J Membrane Biol (2011) 240:159–164
DOI 10.1007/s00232-011-9354-x

Channels Formed by Amphotericin B Covalent Dimers Exhibit Rectification

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Received: 7 January 2011 / Accepted: 1 March 2011 / Published online: 20 March 2011

Abstract Amphotericin B (AmB) is a widely used antifungal antibiotic with high specificity for fungi. We previously synthesized several covalently conjugated AmB dimers to clarify the AmB channel structure. Among these dimers, that with an aminoalkyl linker was found to exhibit potent hemolytic activity. We continue this work by investigating the channel activity of the dimer, finding that all channels comprised of AmB dimers show rectification. The direction of the dimer channel in the membrane depended on the electric potential at which the dimer channel was formed. On the other hand, only about half the monomer channels showed rectification. In addition, these channels were easily switched from a rectified to a non-rectified state following voltage stimulation, indicating instability. We propose a model to describe the AmB channel structure that explains why AmB dimer channels necessarily show rectification.

Keywords Amphotericin B · Antifungal antibiotic · Ion channel · Channel formation · Rectification

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Introduction

Amphotericin B (AmB) is a popular antifungal antibiotic with high specificity for fungi (Gallis et al. 1990; Hartsel and Bolard 1996). This specificity is a result of AmB having a much higher affinity for ergosterol, an abundant sterol in fungal membranes, than for cholesterol, a major constituent of mammalian plasma membranes (Readio and Bittman 1982; Cohen 1992). However, the molecular mechanism for AmB's therapeutic effects remains unknown. In general, it is thought that AmB exerts its selective toxicity by forming ion channels that permeabilize plasma membranes in a sterol-specific manner. This suggests that the mechanism of channel formation by AmB and associated sterols is essential for understanding AmB toxicity (de Kruijff and Demel 1974; Cotero et al. 1998; Matsumori et al. 2004a).

As a preliminary step to clarify AmB channel formation, we investigated binary interactions between previously described AmB monomers (Yamaji et al. 2002; Matsumori et al. 2002, 2004b) by synthesizing several covalently conjugated AmB dimers. We found that, among these, channels formed by AmB dimers with an aminoalkyl-linker (Fig. 1) exhibited especially potent hemolytic activity, consistent with other reports (Matsumori et al. 2004b). These aminoalkyl-linked AmB dimer channels had an EC_{50} of 0.03 μM, surpassing not only AmB monomer channels (EC_{50} 1.3 μM) but also all other AmB dimer channel types. In addition, UV and CD spectra of the aminoalkyl-linked AmB dimer channels showed they were more stable than AmB monomer channels. We therefore speculated that the AmB dimer could provide important clues about the AmB channel formation mechanism. Here, we investigated the properties of aminoalkyl-linked AmB dimer channels and propose a model for the channel formation.

