Effect of pH on the interaction of botulinum neurotoxins A, B and E with liposomes

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The interaction of botulinum neurotoxin serotypes A, B and E with membranes of different lipid compositions was examined by photolabelling with two photoreactive phosphatidylcholine analogues that monitor the polar region and the hydrophobic core of the lipid bilayer. At neutral pH the neurotoxins interacted both with the polar head groups and with fatty acid chains of phospholipids. At acidic pHs the neurotoxins underwent structural changes characterized by a more extensive interaction with lipids. Both the heavy and light chain subunits of the neurotoxins were involved in the process. The change in the nature and extent of toxin–lipid interaction occurred in the pH range 4–6 and was not influenced by the presence of polysialogangliosides. The present data are in agreement with the idea that botulinum neurotoxins enter into nerve cells from a low pH intracellular compartment.

INTRODUCTION

The neuromuscular disease botulism is caused by the most poisonous protein neurotoxin (NT) produced by the bacterium *Clostridium botulinum* (Sugiyama, 1980; Sakaguchi, 1983). Botulinum neurotoxins (BoNT) block acetylcholine release at the neuromuscular junction and thereby cause a flaccid paralysis (Habermann & Dreyer, 1986; Simpson, 1986). The seven BoNT serotypes share a similar macro structure and mechanism of action, but are distinguishable by their different immunological, biochemical and neuropathological properties. The three NT serotypes most frequently associated with human botulism are A (BoNT-A), B (BoNT-B) and E (BoNT-E) (Sugiyama, 1980; Sakaguchi, 1983).

As schematically depicted in Fig. 1(a), BoNTs are synthesized as a single chain (unnicked NT, 150 kDa) and are nicked by a proteolytic cleavage that generates a heavy chain (H, 100 kDa) and a light chain (L, 50 kDa); the subunit chains remain held together by interchain disulphide bridge(s) and non-covalent forces (nicked NT) (Habermann & Dreyer, 1986).

Experimental work and a supposed similarity with diphtheria toxin suggest that the cell intoxication by BoNT proceeds in three main steps: (a) binding, (b) uptake and (c) intracellular metabolic lesion that blocks neurotransmitter release (Simpson, 1986). Growing evidence suggests that the first step is due, at least in part, to the ability of BoNTs to bind polysialogangliosides of the G_{1b} series (Simpson & Rapport, 1971; Kitamura *et al.*, 1980; Agui *et al.*, 1983, 1985; Kozaki *et al.*, 1984; Bigalke *et al.*, 1986; Kamata *et al.*, 1986; Ochanda *et al.*, 1986). These gangliosides are highly concentrated in the nerve tissues (Wiegandt, 1985) and this helps to explain the neurospecificity of BoNT. Recent findings suggest that a protein is also involved in binding BoNTs to nerve cells (Agui *et al.*, 1983, 1985; Williams *et al.*, 1983; Evans

et al., 1986), and the existence of a double receptor formed by lipids and a membrane protein has been proposed (Montecucco, 1986, 1988a). Binding appears to be followed by a receptor-mediated endocytosis that brings the NT into closed vesicles within the nerve terminal (Dolly *et al.*, 1984; Black & Dolly, 1986). The effect of lysosomotropic substances (Simpson, 1982, 1983) and the ability of BoNT and of the N-terminal half of its heavy chain to form ion-conducting channels at low pH (Hoch *et al.*, 1985; Donovan & Middlebrook, 1986; Shone *et al.*, 1987; Blaustein *et al.*, 1987) promoted the idea of a low-pH-driven escape of the NT from an endosomal compartment, similar to that found with diphtheria toxin (Olsnes & Sandvig, 1985).

At low pHs, diphtheria toxin changes conformation with the exposure of hydrophobic surfaces (Blewitt *et al.*, 1985), and this enables it to interact with the hydrocarbon chains of lipids as shown by hydrophobic photolabelling (Montecucco *et al.*, 1985; Papini *et al.*, 1987). Similar studies were lacking for the BoNTs. We examined, by hydrophobic labelling with photoactive phosphatidylcholine analogues (Bisson & Montecucco, 1985, 1986; Montecucco, 1988b), the membrane interaction of NT as a function of pH to evaluate if, at low pH, NT becomes more hydrophobic and thus conducive to interaction with the hydrocarbon chains of phospholipids. BoNT-A, BoNT-B and BoNT-E were studied to ascertain whether conclusions drawn from one serotype are applicable for another serotype.

