Genistein Can Modulate Channel Function by a Phosphorylation-Independent Mechanism: Importance of Hydrophobic Mismatch and Bilayer Mechanics†

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Received May 27, 2003; Revised Manuscript Received September 9, 2003

ABSTRACT: Genistein, a generic tyrosine kinase inhibitor, has been used extensively as a tool to investigate the possible regulation of membrane function by tyrosine phosphorylation. Genistein, in micromolar concentrations, alters the function of numerous ion channels and other membrane proteins, but only in few cases has it been demonstrated that the changes in membrane protein (ion channel) function are due to changes in a protein's phosphorylation status. The major common denominator characterizing proteins that are modulated by genistein seems to be that they are imbedded into, and span, the bilayer component of the plasma membrane. We therefore explored whether genistein could alter ion channel function by a bilayer-mediated mechanism and examined genistein's effect on gramicidin A (gA) channels in planar phospholipid bilayers. gA channels form by transmembrane dimerization of two nonconducting subunits, and genistein potentiates gA channel activity by increasing the appearance rate and prolonging the lifetime of bilayer-spanning gA dimers. That is, genistein shifts the equilibrium between nonconducting monomers and conducting dimers in favor of the bilayer-spanning dimers; the changes in channel activity therefore cannot be due to changes in bilayer fluidity. To obtain further insights into the mechanism underlying this modulation of gA channel function, we examined the effects of genistein on channels formed by gA analogues that differ in amino acid sequence. For a given channel length, the effects of genistein on gA dimerization do not depend on the specific sequence, or the chirality, of the channel-forming gA analogues. In contrast, when we change the channel length (by decreasing or increasing the number of amino acid residues in the sequence), or the bilayer thickness (by changing methylene groups in the acyl chains), the magnitude of genistein's effect increases with increasing hydrophobic mismatch between the channel length and the bilayer thickness. These results strongly suggest that genistein alters bilayer mechanical properties, which in turn modulates channel function. This bilayer-mediated mechanism is likely to apply to other pharmacological reagents and membrane proteins.

The high throughput ion movement through membrane-spanning channels, which permits the real-time observation of single molecule function, demands that ion channel function in vivo be tightly regulated (*1*). Changes in biological function similarly tend to involve tightly regulated changes in the function of specific ion channels and other membrane proteins, and numerous pharmacological reagents modulate ion channel function. Although the exact mechanism by which a given pharmacological compound alters channel function may not be known, it generally is assumed that the compound either binds to the channel protein (or perhaps an accessory protein) or perturbs one or more signal transduction pathways that regulate the channel's function. Among the regulatory mechanisms that have been implicated in the control of ion channel function, regulation by protein phosphorylation and dephosphorylation appears to be particularly widespread (*2*).

The isoflavonoid genistein frequently is used as a tool to alter ion channel function (e.g., ref *3*). Genistein inhibits tyrosine autophosphorylation of the epidermal growth factor receptor (EGFR) with 50% inhibition at ∼25 μM genistein (*4*), and low micromolar concentrations of genistein modulate physiological functions as diverse as long-term potentiation (*5*, *6*), smooth muscle contraction (*7*), and epithelial secretion (*8*, *9*). These effects are mediated through genistein's action on different classes of ion channels, including calcium (*10*), potassium (*11*), chloride (*3*), and ligand-gated (*12*) channels. But, although inhibition of tyrosine phosphorylation may be involved in the modulation of channel function, a direct link between tyrosine phosphorylation and ion channel modulation by genistein usually is missing. Given that genistein modulates such a diverse set of ion channels, whose only common denominator appears to be that they are imbedded into, and span, the membrane's bilayer component, we decided to explore whether genistein could alter channel function by a bilayer-mediated mechanism, using the grami-

† This work was supported by NIH Grants GM21342, GM34968, HL53445, DK55835, and RR15569 and NSF Grant MCB 9816063.

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10.1021/bi034887y CCC: $25.00
Published on Web 11/01/2003

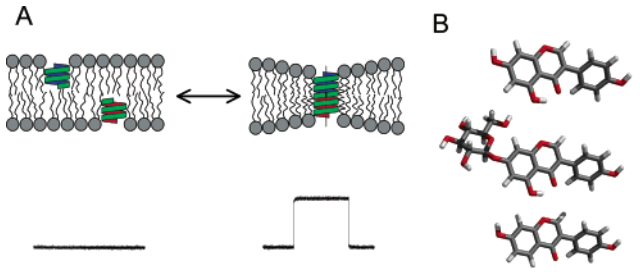

FIGURE 1: Gramicidin channel formation and structures of genistein and its analogues. (A) Formation of an ion-conducting channel by the transbilayer dimerization of gramicidin A subunits. Due to a mismatch between the channel length and the bilayer thickness, channel formation causes a local compression and bending of the two lipid leaflets. Current traces indicate that each opening and closing of the channel corresponds to association/dissociation of channel monomers. (B) From top to bottom: structure of genistein, as well as genistin and daidzein, two inactive analogues.

cidin A (gA) channel to probe changes in bilayer properties (*13*−*18*).

The linear gramicidins, exemplified by gA, are a group of antibiotic peptides produced by *Bacillus brevis* that form cation-permeable channels in biological membranes and lipid bilayers (*19*). gA channels are miniproteins of known structure (*20*−*23*). The channels are symmetric dimers of two right-handed $\beta^{6.3}$-helical subunits, having an alternating D- and L-amino acid sequence, in which the hydrogen-bonded peptide backbone lines a pore, which is 4 Å in diameter and 25 Å in length. The channels form by the reversible transmembrane dimerization of subunits residing in each bilayer leaflet (*24*); channel formation and disappearance (the monomer ↔ dimer equilibrium) thus represent a well-defined molecular transition (Figure 1A), which can be detected electrophysiologically using single-channel recording methods.

The gA channel's hydrophobic length is less than the hydrophobic thickness of an unperturbed bilayer, which means that the energetic penalty associated with exposure of hydrophobic residues to water will force the bilayer hydrophobic core to adjust (or deform) in an attempt to match the channel's hydrophobic exterior (*25*, *26*). That is, channel formation causes a bilayer deformation, which involves a local compression and bending of the two monolayers surrounding the bilayer-spanning channel. Because lipid bilayers are liquid crystals with material properties (area-compression and bending moduli), as opposed to simply being thin sheets of liquid hydrocarbon, this bilayer deformation incurs an energetic cost that varies with changes in bilayer thickness as well as the bilayer area-compression and bending moduli (*26*−*30*). Conversely, channel dissociation causes a release of the bilayer deformation. Overall, these features mean that the channel formation rate and lifetime will vary as a function of the bilayer material (or elastic) properties. This rather unique situation makes gA channels particularly well suited for use as molecular force transducers to study the effects of pharmacological reagents on lipid−protein interactions (*15*, *16*), as maneuvers that alter the magnitude of the energetic penalty associated with channel formation will express themselves as changes in the gA channel association and dissociation rate constants.

Genistein indeed potentiates gA channel activity by increasing the appearance rate and prolonging the lifetime of gA dimers, meaning that genistein increases the gA dimerization constant. Neither the amino acid sequence nor the helix sense of the gA channel has a significant impact on genistein's ability to alter channel function. The magnitude of genistein's effects, however, increases with increasing hydrophobic mismatch between channel length and bilayer thickness. Moreover, genistein's effect is specific, in that genistein analogues that do not inhibit EGFR autophosphorylation (Figure 1B) also have little or no effect on gA channel function. We conclude that genistein modulates gA channel function by altering bilayer mechanical properties. Similar bilayer-mediated channel modulation is likely to apply to other pharmacological reagents and membrane proteins.

MATERIALS AND METHODS

gA analogues of different length and amino acid sequence were synthesized using Fmoc chemistry and purified as described previously (*31*). A gA analogue in which the four Trp residues at positions 9, 11, 13, and 15 were replaced by Tyr [gT (*32*)] was a generous gift from Yves Trudelle (CNRS, Orleans, France). The sequences and abbreviations used for the different analogues are listed in Table 1.

The analogues were stored as ethanolic stock solutions at −20 °C before use.

Genistein, genistin, daidzein, and phloretin were obtained from Sigma Chemical Co. (St. Louis, MO). They were used without further purification and stored as DMSO stock solutions before use.

