 1 as the time to reach the half-maximal incorporated amount, $t_{\frac{1}{2}max}$). The value of $t_{\frac{1}{2}max}$ of TMAP-DPH was approximately tenfold that of TMA-DPH in the platelets and approximately fivefold in the cells.

We examined the transbilayer transport of TMAP-DPH at 25°C in platelets to compare the results of TMA-DPH (Kitagawa et al 1991). The inward transport of TMAP-DPH was significantly dependent on the temperature. At 25°C, the rate of incorporation into the inner leaflet was much less (Figure 2). The incorporated TMAP-DPH after 240-min incubation was only approximately 11% compared with that at 37°C. Therefore, temperature dependency of the incorporation was more significant than that of TMA-DPH (approximately 37% after 60-min incubation (Kitagawa et al 1991)).

Dose-dependency of transbilayer transport of TMAP-DPH

To clarify the characteristics of the transbilayer transport of TMAP-DPH, we examined its transbilayer transport in bovine blood platelets at different concentrations. TMAP-DPH appeared on the cytoplasmic side more quickly at 0.5 μM than at 1.5 μM (Table 1). The transfer proceeded more rapidly at 0.2 μM. The value of $t_{\frac{1}{2}max}$ decreased by approximately one-third, accompanied by a decrease in the concentration from 1.5 to 0.2 μM. Therefore, at lower concentrations the cationic probe showed quicker transfer across the membrane, and vice versa.

Table 1 Time to reach the half-maximal amount, $t_{\frac{1}{2}max}$, of TMA-DPH and TMAP-DPH incorporated into the cytoplasmic sides of bovine blood platelets and bovine adrenal chromaffin cells at 37°C.

Probe	Concn (μM)	$t_{\frac{1}{2}max}$ min^{-1}		Maximal incorporated amount/(10^5 molecules/platelet) or (10^8 molecules/cell)	
		Platelets	Chromaffin cells	Platelets	Chromaffin cells
TMA-DPH	1.5	14±7	15±1	6.47±0.20	1.09±0.27
TMAP-DPH	0.2	43±4[c]	–	0.98±0.19	–
	0.5	66±8[c]	–	2.17±0.46	–
	1.5	136±35[a]	80±25[b]	5.95±0.30	0.96±0.13

Data are expressed as means±s.d. of triplicate samples from three experiments. [a]$P < 0.01$, [b]$P < 0.05$ compared with the values for TMA-DPH. [c]$P < 0.05$ compared with the value for 1.5 μM TMAP-DPH.

Effects of *N*-ethylmaleimide and benzyl alcohol on transbilayer transport of TMAP-DPH

The membrane transport of various substrates can be modified by various compounds such as sulfhydryl-reactive reagents and by benzyl alcohol-induced perturbation of the membrane lipid bilayer. To compare the effects of these compounds on the transbilayer transport of TMAP-DPH with those on the transport of aminophospholipids, we examined their effects on the transbilayer transport of TMAP-DPH in chromaffin cells.

Treatment of the cells with 1 mM *N*-ethylmaleimide markedly stimulated the transbilayer transport of TMAP-DPH, and equilibrium was reached at 60 min after addition of the probe at a concentration of 1.5 μM (Figure 3).

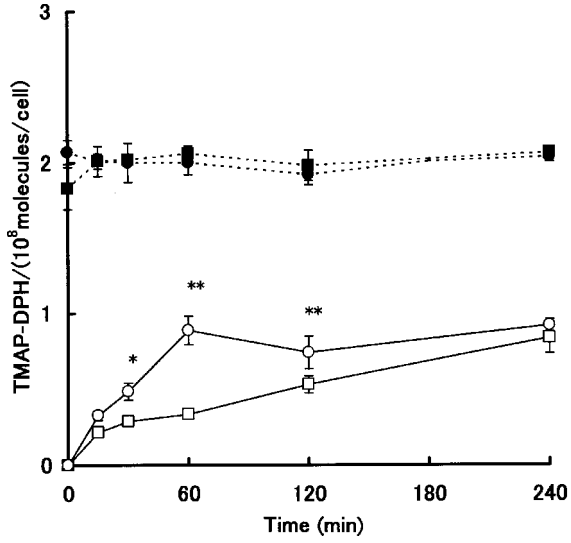

Figure 3 Time courses of total binding (closed symbols) and amount of TMAP-DPH incorporated into the cytoplasmic side (open symbols) of bovine adrenal chromaffin cells at 37°C by the treatment with 1 mM *N*-ethylmaleimide for 10 min (●, ○) or control (■, □). Cells were incubated with 1.5 μM TMAP-DPH. Data present are means±s.d. of three experiments. *$P < 0.05$, **$P < 0.01$ compared with control values.

