DYNAMIC STUDIES OF CERAMIDE ION CHANNELS ENABLED BY A RAPID-PERFUSION PLANAR LIPID MEMBRANE CHIP

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ABSTRACT

A microfluidic system supporting rapid perfusion of reagents to an on-chip bilayer lipid membrane (BLM) has been developed to study dynamic response of membrane or membrane-bound proteins to different (bio)chemical boundary conditions. Comprising an open well supporting a simple diffusion painting method for bilayer formation and multilayer microchannel networks for perfusion of reagents to the membrane, this device supports dynamic injection of multiple reagents at different concentrations with perfusion switching times of several seconds. Here we report a series of studies on the formation and dissolution of ceramide ion channels through the perfusion of a ceramide channel inhibitor (La3+) to induce channel disassembly, and a chelator for the inhibitor enabling the study channel reassembly dynamics. We discuss two models to explain the mechanism of La3+ ceramide inhibition. This system can be applied to study membrane or ion channels with similar time constant, allowing important dynamic features to be probed.

KEYWORDS: lipid membranes, perfusion, ceramide, ion channels

INTRODUCTION

Ceramide (N-acylated spingosine) is involved in several important cellular activities, including differentiation, growth suppression, cell senescence and apoptosis. Recent research shows that it may regulate apoptosis by forming stable channels binding to release mitochondrial proapoptotic intermembrane proteins to the cytoplasm [1-3]. Despite compelling evidence for the ceramide channel model (Fig. 1 c, d), there is limited knowledge on the kinetics of ceramide recruitment and dispersion in response to dynamic changes in chemical boundary conditions. Indeed, most existing observations of both lipid channels and lipid-protein interactions are based on measurements with static chemical boundary conditions. Thus the ability to rapidly perfuse chemicals to membrane-bound channels by pressure-driven convective transport would open the door to a wide range of ion channel studies which are not presently feasible.

Here we demonstrate rapid perfusion in a thermoplastic microfluidic chip to investigate the response of ceramide ion channels under application of time-varying concentrations of La3+ and ethylenediaminetetraacetic acid (EDTA). La3+ has been shown to inhibit ceramide channel in both solvent free membrane and annulus supported membrane, but the mechanism of inhibition remains elusive. Understanding how La3+ inhibit ceramide channel would provide important information about the dynamics of ceramide channel. We studied the relationship between initial disassembly rate and size of channel, the correlation between delay of reassembly by chelator EDTA and incubation time of La3+, and relationship between channel conductance before and after La3+, leading to two interesting models to explain the inhibition.

FABRICATION

The BLM chips used in this study were fabricated as previously reported [4]. Briefly, key components of the perfusion chip include a polyvinylidene chloride (PVDC) film containing an aperture with 80 ~ 150 μm diameter, an open well for painting a bilayer across the aperture [5], a bottom channel network connecting multiple perfusion inlets, and two Ag/AgCl electrodes sealed to the chip by adhesive wax, see Fig. 1(a,b). The top and bottom polycarbonate (PC) wafers containing microchannels are thermally bonded on either side of the PVDC film. With three perfusion inlets and a mixer, shown in Fig. 1(a), flow rates of two reagents and one buffer solution can be tuned to achieve different concentration for each of the chemicals at the lipid membrane.

RESULTS AND DISCUSSION

A bilayer is formed by manually painting a lipid solution across the PVDC aperture as shown in Fig. 1(d). In all tests described here, the lipid solution contains 5 mg/mL 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 5 mg/mL asolectin, 0.5 mg/mL cholesterol, and 0.13 mg/mL C16 ceramide in 10:1 v/v hexanol/hexadecane. Hexanol diffuses into the buffer promptly after lipid plug is painted over the aperture, promoting the bi-

Figure 1: (a) A fabricated BLM chip with three perfusion inlets. (b) Schematic of the integrated device, from [4]. (c) Model of a ceramide channel consisting of 24 columns of ceramide molecules, with a cytochrome C molecule inside the ceramide channel. (d) Transmembrane conductance trace revealing ceramide channel formation. The BLM was formed by diffusive painting (inset).
layer thinning process. Salt buffer includes 1 M KCl, 10mM piperazine-N,N-bis(2-ethanesulfonic acid) (PIPES), 1mM MgCl₂.