Planar bilayers were formed using the pipet method described previously (*33*) from *n*-decane solutions (25 mg/mL) of diphytanoylphosphatidylcholine (DPhPC), dioleoylphosphatidylcholine ($DC_{18:1}PC$), dieicosenoylphosphatidylcholine ($DC_{20:1}PC$), or dierucoylphosphatidylcholine ($DC_{22:1}PC$) (all from Avanti Polar Lipids, Alabaster, AL) across a hole (∼1.6 mm diameter) in a Teflon partition that separates two aqueous solutions of 1 M CsCl (buffered to pH 7 with 10 mM HEPES and CsOH). (A few experiments were done with 1.0 M NaCl, buffered to pH 7.0 with HEPES and NaOH.) The hydrophobic thickness (d_0) of bilayers formed by these different phospholipids is summarized in Table 2. The elastic moduli have been measured for only hydrocarbon-free $DC_{18:1}PC$ bilayers (*34*). On the basis of the results of Rawicz et al., the bilayer compression modulus (K_a) should be ∼250 pN/nm, and the bending modulus (K_c) should be ∼80 pN·nm for hydrocarbon-free bilayers formed by the three monounsaturated phospholipids.

The gramicidins were added from ethanolic stock solutions to the electrolyte solution on either side of the bilayer. The final ethanol concentration was less than 0.1%, or 20 mM, a concentration that has no effect on gA channel function. Genistein (or one of its analogues) was added from stock solutions prepared in DMSO; the final DMSO concentration was less than 0.4%, or 56 mM, a concentration that has no effect on gA channel function (*35*). To ensure that DMSO did not have an effect on genistein's action, we doubled the DMSO concentration to 112 mM, while maintaining the genistein concentration constant (at 40 μM), which had no effect on the channel lifetime. Single-channel experiments were done at 25 ± 1 °C using the bilayer punch method with pipet tip diameters of ∼30 μm (*36*) and an AxoPatch

Table 1: Sequences of the Gramicidins Used in This Study[a]

analogue	sequence	hydrophobic channel length (nm)
gA	f-V-G-A-L-A-V-V-V-W-L-W-L-W-L-W-ea	2.2[b]
gA$^-$	f-V-G-A-L-A-V-V-V-W-L-W-L-W-L-W-ea	2.2
AgA	f-A-G-A-L-A-V-V-V-W-L-W-L-W-L-W-ea	2.2
gT	f-V-G-A-L-A-V-V-V-T-L-T-L-T-L-T-ea	2.2
gA(13)	f-A-L-A-V-V-V-W-L-W-L-W-L-W-ea	1.9
gA(17)	f-G-A-V-G-A-L-A-V-V-V-W-L-W-L-W-L-W-ea	2.5

[a] The underlined residues are D-amino acids; f = formyl; ea = ethanolamide; the number in parentheses denotes the number of amino acids in the sequence. When no sequence length is noted, the analogue is 15 residues long, but we denote specifically the sequence length in experiments where we compare the effects of changing the sequence length. [b] Based on results of Elliott et al. (*25*). The hydrophobic length of channels formed by the 15 amino acid residue gA analogues is estimated to be equal to that of the gA channel. The hydrophobic length of channels formed by the sequence-lengthened or -shortened analogues is obtained by adding/subtracting 0.3 nm per L-D pair of residues.

Table 2: Hydrophobic Thickness of Bilayers Formed by Different Phospholipids

phospholipid	thickness: *n*-decane-containing bilayer[a](nm)	thickness: hydrocarbon-free bilayer (nm)
diphytanoylphosphatidylcholine (DPhPC)	4.2[a]	2.7[d]
dioleoylphosphatidylcholine ($DC_{18:1}PC$)	4.8[b]−5.0[c]	2.7[e]
dieicosenoylphosphatidylcholine ($DC_{20:1}PC$)	5.4[b]	
dierucoylphosphatidylcholine ($DC_{22:1}PC$)	5.8[b]	3.4[e]

[a] Results from Redwood et al. (82); the bilayer thickness is calculated from the specific capacitance, using a dielectric constant of 2.0. The same thickness was determined on the basis of the capacitance measurements in this study. [b] Results from Benz et al. (*83*). [c] Results from Lundbæk et al. (*17*). [d] Results from He et al. (*84*). The hydrophobic thickness is calculated from the phosphate peak-to-peak distance, 3.8 nm, and subtracting 1.1 nm (*85*). [e] Results from Lewis and Engelman (*85*).

1C patch clamp amplifier (Axon Instruments, Foster City, CA). The current signal was filtered at 500−1000 Hz with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA), digitized, and analyzed with a PC computer.

Single-channel current transitions were detected using the algorithm described by Andersen (*36*) implemented in software written in AxoBasic (Axon Instruments, Foster City, CA). Single-channel current transition amplitude histograms and lifetime histograms were constructed as previously described (*13*, *36*). The lifetime histograms were transformed into survivor distributions, and the average channel lifetimes (τ) were determined by fitting a single exponential distribution, $N(t) = N(0)\exp(-t/\tau)$, where $N(t)$ denotes the number of channels with a lifetime longer than time t, to each histogram (*13*, *37*).

Genistein increases not only the channel lifetime but also the channel appearance rate manyfold. After genistein addition it therefore becomes difficult to obtain traces with only a few channel openings or to observe the current baseline when no channels are conducting, a necessary condition for the lifetime analysis. Thus, for lifetime analysis, we routinely break and re-form the large membrane after the addition of genistein. To quantify the effect of genistein on the channel appearance rate, we initially recorded continuous current traces in bilayer membranes formed across 0.1−0.3 mm holes in the partitions before and after genistein addition. (We only report results in which we in the same bilayer could record the channel activity before and after genistein addition.) We also used the bilayer punch method on the same membrane before and after the addition of genistein in order to isolate smaller bilayers, with a lower number of conducting channels. For either analysis, VCR-recorded single-channel traces were played back and digitized into a PC computer using Axoscope (Axon Instruments, Foster City, CA), and the appearance rate was calculated by counting the number of channel appearances per minute over a 10 min period.

The bilayer capacitance was measured by applying a saw-tooth voltage train across bilayers with an area of ~1.2 mm^2; the bilayer area was measured with a calibrated reticule.

The final results for a given experimental condition are based on the mean ± standard deviation based on at least three independent measurements.

RESULTS

Genistein Increases the Lifetime of Gramicidin Channels. The effects of genistein on gA channels were examined first in DPhPC bilayers. Figure 2A shows current traces recorded in the absence and presence of different concentrations of genistein.

The channel lifetime becomes longer as the concentration of genistein is increased, a conclusion that is verified in the lifetime histograms (Figure 2B). Survivor plots of the channel lifetime distributions are well fitted by single exponential distributions in both the absence and presence of genistein (Figure 2B), indicating that genistein causes a uniform alteration in gA channel function rather than inducing a new population of gA channels with distinct kinetic properties. The increase in channel lifetime is a fairly linear function of the genistein concentration (Figure 3), and there is no indication of a saturation of genistein's effect over the concentration range tested.

To ascertain whether the genistein-induced changes in gA channel function could be due to direct gA−genistein interactions, we determined the dose−response curve for both gA, which forms right-handed $\beta^{6.3}$-helical channels, and gA$^-$, which forms left-handed channels. The results are indistin-

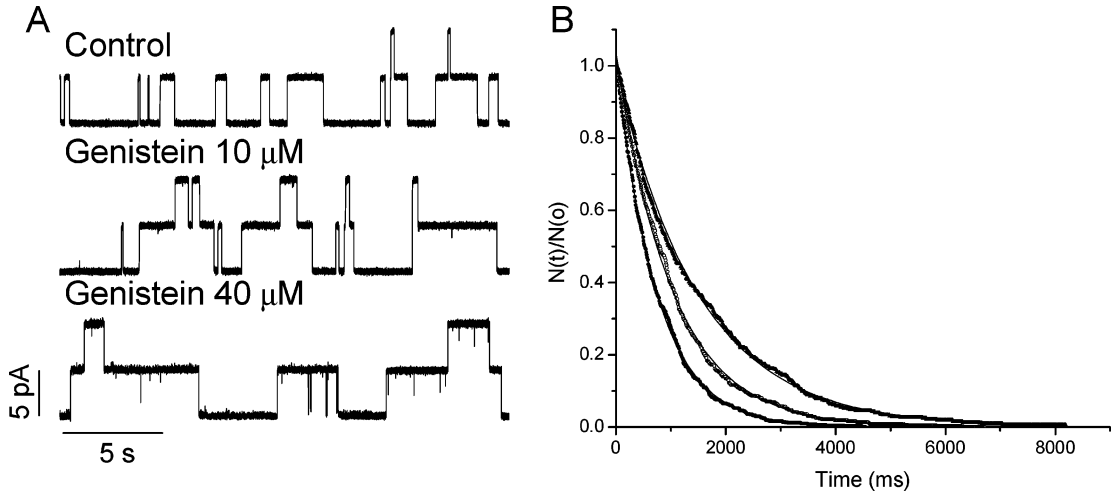

FIGURE 2: Prolongation of gA channel opening by genistein. (A) Current traces recorded in the absence and presence of either 10 or 40 μM genistein. (B) Lifetime distributions, plotted as survivor plots, which are fit by a single exponential distribution at each [genistein]. Dashed lines denote fits of a single exponential distribution to the results. The mean lifetimes calculated from the curve fits are 739 ± 2, 1022 ± 3, and 1502 ± 5 ms, respectively.

