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Multiple Binding Sites for Fatty Acids on the Potassium Channel KcsA

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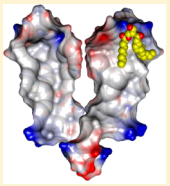
Supporting Information

ABSTRACT: Interactions of fatty acids with the potassium channel KcsA were studied using Trp fluorescence quenching and electron paramagnetic resonance (EPR) techniques. The brominated analogue of oleic acid was shown to bind to annular sites on KcsA and to the nonannular sites at each protein−protein interface in the homotetrameric structure with binding constants relative to dioleoylphosphatidylcholine of 0.67 ± 0.04 and 0.87 ± 0.08, respectively. Mutation of the two Arg residues close to the nonannular binding sites had no effect on fatty acid binding. EPR studies with a spin-labeled analogue of stearic acid detected a high-affinity binding site for the fatty acid with strong immobilization. Fluorescence quenching studies with the spin-labeled analogue showed that the binding site detected in the EPR experiments could not be one of the annular or nonannular binding sites. Instead, it is proposed that the EPR studies detect binding to the central hydrophobic cavity of the channel, with a binding constant in the range of ∼0.1−1 μM.



Fatty acids, particularly polyunsaturated fatty acids, have been reported to affect the functions of many types of ion channels.[1,2] For example, arachidonic acid (C20:4) increases the rate of inactivation of delayed rectifier potassium channels; similar effects are seen with linoleic acid (C18:2), with oleic acid (C18:1) having a slightly weaker effect but stearic acid (C18:0) having no effect.[1] Similarly, arachidonic acid and a variety of other fatty acids, including the saturated fatty acid myristic acid, inhibit the Ca^{2+}-activated K^+ channel hIK1.[3,4]

In principle, fatty acids could bind to a number of different types of sites on an ion channel to affect function, and it is possible that more than one mechanism will be required to explain the range of observed effects. The functions of many membrane proteins have been shown to depend on the structures of the lipid molecules in the lipid annulus.[5,6] Because most annular lipid binding sites show little structural specificity, a fatty acid molecule present in the lipid bilayer could replace a lipid molecule at an annular site and lead to changes in function.[5] Additional binding sites for phospholipids and other hydrophobic molecules have been suggested to be buried within a membrane protein, at protein−protein interfaces in oligomeric membrane proteins, or between transmembrane α-helices; these sites have been termed nonannular sites to distinguish them from the annular lipid binding sites.[5] A clear example is provided by the homotetrameric potassium channel KcsA that has been crystallized with one molecule of the anionic phospholipid phosphatidylglycerol bound at each protein−protein interface.[7] Occupation of the site by anionic lipid molecules has been shown to be important for the function of KcsA.[7,8] Binding of the anionic lipid at the nonannular site was shown to be relatively weak,[8] but the structural specificity of the site has not previously been explored in detail. It is important to realize that that even relatively low association constants for fatty acids at annular or nonannular sites can give rise to relatively high occupancies on the channel protein because of the high local concentration of a fatty acid partitioned into a membrane.

A feature of KcsA and other ion channels is the marked hydrophobicity of the residues lining the central cavity, a feature thought to be important in ensuring a rapid flow of K^+ ions through the channel.[9] This hydrophobic lining provides, of course, potential binding sites for small hydrophobic molecules, and indeed, ions such as the tetrabutylammonium ion have been shown to bind to the cavity wall with one ion binding per tetramer close to the entrance to the selectivity filter, blocking entrance of ions to the filter.[10,11] Inhibition of hIK1 and Kv1.1 potassium channels by polyunsaturated fatty acids has also been attributed to binding to the central cavity.[4,12] Decher et al.[12] found that mutations in just one of the four subunits making up the tetrameric Kv structure were sufficient to block the effect of the fatty acids, suggesting a single fatty acid binding site per channel.

Our aim here is to characterize binding of a fatty acid to KcsA. We have shown that relative lipid binding constants at annular and nonannular sites on KcsA can be determined using fluorescence quenching approaches.[8,13] Quenching of Trp fluorescence by brominated lipid molecules is short-range, so that only those molecules bound close to a Trp residue will quench its fluorescence, quenching efficiency showing a sixth power dependence on the distance of separation.[13] Because the time for two lipid molecules to exchange between the bulk phase and a binding site on a protein is much greater than the Trp fluorescence lifetime,[14,15] the level of fluorescence quenching observed for a protein reconstituted in a mixture of a normal lipid X and a lipid Y containing brominated fatty acyl chains will be proportional to the fraction of binding sites occupied by lipid Y and so will depend on the binding constant of lipid Y compared with that for lipid X. KcsA contains five Trp residues per monomer. Three of the Trp residues, Trp-26, Trp-87, and Trp-113, are exposed to the lipid bilayer and so can

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be quenched by brominated molecules binding to the annular sites but are too far from the nonannular sites to be quenched by a brominated molecule bound to a nonannular site.[8,13] The two remaining Trp residues, Trp-67 and Trp-68, are part of the central P-loop and are too far from the annular sites to be quenched by brominated molecules bound at such sites. However, each Trp-67 residue is close to a nonannular lipid binding site, and binding of a brominated molecule results in very efficient quenching, allowing the determination of binding constants at the nonannular sites.[13]

Efficiencies of quenching can be estimated for a brominated lipid binding in the central cavity of KcsA. A key residue in binding of polyunsaturated fatty acids and other hydrophobic molecules to IKCa1 is Val-275,[4,16] equivalent to Ile-100 in KcsA (Figure 1), and the corresponding residue in Kv1.1,

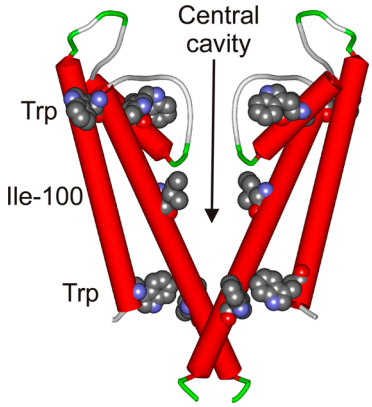

Figure 1. Structure of KcsA. A cross-sectional view in schematic representation showing two of the monomers making up the homotetrameric structure and showing, in space-fill representation, the location of Ile-100 in the central pore and the two bands of Trp residues at the extracellular (top) and intracellular (bottom) sides. The coordinates were from Protein Data Bank entry 1K4C.

