Electrical activity of artificial ion channels incorporated into planar lipid bilayers

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The ion transport ability in a planar lipid bilayer of a 21 amino acid peptide bearing six 21-crown-7 side chains is reported. The compound was designed to form an artificial ion channel by stacking the crown rings, when adopting an α-helical conformation in a bilayer membrane. All the derivatives of 1 exhibited membrane conductivity with the best results obtained with the unprotected analog 4. The latter showed typical single channel activity.

Introduction

Ion channel proteins are complex membrane proteins that control and regulate the flow of ions across cell membranes. Because of their involvement in several diseases and their potential uses in biosensors, these proteins have attracted considerable attention in the past decade. Unfortunately, there are several problems associated with natural ion channel proteins that severely limit their utility as both therapeutic agents and components of biosensors. Firstly, these transmembrane proteins are difficult to isolate in their pure functional form and they tend to denature easily. Secondly, they are very sensitive to degradation, and thirdly, their chemical modification into more elaborated molecular systems is almost impossible owing to their complex structure.

To overcome these difficulties, we sought to develop relatively small molecular systems that could mimic the transport properties of ion channel proteins. Several approaches towards artificial ion channels have been reported in the literature.[1] Most of the molecular systems described have not been thoroughly characterized and have not been shown to be the functional channel in planar lipid bilayer membranes (PLB). Recently, sophisticated molecules were reported to be functional and their active structure has been probed in detail.[2] However, none of these approaches allows easy post-synthesis chemical modification into more useful molecular devices.

Recently, we have described a general strategy to prepare supramolecular devices bearing multiple effectors with a defined and predictable structure.[3] The strategy was used to prepare an artificial ion channel shown to be functional using a vesicle assay.[4] Herein we describe our results of a study of the transport ability of one such device, a 21 amino acid peptide **1** designed to form an artificial ion channel by aligning six 21-crown-7 side chains as illustrated schematically in Fig. 1.

The specific objective of our research program is to develop artificial channels that might operate on their own and not through aggregation, in order to more easily assess their detailed mechanism and functional structure. Also we focus on molecular systems that might be elaborated further into more sophisticated devices.

Results and discussion

Different behaviors were observed with the analogs of **1**. The fully protected and neutral **1** incorporated the bilayer membrane with difficulty when added directly to the buffer solution from the DMSO stock solution. Upon incorporation, irregular transmembrane currents were detected demonstrating that **1** was able to transport ions quite efficiently under large electrical

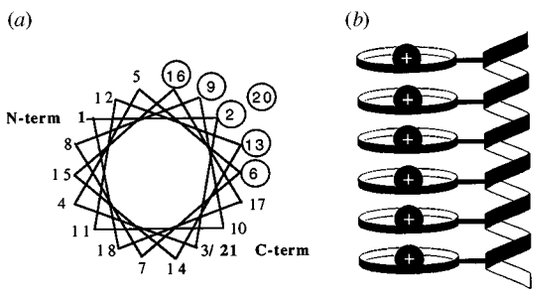
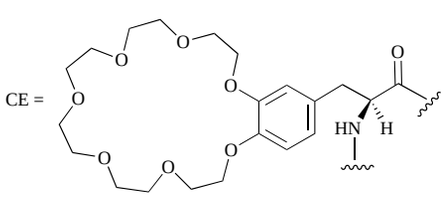


Fig. 1 (*a*) Axial projection of the α-helical structure of the hexacrown peptides **1**–**4** with the positions of the crown residue indicated by the circles and (*b*) the working hypothesis: the helical conformation of **1**–**4** produces an array of crown ethers that might form an artificial ion channel. The N- and C-terminal groups are not shown for clarity. (Reproduced with permission from ref. 4.)