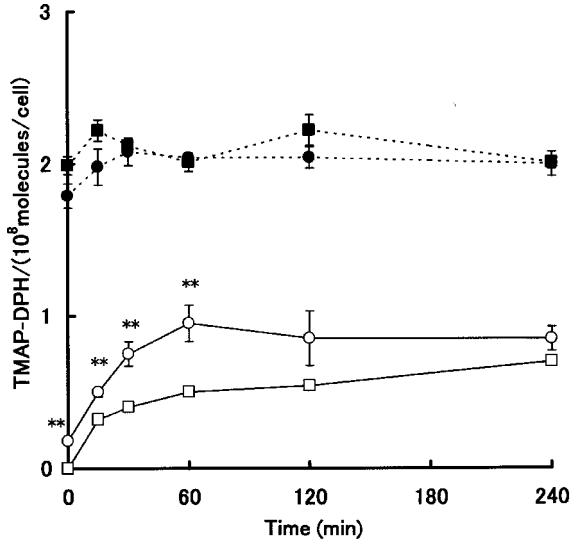

Figure 4 Time courses of total binding (closed symbols) and amount of TMAP-DPH incorporated into the cytoplasmic side (open symbols) of bovine adrenal chromaffin cells at 37°C in the presence of 50 mM benzyl alcohol (●, ○) or in its absence (■, □). Cells were incubated with 1.5 μM TMAP-DPH. Data present are means±s.d. of three experiments. **$P < 0.01$ compared with control value.

Likewise, benzyl alcohol stimulated the transport of TMAP-DPH (Figure 4). Significant increase in the fluidity of the lipid bilayer of the cells was observed by the addition of 50 mM benzyl alcohol, as indicated by the decrease in the apparent rotational correlation time of 5-doxylmethylstearate (Table 2). Treatment with 1 mM *N*-ethylmaleimide induced no significant changes in the fluidity.

Discussion

Amphiphilic quaternary ammoniums first bind to the outer leaflet of the plasma membrane and then gradually penetrate across to the inner leaflet by a flip process, distributing to both membrane leaflets but mainly to the side with a more negative surface charge. The changes in the

Table 2 Effects of benzyl alcohol and *N*-ethylmaleimide treatment on apparent rotational correlation time, τ_0, of 5-doxylmethylstearate-labelled chromaffin cells.

Compound	Concn (mM)	$\tau_0/10^{-9}$ s
Control		2.96±0.13 (7)
Benzyl alcohol	50	2.77±0.11 (8)*
N-Ethylmaleimide	1	3.00±0.12 (8)

Data are means±s.d. of three experiments. Numbers in parentheses indicate number of replicate experiments. *$P < 0.05$ compared with control value.

location of the amphiphilic quaternary ammoniums have been confirmed by the changes in the shape from echinocytes to stomatocytes (Sheetz & Singer 1976; Isomaa et al 1987). Similar shape changes in the erythrocytes were observed also by incubation with TMA-DPH or TMAP-DPH (data not shown). The inward transport rates seem to be different among the different quaternary ammoniums (Kitagawa & Takegaki 1992). As demonstrated in this study, the inward transport rate of TMAP-DPH, which has an additional three-carbon spacer between the fluorophore and the trimethylammonium substituent, is much slower than that of TMA-DPH. Therefore, the flip rates of the amphiphilic quaternary ammoniums seem to be influenced by the molecular size as well as the delocalization of the positive charge of trimethylammonium by the aromatic rings.

It has been suggested that there are flip sites for transbilayer inward movements of the amphiphilic compounds such as TMA-DPH (Bevers et al 1990), although the nature of the sites is unclear. The saturation phenomenon suggests the presence of a regulatory process in the transbilayer transport of TMAP-DPH. One possibility is that membrane proteins are involved in the transport process. However, there is another possibility. Binding of a relatively large quantity of TMAP-DPH to the flip sites may change the surface charge and surface potential around the sites, and thus inhibit the binding of other molecules to the sites by electrostatic repulsion.

The transbilayer transport of TMAP-DPH was stimulated by *N*-ethylmaleimide treatment and by the addition of benzyl alcohol. The stimulatory effect of *N*-ethylmaleimide was in contrast to the inhibitory effects of this reagent on aminophospholipid translocase reported previously (Zachowski et al 1987; Shroit 1994). Therefore, the transport mechanism seems to differ from that of the aminophospholipids. Since 1 mM *N*-ethylmaleimide did not alter the fluidity of the lipid bilayer in chromaffin cells, modification of the membrane protein structure by reaction with sulfhydryl groups may affect the transbilayer mobility of the cationic probe by reducing constraints against the formation of flip sites.

There is a possibility also that TMA-DPH analogues are internalized by endocytosis depending on cell types after binding to the plasma membrane (Illinger et al 1990). However, stimulation of the incorporation of TMAP-DPH by *N*-ethylmaleimide treatment at 37°C and marked differences between the transport rates of TMA-DPH and TMAP-DPH suggested that the contribution of endocytosis to the incorporation of TMAP-DPH was limited in the cells used in this study.

TMA-DPH and TMAP-DPH bind to the outer leaflet of the plasma membrane first. The slow transbilayer transport of TMAP-DPH demonstrated here would be favourable for further study to demonstrate the transport mechanism of amphiphilic quaternary ammoniums. Also, it would be favourable to observe the fluidity of the outer leaflet of the plasma membrane by measuring fluorescence anisotropy just after the addition of the probe.

Conclusions

TMAP-DPH slowly crossed into the cytoplasmic layers of platelet and adrenal chromaffin cell membranes. Times to reach the half-maximal incorporated amount were five- and ten-times longer than those of TMA-DPH. Transbilayer transport of TMAP-DPH showed a saturation phenomenon. It was enhanced by benzyl alcohol-induced perturbation of the membrane lipid bilayer and modification of sulfhydryl groups by *N*-ethylmaleimide. The saturation phenomenon suggested the presence of a regulatory process in the transbilayer transport of TMAP-DPH.

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