On the Origin of Closing Flickers in Gramicidin Channels: A New Hypothesis

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ABSTRACT The submillisecond closing events (flickers) and the single channel conductances to protons (g_H) were studied in native gramicidin A (gA) and in the SS and RR diastereoisomers of dioxolane-linked gA channels in planar bilayers. Bilayers were formed from glycerylmonooleate (GMO) in various solvents. In GMO/decane (thick) bilayers, the largest flicker frequency occurred in the SS channel (39 s^{-1}), followed by the RR (4 s^{-1}) and native gA channels (3 s^{-1}). These frequencies were attenuated in GMO/squalene (thin) bilayers by 100-, 30-, and 70-fold in the SS, RR, and native gA channels, respectively. In thin bilayers, the average burst duration of native gA channels was 30-fold longer than in thick bilayers. The RR dioxolane-linked gA dimer "inactivated" in GMO/decane but not in squalene-containing bilayers. The mean closed time of flickers (~0.12 ms) was essentially the same in various gA channels. In thin bilayers, g_H values were larger by ~10% (SS), 30% (RR), and 20% (native gA) in relation to thick bilayers. It is concluded that flickers are not related to pre-dissociation or dissociation states of gA monomers, and do not seem to be caused by intrinsic conformational changes of channel proteins. It is proposed that flickers are caused by undulations of the bilayer that obliterate the openings of gA channels. Differences between flicker frequencies in various gA channels are likely to result from variations in channel geometries at the bilayer/channel interface. The smaller g_H in thick bilayers suggests that the deformation of these bilayers around the gA channel creates a diffusional pathway next to the mouths of the channel that is longer and more restrictive than in thin GMO bilayers. A possible molecular interpretation for these effects is attempted.

INTRODUCTION

Gramicidin A (gA) is a pentadecapeptide whose primary structure consists mostly of an alternating sequence of D- and L-amino acids (Sarges and Witkop, 1965). In lipid bilayers, the association via H-bonds between the amino termini of two gA monomers located in distinct monolayers causes the formation of an ion channel that is selective for monovalent cations only (Andersen, 1984; Hladky and Haydon, 1972; Urry et al., 1971). The average lifetime of native gA channels in the open state depends on experimental conditions that ultimately affect the dimerization process. Such conditions include the qualitative and quantitative composition of ionic solutions, the nature of lipid bilayers, temperature, and transmembrane voltage. The average open state in native gA channels is within the time scale of tens of milliseconds to seconds (Elliot et al., 1983; Hladky and Haydon, 1972; Kolb and Bamberg, 1977; Ring and Sandblom, 1988a, b; Sigworth and Shenkel, 1988). The closed time of native gA channels can not be measured reliably because it depends on several unknown parameters, such as the concentration and dynamics of gA monomers in distinct monolayers. A distinct gating mode with considerably shorter open and closed times is present in recordings of native gA channels. Once the functional gA channel is assembled, fast closure events (flickers) occur in the submillisecond time scale (Ring, 1986; Sigworth and Shenkel, 1988; Sigworth et al., 1987). Overall, the gating of native gA channels occurs in bursts of activity that resembles several other biological ion channels (Ring, 1986).

gA molecules can be covalently linked using simple chemical groups. These proteins also form ion channels in lipid membranes. Desformylated gAs were covalently linked via a malonyl (Bamberg and Janko, 1977; Urry et al., 1971) or glutaryl (Rudnev et al., 1981). As anticipated (Urry et al., 1971), the average lifetime of the open state of these dimers is several orders of magnitude longer than in native gA channels. Stankovic et al. (1989) devised an interesting and insightful approach to dimerize gAs. These authors have used a dioxolane linker to bridge two gA molecules. In these semisynthetic channels, two chiral carbons in the dioxolane are present. This permits the individual synthesis of two distinct diastereoisomers, namely the SS and RR dioxolane-linked gA channels (for the sake of simplicity these channels will be referred to in this study as the SS and RR channels; Cukierman et al., 1997; Quigley et al., 1999; Stankovic et al., 1989, 1990). These proteins form ion channels in lipid bilayers with distinct properties. As with other covalently linked gA channels, the average open time of both the SS and RR channels is considerably longer than in native gA channels. Even though the open probabilities of the SS and RR channels are $> 95\%$, closing flickers with an average duration in the submillisecond time scale were identified (Armstrong et al., 2001; Cukierman et al., 1997; Quigley et al., 1999; Stankovic et al., 1989, 1990).

Concerning the gating of dixolane-linked gA channels, results from our laboratory have been in apparent experimental conflict with Stankovic's results. First, Stankovic et

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al. (1989, 1990) demonstrated that in HCl solutions, the RR is apparently stable in lipid bilayers. In our experiments, once the RR channel is detected in lipid bilayers of various compositions, it remains in the open state for 1–5 min and dwells in an apparently closed "inactivated" state (Quigley et al., 1999). This does not occur, however, in CsCl or KCl solutions (Armstrong et al., 2001). Second, Stankovic et al. identified flickers in the RR but not in the SS channel. The origins of these flickers were attributed to the rotation of the RR dioxolane inside the pore of the channel that would block ion permeation. In single-channel recordings, these blocking events are seen as channel closures (Crouzy et al., 1994; Stankovic et al., 1990). In contrast, Armstrong et al. (2001) demonstrated that the fast flickers in the RR channel can not be caused by the flipping of the dioxolane group inside the channel. Third, in our hands the SS channel has intense flickering activity (Cukierman et al., 1997; Quigley et al., 1999). As with the RR, those flickers can not be attributed to the flipping the dioxolane linker inside the pore of the channel (Armstrong et al., 2001). One significant methodological difference between Stankovic's work and ours concerns the use of the bilayer solvent. Decane has been systematically used in our experiments whereas Stankovic et al. used squalene.

