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Identification of an ion channel-forming motif in the primary structure of tetanus and botulinum neurotoxins

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Synthetic peptides with amino acid sequences corresponding to predicted transmembrane segments of tetanus toxin were used as probes to identify a channel-forming motif. A peptide denoted TeTx II, with sequence GVVLLLEYIPEITLPVIAALSIA, forms cation-selective channels when reconstituted in planar lipid bilayers. The single channel conductance in 0.5 M NaCl or KCl is 28 ± 3 and 24 ± 2 pS, respectively. In contrast, a peptide with sequence NFIGALETTGVVLLLEYIPEIT, denoted as TeTx I, or a peptide with the same amino acid composition as TeTx II but with a randomized sequence, do not form channels. Conformational energy calculations show that a bundle of four amphipathic α-helices is a plausible structural motif underlying observable pore properties. The identified functional module may account for the channel-forming activity of both tetanus toxin and the homologous botulinum toxin A.

Tetanus toxin; Botulinum toxin; Ionic channel; Protein design; Lipid bilayer; Signal transduction

1. INTRODUCTION

Tetanus toxin (TeTx) and botulinum neurotoxin serotype A (BoTxA) secreted by *Clostridium tetani* and *Clostridium botulinum*, respectively, are two of the most toxic proteins known to humankind: only ~100 pg of pure toxin constitutes one lethal dose [1–3]. Although it has been known for many years that these neurotoxins affect synaptic transmission in nervous tissues, their mode of action at the molecular level remains elusive. TeTx is the agent responsible for the spasticity and convulsions characteristic of human tetanus [1–3], and is more potent in the central nervous system where it blocks the release of the inhibitory neurotransmitters γ-aminobutyric acid and glycine [3]. BoTxA blocks acetylcholine release in peripheral nervous system synapses [1,2] and produces paralysis.

These toxins are structurally homologous [4–8]. Both are aqueous soluble proteins with an apparent M_r = 150,000. They are formed from two disulfide-linked chains: the light chain with M_r = 50,000 and the heavy chain, M_r = 100,000. Proteolytic cleavage produces three distinct fragments [4], A, B and C. A is the light chain, B is a proteolytic fragment of the heavy chain, the N-terminal half with M_r = 50,000 known as β2 and H2 for TeTx and BoTxA, respectively, and C is the C-terminal half of the heavy chain with M_r = 50,000, known as fragment C and H1 for TeTx and BoTxA, respectively [4,9].

The tripartite modularity of the toxins is consistent with the hypothesis of Simpson [1,2] of a three step mechanism for intoxication. Receptor binding (step 1) is considered localized to fragment C, as evidenced by specific binding to gangliosides and by competitive block of the neurotoxic effect of intact toxin [1,4,10–13]. The ganglioside toxin complex is internalized and sequestered into an endocytic vesicle [1,2,11,12,14]. This process (step 2) requires fragment B. The low pH in the vesicle activates insertion of the toxin into the vesicle membrane [1,2,9,12,14,15]. Fragment B contains the channel forming domain, as evidenced by single channel and macroscopic current recordings obtained from lipid bilayers exposed to either intact BoTxA or TeTx toxins or to the TeTx B fragment under conditions that mimic the pH gradient across endocytic vesicles [16–23]. Blockade of transmitter release (step 3) is a property of the light chain, as demonstrated by intracellular injection of isolated light chain [9,15,24–26], by exposure of permeabilized chromaffin granules or PC 12 cells to light chain [21,27–30], or by intracellular delivery of light chain entrapped inside liposomes after fusion with mammalian motor nerve endings [31].

Of the three functional motifs, only the channel-forming activity contained within fragment B [18–20,22,23], has been characterized. A bundle of amphipathic α-helices could form the pore structure, akin to the proposed motif for the pore of ligand- and voltage-gated

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Abbreviations: TeTx, tetanus toxin; BoTxA, botulinum neurotoxin serotype A; PC, 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine; τ_o, open times; τ_c, closed times.

