Covalently Linked Gramicidin Channels: Effects of Linker Hydrophobicity and Alkaline Metals on Different Stereoisomers

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ABSTRACT The direct role of the dioxolane group on the gating and single-channel conductance of different stereoisomers of the dioxolane-linked gramicidin A (gA) channels reconstituted in planar lipid bilayers was investigated. Four different covalently linked gA dimers were synthesized. In two of them, the linker was the conventional dioxolane described previously (SS and RR channels). Two gAs were covalently linked with a novel modified dioxolane group containing a retinal attachment (ret-SS and ret-RR gA dimers). These proteins also formed ion channels in lipid bilayers and were selective for monovalent cations. The presence of the bulky and hydrophobic retinal group immobilizes the dioxolane linker in the bilayer core preventing its rotation into the hydrophilic lumen of the pore. In 1 M HCl the gating kinetics of the SS or RR dimers were indistinguishable from their retinal counterparts; the dwell-time distributions of the open and closed states in the SS and ret-SS were basically the same. In particular, the inactivation of the RR was not prevented by the presence of the retinal group. It is concluded that neither the fast closing events in the SS or RR dimers nor the inactivation of the RR are likely to be a functional consequence of the flipping of the dioxolane inside the pore of the channel. On the other hand, the inactivation of the RR dimer was entirely eliminated when alkaline metals (Cs⁺ or K⁺) were the permeating cations in the channel. In fact, the open state of the RR channel became extremely stable, and the gating characteristics of both the SS and RR channels were different from what was seen before with permeating protons. As in HCl, the presence of a retinal in the dioxolane linker did not affect the gating behavior of the SS and RR in Cs⁺- or K⁺-containing solutions. Alternative hypotheses concerning the gating of linked gA dimers are discussed.

INTRODUCTION

Gramicidin A (gA) is a hydrophobic pentadecapeptide secreted by Bacillus brevis. Its alternating sequence of D and L amino acids (Sarges and Witkop, 1965) defines a right-handed single-stranded β6,3 helical motif (Arseniev et al., 1985; Ketchem et al., 1993, 1997; Urry, 1971; see Burkhart et al., 1998, for different results and interpretation, and the discussions by Andersen et al., 1999, Cross et al., 1999, and Burkhart and Duax, 1999, on gA structure). In lipid membranes, the association between the amino termini of two gAs in lipid bilayers is established by six intermolecular H-bonds. The resulting dimer forms an ion channel in lipid membranes that is selective for monovalent cations only (Busath, 1993; Cross, 1997; Koepp and Andersen, 1996). The closing of this channel is thought to result from the dissociation of monomers following the disruption of one or more intermolecular H-bonds.

Two gA molecules have been covalently connected via their N-termini using different linkers (Bamberg and Janko, 1977; Urry et al., 1971). As predicted, the mean open times in those channels are considerably longer than in native gA. This is taken as evidence for the dimeric nature of gA channels in lipid bilayers (Urry et al., 1971). Stankovic et al. (1989) have used a dioxolane group to covalently link two gAs. Two different stereoisomers of the dioxolane-linked gA dimers can be synthesized (the SS and RR dimers). This ingenious and novel approach is appealing. First, because these different channels can be investigated in various experimental conditions. Second, discrete atomic modifications can be introduced in or to the dioxolane linker without a major impact on the overall protein structure or on its channel-forming capability (see Stankovic et al., 1990; present results). And third, because of its relatively small number of atoms, gA dimers can be modeled at a relatively high level of detail (Chiu et al., 1999; Duca and Jordan, 1997; Lee and Jordan, 1984; Mackay et al., 1984; Roux and Karplus, 1991a,b; Woolf and Roux, 1996). Consequently, the functional differences resulting from those simple manipulations could in principle be rationalized at the atomic level. Thus, dioxolane-linked gA is a useful model to probe the fine physical chemistry of structure-function relationships in ion channels.

The biophysical properties of the SS and RR dimers have been studied in our laboratory under different experimental conditions (Cukierman, 1999, 2000; Cukierman et al., 1997; Quigley et al., 1998, 1999, 2000). In particular, we have been dissecting the properties of H⁺ currents in different planar lipid bilayers. Our experimental results can be partially summarized as follows. 1) The single-channel conductance to protons (gH) in the SS dimer is considerably larger than in the RR. Qualitative differences between the current-voltage relationships in the SS and RR were also noted (Cukierman, 2000; Quigley et al., 1999). 2) The SS...
dimer is stable in planar lipid membranes and has very fast and incompletely resolved closing events. The average lifetime of a functional SS channel is essentially determined by the integrity of the lipid bilayer (Cukierman et al., 1997; Quigley et al., 1999). 3) In contrast, the RR dimer is not stable in bilayers. The average lifetime of a conducting RR channel in planar lipid bilayers is \( \sim 2-4 \) min in HCl solutions (Cukierman, 1999; Quigley et al., 1999).

