BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

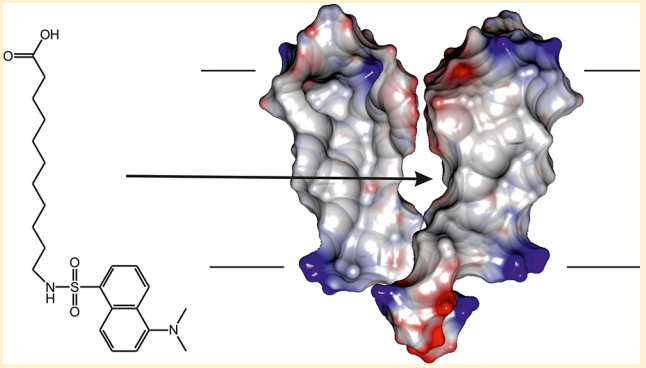
pubs.acs.org/biochemistry

Characterizing the Fatty Acid Binding Site in the Cavity of Potassium Channel KcsA

Natalie Smithers, Juan H. Bolivar, Anthony G. Lee, and J. Malcolm East*

Centre for Biological Sciences, Life Sciences Building, University of Southampton, Southampton SO17 1BJ, U.K.

ABSTRACT: We show that interactions of fatty acids with the central cavity of potassium channel KcsA can be characterized using the fluorescence probe 11-dansylaminoundecanoic acid (Dauda). The fluorescence emission spectrum of Dauda bound to KcsA in bilayers of dioleoylphosphatidylcholine contains three components, which can be attributed to KcsA-bound and lipid-bound Dauda together with unbound Dauda. The binding of Dauda to KcsA was characterized by a dissociation constant of 0.47 ± 0.10 μM with 0.94 ± 0.06 binding site per KcsA tetramer. Displacement of KcsA-bound Dauda by the tetrabutylammonium (TBA) ion confirmed that the Dauda binding site was in the central cavity of KcsA. Dissociation constants for a range of fatty acids were determined by displacement of Dauda: binding of fatty acids increased in strength with an increasing chain length from C14 to C20 but then decreased in strength from C20 to C22. Increasing the number of double bonds in the chain from one to four had little effect on binding, dissociation constants for oleic acid and arachidonic acid, for example, being 2.9 ± 0.2 and 3.0 ± 0.4 μM, respectively. Binding of TBA to KcsA was very slow, whereas binding of Dauda was fast, suggesting that TBA can enter the cavity only through an open channel whereas Dauda can bind to the closed channel, presumably entering the cavity via the lipid bilayer.



Potassium ions moving from the inside to the outside of a cell through a potassium channel first move into a water-filled cavity within the channel and then pass through the narrow selectivity filter that defines the ion selectivity of the filter before being released to the outside (Figure 1).[1] The central cavity is lined by hydrophobic amino acids, ensuring a rapid flow of K^+ ions through the cavity.[1] The hydrophobic lining of the central cavity provides a potential binding surface for small hydrophobic molecules, and X-ray crystallographic studies have shown that tetrabutylammonium (TBA) ions block potassium channel KcsA by binding in the cavity, blocking entry of K^+ ions into the selectivity filter.[2,3] Increasing the length of the acyl chains in a tetraalkylammonium ion increases its binding affinity, showing the importance of hydrophobic interactions for binding.[4] The linings of the central pores of pentameric ligand-gated ion channels have also been shown to be hydrophobic; blockage of these channels by quaternary ammonium ions and by hydrophobic, positively charged drugs such as lidocaine again follows from binding to the hydrophobic surface of the central pore.[5]

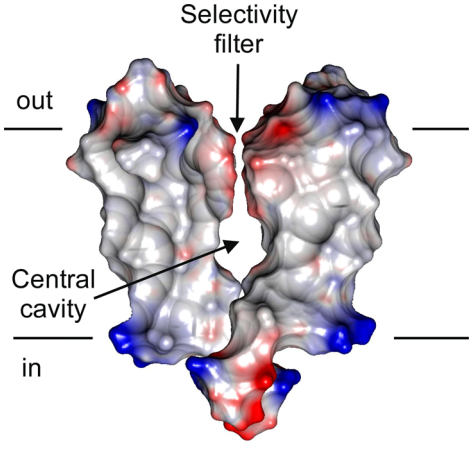


Figure 1. Structure of KcsA. Cross sectional view of the KcsA tetramer in a surface representation showing the selectivity filter and the central cavity. The surface is colored by polarity: gray, hydrophobic; blue, positive; red, negative. The horizontal lines show the approximate position of the surrounding lipid bilayer. The coordinates were from Protein Data Bank entry 1K4C.

Fatty acid molecules have also been suggested to bind in the central cavities of potassium channels, blocking ion flux through the channel.[6−8] The observation that mutations in just one of the four subunits of potassium channel Kv1.1 led to a loss of block by fatty acids suggested that there was a single binding site per channel.[7] We recently used electron paramagnetic resonance (EPR) to study binding of the spin-labeled fatty acid 14-(4,4-dimethyloxazolidinyl-*N*-oxyl)stearic acid (14-SASL) to KcsA.[8] We observed a strongly immobilized signal that we

Received: July 10, 2012
Revised: September 10, 2012
Published: September 12, 2012

   dx.doi.org/10.1021/bi3009196 | *Biochemistry* 2012, 51, 7996−8002

attributed to fatty acid bound in the cavity but were unable to determine the number of binding sites per channel; assuming one site per channel gave a binding constant in the range of ~0.1−1 μM.[8]