In a study of how perfusion back pressure acts on the bilayer, the presence of a reservoir of excess lipid within the membrane annulus was found to be an important factor impacting the perfusion limits. As revealed in a membrane capacitance measurements shown in Fig. 2, disturbance of the membrane was negligible for flow rates up to 2.5 μL/min, with specific capacitance remaining nearly constant at 0.37 μF/cm², and direct optical observation of the membrane revealed that the bilayer diameter remained unchanged over this range of perfusion flows. However, for flow rates above this level, the boundary of the bilayer was observed to expand toward the edge of the aperture together with a corresponding increase in membrane capacitance. This can be interpreted as a combination of membrane bulging and consequent annulus thinning, with lipids and solvent in the annulus continuously pulled out of the annulus and taken up by the bilayer. Indeed, halting the perfusion resulted in a rapid return to the initial capacitance level as lipids within the membrane were allowed to return to the annulus reservoir. However, for flow rates at or above 3.5 μL/min, continuous pumping eventually led to membrane rupture, with a maximum membrane burst capacitance of 280 pF (1.82 μF/cm²). For shorter perfusion cycles, however, the membrane was found to remain stable at significantly higher flow rates up to 20 μL/min.

Fig. 3 shows a typical response of ceramide channel to cyclic injection of La³⁺ and EDTA. The perfusion flow rate is 10 μL/min. The restoration by EDTA can be fitted into two phase process.

The ability of the chip to resolve kinetic responses of ion channels to changes in chemical boundary conditions is determined by the rate at which perfused solutes can be delivered to the BLM site. By confining the fluid behavior within the Taylor regime for La³⁺, we found that using a flow rate of 20 μL/min allowed La³⁺ to initially reach the BLM site within 800 ms, and the time needed for the local concentration to switch from 5% to 95% of the final value is only 5.8 s [4]. This is significantly faster than traditional diffusive perfusion using macro-scale BLM systems, in which switching times are on the order of minutes, preventing rapid kinetic events from being captured. Furthermore, unlike diffusive delivery of reagents, the microfluidic perfusion scheme allows time profile of the reagent concentrations to be precisely defined.

The rapid perfusion ability of this microfluidic BLM platform offers unique opportunities to study lipid-lipid and lipid-protein interactions in response to well controlled (bio)chemical boundary conditions. A series of perfusion tests were conducted, with the delivery of La³⁺ to a stable bilayer membrane containing a ceramide ion channel formed by introducing a fixed concentration of ceramide to the initial lipid mixture. Through these
tests, the initial La\(^{3+}\) induced disassembly rate was found to be proportional to the initial channel size (Fig. 4(a)), and the calculated initial rate of column loss (columns/min) is proportional to the starting circumference of columns (Fig. 4(b)). Likewise, the initial rate of channel reassembly (nS/min) is proportional to the conductance (nS) just before EDTA perfusion (Fig. 4(c)), and the calculated initial rate of column reassembly (columns/min) is proportional to the circumference of columns before EDTA perfusion (Fig. 4(d)). We also vary the time interval between La\(^{3+}\) and EDTA injection to study whether there is different behavior in ceramide channel restoration. found that delay time of EDTA restoring. In Fig. 5(a), each curve represents cyclic injection of La\(^{3+}\) and EDTA, while in Fig. 5(b) the incubation times are grouped into as follows: <10 minutes, 10-30 minutes, >30 minutes. Overall, we found that the incubation time of La\(^{3+}\) strongly affects the channel recovery dynamics. A disassembly-reassembly model, in which ceramide channel collapses upon treatment of La\(^{3+}\), and ceramide molecules diffused away from the channel site, can explain results in Fig 3 and 4. Since larger ceramide channel has more possible disassembly/reassembly site, it has larger initial rate of disassembly/reassembly. Meanwhile, because ceramide channel pieces diffuse in proportion to the square root of time after disassembly, delays in channel recovery are expected to similarly correlate to incubation time of La\(^{3+}\).

We also found that residual channel conductance after La\(^{3+}\) inhibition is proportional to initial value both in terms of channel conductance and channel circumference. Fig. 6 presents inhibition incidents from different experiments, showing a strong linear correlation. This can be explained by a distortion model, where presence of La\(^{3+}\) distorts the cylinder ceramide channel into a slot. This effect is consistent with known interactions between lanthanides and stretch-sensitive channels. To valid this model, further experiments are being carried out including measurements of selectivity in both the cylinder and slot state and ceramide channel fluorescence detection using a modified chip supporting correlated electro-optical measurements.

CONCLUSION
The microfluidic BLM system reported here enables continuous and cyclical perfusion resulting in rapid delivery of precise concentrations of multiple active agents to an on-chip bilayer lipid membrane. We have used the system to explore the dynamic interactions of ceramide with a channel inhibitor, La\(^{3+}\), leading to new insights into the kinetic processes of disassembly and reassembly of ceramide channels. The results suggest a potential channel distortion model to explain how ceramide channels are inhibited by La\(^{3+}\). More generally, the microfluidic platform can be used to investigate the dynamic aspects of other interesting membrane phenomenon and dynamic processes with time constants of just a few seconds, such as lipid phase separation and lipid-protein interactions.

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REFERENCES

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