The present study was motivated by the suggestion of Stankovic et al. (1989) concerning the fast closing events in the RR dimer (in their work, the SS dimer did not show a significant number of closures, see Discussion). Those authors proposed that the rotation of the RR dioxolane linker from outside to the inside of the pore lumen (trans-isomerization reaction (Crouzy et al., 1994)) could provide a structural basis for the fast closures in the RR dimer. The presence of the dioxolane linker inside the channel would obstruct the lumen of the pore interrupting the transport of water and cations (see discussion in Cukierman, 2000; Quigley et al., 1999). The rotation of the dioxolane from outside to inside the pore can be prevented by the high energetic cost of accommodating a bulky and modified dioxolane linker inside the hydrophilic pore. In consonance with this idea, Stankovic et al. (1990) synthesized an RR dioxolane linker modified by the addition of two methyl groups. The resulting covalently linked \((\text{CH}_3)_2\)-RR dimer exhibited a significant decrease in the number of open→closed transitions in relation to the regular \(\text{H}_2\)-RR dimer. It was concluded that the fast closing events in the RR dimer may be caused by the rotation of the RR dioxolane linker inside the pore of the channel. Subsequently, Crouzy et al. (1994) performed a molecular dynamics study of the trans-isomerization process of the dioxolane linker in the SS and RR dimers. They concluded that the energy for the rotation of the SS dioxolane from outside to inside the pore is very high, thus explaining the lack or reduced number of closures in the SS dimer as shown by Stankovic et al. (1989) in their bilayer experiments (see end of Discussion). In the RR, however, said rotation is feasible at room temperature, thus explaining the closures of the RR in the experiments of Stankovic et al. (1989, 1990).

In the present study, a \textit{trans}-retinal molecule was attached to both the SS and RR dioxolane-linked gA dimers (ret-SS and ret-RR, respectively). The massive and hydrophobic nature of the retinal group (see Fig. 1) would prevent the dioxolane linker from partitioning inside the pore. Thus, if the closing of the channel in the SS or in the RR dimers and/or the inactivation of the RR channel is indeed caused by the flipping of the dioxolane to the inside of the pore, then the attachment of a retinal molecule to that linker would prevent said gating phenomena. The results presented in this paper are divided in two parts. In the first part, the effects of inserting a \textit{trans}-retinal molecule in the dioxolane linker in both the SS and RR dimers are described. We show that \(g_{\text{H}}\) values in the different dioxolane-linked dimers are basically the same or slightly larger than in the retinal-attached dioxolane dimers. Most importantly, perhaps, are the experimental observations that both the fast closing events in the SS and RR dimers as well as the inactivation of the RR dimer still persist in those covalently linked dimers with the retinal attachment. In the second part, it is shown that in CsCl (or KCl) solutions the inactivation of the RR dimer is absent. We hypothesize that inactivation is a property of the RR channel when occupied by a proton. Quite interestingly, in contrast to the behavior in HCl solutions, the open state of the RR dimer in KCl or CsCl is extremely stable, even more than the open state of the SS channel itself.

MATERIALS AND METHODS

Methods were essentially the same as described in our previous studies (Cukierman et al., 1997; Quigley et al., 1999). In brief, planar lipid bilayers were formed from a mixture of 4 parts of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (PE) and 1 part of 1-palmitoyl-2-oleoyl-phosphatidylcholine (Avanti Lipids, Alabaster, AL). The final phospholipid concentration was \(60\) mM in decane. Experiments were performed at room temperature (22–24°C). Both sides of the bilayer were connected to a List EPC-7 amplifier (List-Electronic, Darmstadt, Germany) via Ag/AgCl wires. Voltage clamp pulses applied across the membrane were either DC or voltage ramps from 0 to \(-350\) mV in \(-7\) s. Single-channel conductances were

![FIGURE 1 Structural formulas of the SS and RR dioxolane-linked gramicidin A and a retinal-attached dioxolane-linked gA dimer.](image-url)
measured from the initial portion of the current-voltage \((I-V)\) relationships. These \(I-V\) plots were obtained after subtracting the lipid bilayer contribution to the total (single channel plus bilayer) conductance. Dell-time distributions in HCl (or CsCl solutions) of open and closed times were measured in single-channel recordings that were low-pass Bessel filtered at 4 kHz (or 200 Hz) and digitized at 10 kHz (or 500 Hz). Transitions between the open and closed states were defined by a threshold located at 50% of the difference between the open and closed channel currents.

