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Collective behaviour of crown channels

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Abstract Collective behaviour of a crown ether channel, bis[(benzo-15-crown-5)-15-*y*l methyl] pimelate, in a planar lipid bilayer membrane has been studied through electrophysiological methods. A characteristic feature of these channels is their sequential opening, indicated by a uniform stepwise increase in the multi-channel current. The experimental results show that there are three modes of relaxation, of which the slowest one is attributed to the channel–channel interaction. The latter varies with the number of channels incorporated in the bilayer membrane, leading to the interpretation that crown channels behave cooperatively.

Keywords Bilayer electrophysiology · Collective behaviour · Crown ether · Ion channels · Relaxation studies

Introduction

Collective behaviour of membrane channels has been recognized as an important phenomenon in cell biology due to the fact that in a cell membrane in some cases ion channels may exist and function in clusters (Berrier et al. 1992; Valiunas and Weingart 2001). These clusters of channels have been reported to behave cooperatively (Draber et al. 1993; Ghosh 1993; Ghosh and Mukherjee 1993; Kelshian et al. 2000; Valiunas and Weingart 2001). In other words, change in the structure and function of one channel is affecting the neighbouring channels. For channels like porins from bacteria and gap junctions from eukaryotic cells, it is observed that they have multiple or large openings resulting from channel–channel interactions (Berrier et al. 1992; Ghosh et al. 2003). As a result, certain parameters, like channel current, its magnitude and duration, in multi-channel recordings show marked differences from those in single-channel recordings. On the other hand, there are a few reports which claim that synthetic organic compounds also can form ion channels (Neevel and Nolte 1984; Gadhiri et al. 1994; Abel et al. 1997). We have earlier reported that one such synthetic compound, bis[(benzo-15-crown-5)-15-*y*l methyl] pimelate, hereafter referred to as 15C5 crown ether, can form ion channels in artificial lipid bilayer membranes (BLMs), allowing passage of K^+ and Na^+ ions (Vijayvergiya et al. 1999). Investigation of channel properties of the crown ether were done keeping in view the fact that macrocyclic crown ethers form complexes with alkali and alkaline earth cations (Dalley 1978) and thereby affect the permeability of a lipid membrane (Szogyi et al. 1993). In the present work the multi-channel behaviour of the 15C5 crown ether is studied through bilayer electrophysiological experiments. A crown ion channel is formed through stacking of monomers of 15C5 crown ether inside the lipid bilayer (Vijayvergiya et al. 1999). Each monomer consists of two parallel rings separated by a distance of approximately 5 Å; each ring in turn is connected to a benzene ring held together by a hydrocarbon chain (Fig. 1). The aim of this work is to achieve an understanding of collective phenomena in synthetic transmembrane ion channels. Crown ethers, like some other ion transporters, e.g. gramicidin A, valinomycin, etc. (Hille 1984; Gennis 1989; Langs and Trigle 1992), have good potentials as drugs. When drug molecules bind to a cell membrane in vivo, quite often they tend to be in ensemble. Especially in case of 15C5 crown ether the ion channel is formed by several molecules, as mentioned previously (Vijayvergiya et al. 1999). In an ensemble the activities of the ion channels might be very much dependent on collective behaviour rather than on an individual one. Keeping this in view, we have chosen to

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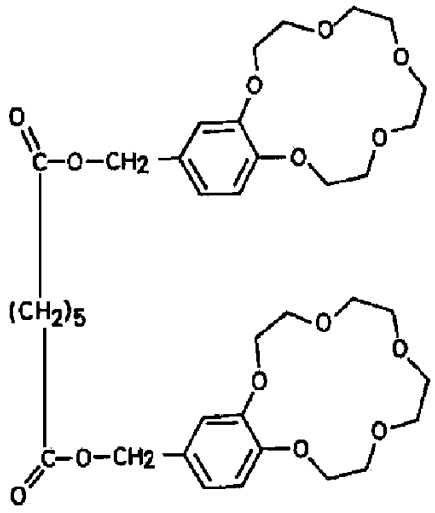

Fig. 1 Chemical structure of 15C5 crown ether

investigate the collective behaviour of 15C5 crown ether. Here, we have addressed the question if crown multi-channels in a BLM are independent or cooperative.

Materials and methods

Chemicals

Diphytanoylphosphatidylcholine (DPhPC) and bovine brain phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabama, USA). Cholesterol, Trizma base, HEPES and potassium chloride were purchased from Sigma (St. Louis, Mo., USA). 15C5 crown ether was obtained from Aldrich (USA). All other chemicals used were of the highest analytical grade available commercially.

