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Spectroscopic Studies of PhTX Facilitated Cation Movement Across Membranes

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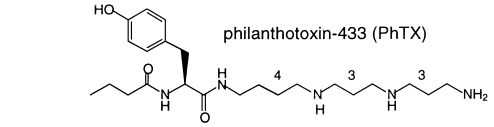
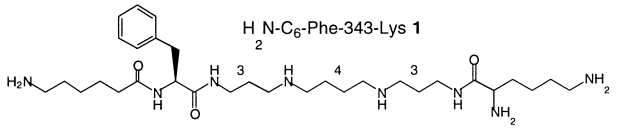
Abstract—Philanthotoxins, noncompetitive inhibitors of the nicotinic acetylcholine receptor and various glutamate receptors, were found to be capable of mediating cation transport across lipid bilayers. With respect to the relatively weak binding constants of these amphiphilic polyamines to neuronal receptor proteins, this finding implies that their interaction with cell membranes might have to be considered in addition to that with protein receptors to fully understand the molecular mechanism of these neurotoxins.

Introduction

The wasp venom neurotoxin philanthotoxin-433 (PhTX), a member of the polyamine amides family of neurotoxins isolated from the venoms of funnel-web spiders,[1–5] is used by wasps to paralyze victims by inhibiting ionic conductance through the nicotinic acetylcholine receptor (nAchR) and glutamate receptors (GluR); however, at low concentrations it exhibits agonist activity. The mechanism of agonist effect at low concentrations is not very well understood.





Many amphiphilic anaesthetics including chlorpromazine, procaine, tetracaine, etc. interact with nerve cell membranes[6–8] (and/or neuronal receptors[9]) to fulfill their functions. Thus it was conceivable that in addition to its direct interactions with receptors, the amphiphilic PhTX might also affect nAchR indirectly by interacting with cell membranes. We have performed studies on the interaction of PhTX with reconstituted vesicle membranes made from egg yolk phosphatidylcholine (PC) using NMR and fluorescence spectroscopy. Our results strongly imply that the mode of action of these neurotoxins could be more complex than previously anticipated because their interaction with cell membranes might also have to be considered in addition to and perhaps together with their extensively studied interaction with neuronal receptor proteins.

Results and Discussion

Sodium ion movement across the membranes was followed by ^{23}Na NMR spectroscopy according to the system designed by Lehn and co-workers.[10,11] Typically, aqueous LiCl buffer containing a polyanionic paramagnetic shift reagent (i.e., Dy(III) tripolyphosphate[12]) was prepared. Dilution of this buffer with aqueous NaCl buffer creates a sodium gradient across the membrane. Because of the bulk size of the shift reagent, it cannot cross the lipid bilayer. Therefore, sodium ions outside the vesicles will be upfield shifted compared to those inside; thus, two distinct peaks could be observed in ^{23}Na NMR corresponding to the sodium outside and inside the vesicles, the concentrations of which can be determined from integrations of respective NMR peaks (Fig. 1(a)). Rates of sodium influx can then be deduced from changes of these integrations after addition of the compound of interest. The collapse of the sodium gradient in the presence of PhTX, analogue **1**[13] and spermine was followed by ^{23}Na NMR (Figure 1(b)). With 120 μM and 80 μM of PhTX, equilibria of sodium across the membrane were reached over a period of 24 and 36 h, respectively, which were much longer than that of gramicidin, a well known ion channel former[14] (Figure 1(b)). Comparison between the rates of 120 μM and 80 μM solutions of PhTX implied that the apparent flux rate is first order to PhTX concentrations around 100 μM. Although PhTX analogue **1** does not inhibit

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GluR (unpublished results), it exhibited almost identical ionophoric activity to that of PhTX in conducting sodium ions, presumably due to its similar (bola) amphiphilic structural features. However, spermine showed no activity under the conditions employed. This demonstrates that the hydrophobic moiety of the PhTX molecule is essential presumably to provide an anchor for PhTX in the membrane.

After sodium equilibrium across the membrane was established over 24 h with 120 μM PhTX, as depicted in Figure 2, the same amount of fresh vesicles without PhTX was added, which caused the percentage of Na inside to drop to 50% due to the increased volume. However, the exchange was completed after another 24 h. This experiment demonstrates that like valinomycin, a known cation carrier, PhTX is able to equilibrate between vesicles and that the intramembrane structure formed is labile.