The observation that 14-SASL was strongly immobilized on KcsA suggested that it might also be possible to study fatty acid binding using fluorescent analogues of fatty acids, because fluorescence emission spectra can be sensitive to environmental mobility as well as to environmental polarity.[9] In particular, the fluorescence emission spectrum of the dansyl probe shows a marked time dependence on the nanosecond fluorescence time scale, because of solvent relaxation around the excited state dansyl group, resulting in a shift of the emission spectrum to longer wavelengths with increasing times after excitation.[10] The extent to which solvent can relax around a dansyl group during the time it remains in the excited state depends on the mobility of the solvent; large shifts in the fluorescence emission spectrum to long wavelengths are expected when the solvent is mobile, but only small shifts are expected for a rigid solvent. The environment of a dansyl group bound to a site on a protein will consist of, at least in part, amino acid residues whose mobility is likely to be limited on the nanosecond fluorescence time scale; in contrast, a dansyl group embedded in a lipid bilayer will experience an environment with much greater mobility. This suggests that the fluorescence emission spectrum for a dansyl-containing probe bound to a reconstituted membrane protein may contain separate components because of protein-bound and lipid-bound probe. We show here that this is the case for 11-dansylaminoundecanoic acid (Dauda) bound to KcsA and that Dauda can be used to characterize the fatty acid binding site in the cavity of KcsA.

EXPERIMENTAL PROCEDURES

Dioleoylphosphatidylcholine (DOPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Dauda was obtained from Axxora (San Diego, CA). Fatty acids were obtained from Sigma, and tetrabutylammonium bromide was obtained from Aldrich.

Purification and Reconstitution of KcsA. KcsA was purified as described by Marius et al.[11] It was reconstituted into lipid bilayers by mixing lipid and KcsA in cholate at a DOPC:KcsA tetramer molar ratio of 40:1, 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 to re-form membranes.[11]

Fluorescence Measurements. Fluorescence was recorded on a model 8000C fluorimeter (SLM, Urbana, IL) at 25 °C. Dauda was added directly to the fluorescence cuvette containing reconstituted KcsA from a 2 or 0.2 mM stock solution in methanol. Concentrations of Dauda and KcsA were determined using molar extinction coefficients of 4800 and 34850 M^{-1} cm^{-1} for Dauda at 335 nm and KcsA monomer at 280 nm, respectively. Fluorescence intensities were measured at 450 nm with excitation at 345 nm, unless otherwise stated. Values for the intensity of the signal measured in the absence of Dauda were subtracted from those measured in the presence of Dauda to give the fluorescence intensity caused by Dauda emission.

The significant light scatter observed in samples containing high concentrations of protein resulted in a decrease in the observed intensity of Dauda emission. This was corrected for using NADH as a nonbinding fluorescence molecule with excitation and emission characteristics similar to those of Dauda;[9] the fluorescence intensity of NADH (10 μM) was measured in the absence and presence of KcsA with excitation and emission wavelengths of 345 and 450 nm, respectively, and a set of correction factors was generated by comparing the measured fluorescence intensity in the presence of a given concentration of KcsA to that in the absence of KcsA.

It was also necessary to correct for the inner filter effect[9,12] observed at high Dauda concentrations. Fluorescence intensities were measured for Dauda solutions in methanol as a function of Dauda concentration, with excitation and emission wavelengths of 345 and 450 nm, respectively. At low Dauda concentrations, fluorescence intensities increased linearly with an increasing Dauda concentration, but at high concentrations, the fluorescence intensity was reduced because of the inner filter effect; comparison of the observed fluorescence intensities at high concentrations with those expected by extrapolation of the values observed at low concentrations gave the required set of correction factors.

The reported fluorescence intensities represent averages of triplicate measurements from two or three separate reconstitutions.

Analysis of Fluorescence Titrations. As described later, titrations measuring fluorescence intensities of Dauda at 450 nm were fit to the sum of a saturable and a nonsaturable component, corresponding to binding to the cavity of KcsA and to partitioning into the lipid bilayer, respectively. Binding of the saturable component was described by the equation[12]

$$L^b = \left\{ nP^t + L^t + K_d - \left[(nP^t + L^t + K_d)^2 - 4nP^tL^t \right]^{0.5} \right\} /2 \quad (1)$$

where L^t and P^t are the total concentrations of Dauda and KcsA tetramer, respectively, n is the number of saturable binding sites per KcsA tetramer, K_d is the dissociation constant for binding of Dauda to the saturable sites, and L^b is the concentration of Dauda bound to the saturable sites. The observed fluorescence intensity measured at 450 nm, F^{obs}, is then given by

$$F^{obs} = C^sL^b + C^{ns}P^t(L^t - L^b) \quad (2)$$

Here the first term refers to the saturable component, and C^s is the constant relating fluorescence intensity to the concentration of Dauda bound to the saturable sites. The second term refers to the nonsaturable component due to partitioning into the lipid bilayer, the extent of which will depend on the unbound concentration of Dauda ($L^t - L^b$) and on the concentration of lipid, given by the concentration of protein P^t and the molar ratio of lipid:protein; the constant C^{ns} is a composite, including a term relating the fluorescence intensity to the concentration of lipid-bound Duada, the partition coefficient, and the lipid:protein molar ratio, and is treated simply as a variable in the fitting procedure.

Titrations were performed as a function of KcsA concentration at a fixed Dauda concentration and as a function of Dauda concentration at a fixed KcsA concentration, and a global fit of the fluorescence intensities to eq 2 was performed using the nonlinear least-squares routine in SigmaPlot (SPSS Inc., Chicago, IL).