gA channels are ~25 Å long, of which ~22 Å corresponds to the hydrophobic length of the channel (Elliot et al., 1983). The thickness of glycerylmonooleate (GMO) membranes depends on the solvent used to form the planar lipid bilayer. It is ~37 Å in squalene, 40 Å in hexadecane, and 58 Å in decane (Dilger, 1981; Dilger and Benz, 1985). The corresponding hydrophobic lengths of those bilayers are ~25, 33, and 48 Å, respectively. Evidently, the function of gA channels (and of virtually any transmembrane protein) depends on the appropriate matching of the hydrophobic moieties of both gAs and the lipid bilayer (Hendry et al., 1978; Hladky and Haydon, 1972; Helfrich and Jakobsson, 1990; Huang, 1986; Elliot et al., 1983; Killian et al., 1998; Kolb and Bamberg, 1977; de Planque et al., 1998; Ring, 1996; van der Wel et al., 2000). Considering that the thickness of GMO bilayers is considerably larger than the length of a gA channel, the matching of the hydrophobic portions of membrane and channel must occur at the expense of a significant deformation of regions of the lipid bilayer adjacent to the mouths of gA (Helfrich and Jakobsson, 1990; Huang, 1986; Killian et al., 1998; de Planque et al., 1998; Lundbæk and Andersen, 1994; Lundbæk et al., 1996, 1997; Ring, 1996). The influence of bilayer deformation on the average duration of bursts in native gA channels has been well documented. Experimental manipulations that would decrease the deformation energy of lipid bilayers significantly enhance the average burst duration of open native gA channels (Lundbæk and Andersen, 1994; Lundbæk et al., 1996, 1997).

In this study, dioxolane-linked gA channels have been reconstituted in GMO bilayers formed with either decane or squalene as the solvent, and in a few experiments with hexadecane. Our major question concerned the molecular basis for the flickering activity in the SS and RR channels. The inactivation of the RR channel in HCl solutions in GMO bilayers was also addressed. Although it has been previously shown that flickering activity in native gA channels is modulated by bilayer/solvent thickness (Ring, 1996; Sigworth and Shenkel, 1988), no such studies were conducted with covalently linked gA channels. Because these channels can not dissociate, they offer an interesting opportunity to ponder on the interactions between gA monomers for the flickering activity in gA channels.

MATERIALS AND METHODS

Bilayers

GMO was purchased either from Sigma (St. Louis, MO) or from Nu-Check (Elysian, MN). Indistinguishable results were obtained regardless the source of GMO. Decane, squalene, and hexadecane were purchased from Sigma. The solvents were further purified in a column consisting of neutral (bottom of the column), acid and basic alumina (Sigma). GMO was prepared at a concentration of ~60 mg/ml. Planar lipid bilayers were formed by the painting method on a circular hole (~0.15 mm) in a plastic partition separating two compartments containing 1 M HCl. Experiments were performed at 23–25°C.

gA channels

Native gA channels were obtained from Fluka (Milwaukee, WI). The SS and RR dioxolane-linked channels were synthesized, purified, and characterized as previously described (Cukierman et al., 1997; Quigley et al., 1999; Stankovic et al., 1989). These channels were added to the experimental bath from a methanol stock solution.

Electrical recordings

Single-channel recordings were obtained with a List EPC7 (List Elektronic, Darmstadt, Germany) in the voltage-clamp mode with an applied transmembrane voltage of 50 mV. Recordings were low-pass Bessel filtered (Frequency Devices, Haverhill, MA) at 3–5 kHz and transferred to a videocassette recorder for offline analysis.

Dwell time distributions

Open and closed events were defined by a threshold located at half amplitude of the single-channel current. Only events from a single channel were analyzed for the SS or RR channels. The number of events for each analysis of these channels was >2000. For native gA channels, the open and closed events were analyzed inside each burst of activity. In those cases, the number of events was considerably smaller than for the SS or RR channels (~75 events on average). Single-channel recordings were low-pass Bessel filtered at 3 kHz and digitized at 7.5 kHz. For the analysis of flickers, closing events were binned at 60 μs durations, and single exponentials were fit to the bins in the range of 0.06–1.5 ms durations (see Fig. 5).