Fluorescence kinetics have been used in studies of ionophoric properties of many natural and unnatural compounds.[15] Usually, a pH-sensitive fluorescent dye (e.g. 5-carboxyfluorescein[16]) is trapped within the vesicles. After a pH gradient is introduced across the membrane, the dissipation of the gradient is followed by fluorescence in the presence of the compound studied. Because of the availability of pH-sensitive fluorescence dye and higher sensitivity of fluorescence compared to ^{23}Na NMR, the ionophoric property of PhTX was also studied using fluorescence kinetics. With 1 μM PhTX, a significant fluorescence decrease was observed over background[17] with an almost identical rate to that of 1 μM valinomycin (Fig. 3). With higher concentrations of PhTX, faster fluorescence decreases were observed, although the total amount of the fluorescence drop was about the same as with 1 μM PhTX (only curves resulting from 1, 10, and 80 μM PhTX are depicted in Fig. 3 for the sake of clarity). Almost identical curves with MeOH and 120 μM spermine compared to the background curve (Fig. 3, curve 1) proved the absence of solvent-induced disruption of membranes and the previously observed inactivity of spermine, respectively.

Although the correlation of transport mechanism and molecular structure is controversial and dependent on the experimental conditions,[18] two types of ion transporters are usually distinguished: channel formers such as gramicidin and ion carriers such as valinomycin. The dependence of ionophore-mediated ion transport on temperature has been useful for mechanistic studies for

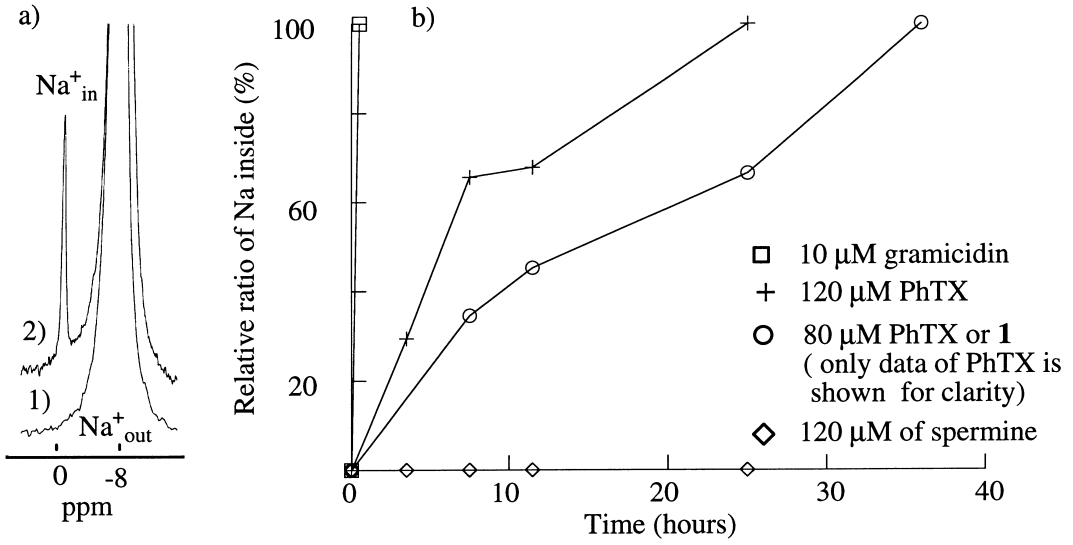

Figure 1. (a) ^{23}Na NMR spectra of PC vesicles with 120 μM PhTX: (1) immediately after addition of 120 μM PhTX; (2) 2 h afterwards; (b) influx of ^{23}Na ions into vesicles with gramicidin, spermine, and PhTX.

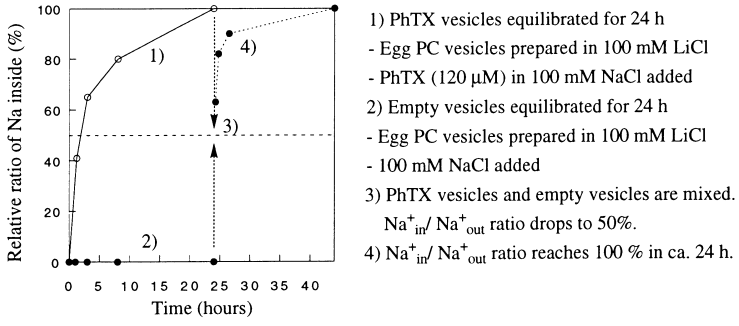

Figure 2. Intervesicular transfer of PhTX molecules.