N-Fmoc-Ala-CE-Ala$_3$-CE-Ala$_2$-CE-Ala$_3$-CE-Ala$_2$-CE-Ala$_3$-CE-Ala-O-Bu^t **1**
H_2N-Ala-CE-Ala$_3$-CE-Ala$_2$-CE-Ala$_3$-CE-Ala$_2$-CE-Ala$_3$-CE-Ala-O-Bu^t **2**
N-Fmoc-Ala-CE-Ala$_3$-CE-Ala$_2$-CE-Ala$_3$-CE-Ala$_2$-CE-Ala$_3$-CE-Ala-OH **3**
H_2N-Ala-CE-Ala$_3$-CE-Ala$_2$-CE-Ala$_3$-CE-Ala$_2$-CE-Ala$_3$-CE-Ala-OH **4**
Ala = L-alanine *N*-Fmoc = Fluorenylmethoxycarbonyl-



gradients. The same irregular activity was obtained immediately when the bilayer was formed in the presence of **1**. In both cases the transport activity was detected at high applied voltages (<-60 and $>+60$ mV), which suggests that the fully protected peptide **1** adsorbs on the PLB and incorporates transiently when the bilayer is subjected to large voltage changes. Control experiments showed that the membrane conductance of the bare bilayer was stable with 10^{-3} M DMSO alone. On the other hand, addition of 1 mM guanidinium chloride to the solution led to a noticeable lowering of the membrane conductivity. Guanidinium ions are known to be bound tightly by ligands of the 21-crown-7 type. An interesting feature of crown peptide **1** is the fact that once it is incorporated, it stays in the bilayer until the latter is broken. On the other hand, in a complementary experiment with a Na^+ piperazine-1,4-bis(ethanesulfonate) (Na-PIPES) solution instead of a NaCl solution, peptide **1** generated ionic currents demonstrating that Na^+ is the charge carried and not Cl^-.

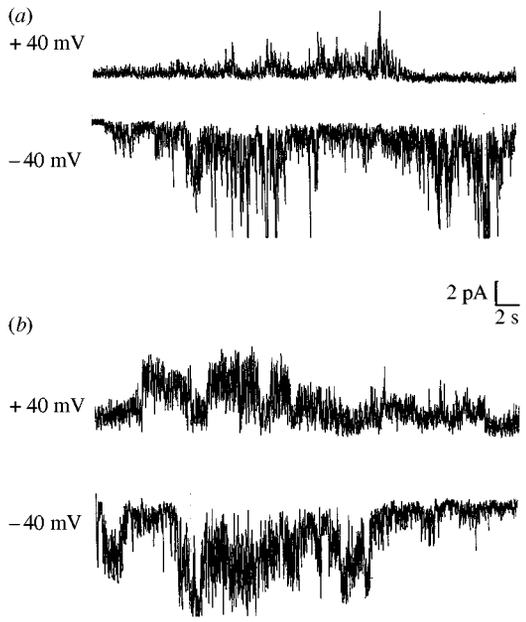


Fig. 2 Typical membrane conductivity observed with peptides (*a*) **2** and (*b*) **3** when using symmetrical NaCl solutions (100 mM on both sides of the bilayer) and applying ±40 mV potentials. Ion transport results in rapid increases of membrane conductivity.

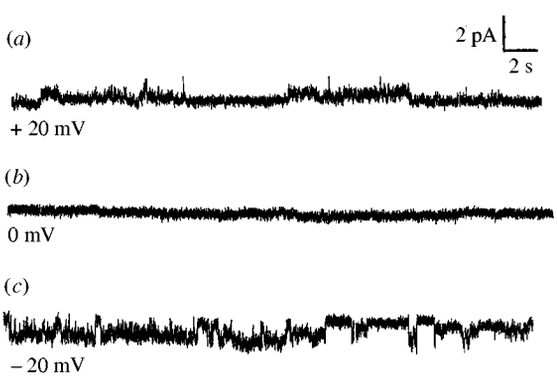


Fig. 3 Typical membrane conductivity observed with the unprotected peptide **4** at positive (*a*) and at negative (*c*) potentials using symmetrical NaCl solutions (100 mM on both sides of the bilayer). The conductivity behavior of **4** is typical of natural ion channels with preferred current levels (1–2 pA). Note the absence of unitary current at 0 mV (*b*).