Competition between TBA and Fatty Acids. Assuming a single site at which Dauda and TBA can bind to the KcsA tetramer, the binding equilibria can be written as

$$P + Dauda \leftrightarrows P{\cdot}Dauda$$

dx.doi.org/10.1021/bi3009196 | Biochemistry 2012, 51, 7996−8002

$$P + TBA \leftrightarrows P{\cdot}TBA$$

with dissociation constants of K_1 and K_2 for binding of Dauda and TBA, respectively. In the absence of TBA and under conditions where Dauda is in excess over KcsA so that the free concentration of Dauda is equal to the total concentration, the fraction of the sites on KcsA occupied by Dauda, $[P{\cdot}Dauda]/P^t$, is given by

$$\frac{[P{\cdot}Dauda]}{P^t} = \frac{[Dauda]}{K_1 + [Dauda]} \quad (3)$$

where [Dauda] is the total Dauda concentration. In the presence of TBA, this becomes

$$\frac{[P{\cdot}Dauda]}{P^t} = \frac{[Dauda]}{K_{eff} + [Dauda]} \quad (4)$$

where

$$K_{eff} = K_1\left(1 + \frac{[TBA]}{K_2}\right) \quad (5)$$

and where it is again assumed that the concentration of TBA is much higher than that of KcsA so that the free concentration of TBA is equal to its total concentration, [TBA].

As described below, competition between TBA and Dauda was studied by incubating KcsA with a series of fixed concentrations of TBA and then titrating with Dauda. The Dauda titration data were then fitted to eq 2 to give the corresponding values of K_{eff}, assuming a value for n of 1. The values for K_{eff} as a function of TBA concentration were then fit to eq 5 to give the dissociation constant for TBA, K_2.

Competition between Dauda and nonfluorescent fatty acids for binding to KcsA was studied by titration with a fatty acid at a fixed Dauda concentration. The data were then fit to the equation

$$F^{obs} = F_{max}\left(\frac{[Dauda]}{K_{eff} + [Dauda]}\right) + F_{min} \quad (6)$$

with K_{eff} given by eq 5 but with the concentration of TBA replaced by that of fatty acid, and with F_{max} and F_{min} being the fluorescent values for fully KcsA-bound and non-KcsA-bound Dauda, respectively.

Fitting Fluorescence Emission Spectra. Fluorescence emission spectra of the dansyl group have been shown to fit to the equation for a skewed Gaussian:[13]

$$F = F_o \exp(-(\ln 2)\{\ln[1 + 2b(\lambda - \lambda_{max})/\omega_\lambda]/b\}^2) \quad (7)$$

where F_o is the maximal fluorescence intensity, F is the fluorescence intensity at wavelength λ, λ_{max} is the wavelength at the peak maximum, ω_λ is the peak width at half-height, and b is the skew parameter. Fluorescence emission spectra were fit to single skewed Gaussians (eq 7) or to a sum of skewed Gaussians using the nonlinear least-squares routine in SigmaPlot.

RESULTS

Binding of Dauda to KcsA. The fluorescence emission spectrum of Dauda in water is compared to those of Dauda bound to lipid bilayers and to bovine serum albumin (BSA) in Figure 2A. The emission spectrum for Dauda in buffer is of lower intensity and shifted to a longer wavelength than those for Dauda located in the hydrophobic environments provided by a lipid bilayer or by the fatty acid binding sites in BSA. Importantly, however, the fluorescence emission maximum for Dauda bound to BSA is at a lower wavelength than that for Dauda in a lipid bilayer (Figure 2A).

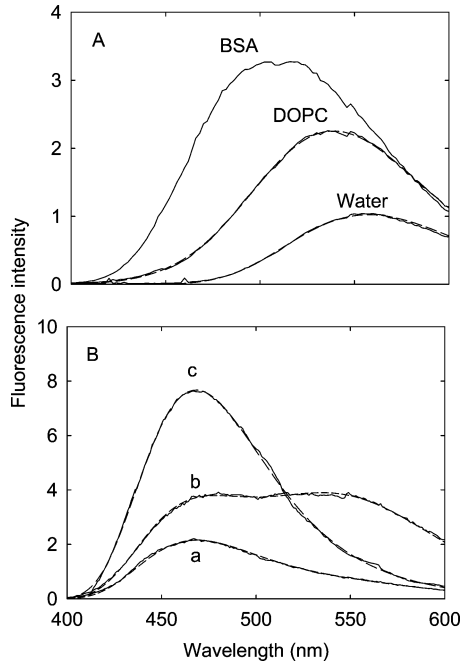


Figure 2. Fluorescence emission spectra of Dauda. (A) Dauda (10 μM) in buffer and in the presence of DOPC (75 μM) and BSA (20 mM). (B) Dauda in the presence of KcsA reconstituted with DOPC with excitation at 345 nm (a and b) or 280 nm (c). The concentration of the KcsA tetramer was 0.19 μM, and concentrations of Dauda were 0.3 (a) and 2 μM (b and c). The dotted lines show best fits to one, two, or three skewed Gaussians, as described in the text, giving the parameters listed in Table 1.

Figure 2B shows fluorescence emission spectra of Dauda bound to KcsA reconstituted in DOPC; the spectra clearly contain more than one component. Fluorescence can be excited directly at 345 nm (curves a and b) or indirectly at 280 nm by the transfer of fluorescence energy from the Trp residues in KcsA (curve c). As shown, when excited at 280 nm, the emission spectrum is dominated by emission at low wavelengths. Because the efficiency of fluorescence energy transfer between donor and acceptor groups is strongly dependent on the distance between the groups,[9] this suggests that fluorescence emission at low wavelengths corresponds to Dauda bound directly to KcsA, for which Trp−dansyl distances will be shorter than for Dauda located in the lipid bilayer component of the membrane.