RESULTS

Fig. 1 shows representative bursts of activity of single gA-channel recordings in GMO/decane bilayers. The aver-

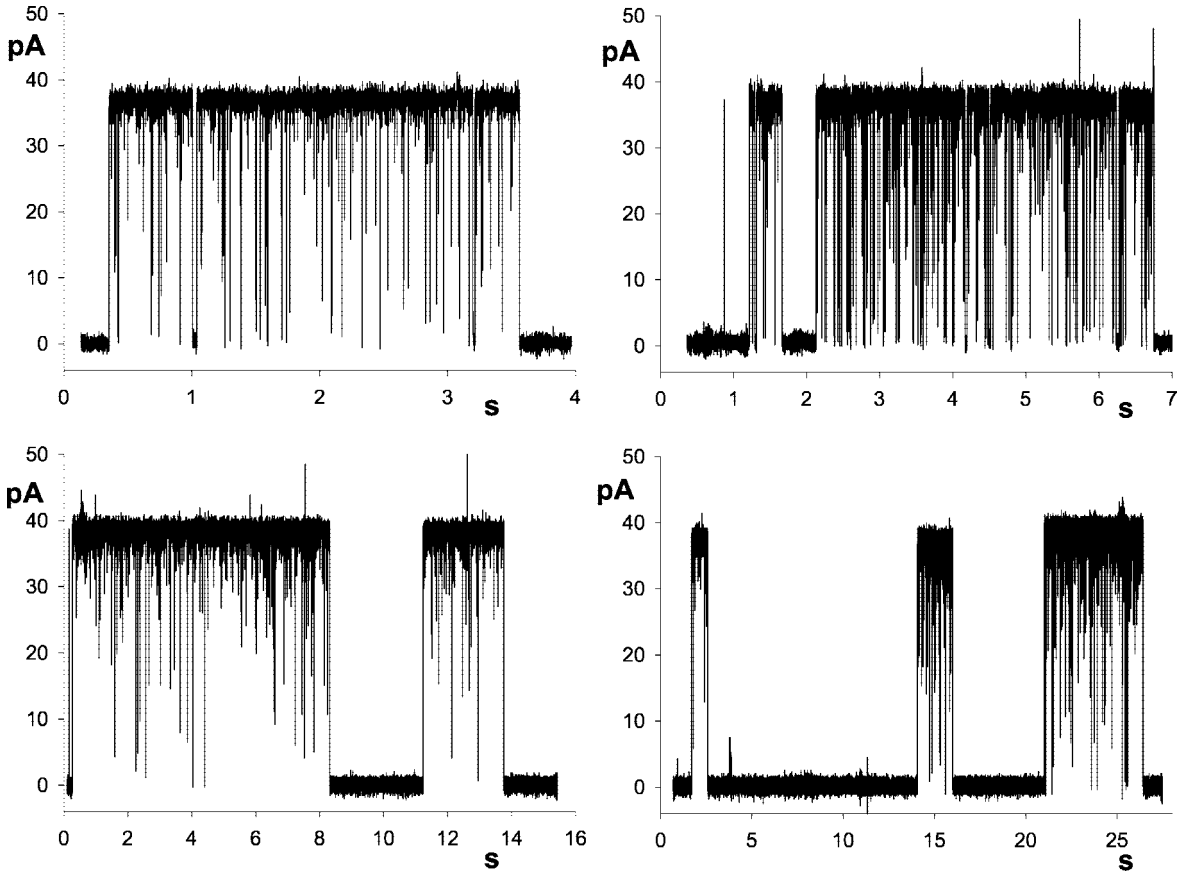

FIGURE 1 Representative bursts of activity in single native gA channels in GMO/decane bilayers in 1-M HCl solutions. Recordings were low-pass filtered at 3 kHz and digitized at 7.5 kHz.

age burst duration was 4.0 ± 0.3 s (mean ± SE, 122 bursts). In good agreement with Ringer's measurements (1986), the average flicker frequency (f) was ~3 s^{-1} (Table 1). Fig. 2 shows recordings of gA channels in GMO/squalene bilayers. Comparing these recordings with those in Fig. 1, the following can be seen. 1) Flickering activity has practically disappeared in bilayers containing squalene. In the upper left panel of Fig. 2 there is the rare presence of flickers even when three gA channels were simultaneously open. In GMO/squalene bilayers f is on average 0.04 s^{-1} (Table 1), which corresponds to a 70-fold reduction as compared with GMO/decane bilayers. 2) The average burst duration of gA

TABLE 1. Nonpaired *t* tests

	1 M HCl	g_H (pS)*	τ_{closed} in ms (n, range)†	f (s^{-1})‡§
SS	GMO/decane	942 ± 26 (8)	0.120 ± 0.004 (5; 0.114–0.125)	39.00 ± 9.9 (9)
	GMO/squalene	1029 ± 23 (7)		0.38 ± 0.11 (8)
RR	GMO/decane	350 ± 12 (18)	0.128 ± 0.020 (4; 0.110–0.150)	4.27 ± 1.42 (14)
	GMO/squalene	462 ± 12 (7)		0.15 ± 0.02 (4)
gA	GMO/decane	791 ± 4 (86)	0.119 ± 0.030 (5; 0.060–0.161)	2.81 ± 0.60 (8)
	GMO/squalene	933 ± 8 (40)		0.04 ± 0.01 (10)

Nonpaired t tests conducted between the following populations:

*$P < 0.039$ (SS in GMO/decane × GMO/squalene); $P < 2.10^{-5}$ (RR in GMO/decane × GMO/squalene); $P < 2.10^{-7}$ (gA in GMO/decane × GMO/squalene).

†Mean closed times were not significantly different between the various gA channels ($P < 0.65 - 0.95$).

‡Comparison between flicker frequencies of any gA channel in either a decane- or squalene-containing GMO bilayer were all found to be significantly different at $P < 10^{-5}$.