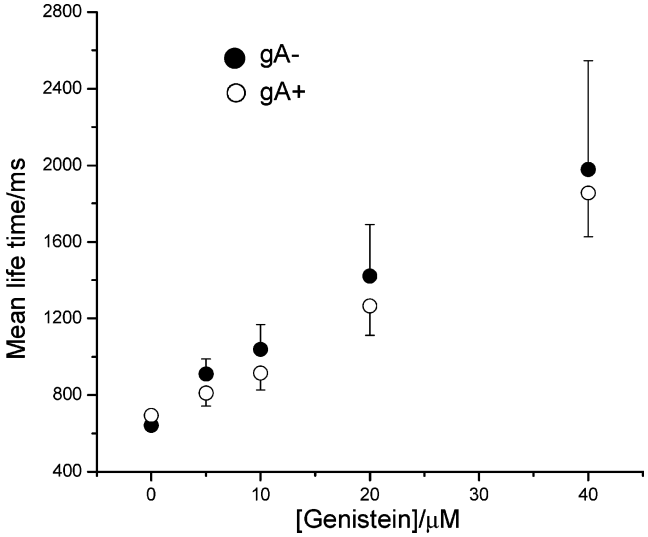

FIGURE 3: Dose−response relationships of genistein on the mean lifetime of gA and gA$^-$ channels. The data points represent mean values determined from four to seven bilayers.

guishable within experimental error, indicating that genistein's effects do not depend on specific binding to the channels.

In contrast to the marked changes in channel lifetime, genistein had no significant effect on the channel conductance, which changed by less than 5% (e.g., Figure 2A). This question is pursued further below.

The lifetime of gA channels varies with changes in bilayer thickness (*38*, *39*). The genistein-induced changes in gA channel lifetime, however, do not result from changes in bilayer thickness, as 40 μM genistein had no significant effect on the specific capacitance of DPhPC/*n*-decane bilayers: in the absence of genistein, the specific capacitance was 4.2 ± 0.1 nF/mm^2; in the presence of 40 μM genistein at both sides of the bilayer, the specific capacitance likewise was 4.2 ± 0.1 nF/mm^2.

Genistein Increases the Appearance Rate of gA Channels. Following the addition of genistein, gA channel activity increases dramatically. Figure 4A shows current traces recorded in the same "large" membrane (diameter ~0.2 mm) before and after the addition of different concentrations of genistein.

In the presence of genistein, not only is the channel lifetime longer but the overall channel activity also is increased. The relationship between the appearance rate and [genistein] is again fairly linear (Figure 4B), but the effect of genistein on the appearance rate is much larger than that on the lifetime (cf. Figures 3 and 4B described above), consistent with the notion that the major bilayer deformation occurs when the subunits associate, whereas channel dissociation should occur when the subunits move only about 1.6 Å apart (cf. refs *29* and *40*). At 20 μM genistein, the highest concentration used for these experiments, the channel lifetime is increased 2-fold (Figure 3), whereas the appearance rate is increased 13-fold. Thus, genistein changes the energetics of gA dimerization.

Effects of Genistein on gA Analogues. A unique advantage of using gA channels to probe whether pharmacologic agents could alter bilayer physical properties is the availability of numerous gA analogues with different amino acid compositions and well-characterized electrophysiological properties. We already demonstrated this advantage in Figure 3, where we compared the effects of genistein on the lifetimes of right-handed (gA) and left-handed (gA$^-$) channels. Figure 5A shows current traces obtained with channels formed by AgA in which Val^1 is replaced by Ala (Table 1) before and after the addition of 40 μM genistein.

Although the lifetime of AgA channels is about 3-fold shorter than that of normal gA channels (233 ± 7 ms vs 694 ± 34 ms), the magnitude of the lifetime increase induced by 40 μM genistein is almost identical to that of gA (about 3-fold; Figure 5B,C). Moreover, when all four tryptophan residues at the channel/solution interface are replaced by tyrosine (gT; Table 1), a similar relative lifetime increase also was observed at 40 μM genistein (Figure 5C). Thus, the specific amino acid sequence of the channel-forming gramicidin molecules does not affect the magnitude of the genistein-induced changes in channel lifetime. Taken together with the results in Figure 3, these results demonstrate that genistein does not exert its action by binding specifically to some site or crevice at the exterior surface of gA channels.

Effects of Inactive Genistein Analogues. Genistein is a member of a large family of isoflavonoids, only some of

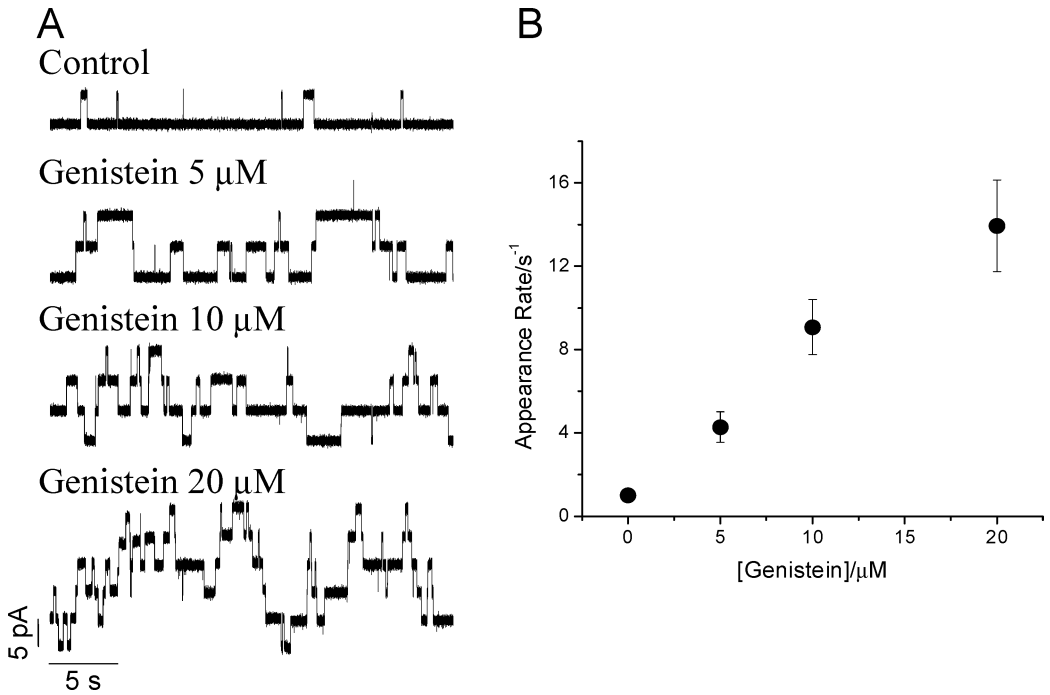

FIGURE 4: Concentration dependence of genistein effects on the appearance rate of gA channels. (A) Current traces obtained in a single bilayer at different [genistein]. (B) Dose−response curve of genistein on the channel appearance rate. Results are from three separate large membranes.

which inhibit EGFR tyrosine autophosphorylation (*4*) or modify the function of other membrane proteins (e.g., ref *41*). To ascertain whether the effect of genistein could be "specific", we examined the effects of genistein analogues that are weak, or inactive, modifiers of integral membrane protein function (Figure 1B) in the hope of identifying a genistein analogue that was inactive in terms of altering EGFR but nevertheless active as a modifier of gA channel function. Genistin has a glucose moiety replacing one of the −OH groups in the polyphenolic ring. It is completely inactive as a modifier of EGFR autophosphorylation (*4*) or CFTR function (*8*), and it also is completely inactive toward gA channels at 40 μM (Figure 5D). Daidzein, an analogue that misses one −OH group at the C5 position in the polyphenolic ring, has variable effects on CFTR function, but its effect is consistently less than that of genistein (*42*, *43*; cf. refs *44* and *45*). Similarly, daidzein has little effect on gA channel lifetime (Figure 5D). The results suggest that the effect of genistein on gramicidin channels indeed is specific in the sense that there is correlation between the ability of genistein and its analogues to alter the function of integral membrane proteins on one hand and of gramicidin channels on the other hand.