We used the two phospholipid derivatives depicted in Fig. 1(b). These lipid analogues are stable in the dark and can be interdispersed in trace amounts among the components of membranes. On illumination with long-wave u.v. light, the photoactive group is converted into highly reactive intermediates able to cross-link and hence label neighbouring molecules in the membrane (Bisson & Montecucco, 1985, 1986; Montecucco, 1988b). PC I

Abbreviations used: NT, neurotoxin; BoNT-A, BoNT-B and BoNT-E, botulinum neurotoxin serotypes A, B and E respectively; PC I, 1-palmitoyl-2-(2-azido-4-nitrobenzoyl)-sn-glycero-3-phospho[³H]choline; PC II, 1-myristoyl-2-{12-[(4-azido-2-nitrophenyl)amino]dodecanoyl}-sn-glycero-3-phospho[¹⁴C]choline.

Fig. 1. (a) Schematic structure of botulinum neurotoxins with the single and dichain forms; (b) structural formulae of the two phospholipid reagents used in the present study

H, heavy chain; L, light chain.

carries its photoactive nitroarylazido moiety near the phosphate group in order to probe the polar region of the membrane, while PC II bears its photosensitive group at the fatty acid methyl terminus, to label the protein surface buried in the hydrophobic core of the membrane. Several lines of evidence indicate that the two reagents indeed probe different regions of the lipid bilayer (Tomasi & Montecucco, 1981; Giraudat *et al.*, 1985; Bisson & Montecucco, 1985, 1986; Montecucco, 1988b), as expected from the different locations of their photoactive group. PC I is ^3H -labelled while PC II is ^{14}C -labelled, and hence they can be used at the same time on the same sample to provide information on the lipid interaction of a protein at different depths of the membrane under the same conditions.

EXPERIMENTAL

Neurotoxins

BoNT serotypes A, B and E were prepared as previously described (DasGupta & Rasmussen, 1983; DasGupta & Woody, 1984; DasGupta & Sathya-moorthy, 1984). BoNT-A was in the dichain form while BoNT-B and BoNT-E were isolated as single-chain molecules. The nicked NTs were prepared before each experiment by trypsin cleavage, as described previously (Sathya-moorthy & DasGupta, 1985), and the reaction was terminated by addition of a 4-fold excess of trypsin inhibitor.

Chemicals

Soya bean asolectin, after repeated acetone precipitation, had the following lipid weight composition: phosphatidylcholine, 24%; phosphatidylethanolamine, 39%; phosphatidylserine, 19%; phosphatidic acid, 6%; phosphatidylinositol, 1%; lyso derivatives, 9%; other phospholipids, 2%. Mixed brain gangliosides and purified bovine brain lipids were kind gifts from Dr. L. Poletti (Fidia Research Laboratories, Abano Terme, Italy). Bovine brain lipids had the following weight composition: gangliosides, 6%; phospholipids, 34%; cerebroside and sulphatides, 48%; cholesterol, 4%; other lipids, 8%; proteins were less than 0.2%. 1-Palmitoyl-2-(2-azido-4-nitrobenzoyl)-sn-glycero-

3-phospho[^3H]choline (PC I, specific activity 2.3 Ci/mmol) and 1-myristoyl-2-{12-[(4-azido-2-nitrophenyl)-amino]dodecanoyl}-sn-glycero-3-phospho[^{14}C]choline (PC II, specific activity 174 Ci/mol) were prepared as previously described (Bisson & Montecucco, 1981).

Incorporation of photoactive labels into liposomes

All operations until illumination were performed under a red light. Liposomes of asolectin, or asolectin plus gangliosides, or bovine brain lipids, plus or minus cholesterol, were prepared by mixing the lipids with PC I and PC II in chloroform/methanol (1:1, v/v) and drying under a stream of N_2 . Diethyl ether was added and the mixture was dried again under N_2 . The lipid/PC I or lipid/PC II molar ratios varied in different experiments in the ranges 675–3300 and 465–1420 respectively. The PC I/PC II ratio was varied in different experiments between 2.1 and 8.4. After removal of the organic solvents *in vacuo*, the lipid mixtures were resuspended either in 100 mM-Tris/acetate/0.5 mM-EDTA, pH 7.4 (Tris buffer) or in 125 mM- NaCl /10 mM-citric acid/10 mM- Na_2PO_4 , pH 7.4 (citrate/phosphate buffer) at the following concentrations: 1.12 mg of asolectin/ml, or 1.05 mg of asolectin plus 70 μg of gangliosides/ml, or 1.06 mg of bovine brain lipids/ml. The samples were ultrasonically dispersed in a bath-type sonicator (Laboratory Supplies, Hicksville, NJ, U.S.A.) until optical clarity was achieved.