Val-505, has also been shown to be important in fatty acid binding.[12] The distances between Ile-100 in KcsA and Trp-67 and Trp-68 in the same subunit are both ~15 Å, with distances between Ile-100 and Trp-67 and Trp-68 in adjacent subunits of ~25 Å and even greater distances to the lipid-exposed Trp residues. A brominated fatty acid such as 9,10-dibromostearic acid (BrSA) bound to the central cavity of KcsA, in a position similar to that suggested for arachidonic acid in Kv1.1,[12] would therefore result in inefficient quenching of Trp residues. As a consequence, Trp fluorescence quenching by brominated fatty acids will report just on binding to annular and nonannular sites.

To study possible binding of fatty acids to the central cavity of KcsA, we have adopted an alternative approach. EPR spectra of nitroxide-labeled lipids are sensitive to molecular mobility and provide a way of distinguishing between lipid molecules in the bulk lipid bilayer component of a membrane and lipid molecules associated directly with membrane proteins.[17] Here we show the presence of a yet more strongly immobilized component in the EPR spectra of 14-nitroxystearic acid bound to KcsA and suggest that this corresponds to fatty acid bound in the channel cavity.

EXPERIMENTAL PROCEDURES

Dioleoylphosphatidylcholine (DOPC) was obtained from Avanti Polar Lipids (Alabaster, AL) and brominated as described previously[8] to give bis(9,10-dibromostearoyl)phosphatidylcholine (BrPC). Oleic acid, methyl oleate, and oleyl alcohol were obtained from Sigma and were brominated similarly to give BrSA, 9, 10-dibromomethylstearate, and 9,10-dibromostearoyl alcohol, respectively. The spin-labeled stearic acid, 14-(4,4-dimethyloxazolidinyl-*N*-oxyl)stearic acid (14-SASL), and the corresponding phosphatidylcholine, 1-acyl-2-[14-(4,4-dimethyloxazolidinyl-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine (14-PCSL), were synthesized as described by Marsh.[17]

Mutagenesis and Reconstitution of KcsA. Site-directed mutagenesis was performed using the QuikChange protocol from Stratagene (La Jolla, CA). In the mutant W67,68, the Trp residues at positions 26, 87, and 113 were replaced with Leu, leaving just the Trp residues at positions 67 and 68. The mutant W67,68R64,89L in which Arg residues at positions 64 and 89 of mutant W67,68 were replaced with Leu was produced. The mutations were confirmed by DNA sequencing.

KcsA and the mutants were purified as described by Marius et al.[8] For fluorescence experiments, KcsA was reconstituted into lipid bilayers by mixing lipid and KcsA in cholate followed by dilution into buffer [20 mM Hepes and 100 mM KCl (pH 7.2)] to decrease the concentration of cholate below its critical micelle concentration and re-form membranes.[8] For EPR measurements, samples were reconstituted in a similar way, followed by dialysis to remove detergent and then pelleting in a benchtop centrifuge.

Fluorescence and EPR Measurements. Fluorescence was recorded on a model 8000C fluorimeter (SLM, Urbana, IL) with excitation at 290 nm, at 25 °C. Fluorescence emission spectra were corrected for light scatter by subtracting a blank consisting of lipid alone in buffer. The reported fluorescence intensities represent averages of triplicate measurements from two or three separate reconstitutions. For experiments with mixtures of DOPC and 9,10-dibromomethylstearate or 9,10-dibromostearoyl alcohol, the components were mixed in the required proportions in organic solvent, dried, and resuspended in cholate before reconstitution with KcsA. Because of its significant aqueous solubility, BrSA in a small volume of methanol was added to the sample after reconstitution; the volume of added methanol never exceeded 4% of the total volume, at which level it had no measurable effect on fluorescence intensity. Fluorescence quenching by BrSA was corrected for the small decrease in fluorescence intensity observed upon addition of an equal concentration of oleic acid.

EPR spectra were recorded on a 9 GHz Varian Century-Line EPR spectrometer with temperature control by thermostated nitrogen gas flow. 14-SASL was incorporated into reconstituted KcsA samples with the required lipid:KcsA molar ratio, at a 14-SASL:lipid ratio of 0.5 mol %, by addition of a concentrated solution of 14-SASL in ethanol. Samples were transferred to 50 μL glass capillaries and placed in a standard 4 mm quartz sample tube. EPR spectra were analyzed by spectral addition or subtraction, as described previously.[14,17] In the spectral addition procedure, spectra were fitted by the weighted sums of two spectral components chosen from a set of spectra for 14-SASL in DOPC and for 14-SASL bound to bovine serum albumin, recorded at 3 °C intervals over the temperature range of 0−37 °C. In fitting a particular experimental spectrum, we first chose an immobile component by matching the observed maximal hyperfine splittings and then used a least-squares procedure to choose the mobile component giving the best fit to the experimental spectrum in the low-field region, where the separation between the mobile and immobile components is most clear. The choice of the immobile component was then

checked by repeating the fitting procedure with a range of immobile components close to that chosen initially. Finally, the calculated composite spectrum was compared to the experimental spectrum to ensure that all features of the spectrum were reproduced faithfully. The reported fractions of mobile and immobile components are the averages of two separate reconstitutions, and the error bars represent the range of values giving acceptable fits to the experimental data.

Analysis of Fluorescence Quenching Data. Fluorescence quenching in a mixture of a brominated phospholipid with its corresponding nonbrominated phospholipid can be described in the simplest case of a single Trp residue and a single type of lipid binding site by the equation

$$F = F_{min} + (F_o - F_{min})(1 - x_{Br})^n \quad (1)$$

where F_o and F_{min} are the fluorescence intensities in nonbrominated and brominated lipid, respectively, F is the fluorescence intensity in the phospholipid mixture when the mole fraction of brominated lipid is x_{Br}, and n is the number of lipid binding sites from which the fluorescence of the Trp residue can be quenched.[13,18] Fitting the experimental quenching data for a chosen phospholipid to eq 1 gives the value of n for that phospholipid.

In a mixture of two different lipid molecules, A and B, equilibrium will be established at each annular lipid binding site:

$$P{\cdot}A + B \leftrightarrows P{\cdot}B + A \quad (2)$$

where P·A and P·B are sites occupied by lipids A and B, respectively, and the binding constant K for lipid B relative to lipid A is given by

$$K = (x_{P\cdot B}x_A)/(x_{P\cdot A}x_B) \quad (3)$$

where the concentrations are in mole fraction units, x, within the membrane. Fluorescence quenching at the annular sites is then described by the equation

$$F = F_{min} + (F_o - F_{min})(1 - f_{Br})^n \quad (4)$$

where f_{Br}, the fraction of sites occupied by brominated lipid, is given from eq 3 by

$$f_{Br} = Kx_{Br}/[Kx_{Br} + (1 - x_{Br})] \quad (5)$$

where K is the association constant for lipid B relative to lipid A. The value of K can therefore be determined from quenching plots if the value of n is known.