Fluorescence emission spectra of the dansyl group have the shape of a skewed Gaussian (eq 7).[13] The emission spectrum for Dauda in water (Figure 2A) was fit to this equation, giving the parameters listed in Table 1. The emission spectrum for Dauda in the presence of DOPC (Figure 2A) was then fit to the sum of two skewed Gaussians, corresponding to Dauda in water and bound in the lipid bilayer, with the parameters for the aqueous component fixed at the values listed in Table 1, giving the values for Dauda in the lipid bilayer (Table 1). The emission spectrum for Dauda in the presence of KcsA with excitation at 280 nm was then fit to the sum of three skewed Gaussians, with the parameters for the lipid-bound and aqueous components fixed at the values listed in Table 1, giving the

dx.doi.org/10.1021/bi3009196 | Biochemistry 2012, 51, 7996−8002

Table 1. Fluorescence Emission Parameters for Dauda[a]

component	λ_{max} (nm)	ω_λ (nm)	b
water	557 ± 3	102 ± 1	0.20 ± 0.01
DOPC	512 ± 1	84 ± 3	0
KcsA	469 ± 1	78 ± 2	0.37 ± 0.02

[a]Fluorescence emission spectra shown in Figure 2 were fit to one or more skewed Gaussians (eq 7) as described in the text. λ_{max} is the wavelength at the peak maximum, ω_λ the peak width at half-height, and b the skew parameter.

values for the KcsA-bound component again listed in Table 1. Finally, the spectra obtained at 0.3 and 2 μM Dauda with excitation at 345 nm (curves a and b, Figure 2B) were fit to the sum of three skewed Gaussians with the parameters fixed at the values given in Table 1; the good fits obtained show that the experimental emission spectra can indeed be represented by the sum of KcsA-bound, lipid-bound, and aqueous components. The amplitudes of the KcsA-bound, lipid-bound, and aqueous components giving the best fits to the emission spectra excited at 345 nm were 2.14 ± 0.01, 0 ± 0.01, and 0.36 ± 0.01, respectively, at 0.3 μM Dauda and 3.40 ± 0.01, 0.39 ± 0.02, and 2.97 ± 0.01, respectively, at 2.0 μM Dauda. The low intensity for the lipid-bound component is consistent with weak binding of Dauda to DOPC, described by an effective dissociation constant (K_d) of ~270 μM.[14]

Confirmation that the blue-shifted peak centered at 469 nm arises from binding of Dauda to the central cavity of KcsA comes from competition experiments with TBA. A single TBA ion binds in the central cavity of KcsA,[2,3] and the effects of fatty acids and tetraalkylammonium ions on channel function are competitive.[7] As shown in Figure 3A, incubation of KcsA with TBA results in a decreased fluorescence emission at low

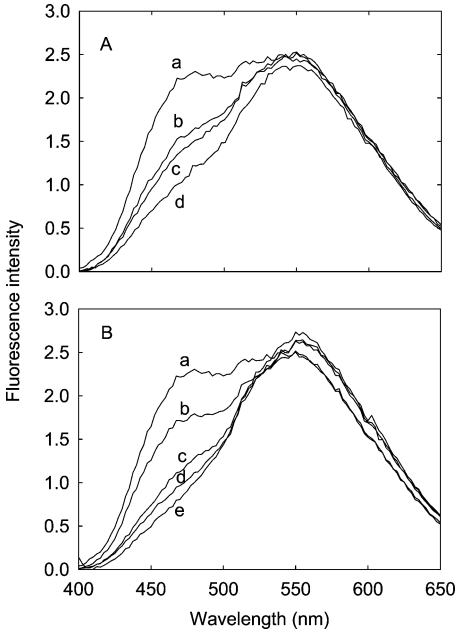


Figure 3. Effects of TBA and oleic acid on fluorescence emission spectra of Dauda in the presence of KcsA. (A) Emission spectra in the absence of TBA (a) and in the presence of 5 (b), 10 (c), and 20 mM TBA (d). (B) Emission spectra in the absence of oleic acid (a) and in the presence of 10 (b), 50 (c), 100 (d), and 300 μM oleic acid (e). The Dauda and KcsA tetramer concentrations were 2 and 0.19 μM, respectively, and fluorescence was excited at 345 nm.

wavelengths, where the spectra are dominated by the KcsA-bound component, with no effects at higher wavelengths; the effects of TBA increase with increasing concentration as expected for simple competition between Dauda and TBA for binding to the central cavity in KcsA. Addition of oleic acid also results in a decrease in intensity for the 469 nm component (Figure 3B), showing that binding of Dauda and oleic acid to the central cavity is also competitive.

Number of Binding Sites for Dauda on KcsA. KcsA was titrated with Dauda, and fluorescence intensities were measured at 450 nm, at which wavelength the spectra are dominated by Dauda bound to the central cavity. Titrations as a function of Dauda concentration (Figure 4B) showed a saturable

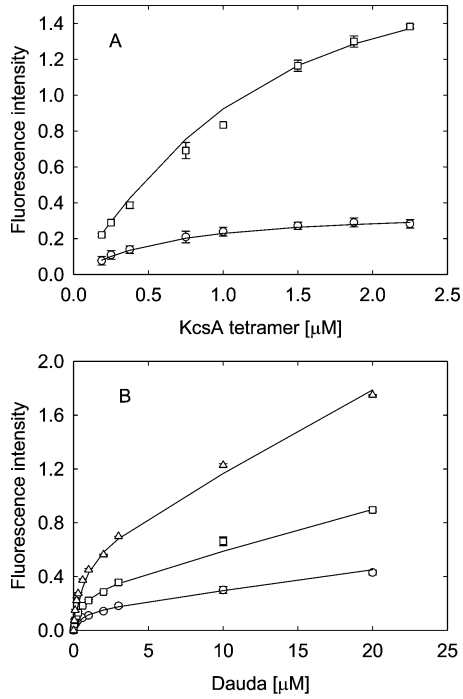


Figure 4. Titrations with Dauda. Fluorescence intensities at 450 nm were measured for (A) 0.2 (○) and 1 (□) μM Dauda in the presence of the given concentrations of the KcsA tetramer and (B) 0.09 (○), 0.19 (□), and 0.37 (△) μM KcsA tetramer in the presence of the given concentration of Dauda. A global fit of the data to eq 2 gave a dissociation constant (K_d) for Dauda of 0.47 ± 0.10 μM and a number of binding sites per tetramer (n) of 0.94 ± 0.06.

component at low concentrations of Dauda and a nonsaturable component resulting in a linear increase in fluorescence intensity at higher concentrations. The data fit to eq 2 with a dissociation constant (K_d) for Dauda of 0.47 ± 0.10 μM and a number of binding sites per tetramer (n) of 0.94 ± 0.06 (Figure 4).