Synaptosomes

Synaptosomes and synaptosomal membranes were purified from the cortex of bovine brains following the procedure of Jones & Matus (1970). PC I and PC II were incorporated into synaptosomes or synaptosomal membranes by following an established procedure (Giraudat *et al.*, 1985). Briefly, the phospholipid probes, dried at the bottom of a glass tube, were dispersed in 200 μl of citrate/phosphate buffer by ultrasonic irradiation. To this was added 250 μg of synaptosomes or synaptosomal membrane proteins [estimated according to Markwell *et al.* (1981), using bovine serum albumin as standard] in 400 μl of citrate/phosphate buffer. The vials were stoppered under N_2 and were

shaken overnight in a rotary shaker at 4 °C in the dark.

Photolabelling and radioactivity determinations

About 10 µg of NT was incubated with 400 µl of liposomes at room temperature for 5 min; in some samples the addition of the toxin was followed by that of buffered reduced glutathione to a final concentration of 5 mM. The mixture was incubated for additional 5 min after adjustment of its pH to the desired value by adding 2.5 % phosphoric acid if citrate/phosphate buffer was used or 25 % acetic acid in the case of Tris buffer. In some samples, the pH was returned to neutrality by adding the appropriate amount of 2 M-Tris. The samples were illuminated with a long-wave u.v. lamp (UVL-56 Ultraviolet Products, San Gabriel, CA, U.S.A.) for 10 min at room temperature with a glass water filter as described before (Montecucco, 1988b). The NT-liposome mixture was transferred to a plastic conical tube and trichloroacetic acid was added to a final concentration of 7 %. After 30 min, the protein was recovered by centrifugation and the pellets were dissolved in 8 % SDS/mM-Tris 50/1 mM-EDTA/3 % β-mercaptoethanol, pH 8. If needed, the pH was adjusted with 2 M-Tris-OH by following the change of colour of Bromophenol Blue. Samples were electrophoresed (Montecucco *et al.*, 1986) on 9 % polyacrylamide gels overlaid with a 1 cm 4 % polyacrylamide gel. The proteins separated on the gels were measured by scanning the Coomassie Blue-stained bands with a Shimadzu CS-930 dual-wavelength densit-

ometer. The gels were divided into 2 mm thick slices. Each of the slices was incubated overnight at room temperature with 300 µl of Packard Soluene 350. After addition of 4 ml of Packard Instagel, radioactivity was determined as d.p.m. in a Packard Tri-Carb 300C scintillation counter. In some experiments, radioactivity was measured by densitometric scanning of Kodak X-Omat films exposed at -80 °C to dried gels previously soaked for 30 min in Amplify (Amersham International, Amersham, Bucks., U.K.).

RESULTS

The Coomassie Blue-stained profiles of the three BoNTs are shown in the top panel of Fig. 2. Their high purity is attested by the presence of only two bands, the heavy and light chains. As documented before (DasGupta & Sugiyama, 1977), the heavy chain of BoNT-A migrates faster than those of BoNT-B and BoNT-E, while the light chain of BoNT-E is faster than those of BoNT-B and BoNT-A. Although these differences were interpreted before as measures of actual sizes of the polypeptides, they could be related to the different amounts of SDS bound by the heavy and light chains of the same size. The latter suggestion is consistent with the known variations in the amino acid compositions and sequences of the three BoNTs (Sathiamoorthy & DasGupta, 1985).

Fig. 2 also shows the photolabelling patterns of the three BoNTs with the surface probe PC I (panels *a*, *b* and

Fig. 2. Hydrophobic photolabelling of botulinum neurotoxins with asolectin vesicles at different pHs

The top panel shows the Coomassie Blue-stained gels of the botulinum neurotoxins used here. Panels (*a*), (*b*) and (*c*) report the patterns of labelling of BoNT-A, BoNT-B and BoNT-E respectively with the superficial probe PC I at neutral pH (○) and at pH 4.5 (●) after incubation with asolectin vesicles and illumination. Panels (*d*), (*e*) and (*f*) show the corresponding patterns of labelling with the deeper reagent PC II obtained under the same conditions.

