KINETICS PROPERTIES OF VOLTAGE INDUCED COLICIN Ia CHANNELS INTO A LIPID BILAYER

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Abstract

The activation kinetics of the ion channels formed by colicin Ia incorporated into a planar bilayer lipid membrane (BLM) was investigated by the voltage clamp technique using different step voltage stimuli. The temporal behaviour of ion channels put in evidence a gain or a loss of memory, revealed by a specific sequence of electrical pulses used for stimulation.

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INTRODUCTION

Planar bilayer lipid membrane (BLM) has been used extensively as a model of biomembranes (Taylor and Schultz 1996, Tien and Ottova 1998). This artificial membrane is a self-assembling system in vitro, which constitutes the fundamental spatial structure of all plasma membranes. In fact, the lipid matrix offers the structural frame in which all the membrane proteins are inserted (i.e., enzymes, transporters, specific receptors, etc.). The advantage of the BLM is that both sides of membrane can easily be altered and probed by electrodes. For long-term investigations and technological applications, planar BLMs can also be made on either gel or metallic supports (Tien and Ottova 1998).

In our experiments, we incorporated colicin Ia into planar BLM. Colicin Ia is a protein toxin secreted by *Escherichia coli*, which kills the other competing bacteria (Konisky 1982). It forms voltage-gated ion-conducting channels both in the inner membrane of target bacteria and in planar BLM (Kienker *et al.* 1997). Colicin Ia is a protein belonging to the class of bacterial toxins that share a common strategy: they are inserted into the membrane of the target cells, punching huge holes into them. *In vivo* these holes allow the entrance of foreign particles and the escape of intracellular components. The dramatic exchange of charged particles have, as a result, the loss of electrochemical membrane potential and the cellular death.

Colicin Ia is a protein of 626 amino acid residues rich in charged residues (conferring to it hydrophylicity), except for a hydrophobic segment of 40 residues near the carboxyl terminus (Wiener *et al.* 1997, Kienker *et al.* 1997). Maybe the amino acid sequence, rich in charged residues, is necessary in the earlier stage of attachment of the colicin Ia molecule to the membrane, whose external surface is also hydrophylic. Then colicin Ia binds with the hydrophobic segment parallel to the membrane and this portion is inserted in a transmembrane orientation (Kienker *et al.* 1997). Colicin Ia translocates a large hydrophilic part of itself completely across a lipid bilayer in conjunction with the formation of a ion-conduction channel (Jakes *et al.* 1998). At least 68 residues flip back and forth across the membrane in association with channel opening and closing (Qiu *et al.* 1996). Channel-forming colicin contacts the inner and outer membranes simultaneously during function (Qiu *et al.* 1996).

The probability of the opening of voltage-gated ion channels is determined by the transmembrane potential (Tzounopoulos *et al.* 1998). This channel activation results from a series of conformational transitions in the channel protein, particularly the movement of charged residues within the membrane electric field (Armstrong and Bezanilla 1973). In this work an electrophysiological method is used in order to demonstrate that it is possible to control the activation kinetics of the colicin Ia incorporated into planar BLM. In this way it was found experimentally that this artificial membrane (BLM + colicin Ia) can gain or can lose its memory.

MATERIAL AND METHODS

The experimental procedure was according to the method previously described (Cássia-Moura 1993). The lipid bilayer was formed by opposing two lipid monolayers across a small hole of 180-200 µm diameter, situated in a Teflon wall separating two aqueous solutions. Lipid monolayers were formed using azolectin (L-α-phosphatidylycholine type II - Sigma
Chemical Co) dissolved in hexane, so that solutions of 1% concentration were realised. During bilayer formation the peak current as response to constant voltage stimulation (amplitude: ±10 mV; duration: 2-4 ms; frequency: 500 Hz) was continuously monitored.

The aqueous solution consisted of 500 mM KCl+5 mM CaCl$_2$+5mM HEPES+1mM EDTA (final pH 7.00). Deionized water, double distilled in glass, was used in the preparation of all solutions. All chemicals were of analytical grade.