§In GMO/decane bilayers, F values were not significantly different between the RR and native gA channels, but were significantly different for the SS versus RR ($P < 10^{-10}$), and SS versus gA ($P < 3 \times 10^{-9}$). In GMO/squalene, f values for the SS and RR channels were not significantly different, and were significantly different for the SS versus gA ($P < 0.004$), and for the RR versus gA ($P < 10^{-4}$).

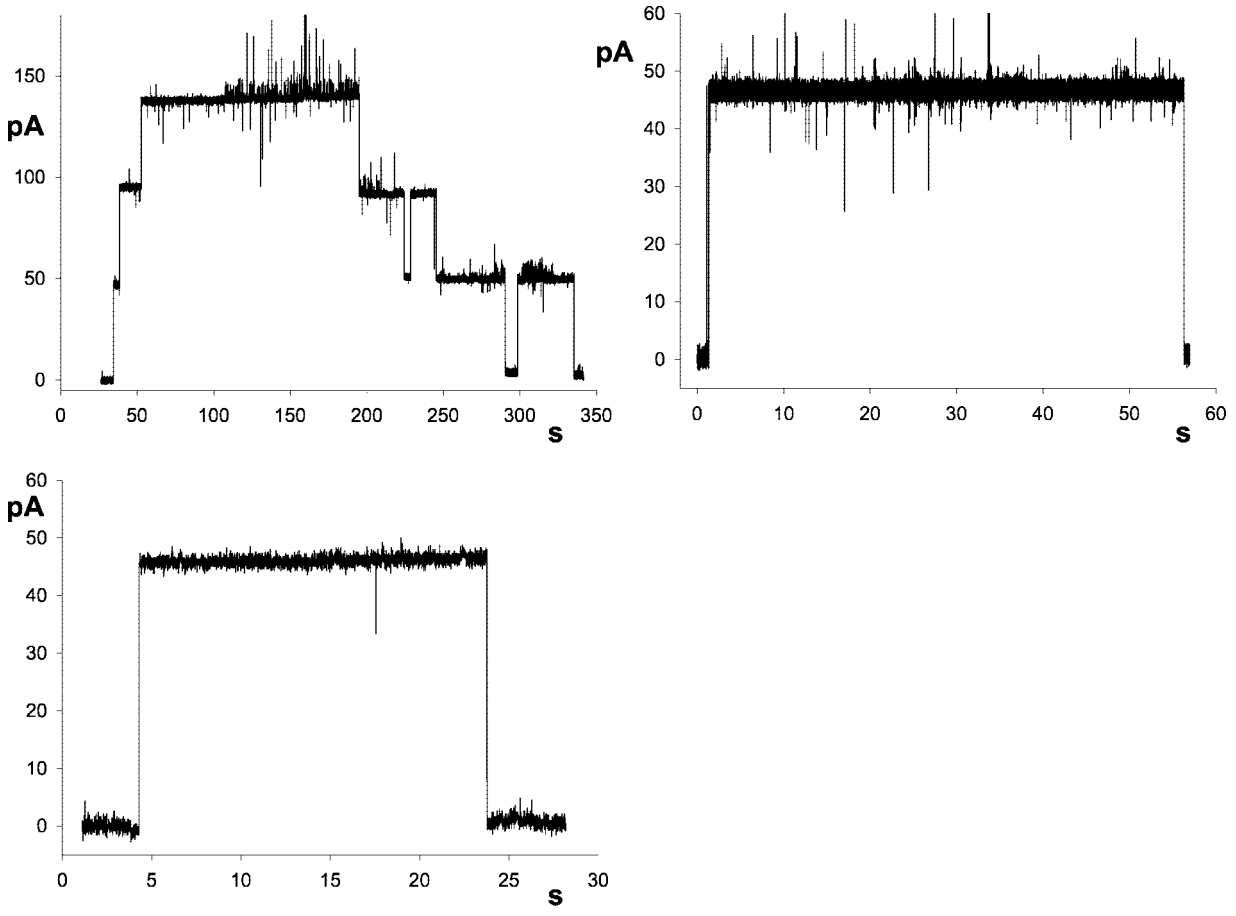

FIGURE 2 Bursts of activity in single native gA channels in GMO/squalene bilayers in 1-M HCl solutions. Notice in the top panel on the left, the overlapping of three open gA channels. Recordings were low-pass filtered at 3 kHz and digitized at 7.5 kHz.

channels in GMO/squalene was 119 ± 22 s (n = 17), which is 30-fold longer than the average duration in GMO/decane bilayers. Interestingly, the flicker frequency is significantly more attenuated (even if only those flickers that cross the 50% level of the single channel current are computed) than the burst duration of gA. 3) The average single channel conductance to protons (g_H) increased by ~18% in squalene in relation to decane-containing membranes (Table 1).

Fig. 3 shows representative segments of recordings of single SS channels in GMO bilayers with decane (*top panels*) or squalene (*bottom panels*). The total duration of the SS channel in the open state is *not* affected by the bilayer solvent. By contrast, and as in native gA, there is a ~100-fold reduction in the average f in squalene in relation to decane-containing GMO bilayers (Table 1). g_H in the SS channel in GMO/squalene is larger than in decane bilayers by ~10% (Table 1).