Fig. 3. pH dependence of hydrophobic labelling of botulinum neurotoxins in the presence of asolectin liposomes

Panels (a), (b) and (c) show the variation as a function of pH of the amount of PC I radioactivity bound to the heavy chain (○) and to the light chain (□) of BoNT-A, BoNT-B and BoNT-E respectively; panels (d), (e) and (f) report the corresponding diagrams obtained with PC II. In order to compare different experiments where different amounts of probes and various lipid/protein ratios were used, radioactivity is expressed as percentage of the maximal labelling obtained in each set of experiments. Points are the average of three to five experiments, and bars represent standard deviations.

c respectively for serotypes A, B and E) and with the deeper probe PC II (panels *d*, *e* and *f* respectively for serotypes A, B and E) at neutral and acidic pHs. It appears that at neutral pH all NTs interact with the asolectin liposomes and that both the heavy and light chains are involved in the lipid interaction. It also appears that the NTs are present both at the polar head group region monitored by PC I and at a deeper level probed by PC II, except for BoNT-E, whose PC II labelling is negligible. It is highly unlikely that either PC I or PC II labelled the toxin from the water phase because we have never in previous studies found evidence for such an occurrence (Bisson & Montecucco, 1985, 1986; Montecucco, 1988*b*). Furthermore, the same result was obtained when the experiment was performed in Tris buffer containing glutathione; in such buffer any aryl azide photogenerated intermediate present in the water phase is expected to be immediately quenched by two powerful nucleophiles such as a substituted amine and a thiol group. On the other hand, a citrate/phosphate buffer was used in most experiments because it is closer to a physiological solution and because such buffer has an almost linear response to the addition of acid in the pH range studied here.

At pH 4.5 both chains of the NTs were extensively labelled by both the surface and deeper probes. Average yields of toxin labelling, expressed as percentage of label

consumed, were: for BoNT-A, 0.39 % with PC I and 0.73 % with PC II; for BoNT-B, 0.1 % with PC I and 0.35 % with PC II; and for BoNT-E, 0.16 % with PC I and 0.35 % with PC II. Within experimental error, identical increases in labelling when going from neutral to acidic solutions were observed in Tris/glutathione buffers. Such low yields of cross-linking are typical of hydrophobic photolabelling and are comparable to those obtained with a range of integral membrane proteins. This is in agreement with the possibility that at low pH BoNTs insert into the lipid bilayer and become true integral components of the membrane.

The presence of polysialogangliosides in the asolectin liposomes caused an increase in the amount of radioactivity bound to the heavy chain after illumination. This ranged from 10 % to 50 % among the three BoNTs, with no change for the light chain (results not shown). This result is in line with previous evidence that the toxin-ganglioside interaction is mediated by the heavy chain. However, the low extent of the additional labelling was unexpected on the basis of the suggested role of polysialogangliosides as receptors for these neurotoxins. (Montecucco *et al.*, 1988).

Fig. 3 shows the pH dependence of the labelling of the subunit chains of BoNT-A, BoNT-B and BoNT-E measured after incubation and illumination of the toxins in the presence of asolectin vesicles tagged with PC I

Fig. 4. pH dependence of PC I/PC II labelling ratio

The change in the $^3\text{H}/^{14}\text{C}$ labelling ratio of the heavy chain (○) and of the light chain (□) of BoNT-A, BoNT-B and BoNT-E in the presence of asolectin vesicles as a function of pH is shown in panels (a), (b) and (c) respectively. Ratios are expressed as percentage of the PC I/PC II ratio present in the liposome preparation used in each experiment. Points are the average of three or more experiments, and bars are standard deviations.

Fig. 5. pH dependence of photolabelling with PC II of botulinum neurotoxins in the presence of liposomes of bovine brain lipids

○, Heavy chain; □, light chain; panels (a), (b) and (c) show the profiles obtained with BoNT-A, BoNT-B and BoNT-E respectively. See legend of Fig. 3 for calculations of percentages.