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channels [32–38], and consistent with the structure of the channel-forming domain of diphtheria toxin determined at 2.5 Å; resolution [39]. Here we report the identification of a plausible pore forming motif in the primary structure of BoTxA and TeTx heavy chain. The strategy involves sequence analysis and molecular modeling to evaluate proposed motifs, followed by synthesis of peptides with sequences representing the identified motifs, and characterization of single channel properties after reconstitution in lipid bilayers. Detailed comparisons with channel properties of the authentic protein toxin establish the fidelity of the design and the suitability of the assay.

2. MATERIALS AND METHODS

2.1. *Conformational energy computations*

Molecular modeling, including energy minimization and molecular dynamics simulations, were conducted on a Silicon Graphics IRIS 4D/21 GTX workstation using the INSIGHT and DISCOVER molecular modeling packages of Biosym (San Diego, CA) [33,38]. Low-energy arrangements of α-helices and four-helix bundles are calculated with semi-empirical potential energy functions and optimization routines, and further refined. Constraints are used for symmetry and to maintain regular helical backbone dihedral angles of $\phi = -45$ and $\psi = -60$ [33].

2.2. *Peptide and protein synthesis, purification and characterization*

Monomeric peptides were synthesized by solid-phase methods, purified by HPLC, and characterized by amino acid analysis, microsequencing, capillary zone electrophoresis and SDS gel electrophoresis [34].

2.3. *Synthetic channel peptides assayed by single channel recordings in lipid bilayers*

Lipid bilayers were assembled by apposition of two monolayers, initially formed at the air–water interface, at the tip of patch pipets [34,40]. Peptides were incorporated into phosphatidylcholine lipid bilayers 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (Avanti Biochemicals, Alabaster, AL) as described [34,40]. Acquisition and analysis of single channel currents were as described [34,40]. Conductance and lifetime values were calculated from recordings with ≥ 300 openings; openings with $\tau_o \leq 0.3$ ms were ignored. Values reported as mean ± S.E.M. where *n* indicates the number of experiments. Bilayer experiments were performed at 24 ± 2°C.

3. RESULTS AND DISCUSSION

3.1. *Sequence analysis*

Alignment of deduced amino acid sequences of TeTx and BoTxA show that the two sequences are highly homologous, with numerous regions of predicted secondary structure. However, only one region is predicted by hydropathy analysis to be a transmembrane domain. This segment encompasses amino acids 659–690 for TeTx and 650–681 for BoTxA. Hydropathic power spectral analysis confirms the assignment and indicates that the structure is an amphipathic α-helix [41,42].

The length of this segment exceeds that necessary to span the bilayer width (32 Å). Accordingly, transmembrane structures may be inferred from calculated low energy conformers. Two segments, I and II, were identified: I extends from amino acid 659 to 680 for TeTx and from 650 to 671 for BoTxA; II extends from residue 668 to 690 for TeTx and from 659 to 681 for BoTxA. These segments are plausible candidates for the pore-forming structure of TeTx and BoTxA. The aligned sequences are:

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TeTx:  I   NFIGALETTGVVLLLEYIPEIT
BoTxA: I   DFVGALIFEGAVILLEFIPEIA

TeTx:  II  GVVLLLEYIPEITLPVIAALSIA
BoTxA: II  GAVILLEFIPEIAIPVLGTFALV
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Due to their low hydrophobicity, neither peptide would be predicted to span the hydrophobic interior of the bilayer as a monomer. However, a tetrameric array of four amphipathic transmembrane helices meeting with their polar surfaces facing inward could form the channel structure. Indeed, the high order dependence of conductance on TeTx and BoTx concentration pointed to the formation of a self-assembled oligomer as the structural entity underlying the conductance events [36–38].

3.2. *Bundles of amphipathic α-helices as the structural motif*

For segment I, the sequences are 82% homologous. The N-terminal half of the sequence contains the OH-containing residue, T9 or S9, respectively, whereas the two rings of negatively charged residues, E16 and E20, are in the C-terminal half of the structure. When modeled as a four-helix bundle, the structure exhibits alignment of pore-lining residues (T9/S9, E16 and E20 indicated in bold type). The occurrence of only one ring of prolines (P19) produces a bundle with a narrow pore diameter (≤ 2 Å) (model not shown); this constriction would render ionic flux unfavorable.