Analysis of the fluorescence quenching results in this way for the mutant W67,68 gives values for n and K for binding at nonannular sites on KcsA, because with this mutant quenching of Trp fluorescence occurs only as a result of binding at the nonannular sites. For wild-type KcsA, quenching will result from binding at both the annular and the nonannular sites. Assuming that all Trp residues have equal unquenched fluorescence intensities and that the three lipid-exposed Trp residues are equivalent,[13] the fluorescence intensity can be written as

$$F = [1 + 3F^{A}_{min} + F^{NA}_{min} + 3(1 - F^{A}_{min})(1 - f^{A}_{Br})^{n_A} + (1 - F^{NA}_{min})(1 - f^{NA}_{Br})^{n_{NA}}]/5 \quad (6)$$

where F^{A}_{min} and F^{NA}_{min} are the minimal fluorescence values for the three lipid-exposed Trp residues and Trp-67, respectively, f^{A}_{Br} and f^{NA}_{Br} are the fractional occupancies by brominated lipids of the annular and nonannular sites, respectively, and n_A and n_{NA} are the values for n describing quenching at the annular and nonannular sites, respectively.

The experimental data were fit to eqs 1−6 using the nonlinear least-squares routine in SigmaPlot (SPSS Inc., Chicago, IL).

Analysis of Binding of 14-SASL to KcsA. As described in Results, EPR spectra for 14-SASL in the presence of KcsA are consistent with fatty acid binding to a single site in the central pore of KcsA. The EPR experiments were performed at a DOPC concentration of ∼85 mM and a total mole fraction of 14-SASL with respect to DOPC (X_{FA}) of 0.005, conditions under which all the 14-SASL will be in the lipid bilayer or bound to KcsA, as described below. Binding of 14-SASL to its binding site on KcsA can then be described by the equilibrium

$$P + FA_L \leftrightarrows P{\cdot}FA \quad (7)$$

where FA_L and P·FA are fatty acid in the lipid bilayer and bound to protein sites, respectively. The association constant K_A for binding of 14-SASL to a single site on KcsA is given from eq 7 by

$$K_A = \frac{x_{P\cdot FA}}{x_{FA}x_P} \quad (8)$$

where concentrations are again expressed as mole fractions, x, within the membrane. Total mole fractions are denoted with the uppercase X, whereas those of bound or "free" species are denoted with the lowercase x. The mole fraction, $x_{P\cdot FA}$, of protein-bound fatty acid (P·FA) is given according to eq 8 by

$$x_{P\cdot FA} = \left[n_s X_P + X_{FA} + 1/K_A - \sqrt{(n_s X_P + X_{FA} + 1/K_A)^2 - 4n_s X_P X_{FA}}\right]/2 \quad (9)$$

where X_P is the total mole fraction of the KcsA tetramer (approximates the tetramer:lipid mole ratio) and n_s is the number of binding sites per KcsA tetramer.

RESULTS

Partitioning of Fatty Acids into the Lipid Bilayer. The significant water solubility of the fatty acids means that only at high lipid concentrations will most of the added fatty acid be in the lipid bilayer. As shown in Figure S1 of the Supporting Information, partitioning of BrSA into bilayers of DOPC is almost complete (>95%) at lipid concentrations of ≥300 μM. Fluorescence quenching experiments to determine the binding constants for KcsA were therefore performed at lipid concentrations of ≥300 μM.

Binding to Nonannular Lipid Binding Sites on KcsA. The W67,68 mutant was used to determine binding constants at the nonannular sites on KcsA. Addition of BrSA to W67,68 reconstituted with DOPC resulted in a marked decrease in fluorescence intensity (Figure 2A), demonstrating binding of fatty acids at the nonannular sites. The observed level of quenching depends on the number of binding sites (n) for BrSA close enough to the Trp residues in W67,68 to cause quenching and on the fractional occupancy of these sites by BrSA (eq 4). Normally, the value for n would be determined from quenching plots of a mixture of a brominated lipid and its nonbrominated analogue (eq 1), but the value of n for BrSA cannot be determined in this way because fatty acids alone do not form bilayers. However, values for n can be obtained from studies of the quenching of KcsA fluorescence in mixtures of

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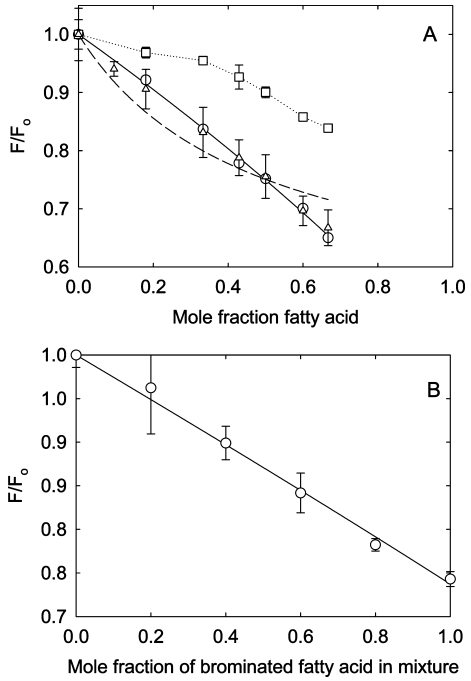

Figure 2. Quenching of the fluorescence of the mutant W67,68 by BrSA. (A) W67,68 was reconstituted into mixtures of DOPC and BrSA, and fluorescence intensities were expressed as F/F_o, where F_o is the fluorescence intensity in DOPC and F is the fluorescence intensity at the given mole fraction of BrSA (○). Fluorescence intensities (○) have been corrected for the decrease in fluorescence intensity observed upon addition of oleic acid (□). Fluorescence intensities (△) are also shown for the mutant W67,68R64,89L. (B) W67,68 was reconstituted into mixtures of DOPC and fatty acid (BrSA and oleic acid) at a total fatty acid:DOPC molar ratio of 2:1, varying the mole fraction of BrSA in the fatty acid mixture (○). The solid lines in panels A and B show fits to the competition model for binding (eqs 4, 11, and 13) giving values for n and K_1 of 0.91 ± 0.24 and 0.87 ± 0.08, respectively. The dashed line in panel A shows the best fit to the simple binding model (eq 14) as described in the text. The DOPC:KcsA monomer molar ratio was 400:1, and the concentrations of KcsA monomer and DOPC were 0.75 and 300 μM, respectively.