The synthesis, purification, and characterization of dioxolane-linked SS and RR dimers were previously described (Cukierman et al., 1997; Quigley et al., 1999; Stankovic et al., 1989). The novel method developed in this study consisted in synthesizing a dioxolane linker with a \(\textit{trans}\)-retinal that covalently linked two gramicidin A molecules (ret-SS and ret-RR).

The native gA used in some of our reactions was thoroughly purified in our laboratory using flash chromatography. Another source of gA (Fluka; Milwaukee, WI) was also used in other reactions. The experimental results obtained with the covalently linked gA were independent of the source of gA. Chemical impurities in the final reaction product were significantly reduced by a long series of solvent extractions during extensive high-performance liquid chromatography (HPLC) runs.

### Synthesis, Characterization, and Purification of ret-SS and ret-RR

The synthesis of diethyl tartrate retinyl acetal (retinal-dioxolane linker) linkers followed a novel and different approach from that previously described for the SS and RR dioxolane linkers (Stankovic et al., 1989; Cukierman et al., 1997). The reaction mechanism of the ring closure differed from the closure of the diol to the diethoxymethane in the RR and SS dioxolane linkers (see Stankovic et al., 1989). The aldehyde group of the \(\textit{trans}\)-retinal was linked in an acetal arrangement with the diols of the diethyl tartrate. The kinetics and yield of this reaction were optimized with cinnamaldehyde. This compound has similar bond conjugation, solvent behavior, and steric hindrance to all-\(\textit{trans}\) retinal. Once the conditions of the reaction were optimized, all-\(\textit{trans}\) retinal was employed for the final synthesis.

Subsequent reactions, purifications, and characterization were performed in the dark. A 1:1 molar ratio of racemic diethyl tartrate and retinal were combined in toluene with catalytic \(\text{NH}_4\text{Cl}\). Reaction was refluxed at 110–120°C for 24 h. The reaction was quenched, cooled, dried with \(\text{K}_2\text{CO}_3\), and filtered. The diethyl tartrate retinyl acetal product was separated from unreacted diethyl tartrate on silica, eluted with 90:100 ether:toluene, and monitored with thin layer chromatography (TLC). The ethyl ester protecting groups of the tartrate were saponified in 1 M \(\text{NaOH}\) and heated at 40–45°C for 1 h. The reaction mixture was neutralized with 0.1 M HCl. The final product (a yellow flaky powder) was extracted with ether. The excess solvent was removed by rotary evaporator. The presence of retinyl in the product and the absence of diethyl tartrate was demonstrated by TLC and NMR.

The gAs were then linked to the diethyl tartrate retinyl acetal (forming the ret-SS and ret-RR dimers) as previously described (Stankovic et al., 1989; Cukierman et al., 1997; Quigley et al., 1999). Retinyl-linked gA was immediately purified by reverse-phase HPLC. The dimers were adequately resolved from gA monomers and desformyl gA. Sequential aliquots from serial HPLC runs were pooled and concentrated. Products were kept in methanol solution in a light-controlled environment at \(-0°C\) until the time of the experiment.

### RESULTS

#### Attachment of retinal to the dioxolane linker: effects on \(g_H\) and gating

Fig. 2 shows continuous single-channel recordings of different SS and RR dimers in planar lipid bilayers. These recordings were obtained in 1 M HCl at a transmembrane potential of 50 mV. Both stereoisomers of the dioxolane-linked dimers and their retinal counterparts share a number of common features. The SS and the ret-SS have very fast (the vast majority of closures cannot be completely resolved) closing flickers (see Fig. 4), their \(g_H\) values are about the same (522 and 519 pS for the SS and ret-SS, respectively; mean \(\pm\) SEM \((n)\): SS, 541 \(\pm\) 9 pS (15); ret-SS, 559 \(\pm\) 7 pS (23)), and they are both stable in lipid bilayers.