Qualitatively similar observations described above for native gA and SS channels also apply to the RR channel. These are shown in Fig. 4 and Table 1. The average f in the RR channel decreased by 30-fold, and g_H increased by 30% in GMO/squalene in relation to GMO/decane bilayers. Most significant is the fact that RR channels do *not* inactivate in GMO/squalene in HCl solutions. Single RR channels in GMO/squalene were followed for periods longer than 15 min. The stability of the open state of single RR channels lasts for ~1–5 min in GMO or phospholipid/decane bilayers (Armstrong et al., 2001; Quigley et al., 1999).

A typical analysis of dwell time distributions is shown in Fig. 5. The mean dwell time distributions of the closed state and the frequency of flickers ($f \approx$ 1/mean open state) were compiled in Table 1. The average of mean closed times was ~0.12 ms and was not significantly different between the various gA channels. Because of the low frequency of closures in the various gA channels in GMO/squalene bilayers, a dwell time analysis could not have been performed as in decane-containing bilayers. However, a close inspection of single closures in various gA channels in GMO/squalene bilayers revealed no marked difference from those recorded in GMO/decane bilayers.

There is a significant variability in the frequency of flickers for any combination of gA channel and lipid bilayer. The standard deviation of f is comparable with its corresponding average. Despite this variability, some gA

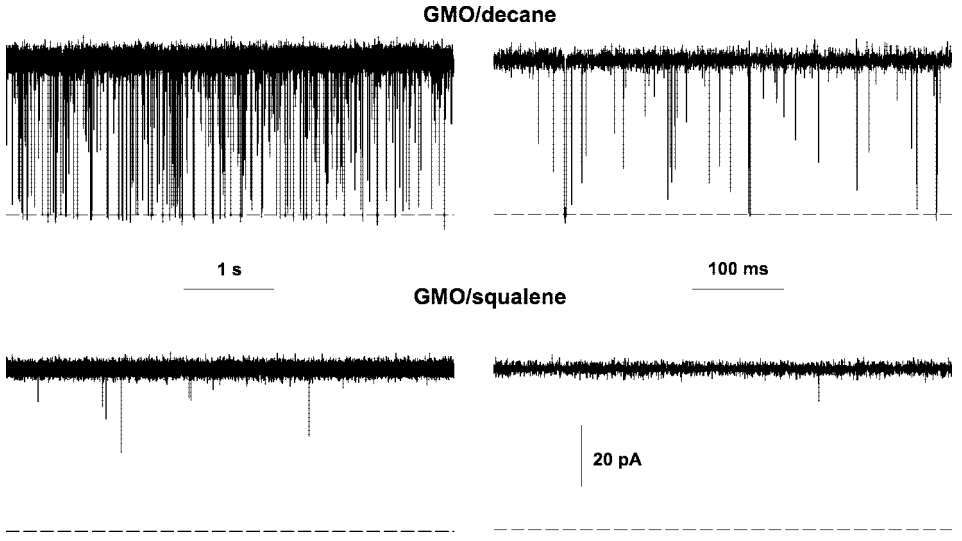

FIGURE 3 Representative recordings of SS channels in GMO/decane (*top recordings*) and GMO/squalene bilayers (*bottom recordings*). Notice the different horizontal calibration marks for the recordings on left and right panels. Recordings were low-pass filtered at 3 kHz and digitized at 7.5 kHz.

channels have significantly larger f values than others, and, most importantly, differences between f values in any gA channel in GMO/decane versus GMO/squalene bilayers are significantly different (Table 1).

The results presented thus far were obtained in GMO bilayers with either decane or squalene as the solvent. To address the issue of a possible effect of squalene on the closing flickers of gA channels, experiments were performed in GMO bilayers with hexadecane as the solvent. The structure of hexadecane is quite different from squalene, and the hydrophobic length of a GMO/hexadecane bilayer lies between GMO/decane and GMO/squalene bilayers (see Introduction). In Fig. 6, a stretch of a recording of a single SS channel is shown in a GMO/hexadecane bilayer. In comparing this with Fig. 3 recordings, it can be seen that f in GMO/hexadecane was larger than in squalene but smaller than in GMO/decane bilayers. For the single-channel recording in Fig. 6, f was $\sim 10\ s^{-1}$. g_H in Fig. 6 was 1040 pS, which is considerably larger than in GMO/decane bilayers and approximately the same as in GMO/squalene. Quantitatively similar results as shown in Fig. 6 for f and g_H were obtained with two other SS channels, and qualitatively similar conclusions also apply for RR channels (results not shown). Whereas the potential effect of solvents on closing flickers can not be entirely eliminated, the experimental results presented in this study are consistent with the idea that the bilayer thickness is the significant variable in determining f (see Discussion).