Allowing for conservative substitutions, the two segment II sequences are 75% homologous. Fig. 1A–D display an energy-optimized structure of a bundle of four identical and aligned peptides corresponding to TeTx II. The minimized four-helix bundle shown in Fig. 1A and B indicates that residues V3, E7, Y8, E11, P15, A19 and A23 are exposed to the pore lumen. The occurrence of prolines at positions 10 and 15 introduces a pronounced kink in the helices and, therefore, an expansion of the pore diameter at this level. A transverse section across the bundle illustrating approximately one turn of helix including E7 (Fig. 1C) and E11 (Fig. 1D) indicates the occurrence of two discrete rings of carboxylates generating a considerable negative charge within the pore. Such rings may underlie the cation selectivity of the toxin channel. In the BoTxA model (not shown), the hydrophilic residue T19 faces the pore lumen, whereas alanines replace non-pore lining hydrophilic residues of the TeTx model, making BoTxA even more

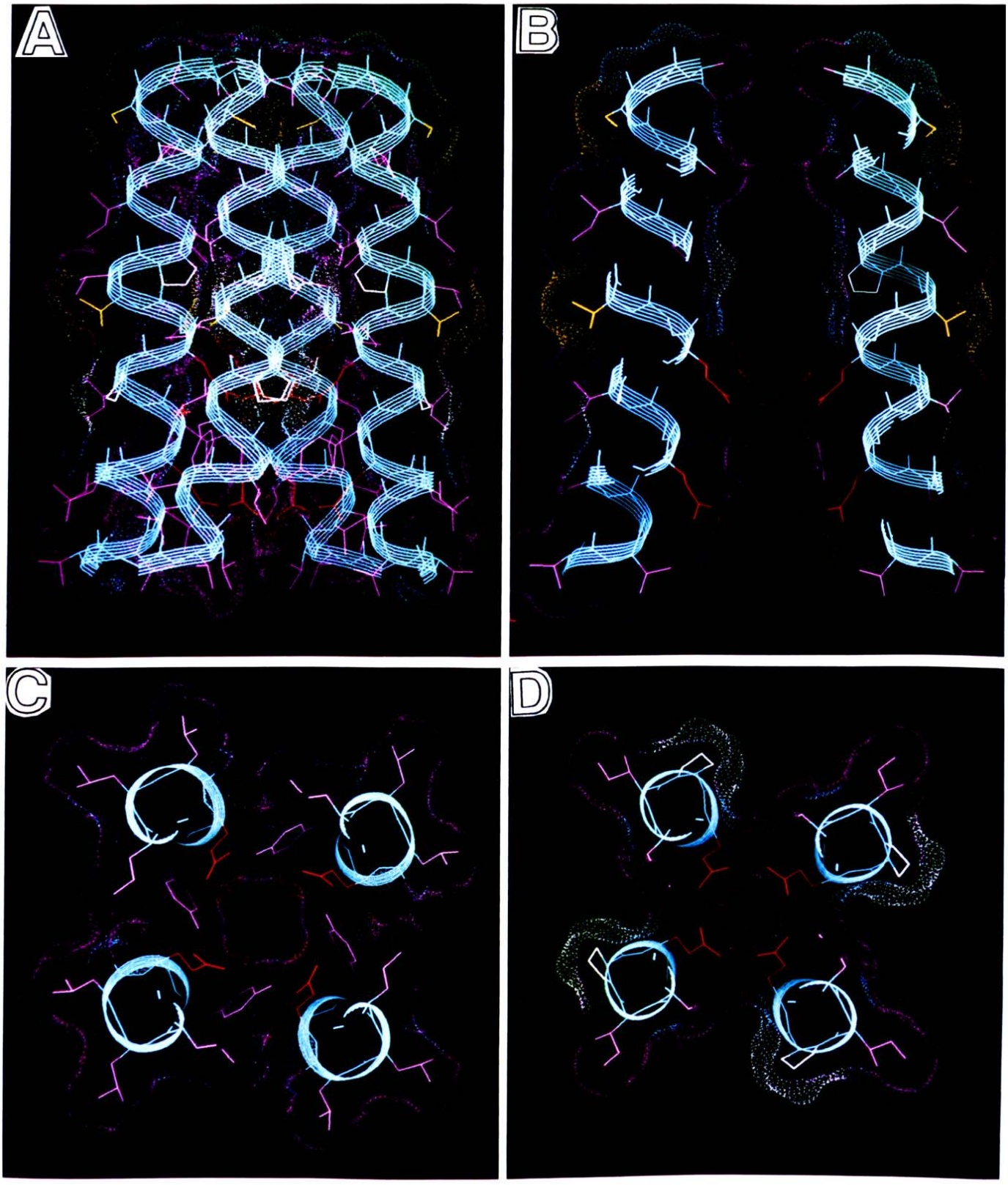

Fig. 1. (A) A bundle of four amphipathic α-helices is a plausible structure for the pore-forming entity of TeTx. Side view of energy optimized molecular model of the pore formed by four parallel helices with sequence corresponding to TeTx II. The N-terminus is at the top. The van der Waals envelope (dotted surface) shows the solvent-accessible surface with ribbon representation of the α-carbon backbone. (B) The central pore lined by negatively charged residues and the location of lipophilic residues (purple) at the exterior of the bundle. The length of the bundle is sufficient to span the bilayer. Note the occurrence of two rings of acidic residues (red) and two rings of prolines (white). (C and D) End-view of a cross-section at the level of E7 (C) and E11 (D) showing approximately two turns of helix. This region corresponds to the narrowest section of the pore with pore diameters of 5 Å (C) and 2.6 Å (D), and is lined by negatively charged carboxylates. Color code: light blue, α-carbon backbone; red, acidic; yellow, polar-neutral; white, proline and glycine; purple, lipophilic residues.

amphipathic. Hence, bundles of amphipathic α-helices fulfill the structural and energetic requirements to form the cation-selective pore identified in reconstitution studies of the intact toxins in lipid bilayers [18–23].