The difficulty in the incorporation of the fully protected peptide **1** led us to believe that analogs with polar and charged terminal groups would penetrate the membrane more easily upon applying a transmembrane potential. In addition, we anticipated that these entities would be more stable than **1** in the bilayer environment. We therefore prepared the partially and the fully deprotected analogs of **1**, compounds **2**, **3** and **4**.

As predicted, the deprotected peptides proved to be more active than **1**, with the best results obtained with the fully deprotected peptide **4**. The amino peptide ester **2** and the *N*-Fmoc-peptide **3** had almost the same activity but were more active than **1**. They showed a more regular membrane conductivity at lower potentials, in the range of −40 to +40 mV (Fig. 2). The addition of guanidinium ions partially blocked the membrane current of **2** and **3**, but the activity reverted to the same levels in time (after around 30 min). This result suggests that the Na^+ ions travel through the channel formed by the alignment of the crown units.

As mentioned, the best results were obtained with the fully deprotected analog **4**. Indeed, typical single channel measurements were observed with preferential current levels at ±20 mV (Fig. 3). Typical amplitudes were in the range of 1 to 2 pA with variable open channel lifetimes. In these preliminary experiments, the gating behavior of the unitary events was not assessed. These results confirmed that after incorporation a molecular hydrophobic entity with the two polar (charged) head groups is anchored more strongly in a bilayer membrane.

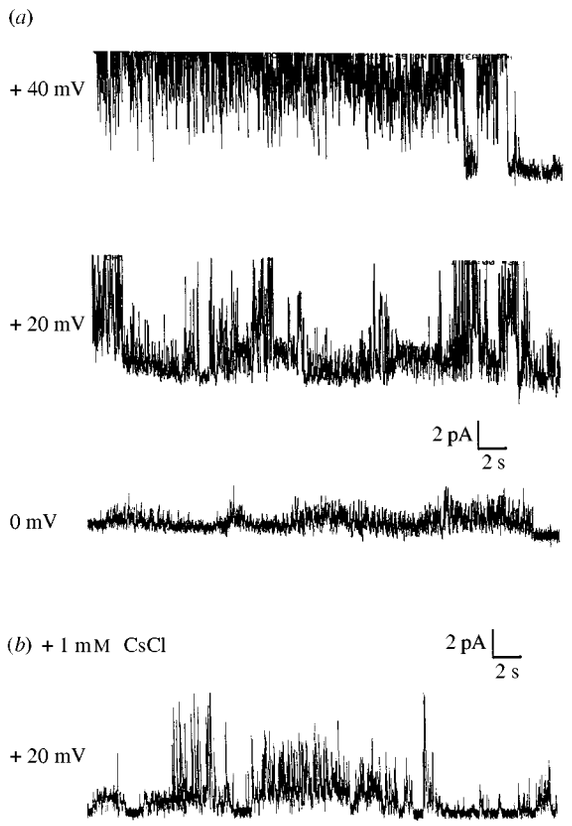


Fig. 4 (*a*) Membrane currents at various holding potentials after incorporation of **4** using different (asymmetrical) NaCl solutions on each side of the bilayer (100 mM *trans*, 300 mM *cis*). The current observed in the absence of potentials (0 mV) is due to the Na^+ transport driven by the ionic gradient; (*b*) as (*a*) but in the presence of 1 mM CsCl in the *cis* chamber to show the partial inhibition of Na^+ transport by the Cs^+ ions.

In order to ascertain that monovalent cations were able to permeate the synthetic channel **4** under their chemical gradient, some experiments were performed using asymmetrical NaCl buffers (*cis* = 300 mM, *trans* = 100 mM; Fig. 4). It is noteworthy that at 0 mV, *i.e.* in the absence of an electrical gradient, positive unitary currents were detected [Fig. 4(*a*), third trace]. The current amplitude and the occurrence of the events appear to be voltage sensitive in this case. Furthermore, the addition of 1 mM Cs^+ in the *cis* chamber partially blocks the electrical activity recorded at +20 mV, suggesting that Cs^+ ions were able to compete with Na^+ ions for the ionic pathway.