Preparation of liposomes

The liposomes containing the crown ether were prepared by the reverse phase evaporation method (Szoka and Papahadjopoulos 1978), as detailed in our earlier communication (Vijayvergiya et al. 1999).

Bilayer experiments

Bilayer experiments were performed as briefly described below. A planar BLM was formed from DPhPC and cholesterol (5:1 w/w, 40 mg/ml in decane) across a 200 μm aperture of a Delrin cup (Warner Instruments, USA), in a symmetric buffer solution of 500 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HEPES, pH 7.4 (Ghosh et al. 1999; Bera and Ghosh 2001). Liposomes containing 15C5 channels were added to the *trans* chamber to fuse with the bilayers spontaneously. Channel currents were recorded with an Axopatch 200A integrating patch-clamp amplifier (Axon Instruments, USA), and analysed using Axodata and Axograph software (Axon Instruments, USA) on a Macintosh computer (Apple Computer, USA), as described in the earlier communication (Vijayvergiya et al. 1999).

Estimation of number of channels open

The maximum number of channels (N_{max}) opening at different applied voltages was determined from all-point amplitude histograms, taking into account the observation that the current levels are equispaced. At a particular voltage, N_{max} is determined from the maximum channel current, I_{max} (corresponding to the furthest peak in the amplitude histogram), divided by the corresponding single-channel current (I_{single}). The single-channel current has been obtained from single-channel experiments. The largest value of N_{max} over the entire range of electrical potential is identified as the total number of channels (N) incorporated in the lipid bilayer.

Relaxation studies

When a voltage is suddenly applied across the membrane, or withdrawn, the channel current immediately jumps to a high value and then gradually relaxes to a stable value (Mathes and Engelhardt 1998). Determination of this relaxation time (τ) of the channel current, when a voltage is applied or withdrawn, is done through the following relation (involving triple exponential functions):

$$I = \sum_{i=1,2,3} I_{i,0} + I_p \exp\left(-\frac{t - t_p}{\tau_i}\right) \quad (1)$$

Here, I_p denotes the peak value of the channel current (positive or negative) at a particular voltage at a time $t = t_p$ (when the voltage is put on or off). It decays exponentially to a stable value $I_{i,0}$ that may or may not be equal to zero.

The time constant τ_i values have been calculated from the experimental data by the best-fit method fitting the triple exponential in Eq. (1) with the help of the software Axodata and Axograph (Axon Instruments, USA). The best-fit τ value for each relaxation curve corresponds to the least chi-square value.

Results and discussion

BLM studies of the crown ether

The 15C5 crown ether exhibits ion channel activity when incorporated in planar lipid bilayers. Figure 2 shows some representative current traces of the channels. At a very low concentration of the crown ether, single-channel activity was observed; the corresponding current–voltage (I–V) characteristics are shown in Fig. 3. At a concentration of about 2 μg/ml of the crown ether, activity of a very few channels was observed (Fig. 2a). Here the channel opening time is usually of the order of several seconds, as observed in the case of gramicidin (Anderson 1999) and the Tris macrocycle (Abel et al. 1997). At higher concentrations of crown ether (200 μg/ml) the channel current shows several discrete levels because of opening and closing of a number of channels (Fig. 2b). Sequential opening of channels indicated by several successive steps of the same height in the current level is a special feature observed in the system. The same height of the steps suggests the presence of identical channels. It may be mentioned here that, for low concentrations of crown ether, the number of distinct steps manually counted from the ion channel current traces is found to be almost equal to the number of channels calculated from the ratio of the maximum current, I_{max}, at a particular voltage and the single-channel current, I_{single} (data not shown). Slight differences observed in the two values may be indicative of either cooperative behaviour among the channels or