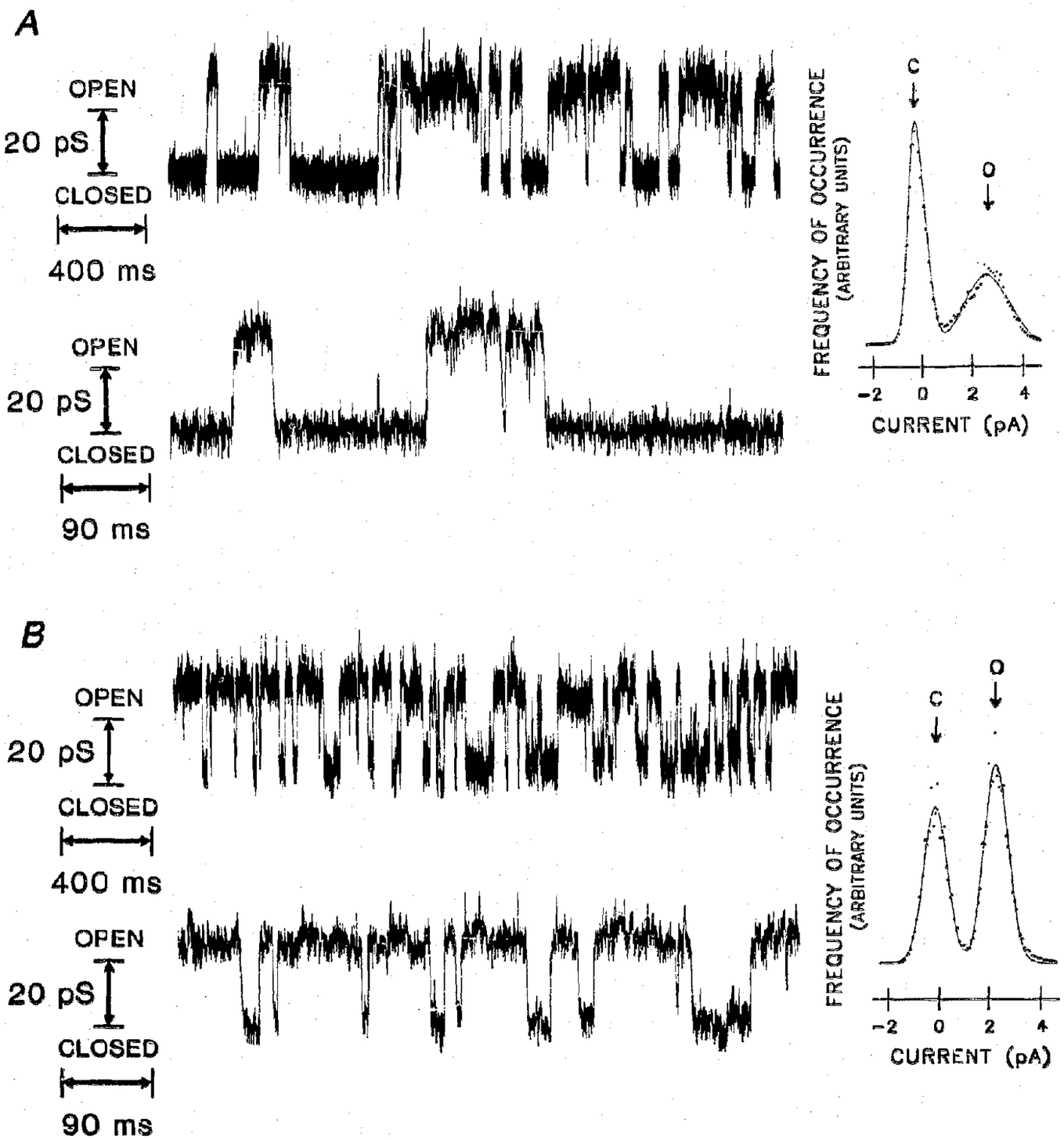

Fig. 2. Single channel currents from phosphatidylcholine bilayers containing the synthetic peptide, TeTx II. Currents were recorded at $V = 100$ mV in symmetric 0.5 M NaCl (A) or KCl (B), 5 mM HEPES, pH 7.0. The lower traces display a section at higher time resolution. Current histograms, generated from segments of records lasting several minutes, are displayed; C and O denote closed and open states, respectively. The probability of the channel being open, P_o, or closed, P_c, is calculated from the area under the corresponding Gaussian curve [22,39]. In NaCl, $P_o = 0.39$; in KCl, $P_o = 0.56$ The single channel current is calculated as the difference between the peaks associated with the closed and open states. The corresponding values for single channel conductances are 28 pS (NaCl) and 24 pS (KCl). Records were filtered at 1 kHz.

3.3. *Channel formation by TeTx II*

TeTx II forms channels in lipid bilayers, as shown in Fig. 2. Conductance events are heterogeneous in both amplitude and channel open and closed times. The most frequent event has a single channel conductance of 28 ± 3 pS (n = 8) in symmetric 0.5 M NaCl (Fig. 2A) and 24 ± 2 pS (n = 7) in 0.5 M KCl (Fig. 2B). The channel is ohmic and cation selective. In NaCl, the channel open times (τ_o) are