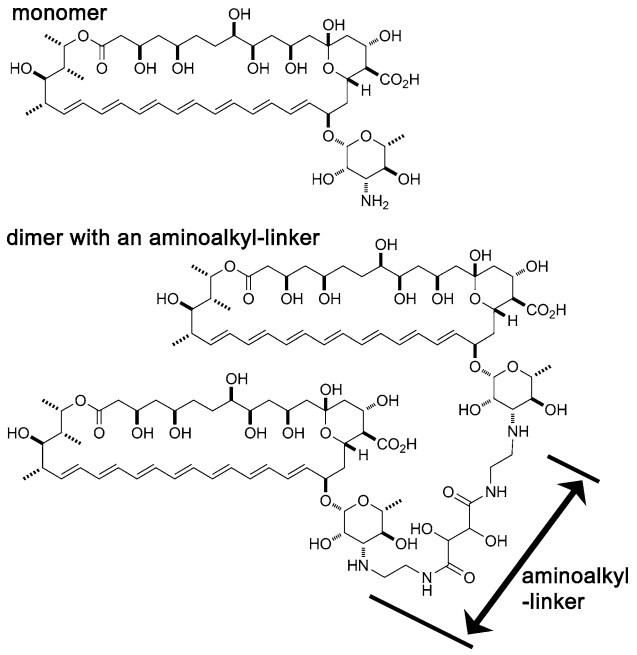

Fig. 1 Structures of the AmB monomer and AmB dimer with an aminoalkyl linker

Materials and Methods

Materials

AmB and ergosterol were purchased from Nacalai Tesque (Kyoto, Japan). Diphytanoylphosphatidylcholine was purchased from Avanti Polar Lipids (Birmingham, AL). Hexadecane and *n*-hexane were purchased from Wako (Osaka, Japan). Aminoalkyl-linker AmB dimers were synthesized as previously described (Matsumori et al. 2004b).

Channel Current Recording

Single-channel recordings from lipid bilayers were obtained using the tip-dip method (Matsumori et al. 2004a; Ehrlich 1992). Briefly, patch-clamp pipettes (GC-150T-10; Harvard Apparatus, Cambridge, MA) with tip diameters of 10 μm were made using a P97 Sutter Instruments (Novato, CA) puller. The same solution was used for both the bath and pipette solution (3 M KCl, 2.5 mM HEPES, 1 mM $CaCl_2$, pH 7.4). Lipid monolayers were formed by spreading n-hexane solutions of mixed lipids onto the surface of the bath solution. For AmB single-channel recordings, diphytanoylphosphatidylcholine, ergosterol and AmB were mixed at a 15,000:3,000:1 molar ratio. A pipette tip precoated with hexadecane was repeatedly passed through the lipid layer of the surface of the solution at +100, −100 or 0 mV until the pipette resistance rose above 5 gigaohms. A lipid bilayer membrane was formed on the tip of the pipette, allowing AmB dimer channels to form in the membrane. Single-channel currents were then recorded by a patch-clamp amplifier (CEZ-2400; Nihon-Kohden, Tokyo, Japan). Current recordings were digitized at 5 kHz and filtered at 1 kHz. Single-channel analyses were carried out using the commercial software pClamp 9 (Axon Instruments, Novato, CA).

Results

Channels Formed by Aminoalkyl-Linked AmB Dimers Always Exhibit Rectification

To examine the activity of channels formed by aminoalkyl-linked AmB dimers (from here on referred to as "AmB dimer channels"), channel currents were measured by the tip-dip method as shown in Materials and Methods (Matsumori et al. 2004a; Ehrlich 1992). Figure 2a shows representative single-channel current traces of AmB dimer channels when they were formed at +100 mV. For these channels, as the voltage became more positive, more current flowed. On the other hand, the current was less sensitive to changes at negative potentials. The corresponding *I–V* curve (*n* ranged 3–15 for different potential values) is shown (Fig. 2b). As seen, more ions flowed at positive potentials for potentials of the same magnitude. The chord conductance was 50 and 17.5 pS at +200 and −200 mV, respectively. We refer to such rectification as "positive rectification." Figure 2c shows that the open probability (P_o) of these channels was lower at positive potentials than negative ones.

Figure 3a shows representative single-channel current traces of AmB dimer channels when they were formed at −100 mV. Unlike the properties seen in Fig. 2, large currents were measured at negative voltages, while much smaller currents flowed at positive ones. Figure 3b shows the corresponding *I–V* curve. The chord conductance was 12.5 and 45 pS at +200 and −200 mV, respectively. We refer to this rectification as "negative rectification."

The P_o of these channels is shown in Fig. 3c. These mirror those seen in Fig. 2. At negative potentials, the P_o was low (0.75 at −200 mV), whereas P_o was high at positive potentials (0.98 at +200 mV). The relationship between conductance and P_o was the same as that seen in Fig. 2; conductance and P_o were inversely related.