Binding of TBA to KcsA. As shown in Figure 3, incubation of KcsA with TBA results in weakened binding of Dauda. However, the decrease in fluorescence intensity seen when TBA is added to KcsA preincubated with Dauda is very slow (Figure 5). The decrease in fluorescence intensity as a function of time fits to a single-exponential process with the rates listed in Table 2. The observation that the rate of displacement of Dauda by TBA is independent of the concentration of TBA (Table 2) shows that the rate of TBA binding is controlled by some initial slow step before the actual binding event. If TBA can enter the central cavity of a potassium channel only when

dx.doi.org/10.1021/bi3009196 | Biochemistry 2012, 51, 7996−8002

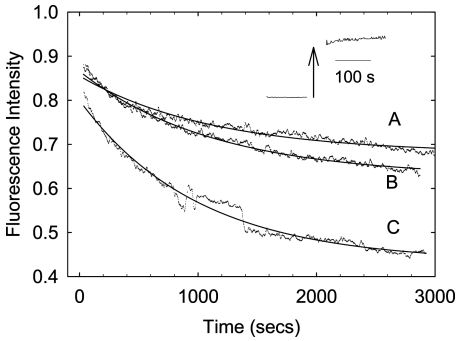


Figure 5. Displacement of bound Dauda by tetrabutylammonium (TBA) ion. KcsA was incubated with 0.2 μM Dauda and the fluorescence intensity measured at 450 nm, with excitation at 280 nm. TBA was then added to give concentrations of 5 (A), 10 (B), and 20 mM (C), and the fluorescence intensity was monitored as a function of time (···). The data were fit to single-exponential decays (—) with the rates listed in Table 2. The inset shows the rapid increase in fluorescence intensity observed upon addition of Dauda (1.0 μM) to KcsA at the time marked by the arrow; the horizontal line corresponds to 100 s.

Table 2. Rate Constants for Displacement of Dauda from KcsA by TBA[a]

[TBA] (mM)	rate constant (s^{-1})
5	0.0009 ± 0.0001
10	0.0009 ± 0.0001
20	0.001 ± 0.0001

[a]The data shown in Figure 5 were fit to single-exponential decays giving the rates listed.

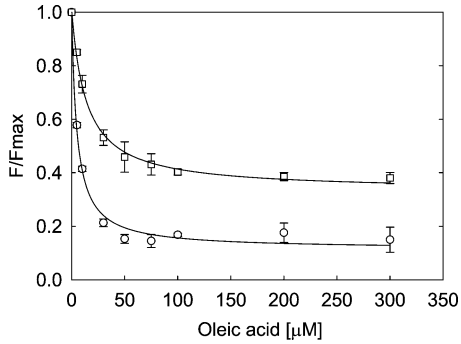


Figure 6. Fluorescence titrations with oleic acid. KcsA (0.19 μM tetramer) was incubated with 0.3 (○) and 2 μM Dauda (□), and fluorescence intensities were then measured after addition of oleic acid to the given concentration. The solid lines show fits to the competitive binding model (see eq 6) with K_d values for oleic acid of 3.02 ± 0.42 and 2.58 ± 0.27 μM calculated from the data at 0.3 and 2 μM Dauda, respectively, assuming a K_d of 0.47 μM for Dauda.

Table 3. Fatty Acid Dissociation Constants for Binding to the KcsA Cavity[a]

fatty acid	chain[b]	K_d (μM)
myristoleic acid	C14:1	27.1 ± 2
palmitoleic acid	C16:1	5.0 ± 0.6
oleic acid	C18:1	2.9 ± 0.2
ecosenoic acid	C20:1	2.6 ± 0.1
erucic acid	C22:1	16.4 ± 2.4
linoleic acid	C18:2	3.0 ± 0.4
linolenic acid	C18:3	5.8 ± 1.4
arachidonic acid	C20:4	3.0 ± 0.4

[a]Determined by displacement of Dauda assuming a dissociation constant for Dauda of 0.47 μM. [b]Chain length followed by the number of double bonds.

the channel is open, this slow step is presumably opening of the channel, which will be slow for KcsA at pH 7.2 as KcsA is a proton-gated channel.[15,16] Interestingly, in contrast to the slow binding of TBA, the increase in fluorescence intensity observed upon addition of Dauda to KcsA is complete within the mixing time of the experiment (Figure 5, inset), so that Dauda does not require the channel to be open for it to bind to its binding site in the cavity.

Determination of Binding Constants for Fatty Acids and TBA. KcsA was incubated with fixed concentrations of Dauda and then titrated with oleic acid to yield a dissociation constant for oleic acid (Figure 6). The data fit to a simple competitive model (see eq 6), giving dissociation constants for oleic acid of 3.02 ± 0.42 and 2.58 ± 0.27 μM measured at 0.3 and 2 μM Dauda, respectively, assuming a dissociation constant of 0.47 μM for Dauda. Similar titrations were performed with a range of other unsaturated fatty acids, giving the dissociation constants listed in Table 3.

Because binding of TBA to KcsA is very slow, the binding constant for TBA was determined by incubating KcsA with TBA overnight, followed by titration with Dauda (Figure 7A). The data were fit to eq 2, giving effective K_d values for Dauda in the presence of TBA, which were then fit to eq 5 giving a dissociation constant for TBA of 1.2 ± 0.1 mM, again assuming a dissociation constant of 0.47 μM for Dauda (Figure 7B).