well fitted by a sum of two exponentials, τ_{o1} = 0.5 ± 0.1 ms and τ_{o2} = 5.2 ± 1.1 ms, and the closed times (τ_c) with a single exponential, τ_c = 1.0 ± 0.2 ms (n = 3). In contrast, in KCl the channel appears in bursts of activity: both open and closed times are best fitted by two exponentials with values τ_{o1} = 0.7 ± 0.1 ms and τ_{o2} = 179 ± 20 ms for the open times and τ_{c1} = 0.8 ± 0.2 ms and τ_{c2} = 19 ± 5 ms for the closed times (n = 3).

3.4. *Sequence specificity and controls*

TeTx I does not form channels after reconstitution in lipid bilayers (n = 5) (Fig. 3A). The perturbations of the baseline, and the occasional distinct conductance steps indicate that peptide I is inserted in the membrane but is not sufficient to generate the discrete channel events that are characteristic of channel proteins. Furthermore, a peptide of the same amino acid composition as TeTx II but with a computer-generated random sequence, predicted to retain propensity for α-helix formation was synthesized. The sequence is EVVLLALIYPALTVPAIISLAE. This peptide incorporates into bilayers, as evidenced by erratic fluctuations in membrane current; however, it does not form discrete conducting events (n = 4) (Fig. 3B). These results support the specificity of the identified sequence as a candidate for the pore.