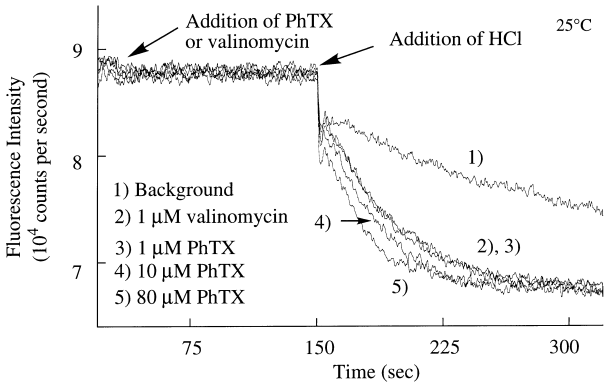

Figure 3. Fluorescence change with valinomycin and various concentrations of PhTX at 25 °C.

the following reasons:[19] since the lipid bilayer becomes less fluid at lower temperatures, the decrease in membrane fluidity should greatly retard the diffusion across the membrane. Thus, if PhTX acts as a channel, lower temperatures should not decrease the rate of ion exchange since transmembrane movement of the ionophore is not required. In contrast if it acts as a carrier, the rate of proton influx should be dropped greatly at lower temperatures. The rates of fluorescence drop decreased at 15 °C at variable concentrations even after initial incubation of PhTX with vesicles at room temperature (only the data of 10 μM PhTX is shown in Figure 4(a) for the sake of clarity), while at 4 °C the rates decreased more significantly. The same trend was observed with valinomycin (Fig. 4(b)), while in contrast the rate of proton transfer with gramicidin actually increased at 4 °C (Fig. 4(c)).

Although these results apparently imply the presence of an ion carrier mechanism, additional contributions from reduced spontaneous proton diffusion (Fig. 3, curve 1), and/or unfavorable binding of PhTX to PC bilayers at low temperatures, to the observed flux rates should not be underestimated. Since the binding of fully protonated PhTX[20] to cations is unlikely, it can be expected that the ion transport mechanism of PhTX is more complex than indicated by the experiments presented here. This concern is further supported by recent studies on the interaction of natural and synthetic polyamines with lipid bilayers which point toward the additional possibilities of either ion channel or pore formation, induced by membrane spanning polyamines[15,21,22] and electrostatic interactions of the polycations with the phosphate anions at the membrane surface,[23] respectively. Detailed studies focusing on these questions are clearly necessary.

Conclusions

The aforementioned results demonstrate the capacity of PhTX to mediate the transport of cations across lipid bilayers. Due to the weak binding between PhTX and the nAchR (K_d~50 μM),[13] and much larger cell membrane surface compared to the area occupied by receptors, these results imply that the interaction of PhTXs with cell membranes might have to be considered in