Table 1 shows the relationship between the potential at which AmB dimer channels were formed and the ensuing channel rectification. When they were incorporated into the membrane at +100 mV, 75% of these channels showed positive rectification. The remaining exhibited negative rectification. When AmB dimer channels were formed in the membrane at −100 mV, 67% had negative rectification

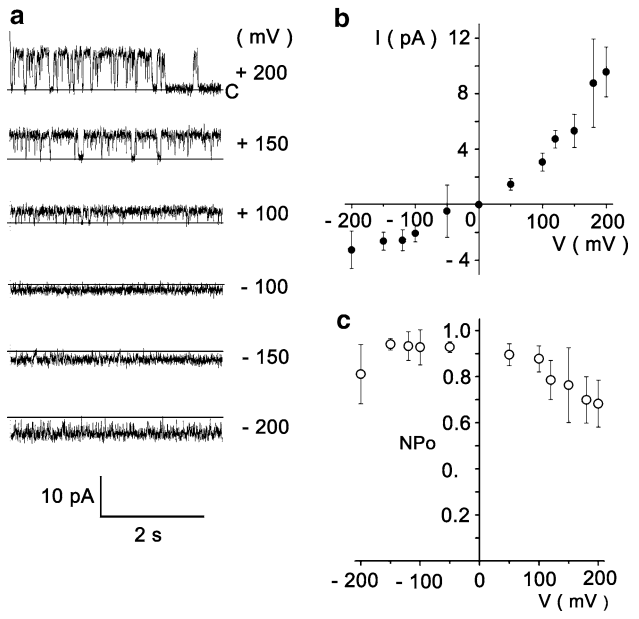

Fig. 2 Activity of an AmB dimer channel when formed in the membrane at 100 mV. **a** Representative current traces. **b** *I–V* relationships. **c** Open probability (P_o). Mean ± SD, $n = 3$–15

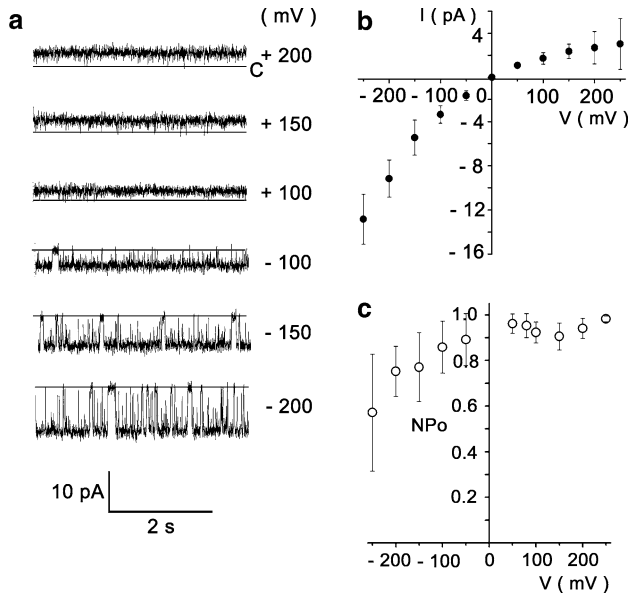

Fig. 3 Activity of an AmB dimer channel when formed in the membrane at −100 mV. **a** Representative current traces. **b** *I–V* relationships. **c** Open probability (P_o). Mean ± SD, $n = 3$–15

Table 1 Channels formed by AmB dimers showed rectification: relationship between the potential at which AmB dimer channels were formed and their rectification ($n = 15$–18)

Potential (mV)	Positive rectification (%)	Negative rectification (%)
+100	75	25
−100	33	67
0	44	56

and the remaining 33% were positive. When incorporated at 0 mV, 44% showed positive and 56% showed negative rectification. Overall, all AmB dimer channels expressed some form of rectification, and for the most part this rectification correlated with the potential at which the AmB dimer channels were formed in the membrane.

Only Some AmB Monomer Channels Show Rectification

Next, we investigated the properties of AmB monomer channels. Figure 4a shows representative current traces of these channels when they were formed in the membrane at +100 mV. Current amplitudes at +200 and −200 mV were similar. Figure 4b, c shows the *I–V* curve and P_o, respectively, for channels exhibiting no rectification. The chord conductance was 40 and 40 pS at +200 and −200 mV, respectively. P_o was independent of the potential.