Colicin Ia was added directly to the aqueous solution in one half of the device realising a final concentration of 1-5 g/ml. For the sake of clarity, we shall refer to the side of the BLM containing colicin Ia as to a cis half, while the colicin-free side will be called trans half. Two Ag/AgCl electrodes were used to connect the electronics to the solutions (one electrode in each half) via salt bridges (2.5% agar in the chamber medium, electrodes immersed in 3 M KCl). A pulse generator with a d.c. voltage of ±200 mV was connected to the cis half. The ionic currents flowing through the artificial membrane were measured under conventional voltage-clamp conditions using an operational amplifier (Burr-Brown model OPA111) in the current-to-voltage converter configuration. The converter input was connected to trans half, and the output was connected to a physiograph (Gilson) either directly or via a digital oscilloscope (Nicolet model 201). Experiments were performed at room temperature, that is at 25 ± 2 °C.

**RESULTS AND DISCUSSION**

More than 2500 recordings of ionic current versus time were obtained from 40 artificial membranes and analysed in this work. The current was produced by pulses of at least 20 s duration of the step voltage stimulus. We do not intend to present all these crude experimental data, but merely their common aspects. The reason is that we noticed a variability of the artificial membrane behaviour, in spite of the fact that the experimental procedure was strictly respected in all the cases.

Although it was observed a slight different quantitative current response of the artificial membranes to the applied voltage pulses, however one can remark, in all cases, the following common features:

- a lack of response to negative pulses (Figure 1a and 1b);
- an inconsistent response to positive pulses less than 50 mV;
- a brief response and an irreversible breakdown of membrane structure to positive pulses greater than 90 mV;
- a “window” of positive pulses (V) between 50 mV and 90 mV, in which all records obtained with the same positive pulse presented a sudden exponential rise of the ionic current and a consecutive longer linear increase phase (Figure 1: $R_1 - R_6$). On the other hand, some of them were different both in the amplitude and time course. At this “window”, a different pattern of responses can be observed for any single voltage value applied to the artificial membrane, but a single stimulus always produce a unique response. The variability in this case is the result of successive repetitive trials on the same preparation, of different measurements on several preparations or of the large time intervals between the measurements. The time required to reach the end of the exponential phase varied from 7 to 50 s.
As concerning the repetitive stimulations, we have put in evidence a critical interval, \( \Delta t_c \), between two identical successive pulses \( V \) interposed by a resting period \( \Delta t \), with the following characteristics:

- it is less than 120 s and is specific to each particular membrane;
- the second pulse applied within \( \Delta t_c \) always performs the same current response if the resting period is maintained. Moreover, the response manifests a greater amplitude if the resting period between the pulses is smaller (Figure 1a: \( R_1 - R_3 \));
- the second pulse applied after \( \Delta t_c \) always conducts to a non controlled current response, both in the exponential phase as in the linear one (Figure 1b: \( R_4 - R_6 \)).

The results of this study were surprising in at least one aspect: namely, from Figure 1 one can observe that if \( \Delta t_1 < \Delta t_2 < \Delta t_3 < \Delta t_c \), then the responses are decreasing in the order \( R_1, R_2, R_3 \), while if \( \Delta t_4 > \Delta t_c \) the response is no more unique.

On the other hand, from Figure 2, it results that when we apply a sequence of two successive pulses (\( P_1 \) and \( P_2 \)) separated by a resting period within \( \Delta t_c \) \((\Delta t_{1-2} < \Delta t_c)\), followed by a third one (\( P_3 \)) separated by a resting period after \( \Delta t_c \) \((\Delta t_{2-3} > \Delta t_c)\), the system will respond stochastically (\( R_3 \)). If by chance the response \( R_3 \) is identical to \( R_1 \) (see bold \( R_3 \)), then the application of a fourth pulse (\( P_4 \)) identical to the second one (\( P_2 \)) and after the same resting period before \( P_2 (\Delta t_{3-4} = \Delta t_{1-2}) \), the last one will induce a response identical to that elicited by the \( P_2 \) pulse. Therefore, the experimental model is “remembering” the previous state (installed after the \( P_1 \) action). Moreover, this state can be induced by a different pulse but pertaining to the mentioned “window”. This is a proof of the memory of this system. The memory here must be interpreted in the general sense: if the actual state of a system depends on its previous states, then this system is endowed with memory.