The Magnitude of Genistein's Effects Depends on the Channel Length. Although the amino acid sequence and channel helix sense do not affect genistein's modulation of the gA channel lifetime, the length of the gA channel (i.e., the number of amino acid residues) does determine the magnitude of the genistein-induced lifetime increase. Figure 6 shows results obtained when a mixture of gA(13) and gA(15) is added to the aqueous solutions on both sides of the bilayer.

In this experiment, one observes three different channel types having different subunit compositions (and lengths): symmetric gA(13) homodimers (with 26 total amino acids in the sequence), gA(15) homodimers (with 30 amino acids), and gA(13)/gA(15) heterodimers (with 28 amino acids). In principle, the gA(13)/gA(15) heterodimeric channels occur in two different orientations with distinct current amplitudes (*40*, *46*, *47*). One therefore may observe up to four different current transition amplitudes, representing the four different channel types. In this experiment, however, the two heterodimer orientations were indistinguishable, most likely due to the limited voltage being applied (cf. ref *47*). Nevertheless, these three different channel types possess characteristic current transition amplitudes and lifetimes. Moreover, because these three different channel types reside in the same lipid bilayer, we are able to examine the effects of genistein on gA dimers with different lengths in the same experimental conditions.

Figure 6A shows current traces generated by three different dimers, with three different single-channel conductance levels, before and after the addition of 40 μM genistein. In the presence of 40 μM genistein, all three channels respond with an increase of the mean lifetime, but the magnitude of enhancement differs (Figure 6B). At 40 μM genistein, the mean lifetime of gA(15) homodimers increases 3-fold (732 $\pm$ 72 vs 2240 $\pm$ 185 ms) as described above; for gA(13)/gA(15) heterodimers 4-fold (56 $\pm$ 3 vs 243 $\pm$ 31 ms), and for gA(13) homodimers 5-fold (33 $\pm$ 3 vs 158 $\pm$ 28 ms).

Considering all of the above results, we conclude that genistein has pronounced effects on the appearance rate (association rate constant) and lifetime (dissociation rate constant) of gramicidin channels. The association and dissociation rate constants are changed in opposite directions, such that the equilibrium between the nonconducting monomers and conducting dimers is shifted in favor of the conducting, bilayer-spanning, dimers. Moreover, though genistein's action is specific in the sense that inactive genistein analogues have little effect on the gramicidin channel lifetime, the genistein-induced effects do not depend on the channels' amino acid sequence or helix sense but do

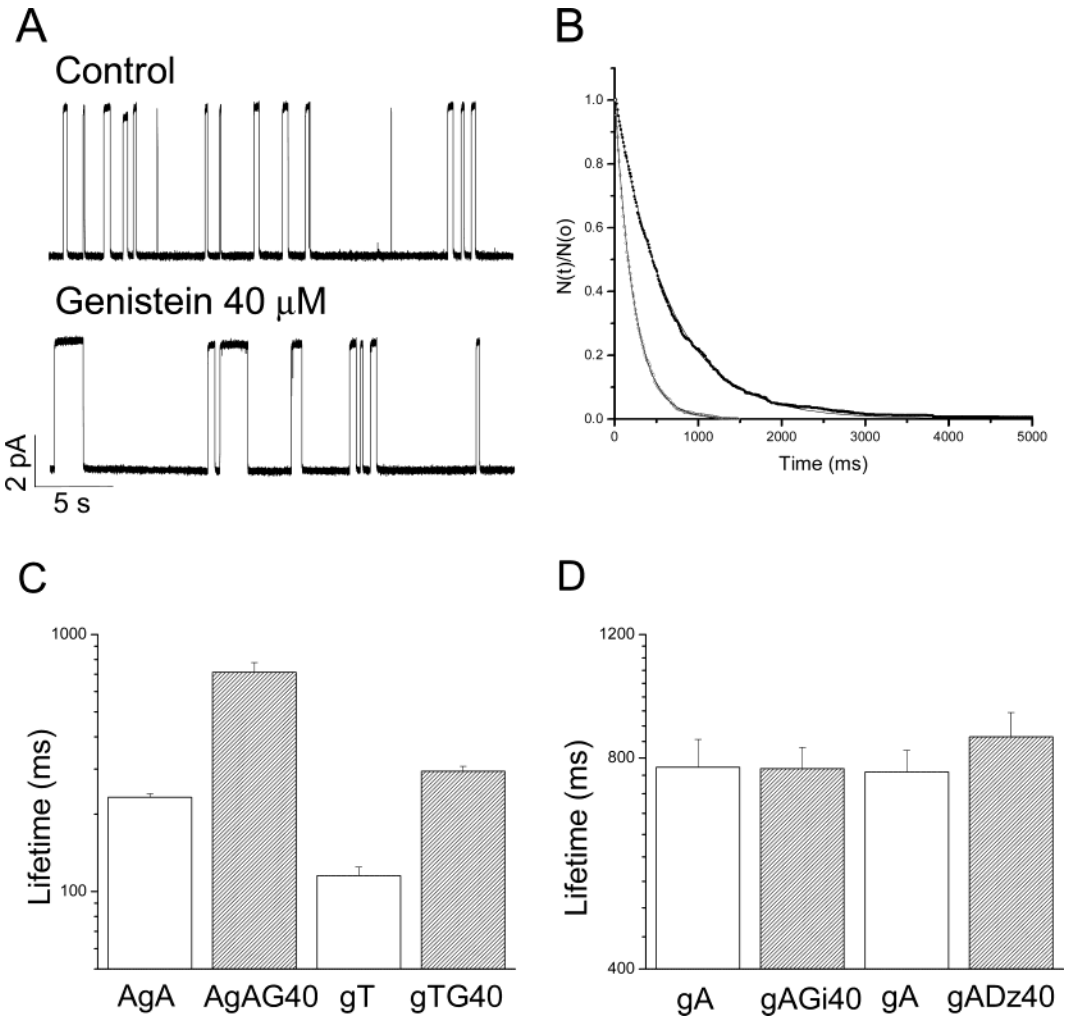

FIGURE 5: Effects of genistein and its analogues on the lifetime of channels formed by gramicidin analogues. (A) Current traces obtained with AgA in the absence or presence of 40 μM genistein. (B) Survivor plot of AgA channel lifetime distributions and a single exponential fit of the distributions. Dashed lines denote fits of a single exponential distribution to the results. The lifetimes obtained from the curve fits are 226 $\pm$ 1 and 1622 $\pm$ 1 ms, respectively. (C) Genistein-induced increases in the lifetime of channels formed by AgA and gT (40 μM genistein). (D) Effects of 40 μM genistin (Gi) and daidzein (Dz) on the lifetime of gA channels.

depend on channel length. The latter two results effectively exclude a direct, specific (stoichiometric) interaction between genistein and the bilayer-spanning channels. By exclusion, the results lead us to suggest that the degree of hydrophobic mismatch is a primary determinant of genistein's effects on gramicidin channel function.

Genistein's Effect Does Not Depend on the Permeant Ion. To address whether genistein's action varied with the choice of permeant ion and whether the changes in channel lifetime could be due to changes in the interfacial dipole potential, we did experiments in 1.0 M NaCl (pH 7.0). Na^+ is less permeant than Cs^+, and we have previously shown that the flavonoid phloretin, which decreases the dipole potential difference at the bilayer/solution interface (*48*) and thereby increases the single-channel conductance of gA channels (*49*), also increases the gA channel lifetime, a result that could be due to the dipole potential change. Table 3 summarizes results obtained with 40 μM genistein, daidzein, and phloretin on AgA(15) as well as gA(13) channels in DPhPC bilayers.

The effects of 40 μM genistein on the channel lifetimes are comparable, whether the permeant ion is Cs^+ or Na^+. The effects of daidzein are larger than would be expected from the gA(15) results in Cs^+ (Figure 5). As expected, daidzein has a larger effect on gA(13) channels than on AgA(15) channels; for both channel types the lifetime changes are almost 2-fold less than is the case with genistein. Both compounds have modest effects on conductance of AgA(15) channels and have no effect on the conductance of gA(13) channels. Phloretin also increases the lifetime of both gA(13) and AgA(15) channels, again with the larger effect on the gA(13) channels; phloretin also produces a 20% increase in the AgA(15) channels' conductance, with modest effects on the conductance of gA(13) channels. In experiments at 10 μM phloretin, the AgA(15) single-channel conductance is 19.6 $\pm$ 0.3 pS, and the lifetimes of AgA(15) and gA(13) channels are similar to those measured in the presence of 40 μM genistein: 648 $\pm$ 155 and 66 $\pm$ 19 ms, respectively. Given the comparable lifetime changes obtained with compounds that have quite different effects on the single-channel conductance and the larger lifetime changes seen with the shorter gA(13) channels, we conclude that the genistein-induced changes in channel lifetime cannot be a primary result of changes in the interfacial dipole potential.