Table 2 shows the relationship between the potential at which AmB monomer channels were formed and their rectification. Regardless of the polarity of the potential

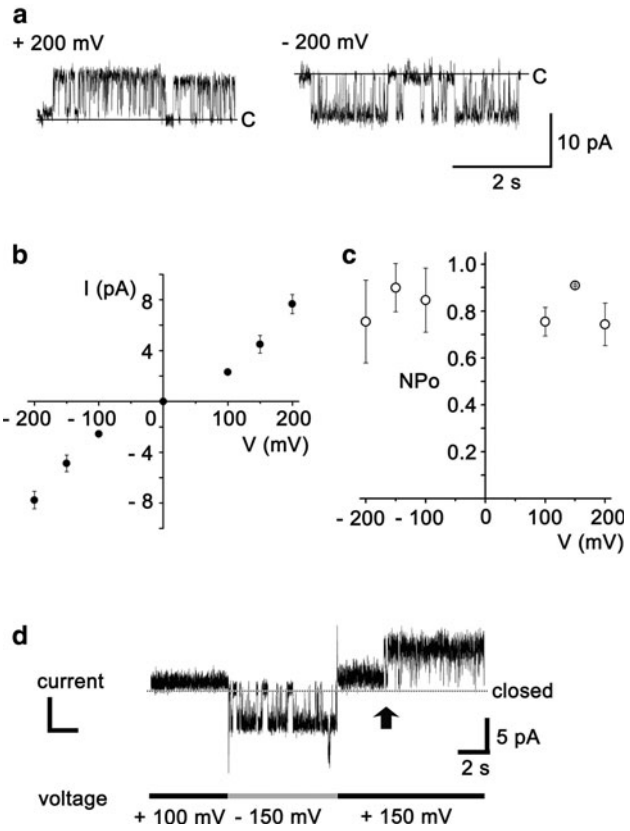

Fig. 4 Activity of AmB monomer channels when formed in the membrane at 100 mV. Half the AmB monomer channels showed no rectification. **a** A representative current trace. **b** *I–V* relationships. **c** Open probability (P_o). **d** A representative current trace showing the transition from a rectification state to a nonrectification state. *Arrow* shows when the transition occurred

Table 2 Channels formed by AmB monomers exhibited a rectified state and a nonrectified state: relationship between the potential at which AmB monomer channels were formed and their rectification ($n = 5$–6)

Potential (mV)	Nonrectification (%)	Positive rectification (%)	Negative rectification (%)
+100	50	33	17
−100	40	20	40

applied during channel formation, half the AmB monomer channels failed to show rectification. For the remainder, the direction was weakly dependent on the potential at which they were formed. Furthermore, it was observed that the rectification of AmB monomer channels was easily removed by a voltage stimulus. Figure 4d shows an AmB monomer channel that had negative rectification suddenly exhibited no rectification after the voltage was changed from −150 to +150 mV (arrow). These results indicate that AmB monomer channels have two states, a rectified state and a nonrectified state, and that the rectified state is unstable. Considering that all AmB dimer channels exhibited rectification and that the direction of rectification depended on the incorporation potential, we conclude that AmB dimer channels are more stable than their AmB monomer counterparts.