![Fig. 2](image-url)
The typical gating pattern illustrated in the top panels of Fig. 2 can last for very long times and is essentially determined by the functionality of the planar lipid bilayer (Cukierman et al., 1997; Quigley et al., 1999). As previously demonstrated for the SS and RR dimers, $g_{H}$ in the ret-RR is also considerably lower than in the ret-SS. In the RR, $g_{H}$ is consistently and significantly larger (~20%) than in the ret-RR (in Fig. 2, 257 vs. 207 pS for the RR and ret-RR, respectively; mean ± SEM (n): RR, 239 ± 4 pS (12); ret-RR 196 ± 5 pS (7); significantly different at $p < 0.01$). Most interestingly, the presence of a retinal attached to the dioxolane linker did not prevent the inactivation of RR channels. Both the RR and the ret-RR have an average lifetime in PEPC bilayers of typically 2–4 min (data not shown).

Single-channel $I$-$V$ plots of different gA dimers are shown in Fig. 3. The essential features of the SS and RR dimers described in previous studies (Cukierman, 2000; Cukierman et al., 1997; Quigley et al., 1999) are conserved in their retinal counterparts. The $I$-$V$ plots in both the SS and ret-SS are sublinear at relatively high voltages (>150 mV) in 1 M HCl solutions in PEPC bilayers. In contrast, both the RR and ret-RR have sigmoid-shaped $I$-$V$ plots. The $g_{H}$ values, as determined by the linear regression of the initial part of the $I$-$V$ plots in this figure (straight lines in $I$-$V$ plots) were, in pS: 529 (SS), 536 (ret-SS), 257 (RR), and 206 (ret-RR). The attachment of a retinal group did not affect overall the morphology of current-voltage relationships in both the SS and RR dioxolane-linked gAs.

In Fig. 4, the dwell-time distributions of the open (top) and closed (bottom) states are shown for the SS (left) and ret-SS (right) dimers. (The relatively short lifetime of the RR channel in a lipid bilayer and the small total number of transitions between the open and closed states precluded a similar dwell-time distribution analysis for the RR dimer.) Dwell-time distributions of both the open and closed states for the different SS dimers could be well fitted with single exponentials, suggesting a minimal gating mode for the SS and ret-SS channels consisting of one open and one closed state (closed $\leftrightarrow$ open). The average mean dwell times for the different SS dimers were as follows: mean open times, 71 ± 3 ms (SS, $n = 5$; range, 67–85 ms), and 67 ± 3 ms (ret-SS, $n = 7$; range, 54–80 ms); and mean closed times, 0.07 ± 0.0005 ms (SS, $n = 5$; range, 0.06–0.09 ms), and 0.06 ± 0.0003 ms (ret-SS, $n = 7$; range, 0.05–0.07 ms). As exemplified by the dwell-time averages, the single experimental result shown in Fig. 4, and the typical recordings in Fig. 2.
(top panels), the differences between average dwell-time distributions in the SS and ret-SS were not meaningful.

**SS and RR channels in CsCl and KCl solutions**

Fig. 5 shows representative samples of continuous recordings of single SS and RR dimers in 1 M CsCl. The middle recording shows a segment of the top recording at an expanded time scale. As shown before in HCl solutions, the SS gated continuously between open and closed states. A significant difference, however, is that most of the closures of the SS channel in CsCl solutions can be completely resolved even at low cutoff frequencies of 200 Hz (the single-channel conductance in Cs+ is considerably smaller than gH, thus demanding a stronger low-pass filtering). The surprising and novel observation was that the RR dimer in CsCl-containing solutions (bottom recording) did not inactivate. In fact, in CsCl, the RR was far more stable in the open state than the SS dimer. Notice that the RR had a smaller number of closing events in relation to the SS dimer. It was not possible to perform a detailed kinetic analysis of the closing events in the RR dimer in the Cs+ solution. Because closures in the RR are rare (Fig. 5, bottom), a very long recording time is necessary to collect a meaningful number of closed events. During those long periods of time, multiple single-channel incorporations into the bilayer occurred, thus precluding the kinetic analysis. Essentially similar results to those shown in Fig. 5 were obtained with retinal-attached gA dimers and in solutions containing K+ instead of Cs+ (results not shown).