(panels *a*, *b* and *c*) and with PC II (panels *d*, *e* and *f*) at different pHs. The same increases in labelling when going from neutral to acidic solutions were observed in Tris/glutathione buffers. The large increase in the interaction of the subunit polypeptides with phospholipids at the surface and deeper level suggests that these NTs at acidic pHs undergo a structural change, with the exposure to phospholipids of previously hidden hydrophobic surfaces. Within this general feature, the three NTs tested differed noticeably in their lipid interaction as function of pH. For example, at neutral pH, while BoNT-E had a nominal interaction with phospholipids only at the level of the glycerol moiety (panel *c* versus *f*), BoNT-B showed a larger interaction and BoNT-A had an intermediate hydrophobic interaction with phospholipids. Also, some variation in the pH transitions seems to exist among the different NTs and among their heavy and light chains.

The PC I/PC II ratio of labelling, measured as the $^3\text{H}/^{14}\text{C}$ ratio of toxin-bound radioactivity after illumination with respect to the starting ratio present in liposomes, changes with pH, and its pH dependence for

the three BoNTs is shown in Fig. 4. These profiles are similar for the three NTs tested and all have a minimum around pH 5. This result cannot be ascribed to a different reactivity of the photogenerated intermediates of PC I and PC II, because Fig. 4 does not report absolute values, it only shows the change with pH of a ratio. The most likely explanation is that the NTs expose amino acid residues of different reactivity at the two levels monitored by PC I and PC II as pH is lowered. These findings also indicate that the increased labelling of NT at acidic pHs is related to a conformational change of the NT, characterized by a more extensive hydrophobic interaction with phospholipids, rather than in a larger number of toxin molecules interacting with lipids.

At all pHs, the $^3\text{H}/^{14}\text{C}$ ratio of protein-bound radioactivity was lower than the one present in the lipid vesicles. Most probably, this is due to the more effective quenching of the photogenerated intermediates by water and buffer components at the surface than in the hydrophobic core of the membrane (Bisson & Montecucco, 1985; Montecucco, 1988a).

The interaction of BoNTs with liposomes depends on

the lipid composition of the membrane, as shown by the increased labelling of the heavy chain caused by polysialogangliosides. Fig. 5 shows the different picture found when BoNTs were illuminated in the presence of vesicles of purified bovine brain lipids. Both subunit chains had extensive hydrophobic interaction with phospholipids already at neutral pH (compare with panels *d*, *e* and *f* in Fig. 3). As before, BoNT-B displayed a greater lipid interaction than BoNT-E. This result does not appear to be related to a different fluidity of the bovine brain lipids with respect to that of the asolectin lipid mixture, because the presence of cholesterol (in the same amount as in the bovine brain cortex) did not change the result (not shown). As with asolectin vesicles, also with bovine brain lipid vesicles: acidic pHs induce a more extensive hydrophobic interaction of all NTs tested as shown by the increase in protein-bound radioactivity of both PC I and PC II.

The lipid interaction of BoNT-A was explored, in preliminary experiments, with a membrane preparation as close as possible to that of the nerve cell. BoNT-A was incubated and illuminated in the presence of synaptosomal membranes purified from rat brain cortex synaptosomes tagged with PC II. Under these conditions, the integral proteins of synaptosomal membranes were labelled and their presence on the gel (SDS/polyacrylamide-gel electrophoresis) did not allow for a quantitative estimation of the labelling of the NT subunits. However, it appears that BoNT-A is labelled at neutral pH and that labelling increases at acidic pHs similarly to what is found with bovine brain lipid vesicles (results not shown).

DISCUSSION

Lipids are thought to be involved in the adhesion of BoNTs to the nerve cell surface (Simpson, 1986; Habermann & Dreyer, 1986). Binding is followed by endocytosis, and the toxin appears inside the nerve endings into smooth vesicles (Simpson, 1982, 1983; Dolly *et al.*, 1984; Black & Dolly, 1986). The internal pH of endosomes drops to values as low as 5 (Mellman *et al.*, 1986) and it has been suggested that a transmembrane pH gradient is required for the translocation of BoNT into the cytoplasm (Simpson, 1986). Studies with intact cells, similar to those performed with diphtheria toxin (Olsnes & Sandvig, 1985), are not feasible at the present time with clostridial NTs because a suitable cell line is not available where the action of NTs can be monitored at the biochemical level. Hence in the present study we have employed different model membranes to monitor the lipid interaction of BoNT.