DISCUSSION

Central Cavity of K^+ Channels. A prominent feature of the structure of potassium channels is the central water-filled cavity lined with hydrophobic residues, located just below the narrow selectivity filter (Figure 1).[1] X-ray crystallographic studies have shown that TBA ions block the channel by binding in the cavity[2,3] with hydrophobic interactions between the butyl chains and the wall of the cavity contributing to the binding affinity.[4] A wide range of charged drug molecules have also been suggested to bind to this same site in many potassium channels, based on mutagenesis experiments.[17−19]

Potassium channels can also be blocked by binding of fatty acids.[20,21] In particular, polyunsaturated fatty acids and endocannabinoids such as arachidonoylethanolamide (anandamide) derived from them have been shown to block potassium channels in the micromolar concentration range.[22−27] Many of these channels are also blocked by simpler fatty acids such as the monounsaturated oleic acid, with oleic acid blocking at lower concentrations than polyunsaturated fatty acids in some cases.[6,26−28] Voltage-gated sodium channels are also blocked by both polyunsaturated fatty acids and oleic acid.[29] Although it has been suggested that the effects of fatty acids on ion channels could be mediated indirectly through effects on the mechanical properties of the lipid bilayer surrounding the channel (reviewed in ref 30), it has also been suggested, on the basis of mutagenesis experiments, that channel block follows from binding to the central cavity.[6,7,25]

Dauda Binding to KcsA. Here we show that the fluorescent fatty acid Dauda can be used to characterize the binding of a fatty acid to the cavity in KcsA. The fluorescence emission spectrum for Dauda in the presence of KcsA contains three components, corresponding to KcsA-bound and lipid-

dx.doi.org/10.1021/bi3009196 | Biochemistry 2012, 51, 7996−8002

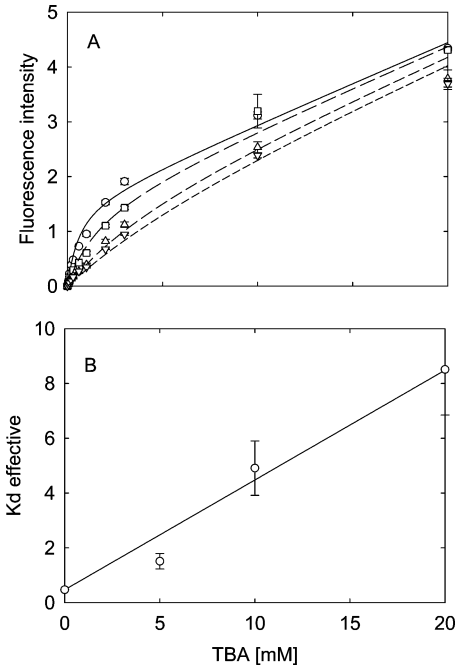


Figure 7. Fluorescence titrations with Dauda in the presence of TBA. (A) KcsA (0.19 μM tetramer) was incubated with the following concentrations of TBA for 3 h: 0 (○), 5 (□), 10 (△), and 20 mM (▽). Fluorescence intensities were then determined in the presence of the given concentrations of Dauda. Each curve was then fit to eq 2 with the number of binding sites per tetramer (n) fixed at 1, giving the effective dissociation constants (K_d, in micromolar) for Dauda plotted in panel B as a function of TBA concentration. The solid line in panel B shows a fit to the competitive binding model (eq 5) giving a value for K_d for TBA of 1.2 ± 0.2 mM, assuming a K_d of 0.47 μM for Dauda.

bound Dauda together with unbound Dauda (Figure 2). The KcsA-bound component is reduced in intensity in the presence of the TBA ion (Figure 3A), showing that Dauda binds in the central cavity of KcsA. Fluorescence titrations (Figure 4) give a number of binding sites for Dauda of 0.94 ± 0.06 per tetramer. The observation of one binding site per KcsA tetramer, together with the competition with TBA ions for binding, suggests a binding site close to the central symmetry axis of the pore and close to the selectivity filter. The positively charged TBA ion binds with its charged nitrogen close to the location of the K^+ ion whose position is resolved in the cavity in the absence of the TBA ion.[3] It is possible that the carboxyl group on Dauda occupies a position similar to that occupied by the nitrogen atom of the TBA ion, interacting with the innermost potassium ion in the selectivity filter, an interaction of the type suggested to be important for the binding of antiarrhythmic chromanol 293B in the cavity of Kv7.1.[18]

The marked shift to lower wavelengths for the emission spectrum for KcsA-bound Dauda relative to lipid-bound Dauda (Table 1) could reflect a difference in hydrophobicity but could also reflect strong immobilization of the bound Dauda on the cavity walls because the emission spectrum of the dansyl group is strongly dependent on environmental mobility;[10] in earlier EPR studies using the spin-labeled fatty acid 14-SASL, we also detected strong immobilization upon binding to KcsA.[8]

Effect of Fatty Acid Structure on Binding in the Cavity. The dissociation constant for Dauda was determined to be 0.47 μM (Figure 4). Dauda is displaced from its binding site by a variety of fatty acids, with the dissociation constants listed in Table 3. The strength of binding of the monounsaturated fatty acids increases with an increasing chain length from myristoleic acid (C14) to ecosenoic acid (C20) but then falls again to erucic acid (C22). The decrease in the dissociation constant from C14 to C16 by a factor of 5.4 (Table 3) is smaller than that expected from simple hydrophobicity arguments; the free energy change of −3.63 kJ/mol per CH_2 group for the partitioning of long chain alcohols into lipid bilayers[31] gives an expected change in the dissociation constant of a factor of 18.7. The changes in dissociation constant from C16 to C20 are even smaller than that between C14 and C16 and, combined with the observed increase in dissociation constant from C20 to C22, suggest that the fatty acid binding site has an optimal size matching a C20 chain. Increasing the number of double bonds in the chain has a relatively weak effect on the dissociation constant, that for arachidonic acid being the same for mono- and polyunsaturated fatty acids (Table 3).