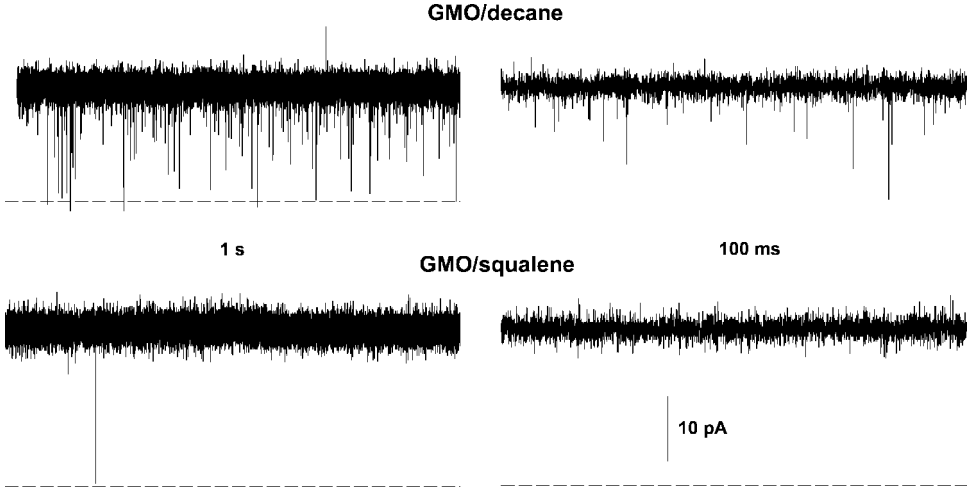

FIGURE 4 Recordings of RR channels in GMO/decane (*top recordings*) and GMO/squalene bilayers (*bottom recordings*). Notice the different horizontal calibration marks for the recordings on left and right panels. Recordings were low-pass filtered at 3 kHz and digitized at 7.5 kHz.

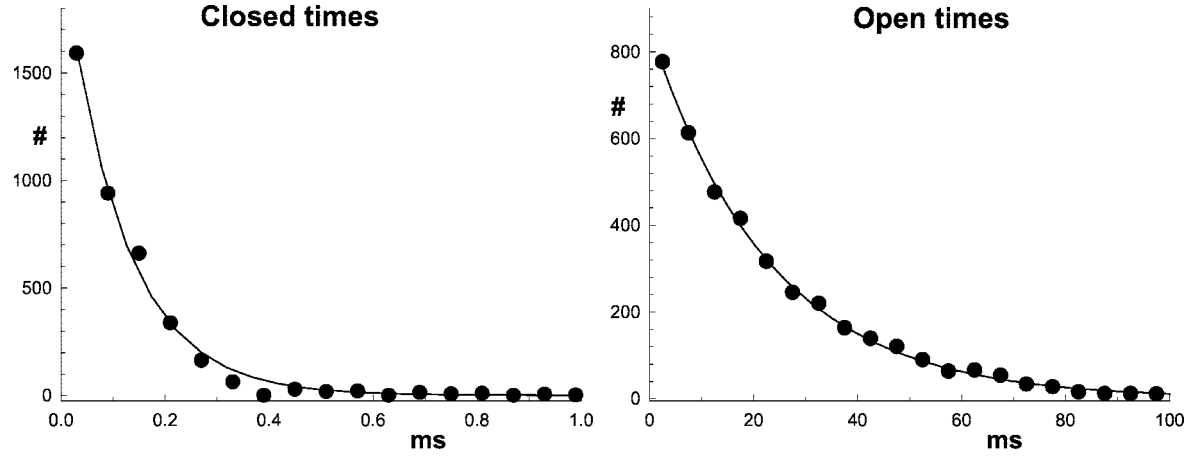

FIGURE 5 Typical dwell time distributions for the closed and open times of an SS channel in a GMO/decane biayer. Exponentials drawn to the dwell times are best fits to experimental points with time constants of 0.114 ms (closed times), and 22.91 ms (open times).

DISCUSSION

The novel experimental results in this study are as follows. 1) The average f in gA channels decreased in the order $f_{SS} > f_{RR} \geq f_{gA}$. 2) In GMO/squalene the average f was attenuated by 100-, 70-, and 30-fold in the SS, native gA channels, and RR channels, respectively, in relation to GMO/decane bilayers. 3) g_H is larger in GMO/squalene than in GMO/decane bilayers. 4) In HCl solutions, the RR channel does not inactivate in GMO/squalene bilayers. 5) The mean closed time of flickers is ~0.12 ms for the various gA channels. We have also confirmed that the average burst duration in native gA channels is 30-fold longer in GMO/squalene than in GMO/decane bilayers.

The hydrophobic lengths of GMO/decane, GMO/squalene bilayers, and gA channels are 48, 25, and 22 Å, respectively (Dilger, 1981; Dilger and Benz, 1985; Elliot et al., 1983; Hendry et al., 1978). The stability of the open state in native gA channels depends on the appropriate matching between the hydrophobic region of bilayer and the side chain residues of gA (Elliot et al., 1983; Hendry et al., 1978; Hladky and Haydon, 1972; Killian et al., 1998; Kolb and Bamberg, 1977; Lundbæk and Andersen, 1994; Lundbæk et al., 1996, 1997; de Planque et al., 1998; Ring, 1996; van der Wel et al., 2000). In thick bilayers, this matching must be achieved by a significant deformation of the bilayer in regions adjacent to the channel's mouths (Helfrich and Jakobsson, 1990; Huang, 1986; Ring, 1996). Because the energy of deformation increases with the difference between the hydrophobic lengths of bilayer and channel, the hydrophobic matching of channel/bilayer is energetically more favorable and likely to occur in GMO/squalene (thin) than in GMO/decane (thick) bilayers.