Hydrophobic Mismatch Determines Genistein's Effects. To examine systematically the importance of hydrophobic

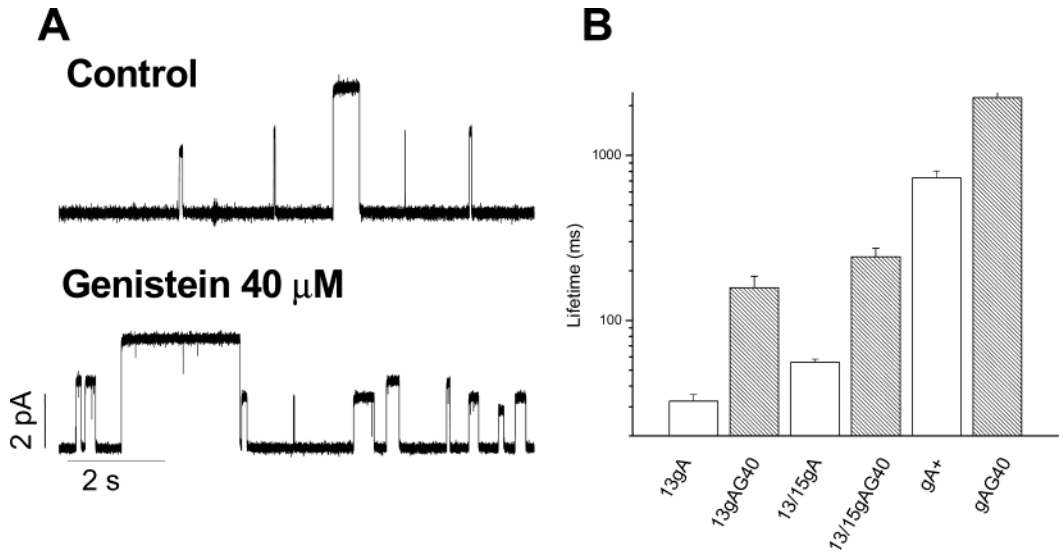

FIGURE 6: The effects of genistein on gramicidin channel lifetimes depend on channel length. (A) Current traces showing channels obtained with a mixture of gA(13) and gA(15), added symmetrically to both sides of the bilayer, where three identifiable channel types are formed. These channels can be distinguished on the basis of their current transition amplitudes, both before and after the addition of 40 μM genistein. (B) Summary of genistein's effects on channel lifetime: 13gA, gA(13) homodimers in the absence of genistein; gA(13)G40, gA(13) homodimers in the presence of 40 μM genistein; gA(13/15), gA(13)/gA(15) heterodimers in the absence of genistein; gA(13/15)G40, gA(13)/gA(15) heterodimers in the presence of 40 μM genistein; gA(15), gA(15) homodimers in the absence of genistein; gA(15)G40, gA(15) homodimers in the presence of 40 μM genistein.

Table 3: Effects of Genistein, Daidzein, and Phloretin on the Conductance and Lifetime of Gramicidin Channels[a]

	genistein		daidzein		phloretin	
gA analogue	conductance (pS)	lifetime (ms)	conductance (pS)	lifetime (ms)	conductance (pS)	lifetime (ms)
gA^{13} control	10.8 ± 0.4	15 ± 2.0	10.8 ± 0.3	16.3 ± 4.0	10.8 ± 0.3	15.3 ± 1.5
gA^{13} + modifier	10.9 ± 0.2	66 ± 16	10.7 ± 0.1	36.7 ± 1.5	11.4 ± 0.6	195 ± 48
AgA^{15} control	16.8 ± 0.6	190 ± 10	17.5 ± 1.0	195 ± 29	16.9 ± 0.5	196 ± 24
AgA^{15} + modifier	17.9 ± 0.3	699 ± 126	17.4 ± 0.3	379 ± 69	20.7 ± 0.5	1650 ± 600

[a] DPhPC bilayers; 1.0 M NaCl, pH 7.0, 200 mV. Each compound was added to a final concentration of 40 μM.

mismatch on genistein's actions, we incorporated gA analogues with different lengths (13, 15, or 17 amino acids) into phosphatidylcholine bilayers formed by lipids with different acyl chain lengths (18, 20, and 22 carbons). By this maneuver, it is possible to change the bilayer thickness, while maintaining the channel length invariant, or to change the channel length, while maintaining the bilayer thickness invariant. Figure 7A shows current traces of gA(15) channels in $DC_{20:1}PC$ bilayers in the absence and presence of 30 μM genistein. Both the frequency of channel openings and the channel lifetime were increased by genistein, as was the case in DPhPC bilayers, and the inactive genistein analogue, genistin, again has negligible effects (Figure 7B). The changes in channel lifetime distributions are shown in Figure 7C,D.

Again, the lifetime distributions are well fit by single exponential distributions, and genistein increased the mean lifetime almost 4-fold (from 72 ± 9 to 267 ± 21 ms); genistin had no effect (63 ± 5 vs 63 ± 7 ms).

Figure 8A shows current traces obtained with gA(17) channels in $DC_{20:1}PC$ bilayers in the absence or presence of genistein; Figure 8B shows similar results obtained in $DC_{22:1}PC$ bilayers.

As expected, from the increased hydrophobic mismatch in the $DC_{22:1}PC$ bilayers, the channel lifetime in the absence of genistein is much shorter than in $DC_{20:1}PC$ bilayers (Figure 8C,D shows the lifetime distributions), and to maintain a similar channel appearance rate, at least 10-fold more of the gA(17) analogue is needed in $DC_{22:1}PC$ bilayers to maintain a channel appearance rate similar to that observed in $DC_{20:1}PC$ bilayers. For gA(17) channels, the mean lifetimes in $DC_{22:1}PC$, $DC_{20:1}PC$, and $DC_{18:1}PC$ bilayers are 6.9 ± 0.5, 66 ± 11, and 3660 ± 400 ms, respectively. These results provide further support for the importance of channel−bilayer mismatch for determining the gramicidin channel lifetime (*29*).

As was the case with gA(15) (Figure 7), genistein enhances the gA(17) channel activity by increasing not only the channel lifetimes but also the appearance rates: panels A and B of Figure 8 show current traces obtained in $DC_{20:1}PC$ and $DC_{22:1}PC$ bilayers, respectively, before and after genistein addition; panels C and D of Figure 8 show the corresponding lifetime distributions. In the presence of 30 μM genistein, the mean lifetimes of gA(17) channels in $DC_{22:1}PC$, $DC_{20:1}PC$, and $DC_{18:1}PC$ bilayers are 26 ± 2 ms ($n = 4$; 3.8-fold increase), 178 ± 8 ms ($n = 5$; 2.7-fold increase), and 7160 ± 600 ms ($n = 5$; 2.0-fold increase), respectively. Comparing these results with those obtained in the absence of genistein, we conclude that genistein's effect on the channel lifetime indeed increases with increasing hydrophobic mismatch.