oleic acid, BrSA, and DOPC, which do form bilayers, with the mole fraction of total fatty acid (oleic acid and BrSA) being fixed but the ratio of oleic acid to 9,10-dibromostearic acid being varied. If the fraction of sites on KcsA occupied by total fatty acid is f_{FA} and the fraction of the total fatty acid molecules that is BrSA is x_{BrFA}, the fraction of sites occupied by brominated fatty acid f_{BrFA} is

$$f_{BrFA} = f_{FA} x_{BrFA} \tag{10}$$

The fluorescence intensity, by analogy with eq 4, becomes

$$F = F_{min} + (F_o - F_{min})(1 - f_{FA} x_{BrFA})^n \tag{11}$$

Values of n and K for BrSA can then be determined by iterative fitting of data sets, fitting quenching data in DOPC/oleic acid/BrSA mixtures to eq 11 and in DOPC/BrSA mixtures to eqs 4 and 5. Because any proportional error in the value of f_{FA} will be smallest when the value of f_{FA} is close to 1, the experiments with DOPC/oleic acid/BrSA mixtures were performed at a high mole fraction of total fatty acid.

Quenching in mixtures of DOPC, oleic acid, and BrSA as a function of BrSA content at a fixed mole fraction of total fatty acid was close to linear (Figure 2B), showing that the value of n was close to 1. The quenching data fit to a competitive binding model in which binding of a molecule of fatty acid displaced a molecule of DOPC from the site; an iterative fit of the two sets of data to the competitive binding model (eqs 4 and 5) gave values for n and the relative association constant K_1 of 0.91 ± 0.24 and 0.87 ± 0.08, respectively (Figure 2). The value for F_{min} of 0.43 ± 0.03 is consistent with the expected extensive quenching of Trp-67 with little quenching of Trp-68.

An alternative to a competitive model for binding of BrSA to the nonannular sites is a simple binding model in which lipid A (DOPC) does not bind significantly to the nonannular sites. In this case, binding of lipid B (BrSA) could be described by a simple binding equation

$$P + B \leftrightarrows P{\cdot}B \tag{12}$$

The equilibrium constant describing competitive binding (eq 2) has no units, whereas the association constant describing the simple binding reaction (eq 12) will have units of reciprocal mole fraction. The two models are related as follows when the competitive binding constant $K > 1$. The fraction f_1 of nonannular sites occupied by lipid B in a mixture of lipids A and B for the competitive binding model is (see eq 5)

$$f_1 = K_1 x_B / [K_1 x_B + (1 - x_B)] \tag{13}$$

where K_1 is the association constant for lipid B relative to lipid A and x_B is the mole fraction of lipid B in the membrane. The fraction f_2 of nonannular sites occupied by lipid B in a mixture of lipids A and B for the simple binding model is

$$f_2 = K_2 x_B / (K_2 x_B + 1) \tag{14}$$

where K_2 is the association constant for lipid B. Now consider the function

$$f_2\left(\frac{1 + K_2}{K_2}\right) = \left(\frac{K_2 x_B}{K_2 x_B + 1}\right)\left(\frac{1 + K_2}{K_2}\right) = \frac{x_B(1 + K_2)}{x_B(1 + K_2) + 1 - x_B} \tag{15}$$

This shows that scaling f_2 by the factor $(1 + K_2)/K_2$ gives a function of the same form as f_1 (eq 13) but with K_1 replaced by $1 + K_2$. The scaling factor accounts for the fact that in the competitive binding model the nonannular site will be fully occupied by lipid B at a mole fraction x_B of 1, whereas this will not be true in the simple binding model where the site might be only partially filled, depending on the value of K_2. The above relationship between the competitive and simple binding models is valid only when $K_1 > 1$ because by necessity $K_2 > 0$ and $K_1 = 1 + K_2$. When $K_1 < 1$, the function f_1 (eq 13) does not have the shape of a simple binding curve.

It was not possible to obtain a good fit of the data shown in Figure 2 to a simple binding model in which the site was either occupied by fatty acid or empty (eqs 12 and 14). A free fit of the data in Figure 2A to eq 14 with a value for n of 0.91 resulted in a value for binding constant K_2 of 0.03 ± 0.15 mole fraction^{-1} with a physically impossible negative value for F_{min} (−16.7), the large negative value for F_{min} being required to give strong quenching when the value for K_2 is so low that the binding site is largely empty. Fixing F_{min} at 0.5 (corresponding to the quenching of just Trp-67) resulted in a very poor fit to the data with a value for binding constant K_2 of 0.2 ± 0.3 mole fraction^{-1} (dashed line, Figure 2A).

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Addition of 9,10-dibromomethylstearate or 9,10-dibromostearoyl alcohol resulted in only low levels of quenching of the fluorescence of W67,68 (Figure 3), showing very weak binding to the nonannular sites on KcsA.

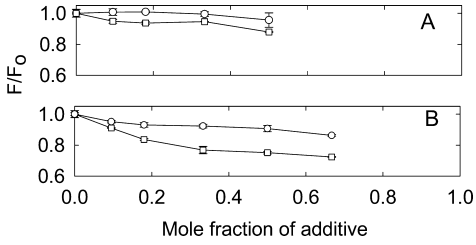

Figure 3. Quenching of the fluorescence of wild-type KcsA and mutant W67,68 by 9,10-dibromostearoyl alcohol (A) and 9,10-dibromomethylstearate (B). Wild-type KcsA (□) and mutant W67,68 (○) were reconstituted into mixtures of DOPC and 9,10-dibromostearoyl alcohol (A) and 9,10-dibromomethylstearate (B), at the given mole fractions of the brominated molecule.

Binding to Annular Lipid Binding Sites on KcsA. Quenching data for wild-type KcsA were analyzed by using the parameters for binding at the nonannular sites determined above to obtain relative binding constants at the annular lipid binding sites. The data fit well to eq 6, giving values of n and K for the annular sites of 4.6 ± 0.2 and 0.67 ± 0.04, respectively, with a value of F^{A}_{min} for the three lipid-exposed Trp residues of 0.27 ± 0.01 (Figure 4). As observed with the W67,68 mutants, the presence of 9,10-dibromostearoyl alcohol resulted in little quenching of the fluorescence of wild-type KcsA (Figure 3A), showing very weak binding at the annular lipid binding sites. With 9,10-dibromomethylstearate, quenching was more marked at the annular sites than for 9,10-dibromostearoyl alcohol, but unlike the case with BrSA, quenching leveled off at a mole fraction of ~0.3, which could reflect the miscibility limit of 9,10-dibromomethylstearate in the lipid bilayer.