Conclusions

Overall, the results reported demonstrate that the hexacrown ether peptide **1** and its analogs incorporate into planar lipid bilayers. Their incorporation induces transmembrane currents of various amplitudes depending on the driving forces applied across the artificial membrane. In the case of the fully deprotected peptide **4**, typical single channel currents were observed. These results demonstrate that compounds **1**–**4** are functional artificial ion channels, although not as efficient so far as the natural ion channel proteins in terms of conductivity. The addition of Cs^+ and guanidinium ions resulted in a partial blockage of the ionic currents, which suggests that Na^+ ions travel in the 'channel' formed by the alignment of the crown ether rings and that **1**–**4** operate in a monomeric form. However, with these preliminary results, it is not possible to completely rule out that channels could be formed alternatively by the aggregation of

multiple helical peptides as in the cases of antibiotic[5] and amphiphilic[2a] peptides. Work is currently underway to characterize further the ion channel activity of **4** and to synthesize analogs with different amino acids and head groups.

Experimental

Synthesis

The hexacrown ether peptide **1** was prepared according to our previously reported procedure.[1] The deprotected analogs of **1** were prepared by the following procedures.

H_2N(Ala-CE-Ala_3-CE-Ala)$_3$O-Bu^t 2. The fully protected peptide **1** (15 mg) was treated for 4 h with a 30% diethylamine solution in DMF. After evaporation of the solvent under vacuum, the crude product was triturated five times with diethyl ether. The remaining white solid was dried under vacuum, then dissolved in glacial acetic acid and lyophilized to yield peptide **2** as a white solid (13 mg, 95%). The product was characterized by reversed-phase HPLC and ^{1}H NMR spectroscopy, which showed the absence of fluorenyl signals.

***N*-Fmoc(Ala-CE-Ala_3-CE-Ala)$_3$OH 3.** Peptide **1** (15 mg) was dissolved in a 10% *p*-cresol solution in neat trifluoroacetic acid and the resulting mixture was stirred at room temperature for 2 h. After evaporation, the oily residue was triturated five times with diethyl ether to yield a white hygroscopic solid. The latter was dissolved in glacial acetic acid and lyophilized to give **3** (13 mg, 90%) which was characterized by reversed-phase HPLC and ^{1}H NMR spectroscopy, which demonstrated the absence of the *tert*-butyl group.

H_2N(Ala-CE-Ala_3-CE-Ala)$_3$OH 4. The fully unprotected peptide **4** was prepared by treating peptide **2** (10 mg) with a 10% *p*-cresol solution in trifluoroacetic acid for 2 h at room temperature. After evaporation of the solvent, trituration with diethyl ether afforded the crude peptide which was dissolved in acetic acid and lyophilized to give the desired peptide **4** (9 mg, 97%). The compound was characterized by the same technique as for **2** and **3**.

Bilayer formation and hexacrown ether peptide incorporation

The planar lipid bilayers (PLBs) were formed at room temperature from a lipid mixture containing phosphatidylethanolamine and diphytanoylphosphatidylcholine (Avanti Polar lipids, Alabama, USA) in a 50:50 ratio. The final lipid concentration was 25 mg ml^{-1} dissolved in decane. A 250 μm diameter hole, drilled in a poly(vinylidene) fluoride (PVF) cup, was pretreated with the same lipid mixture dissolved in chloroform. Using a Teflon stick, a drop of decane lipid mixture was gently spread across the hole to obtain an artificial membrane. Membrane thinning was assayed by applying a triangular wave test pulse and typical capacitance values were 150–250 pF. The experimental chambers (3 ml *cis* and 4.5 ml *trans*) contained 100 mM NaCl, 500 μM $CaCl_2$ and 5 mM Na-PIPES [Na^+ piperazine-1,4-bis(ethanesulfonate)], pH = 7.0. Aliquots of compounds **1**–**4** (3 μl of a 3 mM stock solution in DMSO) were added to the *cis* chamber in the proximity of the bilayer. Incorporations were either spontaneous or achieved by applying negative holding potentials across the lipid bilayer. They were monitored as discrete current fluctuations owing to the presence of synthetic 'channels' in the bilayer. Alternatively, 100 mM LiCl or Na-PIPES buffer solution was used instead of 100 mM NaCl.