3.5. *Comparison with authentic TeTx channels*

To illustrate the similarity between the channel activity featured by TeTx II and that of intact TeTx or its B fragment [22,23], a record obtained in 0.5 M NaCl using fragment B is illustrated in Fig. 4. The single channel conductance is 25 ± 1 pS at V = 80 mV and 29 ± 1 pS at V = −80 mV (n = 3) [22]. Concordant with

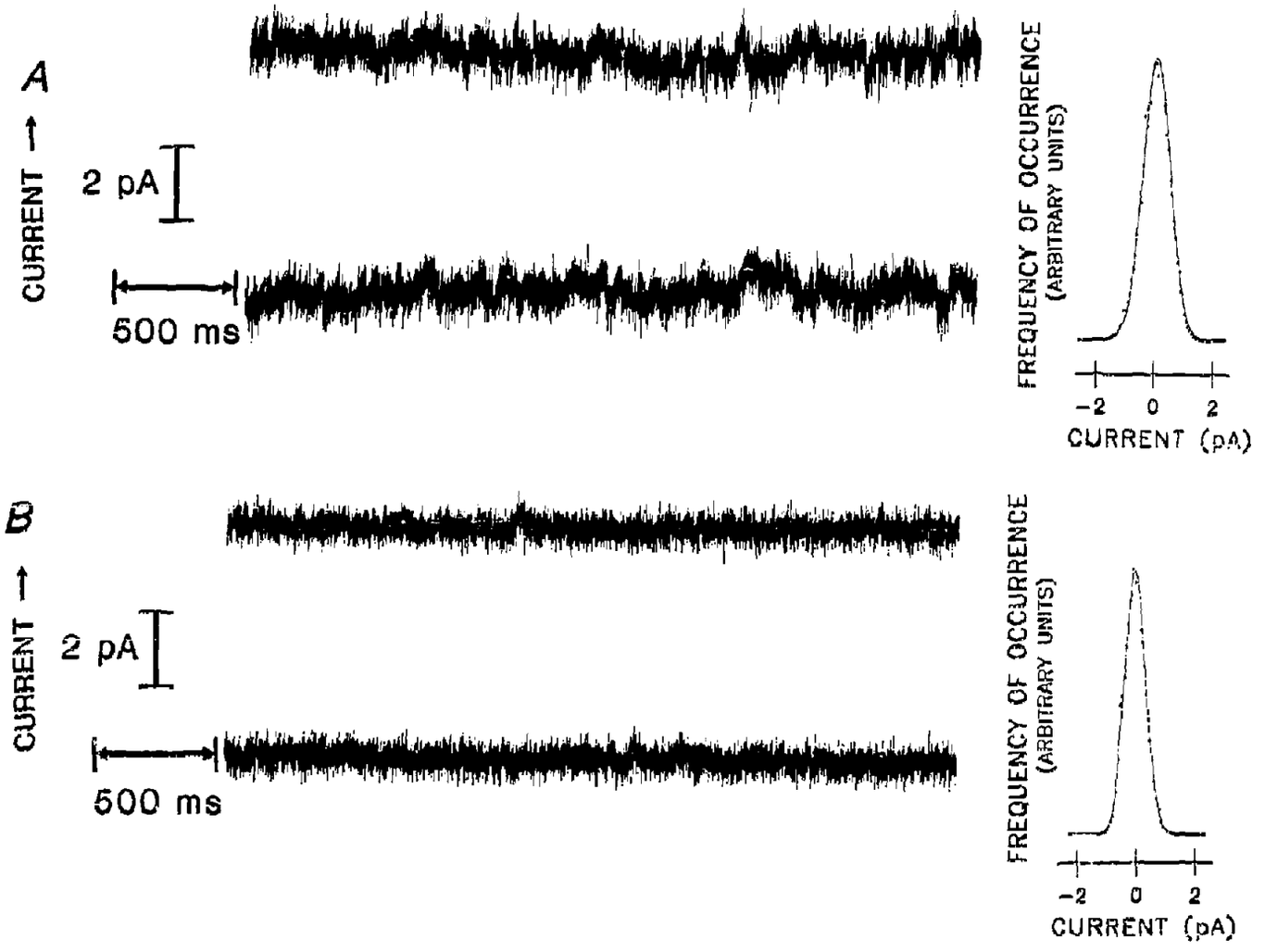

Fig. 3. Membrane currents from phosphatidylcholine bilayers containing the synthetic, TeTx I (A), and the peptide with randomized sequence but identical amino acid composition as TeTx II (B). Currents were recorded at 100 mV in symmetric 0.5 M KCl, 5 mM HEPES, pH 7.0. Records were filtered at 1 kHz. Note the absence of discrete current steps and the occurrence of a single band in the current histograms.

Fig. 4. Single channel currents from phosphatidylcholine bilayers containing the B fragment of TeTx. Currents recorded at $V=-80$ mV in symmetric 0.5 M NaCl, 10 mM HEPES, pH 7.0. Records were filtered at 3 kHz. The corresponding current histogram is shown. Single channel conductance is 29 pS.

expectations, the channel properties of TeTx II match those of intact authentic toxin.

3.6. *Conclusion*

TeTx II emulates several properties of the ion channel formed by authentic tetanus toxin incorporated in lipid bilayers, namely, single channel conductance, cation selectivity and channel open lifetimes in the millisecond time range. Channel activity is sequence specific, as evidenced from the lack of activity by the randomized TeTx II peptide and by TeTx I. The sequence of TeTx II is highly homologous to the corresponding sequence BoTxA II (78%). Conformational energy calculations on both TeTx II and BoTx II indicate that a bundle of four amphipathic α-helices is a plausible structural motif underlying channel activity. The location of pore-lining residues and the overall amphipathicity is compatible with similar function. These results support the notion that this structural motif may represent a functional module associated with channel formation by TeTx and BoTxA.

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REFERENCES

[1] Simpson, L.L. (1986) Annu. Rev. Pharmacol. Toxicol. 26, 427–453.
[2] Simpson, L.L. (1989) in: Botulinum Neurotoxin and Tetanus Toxin (Simpson, L.L. ed.) pp. 153–178, Academic Press, San Diego.
[3] Mellanby, J. and Green, J. (1981) Neuroscience 6, 281–300.
[4] Bizzini, B. (1979) Microbiol. Rev. 43, 224–240.