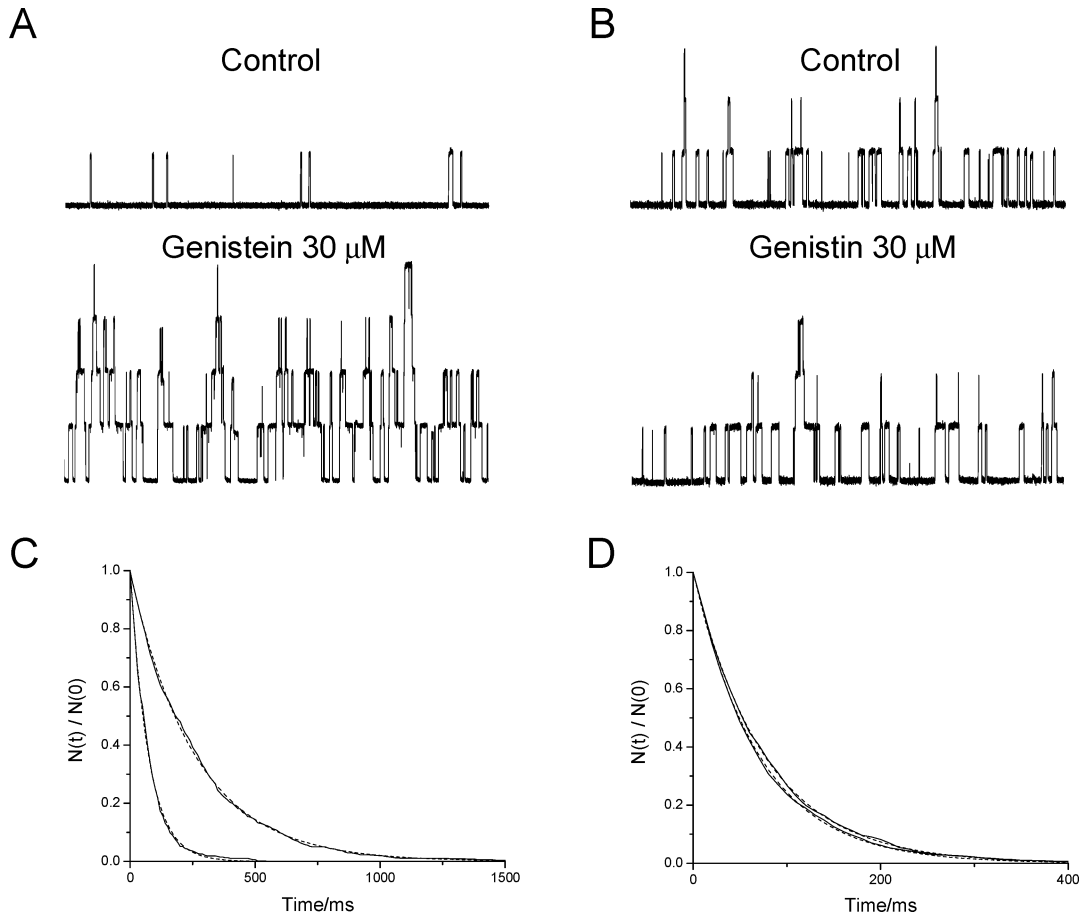

FIGURE 7: Effects of genistein and genistin in bilayers that have increased thickness (so there is larger channel−bilayer hydrophobic mismatch). (A) Single-channel current traces observed with gA(15) in $DC_{20:1}PC$ bilayers in the absence or presence of 30 μM genistein. (B) Single-channel current traces observed with gA(15) in $DC_{20:1}PC$ bilayers in the absence or presence of 30 μM genistin. Each trace in (A) and (B) represents 15 s of continuous recording. (C) Normalized survivor plots of single-channel lifetime distributions of gA(15) channels in the absence or presence of genistein. Dashed lines denote fits of a single exponential distribution to the results. The lifetimes calculated from curve fits are 71 ± 1 and 259 ± 1 ms, respectively. (D) Normalized survivor plots of single-channel lifetime distributions of gA(15) channels in the absence or presence of genistin. Dashed lines denote fits of a single exponential distribution to the results. The lifetimes calculated from curve fits are 70.2 ± 0.3 and 76.4 ± 0.3 ms, respectively.

To examine this question in greater detail, we note that the change in gA channel length per amino acid (~1.6 Å/amino acid; refs *20*−*22*; see also Table 1) is comparable to the change in bilayer thickness per carbon in the acyl chain [~2.0 or ~1.6 Å, respectively, per carbon in *n*-decane-containing or hydrocarbon-free bilayers (Table 2)], so the changes in hydrophobic mismatch that occur as one alters either the amino acid sequence or the acyl chain length can be approximated as the difference between the number of acyl chain carbons (N_C) and the number of amino acids (N_{AA}). So, if genistein's primary effect is to alter the bilayer elastic properties, the magnitude of genistein's effect should scale with $N_C - N_{AA}$. This concept was tested in experiments with gA(13), gA(15), or gA(17) in bilayers formed by $DC_{18:1}PC$, $DC_{20:1}PC$, or $DC_{22:1}PC$ (Figure 9A).

The genistein-induced channel lifetime increases vary with both channel length and bilayer thickness, but they do so in such a way that similar relative changes are observed when $N_C - N_{AA}$ is invariant. Neither the channel length nor the bilayer thickness, per se, determines the effect of genistein. Rather, it is the extent of hydrophobic mismatch that is important!

In several experiments we were able to obtain long stationary recordings of gramicidin channel activity in the same bilayer before and after the addition of genistein. These recordings allow us to quantify the effect of genistein on the channel appearance rate (cf. Figures 7 and 8) for different hydrophobic mismatches (Figure 9B). Again, the relative changes in appearance rate are much larger than the lifetime changes. Moreover, the increase in appearance rate is largest for the largest hydrophobic mismatches [gA(15) in $DC_{20:1}PC$ or gA(17) in $DC_{22:1}PC$]. For a simple monomer−dimer equilibrium (cf. Figure 1A), we are now in a position to estimate the change in the genistein-induced change in the gramicidin dimerization constant using the results in Figure 9A,B and, further, to determine the changes in the free energy of dimerization caused by 30 μM genistein ($\Delta\Delta G$ in Figure 9C). The changes in ΔG, about 3 kcal/mol, are comparable to the making or breaking of a hydrogen bond (*50*). The results in Figure 9 again demonstrate that the genistein-induced changes in channel function depend on the channel−bilayer hydrophobic mismatch but not the bilayer thickness (composition) or the channel length (sequence) per se. We conclude that the effect of genistein on gramicidin

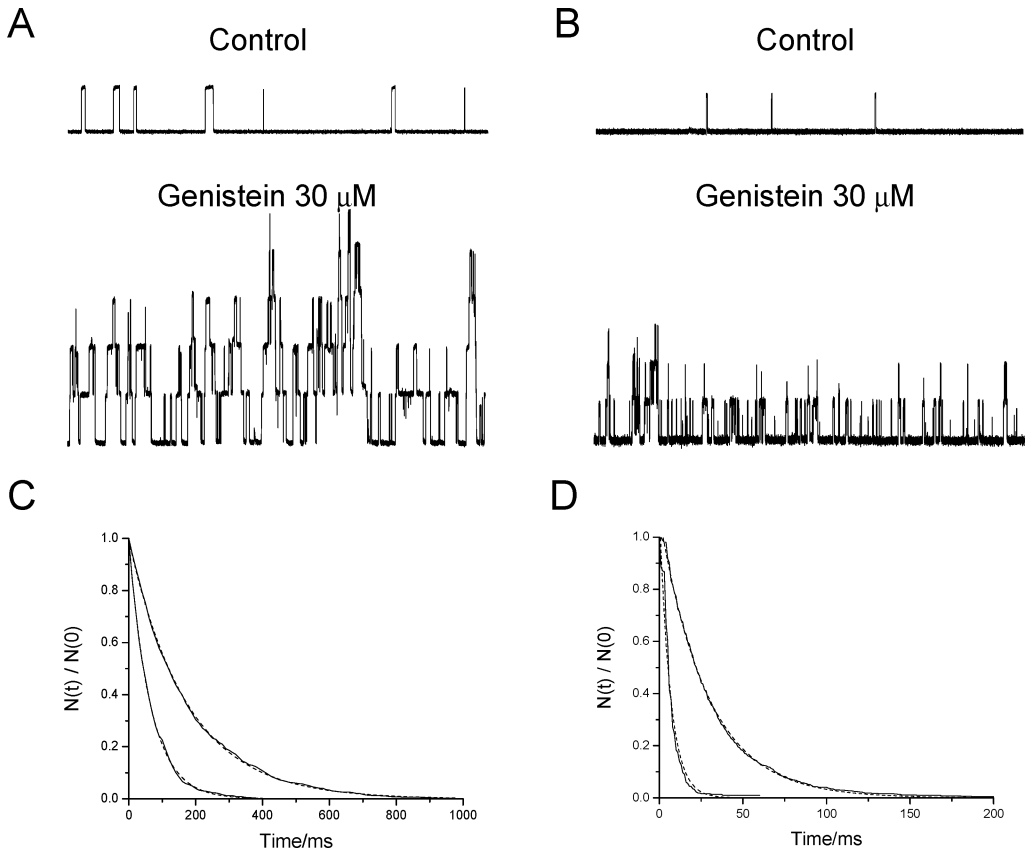

FIGURE 8: The genistein-induced potentiation of gA channel activity depends on the hydrophobic mismatch between channel length and bilayer thickness. (A) Single-channel current traces observed with gA(17) in $DC_{20:1}PC$ bilayers in the absence or presence of 30 μM genistein. (B) Single-channel current traces observed with gA(17) in $DC_{22:1}PC$ bilayers in the absence or presence of 30 μM genistein. Each trace represents 20 s of continuous recording. (C) Normalized survivor plots of single-channel lifetime distributions of gA(17) channels in $DC_{20:1}PC$ bilayers. Dashed lines denote fits of a single exponential distribution to the results. The lifetimes calculated from curve fits are 64 $\pm$1 and 174 $\pm$ 1 ms, respectively. (D) Normalized survivor plots of single-channel duration distributions of gA(17) channels in $DC_{22:1}PC$ bilayers. Dashed lines denote fits of a single exponential distribution to the results. The lifetimes calculated from the curve fits are 6.5 $\pm$ 0.3 and 28.0 $\pm$ 0.1 ms, respectively.

channel function is due to changes in the bilayer deformation energy.