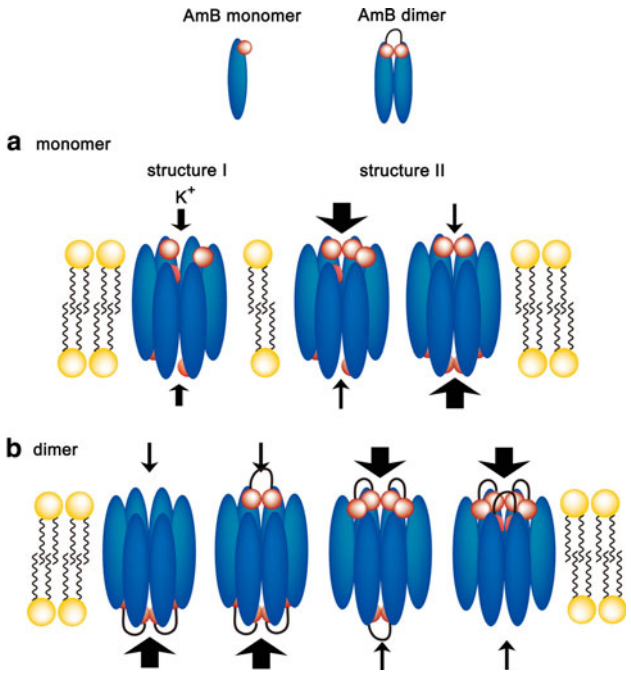

Fig. 5 Structural models for the AmB channels. A channel consists of six AmB monomers. Rectification arises when a majority of AmB monomers point to the same direction. **a** Two AmB monomer channel structures. In structure I, three of six monomers face the same direction (nonrectification state). In structure II, at least four monomers face the same direction (rectification state). **b** AmB dimer channels. Always, a majority of AmB dimers are facing toward one direction, leading to rectification

Discussion

In this study, to better understand the structure of the AmB channel, we measured the activities of channels formed by AmB monomers and aminoalkyl-linked AmB dimers, finding three important features: (1) currents of conductive channels formed by either were similar; (2) channels formed by AmB dimers always exhibited rectification, whereas only half the AmB monomer channels did; and (3) in AmB dimer channels and, to a much lesser extent, AmB monomer channels, the direction of rectification had some dependence on the polarity of the potential applied upon tip-dipping.

The first point means the two channel types have similar single-channel conductance and, therefore, comparable pore sizes. This allows us to assume that the number of AmB units in the two is identical.

The third point can be explained by the charge of the AmB molecule or AmB dimer. pKa values for carboxylic and amino groups in the AmB molecule in water are 3.5 and 9.5, respectively (Mazerski et al. 1990), thus giving a zwitterionic nature to the AmB molecule at neutral pH. It is generally accepted that the NH^{3+} group interacts with the PO^{4-} group of phospholipid molecules, whereas the COO^{-} group is mostly exposed to the water phase (Asandei and Luchian 2008). This water-exposed carboxylate anion may electrostatically induce an orientational preference for AmB molecules in accordance with the polarity of the applied potential.

To explain the second finding, we propose a structural model (Fig. 5). The model makes the following assumptions: Both AmB monomer and dimer channels are constituted of six AmB units, and rectification arises only when an unequal number of monomers orient to one direction. The first assumption seems reasonable because, based on the pore size of the AmB channel, the number of AmB molecules required to form a pore has been estimated at four to 12 (de Kruijff and Demel 1974; Andreoli 1974; Moreno-Bello et al. 1988; Bonilla-Marin et al. 1991), although eight is generally accepted mainly from simple modeling studies (de Kruijff and Demel 1974). More recent spectrophotometric and SFM studies suggested six to eight AmB molecules per channel (Gruszecki et al. 2002), which also supports our use of six AmB molecules. The six-molecule assumption may be further supported by a report that shows the AmB channel pore diameter is 7.2–7.4 Å (Katsu et al. 2008), which was determined by osmotic protection experiments using ergosterol-containing PC liposomes. This size is smaller than the 8 Å (Cass et al.

1970) that has been the basis for the notion of eight molecules per AmB channel. In addition, as discussed above, it is reasonable to assume that the number of AmB units in AmB monomer and dimer channels is identical. The second assumption is also plausible based on the following. Since AmB channels have cation selectivity at neutral pH (Asandei and Luchian 2008), the alignment of an unequal number of AmB molecules to one direction should give rise to an asymmetric electrostatic potential to cations at the mouth and tail of the channel, which thus causes channel rectification.