[5] Thompson, D.E., Brehm, J.K., Oultram, J.D., Swinfield, T.J., Shone, C.C., Atkinson, T., Melling, J. and Minton, N.P. (1990) Eur. J. Biochem. 189, 73–81.
[6] Binz, T., Kurazono, H., Wille, M., Frevert, J., Wernars, K. and Niemann, H. (1990) J. Biol. Chem. 265, 9153–9158.
[7] Eisel, U., Jarausch, W., Goretzki, K., Henschen, A., Engels, J., Weller, U., Hudel, M., Habermann, E. and Niemann, H. (1986) EMBO J. 5, 2495–2502.
[8] Fairweather, N.F. and Lyness, V.A. (1986) Nucleic Acid Res. 14, 7809–7812.
[9] Poulain, B., Mochida, S., Weller, U., Högy, B., Habermann, E., Wadsworth, J.D.F., Shone, C.C., Dolly, J.O. and Tauc, L. (1991) J. Biol. Chem. 266, 9580–9585.
[10] Bagetta, G., Nistico, G. and Bowery, N.G. (1991) Trends Pharmacol. Sci. 12, 285–289.
[11] DasGupta, B.R. (1990) J. Physiol., Paris 84, 220–228.
[12] Dolly, J.O., Ashton, A.C., McInnes, C., Wadsworth, J.D.F., Poulain, B., Tauc, L., Shone, C.C. and Melling, J. (1990) J. Physiol. (Paris) 84, 237–246.
[13] Habermann, E. (1989) in: Structure and function of tetanus toxin. Approaches by DNA and protein chemistry (Ownby, C. and Odell, G.V. eds) pp. 102–109, Pergamon Press, Oxford.
[14] Schmitt, A., Dreyer, F. and John, C. (1981) Naunyn-Schmiedeberg's Arch. Pharmacol. 371, 326–330.
[15] Poulain, B., Mochida, S., Wadsworth, J.D.F., Weller, U., Habermann, E., Dolly, J.O. and Tauc, L. (1990) J. Physiol. (Paris) 84, 247–261.
[16] Boquet, P. and Roa, M. (1985) J. Biol.Chem. 260, 6827–6835.
[17] Boquet, P. and Duflot, E. (1982) Proc. Natl. Acad. Sci. USA 79, 7614–7618.
[18] Borochov-Neori, H., Yavin, E. and Montal, M. (1984) Biophys. J. 45, 83–85.
[19] Donovan, J.J. and Middlebrook, J.L. (1986) Biochemistry 25, 2872–2876.
[20] Hoch, D.H., Romero-Mira, M., Erlich, B.E., Finkelstein, A., DasGupta, B.R. and Simpson, L.L. (1985) Proc. Natl. Acad. Sci. USA 82, 1692–1696.
[21] Ahnert-Hilger, G., Dauzenroth, M.-E., Habermann, E., Henschen, A., Krieglstein, K., Mauler, F. and Weller, U. (1990) J. Physiol. (Paris) 84, 229–236.
[22] Gambale, F. and Montal, M. (1988) Biophys. J. 53, 771–783.
[23] Rauch, G., Gambale, F. and Montal, M. (1990) Eur. J. Biophys. 18, 79–83.
[24] Mochida, S., Poulain, B., Weller, U., Habermann, E. and Tauc, L. (1989) FEBS Lett. 253, 47–51.
[25] Mochida, S., Poulain, B., Eisel, U., Binz, T., Kurazona, H., Niemann, H. and Tauc, L. (1990) Proc. Natl. Acad. Sci. USA 87, 7844–7848.
[26] Penner, R., Neher, E. and Dreyer, F. (1986) Nature 324, 76–78.
[27] Ahnert-Hilger, G. (1989) FEBS Lett. 255, 391–394.
[28] Stecher, B., Waller, U., Habermann, E., Gratzl, M. and Ahnert-