Effect of Arg-64 and -89 on Binding to the Nonannular Sites. Close to each nonannular binding site at the monomer−monomer interfaces in KcsA are two Arg residues, Arg-64 and Arg-89. Because these are the only charged residues close to the nonannular binding sites, they could contribute to fatty acid binding at these sites. However, fluorescence quenching by BrSA is the same for W67,68 and for the mutant W67,68R64,89L in which the Arg residues at positions 64 and 89 have been replaced with Leu residues (Figure 2A). These two Arg residues are therefore unlikely to contribute to binding of the fatty acid to the nonannular sites.

EPR Studies of 14-SASL Binding. EPR spectra for the spin-labeled fatty acid 14-SASL in the presence of a wide range of membrane proteins contain two components, a broad component corresponding to lipids bound at annular sites and a sharp component corresponding to the fluid bilayer regions of the membrane. With the possible exception of the nicotinic acetylcholine receptor,[19] the broad component from the spin-labeled fatty acid is very similar to that observed with spin-labeled phospholipids.[20,21] However, the EPR spectra obtained for 14-SASL and 14-PCSL in the presence of KcsA are very different (Figure 5). The spectrum for 14-SASL is dominated by an immobile component with a maximal hyperfine splitting of 64.6 G, compared to a smaller immobile component for 14-PCSL with a maximal splitting of 60.7 G. The spectrum of the immobile component seen with 14-SASL is very similar to that for 14-SASL bound to bovine serum albumin (Figure 5).

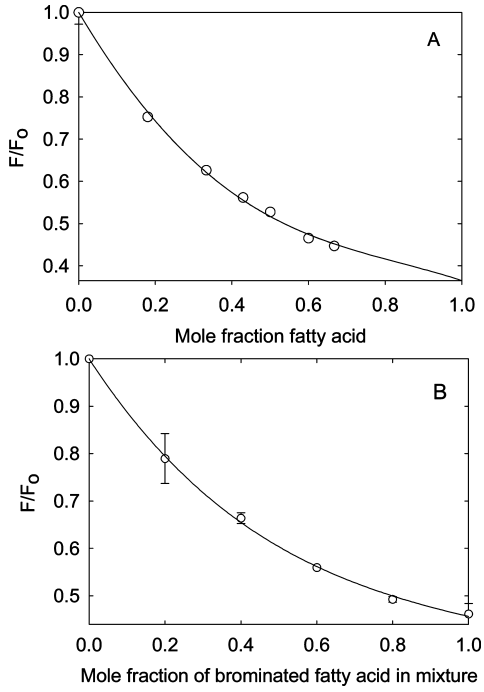

Figure 4. Quenching of the fluorescence of wild-type KcsA by BrSA. (A) KcsA was reconstituted into mixtures of DOPC and BrSA, and fluorescence intensities were expressed as F/F_o, where F_o is the fluorescence intensity in DOPC and F is the fluorescence intensity at the given mole fraction of BrSA (○). Fluorescence intensities were corrected for the decrease in fluorescence intensity observed upon addition of oleic acid. (B) KcsA was reconstituted into mixtures of DOPC and fatty acid (BrSA and oleic acid) at a total fatty acid:DOPC molar ratio of 2:1, varying the mole fraction of BrSA in the total fatty acid mixture (○). The solid lines show fits to the competition model for binding (eqs 2 and 6) with values of n and K for the nonannular sites of 0.91 and 0.87, respectively, giving values of n and K for the annular sites of 4.6 ± 0.2 and 0.67 ± 0.04, respectively. The DOPC:KcsA monomer molar ratio was 400:1, and the concentrations of KcsA monomer and DOPC were 0.75 and 300 μM, respectively.

Both the outer splitting and the line widths indicate that the fatty acid is more strongly immobilized than the phospholipids that are motionally restricted in the annular sites surrounding the protein (see, for example, refs 22 and 23).

Figure 6 shows EPR spectra for 14-SASL as a function of the DOPC:KcsA channel mole ratio at a fixed mole fraction of 14-SASL in the membrane of 0.005. In all cases, the spectra are dominated by the immobile component. The spectra were fit with a sum of two components by using a library of spectra for 14-SASL bound to BSA recorded over the temperature range of 0−37 °C to represent the immobile component, and a library of spectra for 14-PCSL in DOPC over the same temperature range to represent the mobile component, as described in Experimental Procedures.

Figure 7 shows the concentration of the immobile EPR component obtained from the fitting procedure, expressed in terms of the mole fraction of immobilized 14-SASL in the membrane, plotted as a function of the DOPC:KcsA tetramer mole ratio. The proportion of 14-SASL in the immobile component varied from 74 to 90% over the range of DOPC:KcsA tetramer mole ratios from 100:1 to 30:1. Binding of 14-SASL to KcsA was characterized by an association constant K_A for binding of 14-SASL to KcsA from the DOPC bilayer (eq 8). The high fraction of bound 14-SASL meant that it was not possible to

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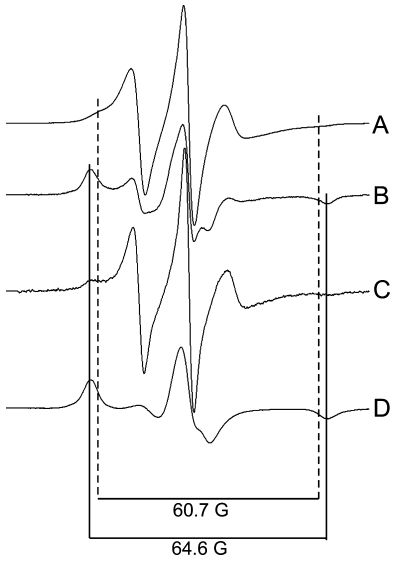

Figure 5. EPR spectra of spin-labeled phosphatidylcholine and spin-labeled fatty acid in the presence of KcsA. (A and B) EPR spectra of 14-PCSL and 14-SASL, respectively, in the presence of KcsA at a DOPC:KcsA tetramer molar ratio of 60:1, at pH 7.2 and 25 °C. (C) Corresponding spectrum for 14-SASL but at pH 3.9. The vertical lines indicate the location of the outer peaks in the 14-PCSL and 14-SASL spectra. The maximal splitting for 14-SASL in KcsA matches that in a sample of 14-SASL bound to BSA at 6 °C (D). For the samples containing 14-SASL, the DOPC:KcsA tetramer molar ratio was 60:1 and the mole fraction of 14-SASL was 0.005, giving a 14-SASL:KcsA tetramer molar ratio of 0.3:1. The first-derivative EPR spectra have all been normalized to equal double-integrated area.