DISCUSSION

In this paper we show that genistein, a commonly used inhibitor of tyrosine kinase activity, has profound effects on gramicidin channel function. Viewed from the perspective of genistein, the action appears to be specific, in the sense that the changes in gramicidin channel function occur over the same concentration where genistein exerts its effects on EGFR autophosphorylation (*4*) and on many different membrane proteins and ion channels (e.g., refs *51*−*53*; also see Table 2 below). Moreover, the inert genistein analogues genistin and daidzein have less, if any, effect on gramicidin channel function. If one did not know otherwise, these results could be interpreted to mean that gramicidin channel function somehow was regulated by tyrosine phosphorylation/dephosphorylation. The obvious fallacy of this suggestion illustrates the difficulties that one may face when interpreting the results of pharmacological manipulations of membrane protein function.

Viewed from the perspective of gramicidin, the action of genistein appears at first sight to be unspecific, in the sense that the changes in channel lifetime do not depend on the channel's amino acid sequence or helix sense. Importantly, however, the genistein-induced changes in gramicidin channel function depend on the degree of hydrophobic mismatch between the bilayer-spanning channels and the host bilayer. That is, genistein alters somehow the energetic consequences of the hydrophobic channel−bilayer coupling.

We first discuss how genistein might be able to modulate membrane protein function by a phosphorylation-independent mechanism and conclude that genistein, in addition to any of its phosphorylation-dependent mechanisms, is a general modifier of membrane protein function due to its effect on bilayer elastic properties. We then briefly discuss some implications for integral membrane proteins and raise the question of whether genistein's effect on EGFR autophosphorylation may involve a similar general alteration of membrane protein function.

Genistein Is a General Modifier of Bilayer Mechanical Properties. How does an alteration of membrane properties modulate ion channel function? What properties of the lipid bilayers do these reagents alter? Ion channel proteins are imbedded in lipid bilayers, which are highly specialized anisotropic environments, in which the proteins' bilayer-spanning domains remain in close apposition to the lipid bilayer's hydrophobic core (*54*). Ion channel gating generally is believed to involve significant protein conformational changes, as the Q_{10} for the gating kinetics is in the range of 2−4, where a Q_{10} of 3 corresponds to an activation energy of 20 kcal/mol (*1*). The relevant gating motion(s) may

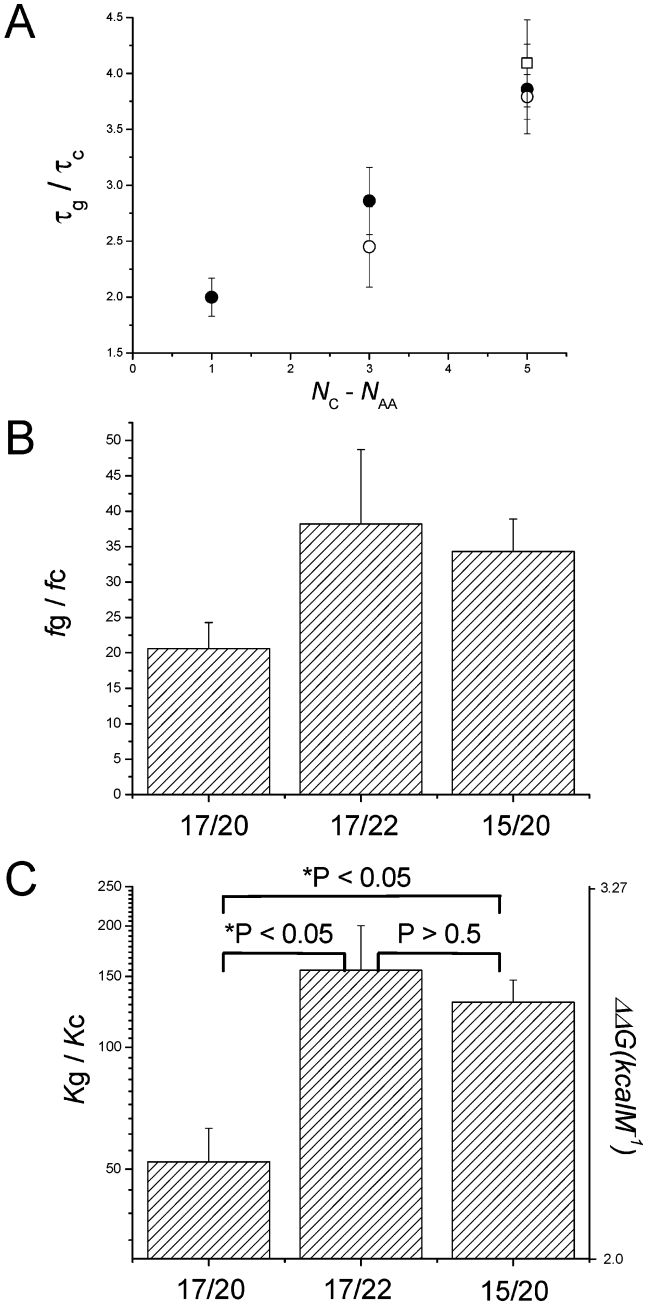

FIGURE 9: Effects of genistein on the gating kinetics of gA. (A) Relative increase of the mean lifetime by genistein. N_C denotes the number of carbons in the phospholipid acyl chains, and N_{AA} denotes the number of amino acid residues in gA; τ_c is the lifetime in the absence of genistein, and τ_g is the lifetime in the presence of 30 μM genistein. Key: closed circles, gA(17); open circles, gA(15); open square, gA(13). (B) Relative increase of the appearance rate induced by 30 μM genistein. f_c is the channel appearance rate in the absence of genistein; f_g is the appearance rate in the presence of 30 μM genistein. Key: 20/17, gA(17) channels in $DC_{20:1}PC$ bilayers; 22/17, gA(17) in $DC_{22:1}PC$ bilayers; 20/15, gA(15) in $DC_{20:1}PC$ bilayers. (C) Effects of genistein on the gA dimerization constant ($f\tau$). K_c is the dimerization constant in the absence of genistein; K_g is the dimerization constant in the presence of 30 μM genistein.

involve rotational or/and translational movements of subdomains embedded in the lipid bilayer, movements that would be expected to perturb the packing of lipids adjacent to the channel protein. Indeed, recent high-resolution structures of three bacterial potassium channels, including H^+-, Ca^{2+}-, and voltage-activated channels (*55*−*58*), of a bacterial chloride channel (*59*), of the sarcoplasmic reticulum Ca^{2+}-ATPase (*60*, *61*), and of the MscL stretch-activated channel (*62*, *63*) all provide evidence for significant reorganizations of the proteins' transmembrane segments, including changing the segment lengths as well as the tilt of bilayer-spanning α-helices, during "normal" channel function. The hydrophobic coupling between the proteins' bilayer-spanning domains and the bilayer hydrophobic core will tend to cause a reorganization of these transmembrane domains to alter the packing of the lipids surrounding the protein in question. This altered lipid packing, or bilayer perturbation, has an energetic cost, in which case the gating equilibrium would be subject to modulation through physical and chemical perturbations of the host lipid bilayer.

In biological membranes, genistein can modulate ion channel function when applied from either side of the membrane (*51*, *64*). Genistein also alters ion channel function in cell-attached patches, meaning that it is membrane permeant (*3*, *65*), and genistein and other isoflavonoids partition into lipid bilayers (*66*). Genistein thus might alter membrane protein function by altering the bilayer fluidity. Though we cannot exclude that genistein alters bilayer fluidity, and fluidity often has been invoked to explain functional consequences of changes in lipid bilayer composition, we can nevertheless conclude that changes in fluidity cannot account for our major result: that genistein increases the channel association rate constant whereas it decreases the dissociation rate constant.[1] This point is crucial because changes in bilayer fluidity will alter the association and dissociation rate constants in the same direction (*67*). For an isolated fluidity change, the equilibrium distribution between conformational states should be unaffected (*68*). Because genistein alters the gA dimerization constant, the results cannot be accounted for by changes in bilayer fluidity.

Moreover, genistein causes modest changes in the interfacial dipole potential, as the Na^+ conductance is altered little by 40 μM as compared to phloretin that causes large changes in the single-channel conductance; cf. Table 3. This observation effectively rules out that the gA channel stabilization could arise from altered electrostatic interactions between the dipolar tryptophan side chains (*69*) and the interfacial dipole potential difference. Similarly, we can exclude that the changes in gramicidin channel function result from changes in bilayer thickness, as 40 μM genistein has no effect on the specific capacitance of DPhPC/*n*-decane bilayers or the membranes of the Calu-3 epithelial cell line or NIH3T3 cells (*70*).