The dissociation constants for KcsA listed in Table 3 are comparable with the concentrations of fatty acids blocking mammalian potassium channels. For example, 50% block of human cardiac Kv4.3 and Kv1.5 channels by oleic acid has been observed at 2.2 and 0.4 μM, respectively, and by arachidonic acid at 0.3 and 1.5 μM, respectively.[26,27] The physiological significance of this block is difficult to assess because the relevant free cellular concentrations of fatty acids are not known and local concentrations could be high where receptor-mediated activation of phospholipases leads to release of fatty acids from membrane phospholipids. However, TRAAK and TREK channels are activated by arachidonic acid and other polyunsaturated fatty acids at concentrations in the micromolar range,[32] implying that these kinds of concentrations of free fatty acids must be physiologically relevant to cell function.

Mode of Binding of TBA and Fatty Acids to the Cavity. The dissociation constant for TBA was determined to be 1.2 ± 0.1 mM (Figure 7). A wide range of dissociation constants for TBA have been estimated from electrophysiological measurements ranging, for example, from 1.5 μM for Kv1.4[2] to ∼0.2 mM for K_{Ca}3.1,[33] 2 mM for ROMK1,[34] and 400 mM for 1RK1,[34] the wide variation being attributed to large differences in the on rates for binding.[3] The large size of the TBA ion (diameter of ∼10 Å) means that it is likely to be able to enter the cavity in KcsA only when the channel is open. This is consistent with the very slow rate of displacement of Dauda by TBA observed at pH 7.2, described by a rate constant of 0.0009 ± 0.0001 s^{-1} (Figure 5 and Table 2). In contrast, binding of Dauda to KcsA is much faster, being complete in the mixing time of the experiment, <1 min (Figure 5). Similarly, displacement of Dauda by added fatty acids is complete within the mixing time of the experiment (data not shown). The implication is that Dauda and other fatty acids can bind directly to the closed KcsA channel, presumably via the lipid bilayer with the bound fatty acid molecules penetrating between the transmembrane α-helices.

AUTHOR INFORMATION

Corresponding Author

*Telephone: 44 (0) 2380 594222. E-mail: j.m.east@soton.ac.uk.

Funding

This study was supported by grants from the Wellcome Trust and a Marie Curie Early Stage Training Fellowship (to J.H.B.)

from the 6th Framework Program of the European Commission.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

14-SASL, 14-(4,4-dimethyloxazolidinyl-*N*-oxyl)stearic acid; Dauda, 11-dansylaminoundecanoic acid; DOPC, dioleoylphosphatidylcholine; BSA, bovine serum albumin; EPR, electron paramagnetic resonance; TBA, tetrabutylammonium.

■ REFERENCES

(1) Doyle, D. A., Cabral, J. M., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) The structure of the potassium channel: Molecular basis of K^+ conduction and selectivity. *Science 280*, 69−77.

(2) Zhou, M., Morais-Cabral, J. H., Mann, S., and MacKinnon, R. (2001) Potassium channel receptor site for the inactivation gate and quaternary amine inhibitors. *Nature 411*, 657−661.

(3) Faraldo-Gomez, J. D., Kutluay, E., Jogini, V., Zhao, Y. X., Heginbotham, L., and Roux, B. (2007) Mechanism of intracellular block of the KcsA K^+ channel by tetrabutylammonium: Insights from X-ray crystallography, electrophysiology and replica-exchange molecular dynamics simulations. *J. Mol. Biol. 365*, 649−662.

(4) Choi, K. L., Mossman, C., Aube, J., and Yellen, G. (1993) The internal quaternary ammonium receptor site of *Shaker* potassium channels. *Neuron 10*, 533−541.

(5) Hilf, R. J. C., Bertozzi, C., Zimmermann, J., Reiter, A., Trauner, D., and Dutzler, R. (2010) Structural basis of open channel block in prokaryotic pentameric ligand-gated ion channels. *Nat. Struct. Mol. Biol. 17*, 1330−1336.

(6) Hamilton, K. L., Syme, C. A., and Devor, D. C. (2003) Molecular localization of the inhibitory arachidonic acid binding site to the pore of hIK1. *J. Biol. Chem. 278*, 16690−16697.

(7) Decher, N., Streit, A. K., Rapedius, M., Netter, M. F., Marzian, S., Ehling, P., Schlichthorl, G., Craan, T., Renigunta, V., Kohler, A., Dodel, R. C., Navarro-Polanco, R. A., Preisig-Muller, R., Klebe, G., Budde, T., Baukrowitz, T., and Daut, J. (2010) RNA editing modulates the binding of drugs and highly unsaturated fatty acids to the open pore of Kv potassium channels. *EMBO J. 29*, 2101−2113.

(8) Bolivar, J. H., Smithers, N., East, J. M., Marsh, D., and Lee, A. G. (2012) Multiple binding sites for fatty acids on the potassium channel KcsA. *Biochemistry 51*, 2889−2898.

(9) Lakowicz, J. R. (1999) *Principles of Fluorescence Spectroscopy*, Kluwer Academic/Plenum Press, New York.

(10) Ghiggino, K. P., Lee, A. G., Meech, S. R., O'Connor, D. V., and Phillips, D. (1981) Time-resolved emission spectroscopy of the dansyl fluorescence probe. *Biochemistry 20*, 5381−5389.

(11) Marius, P., Zagnoni, M., Sandison, M. E., East, J. M., Morgan, H., and Lee, A. G. (2008) Binding of anionic lipids to at least three nonannular sites on the potassium channel KcsA is required for channel opening. *Biophys. J. 94*, 1689−1698.

(12) Lee, A. G. (1982) Membrane studies using fluorescence spectroscopy. *Tech. Life Sci.: Lipid Membr. Biochem. B422*, 1−49.

(13) Rooney, E. K., and Lee, A. G. (1986) Fitting fluorescence emission spectra of probes bound to biological membranes. *J. Biochem. Biophys. Methods 120*, 175−189.