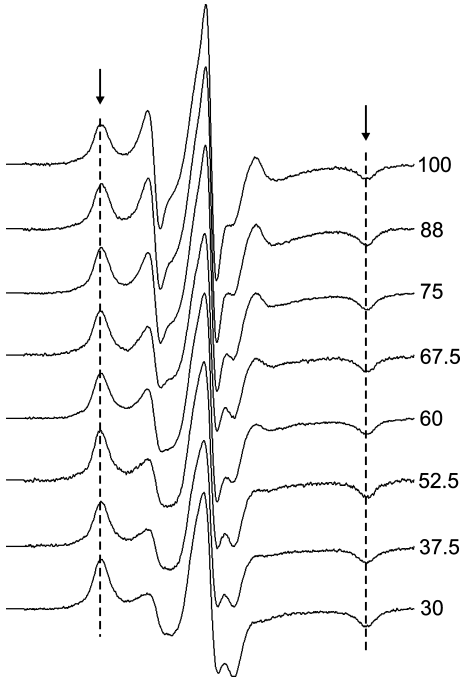

Figure 6. EPR spectra of 14-SASL in the presence of KcsA as a function of DOPC:KcsA tetramer molar ratio, at 25 °C. The outer peaks of the immobile component are shown by the arrows and dashed lines. The first-derivative EPR spectra have all been normalized to equal double-integrated area.

determine separately both the number of binding sites per channel (n_s) and the affinity of the site (K_A). The data in Figure 7

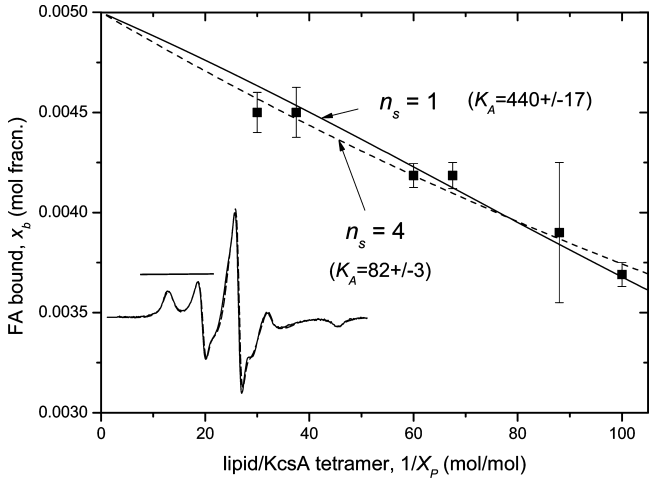

Figure 7. Mole fraction of 14-SASL bound to KcsA as a function of lipid:KcsA tetramer molar ratio. The experimental data (■) for the bound concentration of 14-SASL were obtained by fitting the spectra for 14-SASL shown in Figure 6 and are expressed in terms of the mole fraction of immobilized 14-SASL relative to total lipid in the membrane. The total mole fraction of 14-SASL in the membrane was constant at 0.005. The data were fit by eq 9 with a value for the number of binding sites per channel (n_s) of 1, giving an association constant (K_A) of 440 ± 17 (—), or with an n_s of 4 giving a K_A of 82 ± 3 (---). The inset (—) shows the experimental EPR spectrum for 14-SASL bound to KcsA at a lipid:KcsA molar ratio of 100:1; the dashed line shows the best fit spectrum, with an immobile fraction of 0.738. The horizontal line above the EPR spectrum shows the region of the spectrum where the separation between the mobile and immobile components is most clear.

were therefore fit to eq 9 by assuming an n_s value of 1, giving a value for the association constant K_A of 440 ± 17. A fit for an n_s of 4 is also included in Figure 7, for comparison; the fitted K_A value of 82 ± 3 is correspondingly lower.

When the pH was changed from 7.2 to 3.9, the immobile component of 14-SASL decreased very markedly in intensity and changed in line shape, with a concomitant increase in the intensity of the mobile component (Figure 5). This might conceivably reflect a change in the conformation of KcsA. However, the pK_a for lipid-bound fatty acid is ~7,[24,25] so that at pH 3.9 bound 14-SASL will be present largely in the protonated, uncharged form. This would immediately result in a loss of intensity for the immobile component, if, as is likely, the protonated form of the fatty acid binds only weakly to the site on KcsA to which the charged form binds with high affinity, the fatty acid then being located primarily in the lipid bilayer.

Trp Fluorescence Quenching by 14-SASL. It is possible to compare the results of the EPR studies with 14-SASL and the fluorescence quenching studies with BrSA by making use of the ability of nitroxide spin-labels to quench Trp fluorescence. London and Feigenson[18] have shown that spin-labels quench Trp fluorescence by a contact mechanism with a critical distance of ~11 Å. Figure 8A compares the quenching of the fluorescence of the Trp analogue *N*-palmitoyl-L-tryptophan *n*-hexyl ester (NPTH) in bilayers of DOPC by BrSA and by 14-SASL, showing very similar concentration dependencies for quenching by the two quenchers, but with slightly more efficient quenching with 14-SASL. Quenching of the Trp fluorescence of wild-type KcsA and of W67,68 by 14-SASL is also slightly more marked than quenching by BrSA (Figure 8), but again with a very similar concentration dependence.

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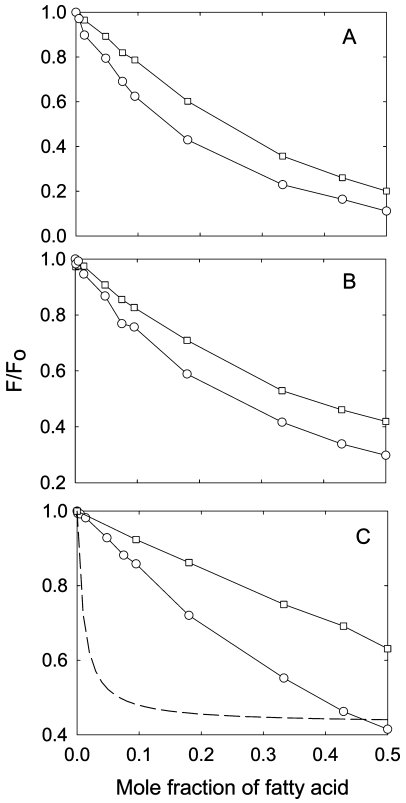

Figure 8. Fluorescence quenching by 14-SASL. Fluorescence intensities are plotted for the Trp analogue NPTH (A), wild-type KcsA (B), and W67,68 (C) in the presence of the given mole fractions of 14-SASL (○) or BrSA (□). Fluorescence intensities are expressed as F/F_o, where F_o is the fluorescence intensity in the absence of fatty acid, and in panels B and C, the data have been corrected for the decrease in fluorescence intensity observed upon addition of oleic acid. For all samples, the concentration of DOPC was 600 μM. For panel A, the DOPC:NPTH molar ratio was 300:1. For panel B, the DOPC:KcsA tetramer molar ratio was 4000:1. For panel C, the DOPC:W67,68 tetramer molar ratio was 1600:1. The dashed line in panel C corresponds to the quenching curve that would be observed if the high-affinity 14-SASL binding detected in the EPR experiments corresponded to binding to the nonannular sites, as described in the text.