Recording instrumentation

The currents were recorded using a low noise operational amplifier DAGAN8900. The currents were then filtered (cut off frequency 10 kHz) and recorded on a video cassette recorder through a modified pulse code modulation device (DAS/VCR 900, UNITRADE). The currents were simultaneously displayed on-line on a chart recorder (DASH II MT, Astro Med.) and on an oscilloscope (Kikusui, 5040). Current recordings were played back, filtered at 500 Hz and sampled at 2 kHz for storage on hard disk and further analysis using a HP-Vectra computer. The open probability values (P_o) were determined from the data stored in 40 s duration files. Applied voltages are defined with respect to the *trans* chamber, which was held at virtual ground. The *cis* chamber was defined as the side where the synthetic molecules were added.

Acknowledgements

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References

1 (*a*) O. Murillo and G. W. Gokel, *Acc. Chem. Res.*, 1996, **29**, 425 and references cited therein; (*b*) G. G. Cross, T. M. Fyles, T. D. James and M. Zojaji, *Synlett*, 1993, 449 and references cited therein; (*c*) L. Julien, T. Lazrak, J. Canceill, L. Lacombe and J.-M. Lehn, *J. Chem. Soc., Perkin Trans. 2*, 1993, 1011; (*d*) U. F. Kragton, M. F. Roks and R. J. M. Nolte, *J. Chem. Soc., Chem. Commun.*, 1985, 1275; (*e*) F. M. Menger, D. S. Davis, R. A. Persichetti and J. J. Lee, *J. Am. Chem. Soc.*, 1990, **112**, 2451; (*f*) I. Tabushi, Y. Kuroda and Y. Yokota, *Tetrahedron Lett.*, 1982, **23**, 4601; (*g*) E. Stadler, P. Dedeck, K. Yamashita and S. L. Regen, *J. Am. Chem. Soc.*, 1994, **116**, 6677; (*h*) O. Murillo, S. Watanabe, A. Nakano and G. W. Gokel, *J. Am. Chem. Soc.*, 1995, **117**, 7665.

2 (*a*) K. S. Åkerfeldt, J. D. Lear, Z. R. Wasserman, L. Chung and W. F. DeGrado, *Acc. Chem. Res.*, 1993, **26**, 191 and references cited therein; (*b*) M. Montal, M. S. Montal and J. M. Tomich, *Proc. Nat. Acad. Sci. USA*, 1990, **87**, 6929; (*c*) M. R. Ghadhiri, J. R. Granja and L. K. Buehler, *Nature*, 1994, **369**, 301; (*d*) Y. Kobuke, K. Ueda and M. Sokabe, *J. Am. Chem. Soc.*, 1992, **114**, 7618; (*e*) Y. Kobuke, K. Ueda and M. Sokabe, *Chem. Lett.*, 1995, 435; (*f*) Y. Tanaka, Y. Kobuke and M. Sokabe, *Angew. Chem., Int. Ed. Engl.*, 1995, **34**, 693; (*g*) K. Ichikawa, M. A. Hossain, T. Tamura and N. Kamo, *Supramol. Chem.*, 1995, **5**, 219; (*h*) M. Engels, D. Bashford and M. R. Ghadiri, *J. Am. Chem. Soc.*, 1995, **117**, 9151.

3 (*a*) N. Voyer, *J. Am. Chem. Soc.*, 1991, **117**, 1818; (*b*) N. Voyer and J. Lamothe, *Tetrahedron*, 1995, **51**, 9241.

4 N. Voyer and M. Robitaille, *J. Am. Chem. Soc.*, 1995, **117**, 6599.

5 U. P. Fringeli and M. Fringeli, *Proc. Natl. Acad. Sci. USA*, 1979, **76**, 3852; see also reference 2(*a*).

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