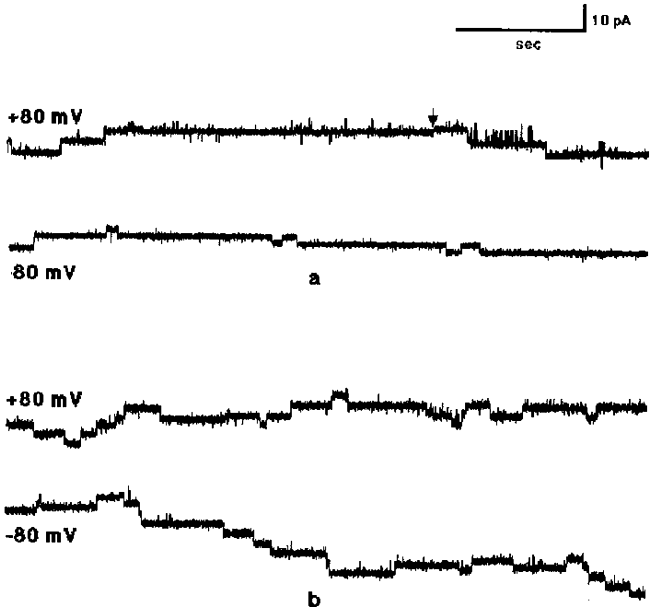

Fig. 2 Current traces at low (**a**) and high (**b**) concentrations of 15C5 crown ether, respectively. The bilayer was formed between symmetric solutions of 500 mM KCl, 5 mM $MgCl_2$, and 10 mM Tris-HEPES at pH 7.4. The signal was filtered at 400 Hz. The *arrow* in **a** indicates the sub-conductance level

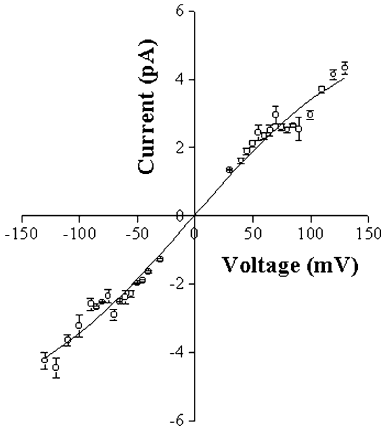

Fig. 3 Single-channel current versus voltage plot of 15C5 in a BLM. *Bars* indicate standard deviations in the mean of eight observations. Other experimental conditions are the same as in Fig. 2

occurrence of sub-conductance levels (as seen in Fig. 2a).

Relaxation studies

The relaxation of the crown ether channel at different applied voltages has been studied with the aim to understand the collective behaviour of the system. It may be mentioned here that the observed current is the sum of the contributions of the full open states and the substates of the crown ether channels. While fitting the experimental data (current–time) for relaxation in the exponential equation, we observed that, instead of a single exponential fit, a combination of three exponentials is better (Fig. 4a). This indicates that there exist three major modes of relaxation of the crown ether channels, each designated by a time constant, e.g. τ_1, τ_2, τ_3. Analyses show that the value of τ_1 lies below 10 ms, that of τ_2 varies from 10 to 20 ms, and for τ_3 it varies from 15 to 300 ms. As per our observations, variations of τ_1 and τ_2 with voltage (V) as well as the number of crown channels incorporated (N) are insignificant. We suggest that the fastest relaxation τ_1 is due to interaction of the crown ether with the aqueous environment. The interaction of the crown ether with the lipid molecules of the membrane is expected to cause relaxation of the channels slower than that of the solvent.

Moreover, the ratio of crown ether incorporated to lipid being small, the aforesaid interaction is not likely to change significantly with the number of channels incorporated. Considering these points, the slower relaxation τ_2 is suggested to be due the interaction of the crown ether with lipids. The crown-to-crown molecular interactions on the bilayer lipid membrane is supposed to help a channel most to remain in the open state, thus delaying the current decay with time significantly. Hence, this is thought to be the slowest relaxation mode, i.e. time constant τ_3. The latter very much depends on the potential across the membrane. The experimental data show that τ_3 is maximized at a particular voltage (near $+80$ mV or -80 mV, data not shown). From the current traces of the multi-crown system we have estimated τ_3 at different total numbers of channels (N) at different voltages. Figure 4b shows the variation of τ_3 with N at different potentials, $+100$, $+80$, -80 and -60 mV. It indicates that τ_3 increases initially with N but tends to decrease at higher N. At lower voltages the variations are inconsistent. As a whole, τ_3 does change with N, although the variation pattern is not definite. It may be noted here that the basis of channel–channel cooperativity is their mutual interaction (direct or indirect). The latter would depend on the number of participating channels, leading to an increasing trend in the τ_3 versus N plot. Hence, on the basis of our experimental data, we conclude that crown ether channels on a lipid bilayer behave cooperatively. However, the quantification of the aforesaid cooperativity is not possible at present.