These fluorescence quenching experiments suggest that 14-SASL and BrSA have similar affinities for the annular sites and nonannular sites. In particular, there is no evidence of a site with a very high affinity for 14-SASL on KcsA as detected by the EPR experiments. For example, if the high-affinity binding observed in the EPR experiments corresponded to binding at the four nonannular sites per KcsA channel, the corresponding value of K_A would be ~82 (see eq 9 and Figure 7), and assuming for the sake of simplicity that the quenching efficiencies of 14-SASL and BrSA are equal, the quenching curve (Figure 8C, dashed line) would show extensive quenching at low 14-SASL concentrations, which is not observed experimentally. We conclude therefore that the high-affinity binding observed for 14-SASL in the EPR experiments does not correspond to binding to either the annular or nonannular binding sites and must correspond to binding to a site too distant from the Trp residues in KcsA to result in any fluorescence quenching.

DISCUSSION

Annular and Nonannular Binding Sites. The results presented here show that fatty acids bind to a variety of sites on KcsA. Binding of phospholipids to the annular sites on KcsA shows limited selectivity with, for example, a binding constant for phosphatidic acid relative to DOPC of ~2.0.[26] Quenching of the fluorescence of the lipid-exposed Trp residues in wild-type KcsA by BrSA (Figure 4) shows that BrSA can also bind to these sites with a binding constant relative to DOPC of 0.67 ± 0.04.

The value of n describing the average number of two-chain lipid molecules binding close enough to a Trp residue in wild-type KcsA to result in quenching (eq 1) varies with phospholipid structure between 1.7 and 2.5;[26] the value of n for BrSA, 4.6 ± 0.2, suggests that two fatty acid molecules will bind to KcsA at the annular sites in exchange for one two-chain phospholipid. An alternative way to describe binding at the annular sites is to express concentrations in the membrane on a chain basis, accounting for the fact that BrSA contains just one chain whereas DOPC contains two. With concentrations expressed in this way, occupancy of the binding sites by fatty acid is still described by eq 5 but with K replaced by $2K$. The binding constant for BrSA relative to DOPC then becomes 1.34, similar to the binding constant for phosphatidic acid relative to DOPC.[26]

Quenching studies with the KcsA mutant W67,68 (Figure 2) show that BrSA can also bind to the nonannular sites on KcsA located one at each protein−protein interface in the homotetrameric structure. These sites show a preference for anionic lipid, and the presence of anionic lipid leads to an increased probability of the channel being in a conducting state.[7,8] The binding constant for BrSA relative to DOPC was determined to be 0.87 ± 0.09 (Figure 2), which can be compared to a binding constant for dioleoylphosphatidylglycerol relative to DOPC of 3.33 ± 0.35 calculated from the data of Marius et al.[8] The value of n for quenching of W67,68 fluorescence by BrSA was 0.91 ± 0.24 (Figure 2), consistent with the crystal structure of KcsA that shows that just one of the two chains of the bound phosphatidylglycerol molecule penetrates into the cleft between adjacent monomers, with the second chain adopting a more peripheral location.[7]

Two Arg residues, Arg-64 and Arg-89, located close to the nonannular binding site probably contribute to the preferential binding of anionic lipid to the nonannular site,[8] and indeed, molecular dynamics simulations show the bound phosphatidylglycerol molecule hydrogen bonding to these two Arg residues.[27] However, mutation of these two Arg residues to Leu resulted in no change in binding of BrSA to the nonannular sites (Figure 2), suggesting that they are not involved in direct interaction with the carboxyl group of the fatty acid. An alternative possibility could be hydrogen bonding to the NH group of the peptide bond between Thr-85 and Leu-86.

Uncharged analogues of BrSA such as 9,10-dibromostearoyl alcohol and 9,10-dibromomethylstearate show very weak binding at the nonannular sites on KcsA (Figure 3). The level of quenching of wild-type KcsA by 9,10-dibromostearoyl alcohol was also low, suggesting limited binding to the annular sites, but for 9,10-dibromomethylstearate, quenching at low mole fractions was more comparable to that seen with BrSA, although quenching leveled off at a mole fraction of ~0.3 (Figure 3B). These results could reflect weak interactions of these molecules with the annular and nonannular sites but could also reflect their mixing properties with the DOPC bilayer. Smaby and Brockman[28] reported miscibility limits for methyl oleate and oleyl alcohol with 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) in monolayers of ~1:4 and ~1:1, respectively. They also report formation of a complex between methyl oleate and oleyl alcohol and POPC with the formation of condensed

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complexes with areas smaller than the sums of the areas of the two components.[28] Limited binding of 9,10-dibromostearoyl alcohol and 9,10-dibromomethylstearate to sites on KcsA could therefore be in part due to strong interaction of these molecules with the phospholipid component of the membrane, competing with binding to the protein. Limited binding would also result if the condensed complexes formed separate domains within the membrane, as suggested for oleyl alcohol,[29] and KcsA partitioned preferentially into the domains enriched with DOPC.

The level of occupancy of the annular and nonannular binding sites by oleic acid can be calculated for a given aqueous concentration of oleic acid from the relative binding constants determined above and the dissociation constant of 17 μM[25] describing binding of oleic acid to a lipid bilayer. For example, at an aqueous concentration of oleic acid of 17 μM, 40% of the annular sites and 47% of the nonannular sites will be occupied by oleic acid.