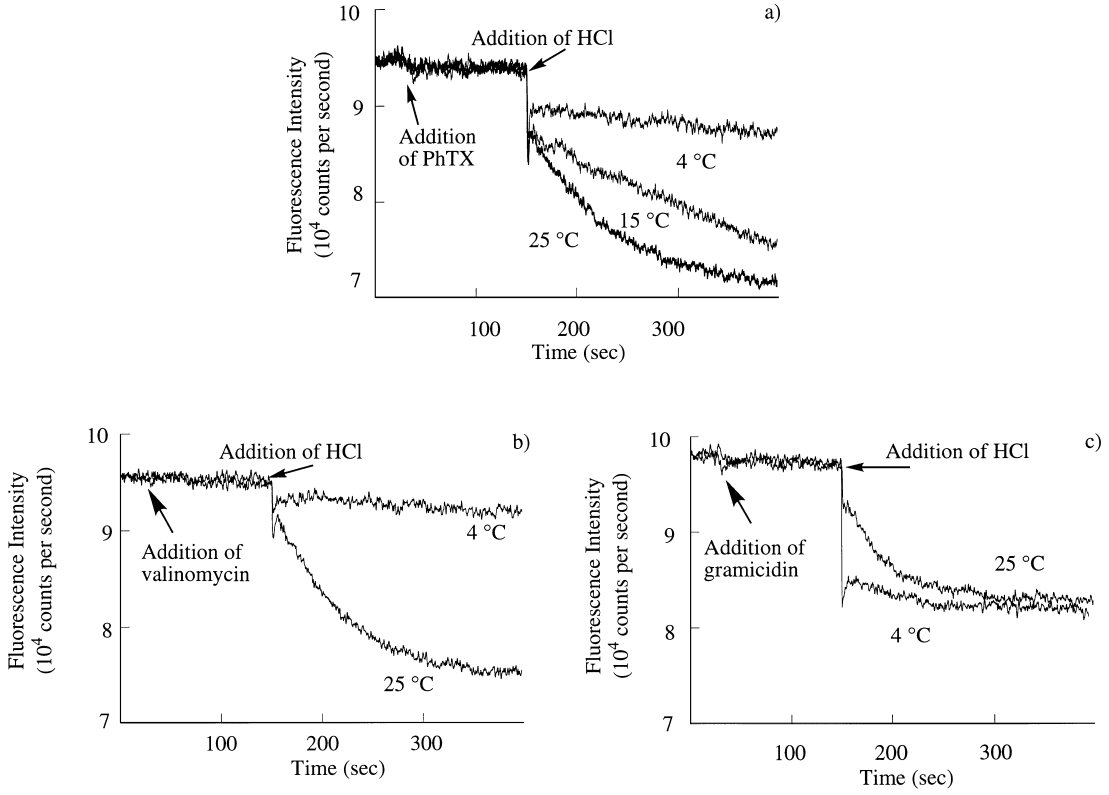

Figure 4. Fluorescence change with (a) PhTX (10 μM); (b) valinomycin (1 μM); (c) gramicidin (6 nM) at variable temperatures.

addition to that with protein receptors to fully understand the molecular mechanism of these neurotoxins. At this stage it is not possible to estimate the importance of either interaction with the membrane, or proteins, or both for neurotoxicity (and antibiotic activity (!)). The additional possibility that PhTX-induced structural changes of the lipid bilayer may account for the observed effects on structure and activity of nAchR and GluR receptors fully reveals the potential mechanistic complexity that is becoming comparable to that with amphiphilic chlorpromazine.[8,9] The observed agonist effect on receptors with low PhTX concentrations in particular may be accounted for by an indirect effect induced by nonspecific binding of the polyamine to the lipid bilayer. However, at higher concentrations, the PhTX molecules may enter the open channel and act as open-channel antagonists, thus blocking the ion flow. This could also explain why **1** is as active an ionophore as PhTX itself, but it does not inhibit ion conductance of GluR. Further studies are underway to determine how PhTX transports cations across lipid bilayers in vitro and in vivo, and how the binding of PhTX to membranes influences the structure of the lipid bilayer and membrane protein receptors as well.

Experimental

Vesicles for fluorescence studies were prepared following the procedures of Reynolds et al:[24] fresh egg yolk PC (30 mg), and octyl β-D-glucopyranoside (180 mg) were dissolved in chloroform/methanol (5 mL 2/1) and the solution was evaporated to a thin film by a rotatory evaporator. After complete removal of organic solvents by vacuum pump (0.2 Torr), the residue was dissolved in 5-carboxyfluorescein (5 μM), LiCl (100 mM), and pH 7.4 Tris (10 mM) aqueous buffer (2 mL). The resulting clear solution was then dialyzed against the same buffer (3×1 L) for 36 h followed by LiCl (100 mM) pH 7.4 Tris (10 mM) aqueous buffer (3×1 L) at room temperature for another 36 h. The lipid concentration of the resulting vesicle solution was estimated by measuring the phosphate content after digestion. Upon fluorescence measurement, vesicle solution (30 μL) was added to LiCl (100 mM) pH 7.4 Tris (10 mM) buffer (3 mL) followed by addition of methanol solution of PhTX, valinomycin or gramicidin (30 μL). After incubation for 2 min, HCl (0.09 M, 30 μL) was added with continuous stirring. Emission of the solutions was followed at 520 nm (excitation 490 nm). For ^{23}Na NMR measurements, vesicles were prepared as described above except without the addition of 5-carboxyfluorescein to the buffer solution. To the vesicle solution (200 μL) were added NaCl (100 mM) pH 7.4 Tris (10 mM) aqueous buffer (200 μL), shift reagent (100 μL, 10 mM $DyCl_3$, 30 mM $Na_5P_3O_{10}$, 10 mM Tris pH 7.4), and methanol solution of PhTX (30 μL). The ^{23}Na NMR spectra of the resulting solutions were then taken.

Acknowledgements

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