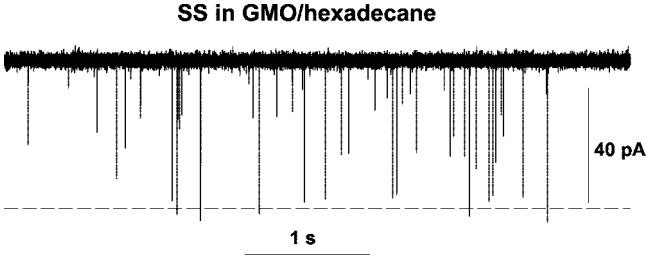

FIGURE 6 (*Top*), Recordings of a single SS channel in a GMO/hexadecane bilayer. Recording was low-pass filtered at 3 kHz and digitized at 7.5 kHz. The flicker frequency in this experiment was ~10 s^{-1}.

In discussing the gating of gA channels, two different phenomena must be considered. The first concerns the duration of a burst in native gA channels. The elastic tension accumulated during the compression of the bilayer around a gA channel is eventually released leading to the local expansion of the bilayer. If this expansion is significant, gA monomers in native gA channels will be pulled apart and dissociate (end of the burst). Experimental maneuvers that decrease the energy of deformation of the bilayer around native gA channels cause a significant increase in the average burst duration (Lundbæk and Andersen, 1994; Lundbæk et al., 1996, 1997). In consonance with other experimental results (Elliot et al., 1983; Hendry et al., 1978; Hladky and Haydon, 1972; Kolb and Bamberg, 1977), we have shown that the average burst duration in native gA channels is 30-fold longer in GMO/squalene than in GMO/decane bilayers.

Because the SS and RR channels can not dissociate and remain in the open state for very long durations (over the years, single SS channels in the open state were tracked for hours), these channels keep the bilayer constantly deformed around their channel mouths. Consequently, the deformation of the bilayer around a dioxolane-linked gA channel is less energetic than the exclusion of the channel from the lipid bilayer or burying the channel inside the membrane.

The second category of gating events is comprised of very brief closing flickers that were identified in native gA

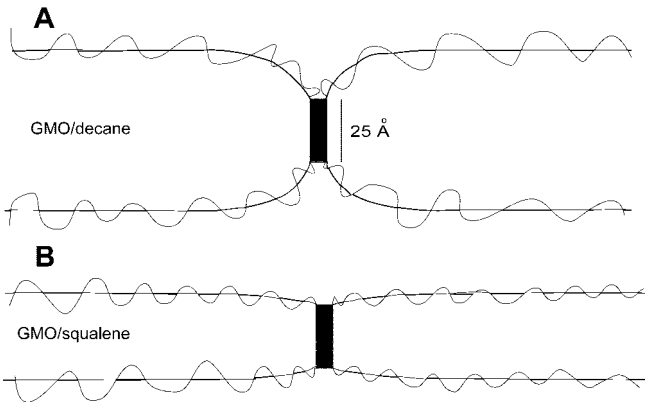

FIGURE 7 Representation of gA channels in thick and thin GMO bilayers. The freely drawn undulations of the bilayer were superimposed on an average thickness. The channel is represented by a black rectangle. (*A*) Two undulations coalesce and obliterate the channel mouth.

channels (Ring, 1986). Their frequency was considerably reduced in thin GMO bilayers (Ring, 1986; Sigworth and Shenkel, 1988). In this study, this has been confirmed. Most significant, however, is that we have now extended these observations to the SS and RR dioxolane-linked gA channels. In this regard, two meaningful conclusions are: 1) closing flickers are not likely to relate to instabilities between gA monomers (pre-dissociation or dissociation states of native gA channel); and 2) flickers in dioxolane-linked (and also native) gA channels result *not* from intrinsic conformational changes in the SS or RR channels (Armstrong et al., 2001), but from interactions between channel and bilayer.

A hypothetical mechanism for flickers in gA channels

There is a considerable compression of a thick bilayer around gA channels (Helfrich and Jakobsson, 1990; Huang, 1986; Ring, 1996). This compression develops relatively long and narrow vestibules connecting the mouths of the pore to the external solutions (Fig. 7 *A*). Bilayers are not static structures with a homogeneous thickness along their length. On the contrary, the energy involved in thermal undulations of the bilayer surfaces is of a few kTs only (Bach and Miller, 1980; Helfrich and Jakobsson, 1990; Hirn et al., 1998; Sackmann, 1994). Considering that the diameter of the mouth of the channel (and of the narrowest portion of the vestibule) is ~4 Å, some of these undulations may be large enough (Hirn et al., 1998; Sackmann, 1994) to obliterate partially or completely a section of the narrow channel vestibules in a thick bilayer (Fig. 7 *A*). The lumen of the vestibule could also be obliterated by overlapping undulations that originate from distinct regions in the vestibule. Thus, single channel currents will be blocked. In contrast, in thin bilayers there would be a small or negligible deformation of the bilayer adjacent to a gA channel (Fig. 7 *B*). The vestibules connecting the mouths of the channel to external solutions are small or nonexistent and, consequently, the probability of a membrane undulation overlapping completely the mouth of the channel would be small compared with a thick bilayer. This could explain the significant attenuation of f in GMO/squalene bilayers.