Binding to the Central Cavity. The lining of the central cavity of potassium channels (Figure 1) has been shown to be hydrophobic and provide a binding site for the tetrabutylammonium ion and its derivatives[10,11] and for the drug clotrimazole.[16] Increasing the length of the alkyl chains in a tetraalkylammonium ion increases its binding affinity, consistent with hydrophobic interactions being important in binding.[30] It has been suggested, on the basis of mutagenesis studies, that fatty acids can also bind to this central hydrophobic cavity[4,12] and binding of tetraalkylammonium ions and fatty acids is competitive.[12]

Binding of BrSA to the central cavity of KcsA would not be detected by fluorescence quenching because the site is too distant from the Trp residues in KcsA to result in quenching. EPR with spin-labeled fatty acids was therefore used to detect binding to the cavity, making use of the sensitivity of EPR spectra of spin-labeled molecules to rotational immobilization. The EPR spectrum for 14-SASL bound to KcsA shows a maximal hyperfine splitting greater than that observed for the spin-labeled phosphatidylcholine 14-PCSL in the presence of KcsA (Figure 5). The maximal splitting observed for 14-SASL bound to KcsA is comparable to that for 14-SASL bound to BSA, and because fatty acids bind to serum albumin in a number of deep pockets within the structure,[31] this implies a buried site for binding of 14-SASL on KcsA.

The proportion of bound 14-SASL registered by the EPR spectra is very high (Figures 5 and 6). It is not possible to obtain estimates for both the number of binding sites and their affinity, but if it is assumed that there is just one binding site per KcsA tetramer, the data fit to an association constant K_A for binding of 14-SASL to KcsA from the lipid phase (eq 9) of 440 ± 17 (Figure 7). Fluorescence quenching studies with 14-SASL confirm that this high-affinity binding site does not correspond to either the annular or nonannular binding sites detected by fluorescence quenching with BrSA (Figure 8). The presence of the nitroxy group on 14-SASL is unlikely to have a major effect on the type of site to which 14-SASL can bind, so that the most likely binding site for 14-SASL is therefore a fatty acid binding site in the central cavity of the potassium channel.

The fatty acid–protein associations studied here are all confined to the membrane, where the appropriate unit of concentration is mole fraction (approximated by mole ratio with respect to lipid). In conventional ligand binding experiments, on the other hand, affinities are expressed by the dissociation constant K_d, with concentrations in moles per liter of the total aqueous volume. These constants are related, as follows. The mole fraction of ligand-free protein, for example, is given by

$$x_P = \frac{[P]}{[L_t]} \tag{16}$$

where brackets denote molar concentrations in water for each species and $[L_t]$ is the total lipid concentration. Expressed in terms of aqueous molar concentrations, eq 8 for the fatty acid association constant therefore becomes

$$K_A = \frac{[P{\cdot}FA][L_t]}{[FA_L][P]} \tag{17}$$

where $[L_t]$ is the total lipid concentration in moles per liter.

The conventional binding experiment is described by the equilibrium between protein-bound 14-SASL and 14-SASL in the aqueous medium

$$P{\cdot}FA \rightleftharpoons FA_w + P \tag{18}$$

where FA_w is free fatty acid in water. From eq 18, the aqueous dissociation constant is thus

$$K_d = \frac{[FA_w][P]}{[P{\cdot}FA]} \tag{19}$$

where brackets again denote molar concentrations in water for each species. From eqs 17 and 19, the conventional dissociation constant is related to the association constant determined in the EPR experiment by

$$K_d = \frac{[FA_w]}{[FA_L]}\frac{[L_t]}{K_A} \tag{20}$$

The quotient $[FA_L]/[FA_w]$ in eq 20 represents enrichment of the fatty acid in the membrane by favorable partitioning from the aqueous phase, the extent of which depends directly on the total aqueous lipid concentration, $[L_t]$.

The partition coefficient K_p of the fatty acid between lipid and water is defined as the ratio of the concentration of fatty acid in the lipid phase, given in units of moles per liter of lipid, to that in the aqueous phase, given in units of moles per liter of water (i.e., $[FA_w]$). If both concentrations are expressed in terms of moles per liter of water, the partition coefficient is then given by

$$K_p = \frac{[FA_L]/([L_t]M_L\bar{\nu}_L \times 10^{-3})}{[FA_w]} \tag{21}$$

where M_L is the molecular weight of the lipid and $\bar{\nu}_L$ (milliliters per gram) is the lipid partial specific volume. The latter, together with the total aqueous lipid concentration $[L_t]$, is needed to convert the concentration of FA_L from moles per liter of water (i.e., $[FA_L]$) to moles per liter of lipid. Via combination of eqs 20 and 21, the relation among the aqueous dissociation constant, the intramembrane association constant, and the lipid/water partition coefficient is

$$K_d = \frac{1}{K_A K_p M_L \bar{\nu}_L \times 10^{-3}} \tag{22}$$

If the fatty acid partition coefficient, K_p, is known, the conventional dissociation constant can then be obtained from the EPR binding results by using eq 22.

A $K_p\bar{\nu}_L$ value of $\approx 3 \times 10^3$ mL/g at pH 7.2 can be calculated for 14-SASL from direct EPR measurements of the partitioning

of spin-labeled myristic acid into lipid bilayers,[24,32] as described in the Supporting Information. Electrophoresis measurements[25] and fluorescence quenching studies[33] give $K_p\bar{v}_L$ values of $\approx 1.3 \times 10^4$ and 2×10^4 mL/g, respectively. Calculated values of K_d then vary between ~0.1 and ~1 μM depending on the value assumed for the partition coefficient. These estimates can be compared with the concentrations of arachidonic acid required for the effects on eukaryotic potassium channels, which vary between ~1 and ~30 μM;[2,4] it has been estimated that free cytosolic concentrations of arachidonic acid are probably approximately $\leq$10 μM under normal conditions but could rise to >50 μM because of the local action of phospholipases.[12]

Effects of nonpolyunsaturated fatty acids vary markedly between channels, with no observed effects on some channels,[1,2] although, for example, the saturated fatty acid myristic acid at 3 μM results in major block of the hIK1 channel.[3] These differences presumably reflect differences in the residues lining the central cavity. Binding could be favored by the presence of multiple *cis* double bonds in the fatty acyl chain because potential energy wells for rotation about C−C bonds adjacent to a double bond are low.[34] Further, a molecular dynamics simulation of the interaction of rhodopsin with lipids containing polyunsaturated fatty acyl chains showed the chains penetrating deeply into the protein core, modifying helix−helix interactions,[35] and similar interactions might occur for a polyunsaturated fatty acid bound to the cavity of a potassium channel.

■ ASSOCIATED CONTENT

Supporting Information

Information about partitioning of fatty acid into lipid bilayers and evidence that partitioning of BrSA is complete at lipid concentrations of >300 μM. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BrSA, 9,10-dibromostearic acid; DOPC, dioleoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; BrPC, bis(9,10-dibromostearoyl)phosphatidylcholine; 14-SASL, 14-(4,4-dimethyloxazolidinyl-*N*-oxyl)stearic acid; 14-PCSL, 1-acyl-2-[14-(4,4-dimethyloxazolidinyl-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine.

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