*I-V* plots of the SS and RR channels in 1 M CsCl solutions are shown in Fig. 6. In both the SS and RR channels, gCs is linear over a considerable range of voltages. We have found that PEPC bilayers in CsCl (or KCl) solutions are less stable at high voltages than in HCl. This usually limited the measurement of single-channel currents to voltages smaller than ~300 mV. Even though the differences between gCs in the RR and SS were rather small (in contrast to gH), gCs on average was consistently larger in the SS than in the RR dimer (32 vs. 28 pS, respectively; mean ± SEM (n): SS, 32.2 ± 1.2 pS (6); RR, 27.8 ± 0.9 (16)), significantly different at *p* < 0.013).

The dwell-time distributions in the SS dimer in CsCl solutions were also considerably different than in HCl. In Fig. 7, the open and closed times were fitted with single exponentials with time constants of 462 and 21 ms, respec-
tively. The means ± SEM of different single-channel experiments were as follows: \( \tau_{\text{open}} \) 423 ± 80 ms \((n = 5; \text{range, 284–731 ms}) \), and \( \tau_{\text{closed}} \) 26 ± 7 ms \((n = 5; \text{range, 16–50 ms}) \).

In moving from a proton to a Cs\(^+\)-containing solution, \( k_{\text{oc}} \) \((1/\tau_{\text{open}}) \) increased from 14 to 31 s\(^{-1}\) whereas \( k_{\text{co}} \) \((1/\tau_{\text{closed}}) \) had a dramatic decrease from \( 14 \times 10^3 \) to 38 s\(^{-1}\). Because of the heavy low-pass filtering in Cs\(^+\) solutions, it is not possible to ascertain that the very short-duration closures in HCl are absent in the former solution. However, the long-duration closures present with Cs\(^+\) were definitely absent in HCl solutions.

**DISCUSSION**

The novel findings in this study are as follows. 1) The gating of the SS or RR channel was not modulated by the presence of a retinal group attached to the dioxolane linker. Dwell-time distributions of the open and closed states were basically the same in the different SS dioxolane-linked gA dimers in different solutions. 2) In particular, the inactivation of the RR dimer in HCl was not prevented by the attachment of a retinal to the dioxolane linker. 3) In Cs\(^+\) or K\(^+\) solutions, the inactivation of the RR dimer was absent, and the open state of the channel was extremely stable. 4) Channel properties of dioxolane linked gA dimers depend on the nature of the permeating cation.

It was proposed that the rotation of the dioxolane linker from the outside to inside the pore of the gA channel could cause channel closures in the RR (Crouzy et al., 1994; Stankovic et al., 1989, 1990). This possibility was here readdressed with a dioxolane linker attached with a retinal. This is a massive and highly hydrophobic linker that prevents the rotation of the dioxolane from outside to inside the pore.
The closures of the SS or RR dimers or the inactivation of the RR dimer would be abolished or significantly attenuated with a (dioxolane plus retinal) linker if the rotation of the dioxolane was uniquely responsible for said phenomena. The rate constants of closing ($1/\tau_{\text{open}} \approx 14 \text{ s}^{-1}$) and opening ($1/\tau_{\text{closed}} \approx 14 \times 10^3 \text{ s}^{-1}$) are remarkably similar in both the SS and ret-SS, indicating that the closed$\leftrightarrow$open transitions in the SS dimers are not determined exclusively by the flipping of the dioxolane linker inside or outside the channel’s pore. The possibility that the inactivation in the RR dimer is caused by the presence of the dioxolane inside the pore of the channel can also be eliminated by an analogous line of reasoning. Thus, alternative molecular mechanisms must be sought to explain channel closures in both the SS and RR dimers as well as the inactivation of the RR dimer in HCl solutions. A potential mechanism that could underlie the fast closures in dioxolane-linked gA dimers relates to the intrapore chain of H-bonded water molecules (water wire, Nagle and Morowitz, 1978). As previously discussed (Cukierman, 2000, and references therein), the transport of protons inside gA channels depends on the distribution of water molecules inside the pore as well as on the proper H-bond connectivity between them. Because water molecules establish H-bonds with carbonyl oxygens from the gA wall, there should be dynamic fluctuations in the water structure inside the pore as a function of the fluctuations in protein structure itself (Pomes and Roux, 1996). It is thus possible that a dynamical equilibrium coexists between two conformations of the water wire: one in which the water wire is intact and able to transfer protons along the pore of the channel and another one in which the water wire cannot transport protons. This dynamical equilibrium could be seen in single-channel recordings as opening-closing events. Perhaps, in long-run molecular dynamics simulations (Pomes and Roux, 1996) such different ensemble conformations of the H-bonded water chain could be demonstrated. Concerning the inactivation of the RR channel in HCl solutions, our preliminary results with simulated annealing of the SS and RR dimers (S. Cukierman and R. Pomes, work in progress) are revealing that the SS dimer can essentially be represented by a single molecular conformation. The same, however, does not hold for the RR. Different energy minima configurations can be seen with the RR dimer. It is possible that the active and inactive states of the RR correspond to different energy minima structures. Future work will definitely address this hypothesis.