Hilger, G. (1989) FEBS Lett. 255, 391–394.
[29] Bittner, M.A., Habig, W.H. and Holtz, R.W. (1989) J. Neurochem. 53, 966–968.
[30] Bittner, M.A., DasGupta, B.R. and Holtz, R.W. (1989) J. Biol. Chem. 264, 10354–10360.
[31] De Paiva, A. and Dolly, J.O. (1990) FEBS Lett. 277, 171–174.
[32] Montal, M. (1990) FASEB J. 4, 2623–2635.
[33] Oiki, S., Madison, V.E. and Montal, M. (1990) Proteins: Struct. Funct. Genet. 8, 226–236.
[34] Grove, A., Iwamoto, T., Montal, M.S., Tomich J.M. and Montal, M. (1992) Methods Enzymol. 207, 510–525.
[35] Oiki, S., Danho, W. and Montal, M. (1988) Proc. Natl. Acad. Sci. USA 85, 2393–2397.
[36] Oiki, S., Danho, W., Madison, V.E. and Montal, M. (1988) Proc. Natl. Acad. Sci. USA 85, 8703–8707.
[37] Montal, M., Montal, M.S. and Tomich, J.M. (1990) Proc. Natl. Acad. Sci. USA 87, 6929–6933.
[38] Grove, A., Tomich, J.M. and Montal, M. (1991) Proc. Natl. Acad. Sci. USA 88, 6418–6422.
[39] Choe, S., Bennett, M.J., Fujii, G., Curmi, P.M.G., Kantardjieff, K.A., Collier, R.J. and Eisenberg, D. (1992) Nature 357, 216–222.
[40] Montal, M., Anholt, R. and Labarca, P. (1986) in: The Reconstituted Acetylcholine Receptor (Miller, C. ed.) pp. 157–204, Plenum Press, New York.
[41] Greenblatt, R.E., Blatt, Y. and Montal, M. (1985) FEBS Lett. 193, 125–134.
[42] Eisenberg, D., Schwarz, E., Komaromy, M. and Wall, R. (1984) J. Mol. Biol. 179, 125–143.