Variation of f in various gA channels

We have found that on average, $f_{SS} > f_{RR} \geq f_{gA}$. There must be a range of intermonomeric distances in which native gA channels remain in the open state (Elliot et al., 1983; Huang, 1986; Ring, 1996). It is also likely that the open state in native gA channels results not from a single but from distinct topological arrangements between gA monomers in the membrane. In contrast, the dioxolane linker provides a *constrained* transition between gA monomers (Quigley et al., 1999; Stankovic et al., 1990). On average, the intermonomeric distance in gA dimers is shorter, its fluctuation constrained, and considerable less degrees of freedom exist between gA monomers in relation to native gA channels. In view of these geometrical distinctions, it may be easier for the bilayer (less deformation around the channel) to hydrophobically shield native gA or RR channels than the SS. This would ultimately reflect in the variability of f among various gA channels.

Inactivation of the RR channel is absent in GMO/squalene bilayers

We have systematically noticed over the years that the typical lifetime of an RR channel in HCl solutions in either GMO or phospholipid/decane bilayers is ~1–5 min. This inactivation does not occur however, when alkalines are the permeating cations (Armstrong et al., 2001). We have learned that in GMO/squalene bilayers and in HCl solutions the RR channel lasts considerably longer and does not seem to inactivate. Evidently, we do not have a complete picture of the inactivation of the RR channel. However, it seems that there is a conformation of the RR channel that is not properly hydrophobically shielded by GMO/decane bilayers. Such a conformation leading to inactivation can not occur when alkaline metals are the permeating cations.

g_H in GMO/squalene bilayers is larger than in GMO/decane

The single channel conductances to alkaline metals in native gA channels are basically the same in GMO bilayers with different thicknesses (Hladky and Haydon, 1972; Kolb and Bamberg, 1977; Rudnev et al., 1981; Sigworth and Shenkel, 1988). In this study, it was found that g_H is larger in GMO/squalene than in GMO/decane bilayers by 10% (SS),

30% (RR), and 20% (native gA channels). Even though a more detailed analysis of this effect is deserved, a brief comment is in order. Because g_H in gA channels is quite high, the access resistances of the channel to H^+ may have a significant effect on it (Cukierman, 1999, 2000; Quigley et al., 1998). Geometries of the vestibules facing the entry and exit sides of gA channels are significantly different between thin and thick bilayers (Fig. 7). A large deformation of thick bilayers would create long vestibules adjacent to the mouths of the channel. If we assume that proton transfer inside gA channels is not affected by the thickness of the bilayer, the relatively smaller g_H in thick bilayers suggests that a longer and more restrictive diffusional path for protons to access the channel is present in these bilayers. In the case of alkalines at relatively high concentrations, differences between access resistances in thick and thin bilayers are small compared with the large intrinsic resistance of the channel to those cations (g_H is ~2–3 orders of magnitude larger than single channel conductance to alkalines). This would explain the lack of effects of bilayer thickness on single channel conductances to alkalines.

Proton transfer in aqueous solutions occurs by a specific hop-and-turn mechanism known as a Grotthuss mechanism (Cukierman, 2000). OH groups from GMO protrude into the lumen of the vestibules created by the deformation of thick bilayers adjacent to the mouths of the channel. The waters inside the vestibules are likely to donate and accept H-bonds from these hydroxyl groups. Considering the width of the vestibule at the narrowest region (~4 Å), these H-bonds would be quite strong. This extensive and strong coordination of vestibule waters with OHs could retard proton hop and/or the reorientation of water molecules (turn step). This would reduce the rate of proton transfer into and out of the channel.

Comparison with previous experimental results

Experimental discrepancies with the original studies by Stankovic at al. (1989, 1990) were pointed out in previous studies (Cukierman, 2000; Cukierman et al., 1997; Quigley et al., 1999). In this paper, the SS and RR channels were studied in GMO/squalene bilayers. Some of the previously identified experimental discrepancies were eliminated: 1) a major reduction (but not abolishment) of flickers in the SS channel in GMO/squalene bilayers; and 2) the RR channel does not inactivate in GMO/squalene bilayers. The continuing experimental discrepancies are: 1) the intense flickering activity reported for the RR channel in HCl (100 s^{-1}, Stankovic et al., 1990) has not been corroborated over the years; and 2) Stankovic et al. (1990) proposed that flickering in the RR channel is caused by a rotation of the RR dioxolane that obliterates the pore of the channel. In contrast, we have found that the flickering activity in the SS or RR channels is essentially a consequence of bilayer deformation around channels. Furthermore, flickers did not disappear when a bulky hydrophobic molecule (retinal) was attached to the dioxolane linker in the SS or RR channels (Armstrong et al., 2001). This would prevent the rotation of the dioxolane inside the lumen of the pore. Whereas our experiments have been performed with planar bilayers, Stankovic et al. have used a variation of the patch-clamp technique. Bilayers formed by this technique are under considerable more tension than planar bilayers. Perhaps, this could account for the remaining experimental disagreements between our experimental results.

In summary, it is proposed that in thick bilayers there is a relatively long and narrow vestibule connecting the mouths of the channel to the external solutions. This restricted diffusional space would account for a decreased g_H in various gA channels in GMO/decane in relation to GMO/squalene bilayers. The undulations of the bilayer in those vestibules could obliterate the access to the channel causing brief flickers. Differences in geometries among various gA channels could ultimately affect the way these channels are shielded hydrophobically by the bilayer, and as such, determine the variability of f in gA channels.

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