At neutral pH BoNT-A and BoNT-B, and to a lesser extent BoNT-E, interact with asolectin liposomes via both their heavy and light chains. The heavy chain was labelled more strongly than the light chain, at variance with what was found with tetanus NT (Montecucco *et al.*, 1986). However, amounts of labelling cannot be related to proportional extents of protein surface exposed to lipids because the amount of radioactivity incorporated in the protein after illumination depends on the reactivity of the amino acid residues exposed to the photoactive group (Bisson & Montecucco, 1985). Given this intrinsic limitation of the method, the results suggest that BoNT-B has more extensive hydrophobic interaction with the lipid bilayer at neutral pH than BoNT-A and BoNT-E. This conclusion is based on the assumption that PC I

does not label water-exposed protein regions and that PC II does not fold back, thus bringing its photoactive group to the membrane surface. We have shown that PC I does indeed behave as a phospholipid and does not label from the water phase (Bisson *et al.*, 1979, 1982; Montecucco *et al.*, 1980, 1983, 1985; Hoppe *et al.*, 1983). Moreover, in several cases we could also show that the fatty acid chain of PC II does not appreciably kink to the membrane surface (Montecucco *et al.*, 1980, 1983; Tomasi & Montecucco, 1981; Giraudat *et al.*, 1985; Ribi *et al.*, 1988). In addition, superimposable results were obtained here when water-soluble quenchers of the photogenerated intermediates were used.

In the presence of synaptosomal membranes and of vesicles of bovine brain lipids, at neutral pH, all BoNTs tested here are labelled more by PC II than in the presence of asolectin. This hydrophobic interaction of BoNTs, whose extent depends on lipid composition, deserves further comment. It indicates that NTs at neutral pH are adsorbed on the liposome surface and that either they insert a portion of their polypeptide chain into the hydrophobic part of the lipid bilayer or they induce a local rearrangement of the bilayer architecture with the induction of kinks in the hydrocarbon chains of phospholipids. We cannot at the present time discriminate between these two possibilities.

With all membranes tested here, as pH is lowered, BoNTs appear to undergo a structural change, involving both subunit chains, with the exposure of hydrophobic surfaces responsible for the clearly enhanced hydrophobic interaction with phospholipids. This is suggested not only by the large increase in labelling at acidic pHs, but also by the change of the PC I/PC II labelling ratio. The latter finding is a clear indication that different, and not only more, residues of NT are exposed to the photoactive groups at low pH.

That a low-pH-driven structural change is at the basis of the variation of lipid interaction is also suggested by the pH dependence of the labelling that comprises the range of pK_a of the imidazole and carboxylic groups of proteins (Tanford, 1962). This can be taken as an indication that the protonation of histidyl, aspartyl and glutamyl residues, whose pK_a values fall in the pH range where the transition occurs, is involved (Tanford, 1962). Fluorescence studies, similar to those performed with diphtheria toxin (Blewitt *et al.*, 1985), can be performed to test this hypothesis.

The change in toxin-lipid interaction, as monitored by hydrophobic photolabelling, occurs in a pH range closely similar to those found for the channel-forming activity of BoNT-A and of its heavy chain *N*-terminal part, and it overlaps the range measured in endosomes of living cells. Thus the present data are in agreement with the idea that BoNT could reach the cytoplasm of nerve cells from a low pH endosomal compartment.

Hoch *et al.* (1985) have suggested that a channel formed by heavy chains at low pH could allow the membrane translocation of the unfolded light chain. According to this 'tunnel' model, the light chain during its passage across the membrane is protected from contact with lipids by the heavy chain. In view of the phospholipid labelling of the light chain of BoNT reported here, renewed thinking in this matter is warranted. The present results agree with a model where both chains of the NT enter into contact with lipids, possibly in an aggregated form as recently suggested for BoNT serotype C and for

diphtheria toxin (Donovan & Middlebrook, 1986; Papini *et al.*, 1987, 1988). The heavy chains could form a hydrophilic cleft that nests hydrophilic groups of the light chain during its passage in the hydrophobic phase, thus reducing the energetic cost of its membrane translocation. After the release of the light chain, the hydrophilic cleft is expected to reduce its size to minimize the interaction with the fatty acid chain of phospholipids, and a proper alignment of hydrophilic residues can give rise to an ion channel. In this 'cleft' model, the formation of an ion channel is a consequence of the translocation process rather than being functional to the process itself as in the 'tunnel' model.

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