(14) Rooney, E. K., East, J. M., Jones, O. T., McWhirter, J., Simmonds, A. C., and Lee, A. G. (1983) Interaction of fatty acids with lipid bilayers. *Biochim. Biophys. Acta 728*, 159−170.

(15) Meuser, D., Splitt, H., Wagner, R., and Schrempf, H. (1999) Exploring the open pore of the potassium channel from *Streptomyces lividans*. *FEBS Lett. 462*, 447−452.

(16) Heginbotham, L., LeMasurier, M., Kolmakova-Partensky, L., and Miller, C. (1999) Single *Streptomyces lividans* K^+ channels: Functional asymmetries and sidedness of proton activation. *J. Gen. Physiol. 114*, 551−560.

(17) Decher, N., Pirard, B., Bundis, F., Peukert, S., Baringhaus, K. H., Busch, A. E., Steinmeyer, K., and Sanguinetti, M. C. (2004) Molecular basis for Kv1.5 channel block. *J. Biol. Chem. 279*, 394−400.

(18) Lerche, C., Bruhova, I., Lerche, H., Steinmeyer, K., Wei, A. D., Strutz-Seebohm, N., Land, F., Busch, A. E., Zhorov, B. S., and Seebohm, G. (2007) Chromanol 293B binding in KCNQ1 (Kv7.1) channels involves electrostatic interactions with a potassium ion in the selectivity filter. *Mol. Pharmacol. 71*, 1503−1511.

(19) Strutz-Seebohm, N., Gutcher, I., Decher, N., Steinmeyer, K., Land, F., and Seebohm, G. (2007) Comparison of potent Kv1.5 potassium channel inhibitors reveals the molecular basis for blocking kinetics and binding mode. *Cell. Physiol. Biochem. 20*, 791−800.

(20) Meves, H. (2008) Arachidonic acid and ion channels: An update. *Br. J. Pharmacol. 155*, 4−16.

(21) Boland, L. M., and Drzewiecki, M. M. (2008) Polyunsaturated fatty acid modulation of voltage-gated ion channels. *Cell Biochem. Biophys. 52*, 59−84.

(22) Poling, J. S., Karanian, J. W., Salem, N., and Vicini, S. (1995) Time- and voltage-dependent block of delayed rectifier potassium chanels by docosahexaenoic acid. *Mol. Pharmacol. 47*, 381−390.

(23) McKay, M. C., and Worley, J. F. (2001) Linoleic acid both enhances activation and blocks Kv1.5 and Kv2.1 channels by two separate mechanisms. *Am. J. Physiol. 281*, 1277−1284.

(24) Guizy, M., David, M., Arias, C., Zhang, L., Cofan, M., Ruiz-Gutierrez, V., Ros, E., Lillo, M. P., Martens, J. R., and Valenzuela, C. (2008) Modulation of the atrial specific Kv1.5 channel by the n-3 polyunsaturated fatty acid, α-linolenic acid. *J. Mol. Cell. Cardiol. 44*, 323−335.

(25) Moreno-Galindo, E. G., Barrio-Echavarria, G. F., Vasquez, J. C., Decher, N., Sachse, F. B., Tristani-Firouzi, M., Sanchez-Chapula, J. A., and Navarro-Polanco, R. A. (2010) Molecular basis for a high-potency open-channel block of Kv1.5 channel by the endocannabinoid anandamide. *Mol. Pharmacol. 77*, 751−758.

(26) Barana, A., Amoros, I., Caballero, R., Gomez, R., Osuna, L., Lillo, M. P., Blazquez, C., Guzman, M., Delpon, E., and Tamargo, J. (2010) Endocannabinoids and cannabinoid analogues block cardiac hKv1.5 channels in a cannabinoid receptor-independent manner. *Cardiovasc. Res. 85*, 56−67.

(27) Amoros, I., Barana, A., Caballero, R., Gomez, R., Osuna, L., Lillo, M. P., Tamargo, J., and Delpon, E. (2010) Endocannabinoids and cannabinoid analogues block human cardiac Kv4.3 channels in a receptor-independent manner. *J. Mol. Cell. Cardiol. 48*, 201−210.

(28) Devor, D. C., and Frizzell, R. A. (1998) Modulation of K^+ channels by arachidonic acid in T84 cells. I. Inhibition of the Ca^{2+}-dependent K^+ channel. *Am. J. Physiol. 274*, C138−C148.

(29) Xiao, Y. F., Ke, Q., Wang, S. Y., Auktor, K., Yang, Y., Wang, G. K., Morgan, J. P., and Leaf, A. (2001) Single point mutations affect fatty acid block of human myocardial sodium channel α subunit Na^+ channels. *Proc. Natl. Acad. Sci. U.S.A. 98*, 3606−3611.

(30) Oz, M. (2006) Receptor-independent actions of cannabinoids on cell membranes: Focus on endocannabinoids. *Pharmacol. Ther. 111*, 114−144.

(31) Franks, N. P., and Lieb, W. R. (1986) Partitioning of long-chain alcohols into lipid bilayers. Implications for mechanism of general anesthesia. *Proc. Natl. Acad. Sci. U.S.A. 83*, 5116−5120.

(32) Fink, M., Lesage, F., Duprat, F., Heurteaux, C., Reyes, R., Fosset, M., and Lazdunski, M. (1998) A neuronal two P domain K^+ channel stimulated by arachidonic acid and polyunsaturated fatty acids. *EMBO J. 17*, 3297−3308.

(33) Banderali, U., Klein, H., Garneau, L., Simoes, M., Parent, L., and Sauve, R. (2004) New insights on the voltage dependence of the $K_{Ca}3.1$ channel block by internal TBA. *J. Gen. Physiol. 124*, 333−348.

(34) Guo, D., and Lu, Z. (2001) Kinetics of inward-rectifier K^+ channel block by quaternary alkylammonium ions: Dimension and properties of the inenr pore. *J. Gen. Physiol. 117*, 395−405.