Under these assumptions, the rectified nature of the AmB dimer channel is accounted for if the channel consists of three dimer molecules (Fig. 5b). This requires an unequal number of molecules facing opposite directions. On the other hand, nonrectified AmB monomer channels occur when an equal number of AmB molecules (three) face opposite directions (Fig. 5a). The probability of nonrectification in AmB monomer channels is approximately ${}_6C_3(1/2)^6 \approx 30\%$, assuming each AmB molecule is independently aligned and the effect of the initially applied potential polarity is not large. As listed in Table 2, however, 40–50% of AmB monomer channels showed nonrectification. This suggests that AmB molecules mutually orientate preferably in an antiparallel manner, which would increase the probability of nonrectification in AmB monomer channels.

Here, we should address the inconsistency between our previous solid-state NMR results (Umegawa et al. 2008) and the current data. Since in the previous solid-state NMR experiments we did not observe dipolar interactions, suggesting antiparallel alignments for AmB–AmB interaction in ergosterol-containing POPC membrane, we concluded that the parallel alignment was dominant for AmB–AmB in the ergosterol membrane. There are some significant differences in experimental conditions which may cause this discrepancy: the difference in membrane lipids used for the current study (DPhyPC, a highly branched lipid) and solid-state NMR experiments (POPC) and the difference of AmB–ergosterol–lipid ratio (1:3,000:15,000 for this experiment and 1:1:10 for the NMR experiment). The membrane thickness of DPhyPC is almost identical to that of POPC (Tristram-Nagle et al. 2010), but hexadecane, which was used for the current recording as a precoating for the tip, is known to increase membrane thickness by approximately 5 Å (Fettiplace et al. 1971). One possible explanation for the discrepancy is the notion that in the thicker DPhyPC membrane containing hexadecane, a channel with antiparallel orientation may more feasibly span the membrane than a parallel one since the polar head groups in the former channel can extend in both directions to reach the membrane surface. Thus, a parallel-aligned channel may not be favored (or nonconductive) in the membrane used.

Moreover, it is also likely that the relatively low signal-to-noise ratios of the solid-state NMR experiments due to 15% ^{13}C enrichment of AmB made it difficult to detect signals due to antiparallel alignment. In recent experiments, we found the coexistence of parallel and antiparallel alignments for AmB–ergosterol interaction (unpublished data), suggesting that antiparallel AmB–AmB interaction could also occur to a certain extent since ergosterol is known to act as a glue between AmB molecules. Hence, we reexamined the intermolecular distance measurements by SSNMR experiments using 100% ^{13}C-enriched AmB to enhance the sensitivity. A more precise channel model will be proposed in due course by combining the current data with the structural information that will be obtained from the NMR measurements.

In conclusion, we found that AmB dimer channels always showed marked rectification, while only half the AmB monomer channels did. These observations can be explained by our model (Fig. 5) in which the AmB channel is presumed to consist of six AmB molecules. Therefore, the current study provides new insight into the structure of AmB channel assembly. This should act as a useful basis for future computational and experimental work seeking to better understand the mechanism of action for AmB.

Conclusions

AmB is a popular antifungal antibiotic because of its high specificity for fungi. Its therapeutic effect is thought to be induced by an ion-permeable channel formed by AmB and sterol in the lipid membrane. It was previously shown that channels formed by aminoalkyl-linked AmB dimers exhibit potent hemolytic activity and are more stable than AmB monomer channels. Hence, in this work, to clarify the AmB channel structure in a membrane, we measured single-channel activity. We found that all AmB dimer channels showed rectification. Additionally, the electric potential at which the AmB dimer channels were formed in the membrane affected the direction of the channels. In contrast, only half the AmB monomer channels showed rectification. Even then, they could be switched to a nonrectified state by applying voltage stimulation, indicating channel instability. From these results, we propose a structural model of the AmB channel where the channel consists of six AmB monomers and rectification arises when a majority point in the same direction.

Acknowledgments We thank Prof. P. Karagiannis for carefully revising the manuscript. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Innovative Nanoscience of Supermolecular Motor Proteins Working in Biomembranes, Molecular Science of Fluctuations toward Biological Functions and Molecular System Life Science).

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