As mentioned in our previous publications, the forces involved in collective gating of the channels are both cooperative and inhibitory. It has been argued that opening of one channel would lead to a change in ion flux, which might affect the gating of neighbouring channels (Ghosh 1993; Ghosh and Mukherjee 1993). For example, a flow of potassium ions through a crown channel would create a diffusion potential against (towards) the externally applied positive (negative) potential, causing closing (opening) of the neighbouring channels. In addition, the chemical structure of 15C5 crown ether, with its stacking posture in the lipid membrane giving rise to channel formation, offers good possibilities for lateral electrostatic interaction of the channels in the membrane, leading to

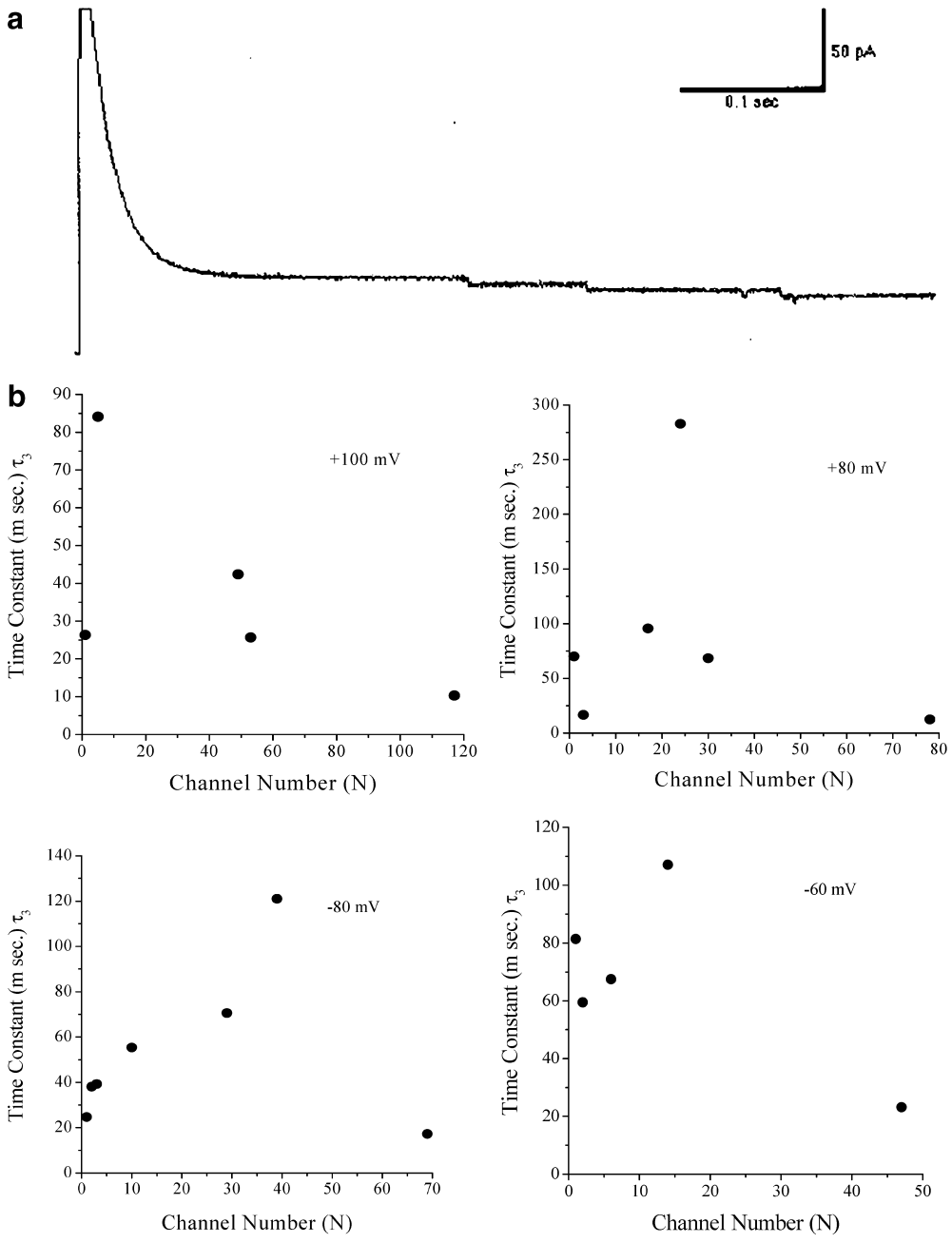

Fig. 4 **a** Representative current decay with time through a crown multi-channel when a particular voltage is applied (+100 mV). **b** Variation of crown–crown relaxation time (τ_3) as a function of the number of active channels (N)

cooperative behaviour. The latter is reflected in our analysis of the relaxation time (τ_3).

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