The experimental results in this study also demonstrated that gating in dioxolane-linked gA dimers depends strongly on the nature of the permeating cation. This is a property of native gA channels (Kolb and Bamberg, 1977; Ring and Sandblom, 1983, 1988). The significant new finding was that alkaline metals caused a marked stabilization of the open state of the RR channel, eliminating completely the inactivation that has been always noticed with conducting protons. In contrast, the closed state of the SS dimer was significantly stabilized by alkaline metals via a sharp decrease in $k_{\text{on}}$. Measurements of single-channel conductances and structural information (see, for example, Burkhart et al., 1998; Cross, 1997; Wallace, 1998) demonstrate that alkaline cations interact quite effectively with the carbonyls of gramicidin channels. At this point we can only speculate that these electrostatic interactions, in addition to contributing to the decrease in the single-channel conductances (in relation to $g_{\text{H}}$), could also be responsible for the considerable differences in gating (including RR inactivation) that is observed between H$^+$- and Cs$^+$-containing solutions. Another possibility that must be discussed is a consequence of the essential qualitative difference between proton transport (via Grotthuss mechanism) and other monovalent cations (via conventional hydrodynamic flow) in gA channels. As discussed above (also see Cukierman, 2000), the integrity of a H-bond network in water molecules inside the pore is essential for a Grotthuss-type mechanism to occur. Thus, if the waters inside the RR channel adopt a very stable conformation characterized by interruptions in the H-bonded...
water chain then this will be seen in single-channel recordings as inactivation of the RR channel. With other monovalent cations, it is not likely that the interruption of the water wire would cause inactivation. These issues will be addressed in future computational studies. Finally, because the gating of the SS and RR dimers in CsCl was not affected by the attachment of a retinal group to the dioxolane (results not shown), we can further conclude that flipping of the dioxolane linker is not uniquely responsible for the relatively slow gating mode of the SS channel in the presence of alkaline metals.

An essential point concerns the ratios between different single-channel conductances ($g_{Cs}$ or $g_{H}$) in the SS and RR dimers. Although $g_{H}$ is 200–400% larger in the SS than in the RR (Quigley et al., 1999; Cukierman, 2000), $g_{Cs}$ is ~10% larger in the SS in 1 M CsCl. Because $g_{H}$ is not limited by the hydrodynamic flow of protons as is the case with other monovalent cations, it is likely that $g_{H}$ is far more sensitive to subtle differences in the distribution of waters and H-bond dynamics between waters and carbonyl groups inside the SS or RR channels (Cukierman et al., 1997; Quigley et al., 1999). This makes the SS and RR dimers useful models to explore the basis of differences in $g_{H}$ in ion channel proteins. These studies are likely to provide insights to possible mechanisms of proton transfer in complex proteins involved in bioenergetics.

**Comparison with other experimental work.**

Our experimental results are in disagreement with previous work by Stankovic et al. (1989, 1990) in a few essential points. 1) Stankovic et al. (1989) demonstrated that the SS is markedly stable in the open state whereas the RR had fast closing flickers. Those experiments were performed in KCl or HCl solutions. 2) There has been no report of inactivation of the RR in HCl (Stankovic et al., 1990). 3) Methylation of the dioxolane in the RR dimer prevented the closures of the RR channel (Stankovic et al., 1990), which supported the idea that rotation of the dioxolane linker is critical for channel gating. The bilayer methodology used in those studies was different from ours. Those bilayers were formed with glycerylmonooleate in squalene at the tip of a glass micropipette and may have been under significant tension (Heinemann, 1990), which is known to modulate the behavior of gA channels (Lundbæk et al., 1997). We have obtained experimental results in glyceryl monooleate/decane bilayers that were qualitatively similar to those shown here for PEPC/decane bilayers. However, we have not investigated the effects of either the solvent (decane versus squalene) or the bilayer-forming technique on the properties of the SS and RR channels. These issues will have to be addressed in the near future.

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