How, then, can genistein alter the free energy difference of the gramicidin monomer ↔ dimer equilibrium? Even though atomic resolution structures of membrane proteins do not provide sufficient information to evaluate the energetic consequences of a change in bilayer mechanical properties, there exists an established model for the energetics of the bilayer deformation associated with gramicidin channel formation (*26*−*28*). The hydrophobic coupling between the

[1] We also note that though phloretin increases the bilayer permeability to small solutes, i.e., increases bilayer fluidity (*48*), the major effect of phloretin is due to changes in interfacial dipole potential (*48*), which is reflected in the single-channel conductance, and changes in bilayer elastic properties, which is reflected in an increased gA channel activity and lifetime (*49*). Again, the changes in bilayer fluidity cannot be causal for the changes in channel activity.

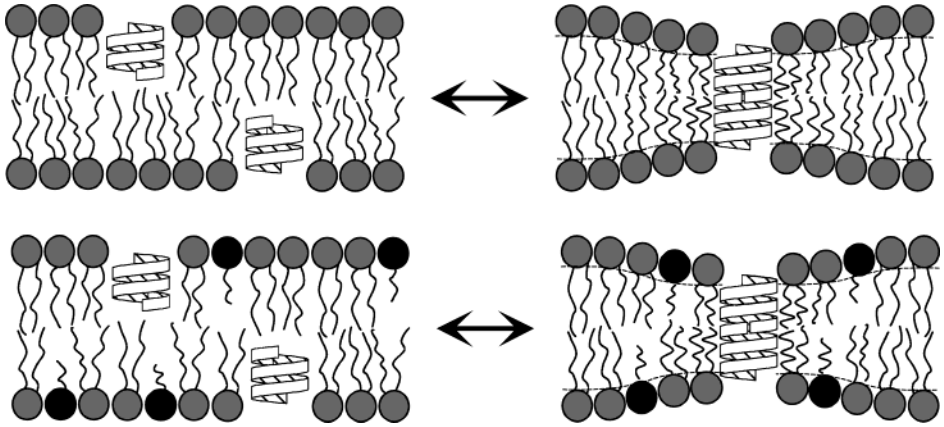

FIGURE 10: Schematic model for the genistein-induced stabilization of bilayer-spanning gramicidin channels. Top: the gramicidin monomer ↔ dimer equilibrium in an unmodified bilayer, where the two monolayers around the channel are deformed, such that the monolayers are compressed and bent. Bottom: the gramicidin monomer ↔ dimer equilibrium in a genistein-modified bilayer. In this case, the hydrophobic volume of each adsorbed genistein molecule is less than that of a phospholipid molecule, which in its own right will establish a curvature stress in the bilayer (e.g., ref *18*). When a gramicidin channel forms in the presence of genistein, the bilayer deformation energy will be reduced, compared to the situation for the unmodified bilayer, because the adsorbed genistein molecules will tend to reduce the energetic cost associated with packing both lipid and genistein molecules around the channel.

channel and bilayer, together with the hydrophobic mismatch, causes gramicidin channel formation to compress and bend the two bilayer leaflets surrounding the channel, and the free energy change associated with this bilayer deformation is a quadratic function of the vertical displacement of the bilayer/solution interface (the hydrophobic mismatch). The gramicidin/bilayer system thus behaves as a simple spring with a spring constant that is determined by the bilayer material properties, the bilayer thickness (d_0), the compression and bending moduli (K_a and K_c, respectively), and the channel dimensions (*71*). The "conformational change", and bilayer deformation, that is associated with gramicidin channel formation may well be specific for gramicidin channels, but while bending and compression are independent modes of bilayer deformation (*26*, *72*), the elastic moduli that characterize the energetic cost of bending and compression are related by the expression $K_c = K_a d_0^2/24$, where d_0 is the bilayer thickness (*34*), and the elastic moduli. That is, the measured changes in gramicidin channel lifetime report changes in general bilayer properties.

According to this elastic bilayer model, maneuvers that alter the spring constant will alter the gA dimerization equilibrium. Furthermore, the magnitude of the genistein-induced changes in channel function will vary with the degree of hydrophobic mismatch. It is in this context important that any compound that adsorbs reversibly into a lipid bilayer will alter the bilayer elasticity, both the bilayer area-compression and bending moduli (*73*−*76*), although the effects will vary as a function of the compound's detailed structure and localization in the bilayer.

The effects of genistein depend on neither the gramicidin sequence nor its chirality; moreover, genistein alters the gramicidin monomer−dimer equilibrium, and the magnitude of the genistein-induced changes in channel properties depends on the channel−bilayer hydrophobic mismatch. The most parsimonious interpretation of genistein's effect on gramicidin channel function thus becomes that genistein adsorbs at the bilayer/solution interface and thereby lowers the bilayer area-compression and bending moduli, which in turn will decrease the stiffness of the spring that describes the energetics of the gramicidin-induced bilayer deformation.

A schematic model of how genistein may act is shown in Figure 10. The adsorption of genistein at the bilayer/solution interface will decrease the work of compressing and bending the two monolayers that surround the bilayer-spanning channel, and that need to deform in order to allow for channel formation, because genistein's contribution to the volume of the bilayer hydrophobic core will be less than its contribution to the interfacial area. Molecules that adsorb deeper into the bilayer, such as may be the case for daidzein, which has one less −OH group, would be expected to have a lesser effect on the bilayer deformation energy associated with channel formation. It is in this context also of interest to note that the ability of halogenated hydrocarbons to act as general anesthetics is correlated with their preference for an interfacial localization (*77*). For the moment, the modest effect of daidzein remains a puzzle, but daidzein's effects on CFTR are variable but consistently smaller than those of genistein (*42*, *43*; cf. refs *44* and *45*). In any event, if a membrane protein's function can be modified by daidzein, in a manner similar to the genistein-induced changes, it would provide strong evidence that these reagents' actions are bilayer-mediated.

Implications for Integral Membrane Proteins. Could a hydrophobic mismatch be important for the gating of ion channels other than gramicidin channels? As noted above, atomic resolution structures of a number of membrane proteins demonstrate significant reorganizations of the proteins' transmembrane segments, which include changing the lengths of segments brought about by tilting the bilayer-spanning α-helices. Moving a transmembrane segment (or part of it) from a vertical to a more oblique position (and vice versa) may not only create a hydrophobic mismatch, however; it also may alter the boundary conditions for lipid packing around the protein, which in its own right will incur an energetic cost (*28*, *71*, *78*). As is the case for gA dimerization, these types of conformational changes make protein function subject to alterations of lipid properties. In fact, genistein modulates the function of a large number of different ion channels, many of which have little sequence similarity (Table 4).

Table 4: Ion Channels Whose Function Is Modulated by Genistein

channel	stimulatory	inhibitory	tyrosine kinase involvement	ref
CFTR	X	X	±	*51*
I_{Cl} (swell)		X	?	*86*
glycine receptor		X	−	*53*
nonselective cation channel		X	?	*87*
cNGC		X	±	*88*
KCNQ2		X	±	*89*
hKv1.4 (A-type)		X	−	*90*
SK	X		+	*91*
BK	X		?	*92*
K_{ATP}	X	X	±	*11*
I_f		X	−	*93*
V-gated Na channel		X	−	*94*
L-type Ca channel		X	±	*95*
N-, T-type Ca channel		X	?	*96*

Only in a few of these cases has a direct involvement of tyrosine phosphorylation been demonstrated. Moreover, other compounds that modulate membrane protein function also alter gA channel function at relevant concentrations (*15*, *16*).

The mechanism we define here, altering membrane function by altering the bilayer elastic properties, is likely to be applicable to many other pharmacological compounds and membrane proteins (see also ref *17*). Going one step further, this mechanism could be relevant for the genistein inhibition of EGFR tyrosine phosphorylation, as EGFR activation is likely to involve the dimerization of two ligand-bound EGFR (*79*). This dimerization step is likely to involve significant changes in protein−bilayer interactions that could be altered by genistein. Indeed, the lateral interactions between two bilayer-spanning gA channels appear to depend on the channel−bilayer interactions (*80*), and EGFR function is very sensitive to perturbations of the membrane lipid composition (*81*). Perhaps it is time to reexamine the mechanism by which genistein acts on EGFR and other membrane proteins.

ACKNOWLEDGMENT

We thank Dr. Shobana Sundaram for helpful discussions and Aung-Kyaw Chi and Sonya E. Tape for technical assistance.

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BI034887Y