In the case of the “window” pulses, applied into an interval smaller than \( \Delta t_c \), the enhancement (see Figures 1 and 2) of response can be attributed to the “memory” of the artificial membrane.

The interpretation of this artificial membrane behaviour to voltage pulses is a very difficult task. However, one could advance some hypotheses concerning this complex behaviour.

- After the stimulation of the artificial membrane, some colicin Ia molecules, which are forming membrane channels do not have enough time to relax themselves (i.e., to perform a transition from the open state to a complete closed one). But, because the order of magnitude of \( \Delta t_c \) is too high as compared with the time of allosteric transition of protein, one could advance the additional hypothesis that the lipid frame of the artificial membrane will prevent a fast transition of these molecules between the two states (open and closed). As we already presented, at least 68 amino acid residues flip back and forth across the membrane.
- Another possibility could be of the increase the density of colicin Ia incorporated into the artificial membrane under the influence of the electric pulses themselves.
- It could also be possible that the voltage pulses induce some colicin Ia molecule aggregation, resulting thus some clusters formation with a higher ionic conductivity than that of the individual channels.
As concerning the stochastic behaviour of the artificial membrane in response to “window” pulses, but applied at intervals longer than $\Delta t_c$, up to now we do not have a plausible explanation. Perhaps one could interpret this strange behaviour also in the term of ionic channels’ memory of a long term type.

All these hypotheses must be confronted in the future with new experimental data. We intend to extend this study to BLM with different chemical compositions and also to biological membranes, in order to have a better understanding of the complex bioelectrogenesis of the specialised tissues (e.g. heart, brain, etc.) and to furnish data for an improved electrodiagnosis and electrotherapy.

**CONCLUSIONS**

We used a quite simple model of biological membranes namely BLM with colicin Ia incorporated, in order to test its kinetics properties. By an electrophysiological method, we demonstrated that it is possible to control the activation kinetics of the experimental model. In this model the ion channel memory is experimentally induced and can be controlled by a specific sequence of pulses used for electrical stimulation.

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**Figure Captions**

FIGURE 1 - The time course response of our experimental model as a function of the pulse characteristics (voltage applied and resting period). Upper curve: the time course of step pulses applied.

a) The responses to pulses applied within $\Delta t_c$. In this case the responses are deterministic.

b) The responses to pulses applied after a resting period greater than $\Delta t_c$. In this case the responses are not predictable ($R_4, R_5, R_6$ or any other responses).

FIGURE 2 - The ionic current time response ($R_1 - R_4$) of the experimental model as a consequence of a specific temporal sequence of voltage pulses ($P_1 - P_4$). The applied voltage $P_3$ are in the range of $P_{3\text{min}} = +50$ mV and $P_{3\text{max}} = +90$ mV (that is, within the “windows”). $P_1, P_2$ and $P_4$ are also in this range as $P_3$ but they are fixed, while $P_3$ has any value in this range. $\Delta t_c$ is specific to each artificial membrane (for details, see the text). Note that $\Delta t_{2-3} > \Delta t_c$ and in this case, there are many uncontrolled possibilities of $R_3$ responses (but at a given pulse a single response is obtained). The response $R_3$ (bolded in the figure) is identical with $R_1$ (although it is possible that $P_3$ that generates it, is not identical to $P_1$). In this case, the stimulation of the experimental model with a $P_4$ pulse after $\Delta t_{3-4} < \Delta t_c$ will produce the response $R_4$ identical to the response $R_2$, if the resting period $\Delta t_{1-2}$ is equal to $\Delta t_{3-4}$, and if the applied voltage $P_4$ is equal to $P_2$. 
FIGURE 2