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Dibucaine–ion-channel interactions in model systems: A study using fluorescence resonance energy transfer

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Abstract

In 1986, Ondrias et al. [Stud. Biophys. 115 (1986) 17] reported that dibucaine and other local anesthetics reduced the conductivity of membranes containing gramicidin ion channels. They attributed this behavior to a local anesthetic induced swelling of the membrane. Then in 1992, Bridal and Busath [Biochim. Biophys. Acta 1107 (1992) 31] re-examined this system using single channel measurements and found that the channel frequency was reduced, but not the conductance of single channels. They suggested that the mechanism involved a reduction of gramicidin membrane concentration owing to local anesthetic solubilization. In both studies, no direct membrane mediated dibucaine–gramicidin interactions were inferred. It is possible to determine conclusively whether or not direct gramicidin–dibucaine interactions take place using fluorescence resonance energy transfer (FRET). To this end, we have examined the interactions between the ion channel, gramicidin and the antiarrhythmic/local anesthetic, dibucaine, in dioleoylphosphatidylcholine (DOPC) liposomes. FRET was observed for liposomes containing relatively high concentrations of gramicidin and dibucaine, but not for lower concentrations. It can therefore be concluded that there is no specific association between gramicidin and dibucaine in DOPC liposomes. The FRET observed can be attributed to the donors and acceptors situated in close proximity due to their high relative concentration in the liposome. To further examine the nature of the FRET-inducing interactions, the donor (tryptophan) and acceptor (dibucaine) chromophores were examined for FRET in aqueous and lipophilic solutions at sufficiently high concentration to make interactions possible. No interactions were observed for lipophilic solutions, but a quenching of tryptophan fluorescence by dibucaine was recorded in aqueous solution. It is therefore possible that dibucaine can solvate gramicidin in aqueous solution, although we did not observe this to happen for the DOPC liposome solution used in the present study.

Keywords: FRET; Dibucaine; Gramicidin; Liposome; Local anesthesia

1. Introduction

A cell membrane is a complex biological interface that functions both as a wall of defense and as a conduit for the exchange of biochemical information. A set of proteins, known as ion-channels, control a cell's permeability to ions with astonishing selectivity and sensitivity. In some unfortunate medical conditions, such as cardiac arrhythmia, certain ion-channels do not function properly. Therapeutic treatment of arrhythmia has been achieved using small molecule channel blocks, that bind to the membrane-bound protein. One such series of therapeutic agents is the class I antiarrhythmics. Moreover, some antiarrhythmics may function as topical local anesthetics, potentially operating via similar ion channel blocking mechanisms. Single channel conductance experiments on bovine cardiac and rat skeletal Na^+ channels [1,2], indicate that channel block may operate via two mechanisms known as fast and slow block. The insertion kinetics of the drug directly into the channel should be governed primarily by a Coulombic interaction with the short lived open state of the channel and therefore represent fast block. A slower kinetic block occurs if the drug were to penetrate the phospholipid bilayer and bind dispersively to the outside of the protein during its long-lived closed state. This binding presumably maintains a structural change in the protein, rendering it inoperative. Although single channel conductance experiments provide excellent insight into the rates of block binding, they do not always reveal the microscopic mechanism.

A possible tool to help observe and understand ion-channel–drug interactions is fluorescence resonance

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energy transfer (FRET). FRET has been used quite extensively to study the interactions of molecules over distances the order of 50–100 Å. FRET is a process whereby an electronic transition of a donor chromophore is stimulated in the presence of an acceptor chromophore. If the acceptor chromophore is in close proximity, there is a finite probability that donor–acceptor energy transfer will take place before the donor can fluoresce. Thus, there will be a decrease of the steady-state fluorescence yield and lifetime of the donor and a commensurate increase in the fluorescence yield of the acceptor. Simultaneous with proximity, the other main criterion for FRET is a significant overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor. Therefore, to study drug–protein interactions using FRET, the species involved should possess aromatic systems. For proteins, tryptophan, tyrosine and phenylalanine residues are potential donors and the quinolinic moiety of the local anesthetic dibucaine is a potential acceptor.

In a study by Gutierrez-Merino et al. [3], fluorescence resonance energy transfer was observed between proteins in skeletal muscle sarcoplasmic reticulum membrane samples and the antiarrhytmic/local anesthetic, dibucaine. They suggest that the inhibition of $(Ca^{2+} + Mg^{2+})$ ATPase by dibucaine is related to a progressive disruption of the lipid annulus surrounding the protein. There have also been several studies of the interactions between local anesthetics (including dibucaine) and phospholipid membranes. Barghouthi and Eftink [4] have used steady-state and time-resolved fluorescence measurements to determine that the rotational motion of dibucaine, in a unilamellar vesicle of dimyristylphosphatidylcholine and dimyristylphosphatidyl glycerol, is slowed considerably compared with its motion in aqueous solution. Louro et al. [5] used fluorescence quenching of dibucaine by iodide and by nitroxide-labeled stearic acids to profile the depth of dibucaine in rabbit skeletal muscle sarcoplasmic reticulum. They determined that the average depth of dibucaine was at the most that of the fourth carbon acid of the fatty acid chain.

To assess FRET as a tool to examine small molecule–ion-channels interactions, a model system would be beneficial. A simple ion-channel model system is gramicidin. Gramicidin is a 15-residue polypeptide that forms a $\beta^{6.3}$ helix in a membrane environment and contains four tryptophan residues. Conductance measurements on gramicidin have been previously used as a model for local anesthetic–ion-channel interactions [6,7]. It was suggested in Ref. [6] that membrane swelling was responsible for the lower membrane conductance observed. In Ref. [7], local anesthetics were observed to lower the channel occurrence frequency. This was attributed to a loss of ion channels from the membrane into solution owing to a solubilization of gramicidin by the local anesthetics. Interestingly, for dibucaine there was an increase in the ion channel lifetime by 16%, coupled with the lowering of mean conductance. This could suggest that membrane-incorporated dibucaine reduces the lateral mobility of the gramicidin monomers by inducing a general increase in membrane viscosity.

The aim of this work is to establish the characteristics of the interactions between the local anesthetic dibucaine and gramicidin using FRET. For this model system, the FRET donor will be tryptophan and the acceptor will be dibucaine. Also, the nature of FRET between dibucaine and tryptophan is examined by holding the donor and acceptor pair in close proximity. This is first accomplished by studying high concentration aqueous solutions of tryptophan and dibucaine. This will be relevant to the fast block mechanism. The second approach is to approximate the membrane mediated blocking by assaying FRET between the tryptophan chromophore model, 3-methylindole, and dibucaine in lipophilic media (octanol or decanol). Finally, gramicidin–dibucaine interactions are examined using a dioleoylphosphatidylcholine (DOPC) liposome as a model membrane.

2. Experimental procedures

2.1. Samples

2.1.1. Materials

Dibucaine hydrochloride, free base dibucaine, L-tryptophan (SigmaUltra minimum 98% (TLC)), sodium dodecyl sulfate (SDS), sodium acetate and acetic acid were obtained from Sigma and used without further purification. Octanol and decanol were purchased from ES Science and were spectroscopic grade. An acetate buffer was prepared with pH $\approx$ 6.5 and an ionic strength of 0.01 M. The buffer was prepared using Nanopure water with a resistance of 18 MΩ.

2.1.2. Sample preparation

Bulk solutions. For the aqueous solution studies, the concentration of tryptophan was 1×10^{-2} M. The dibucaine final concentration range was from 2 to 10 mM. The range for dibucaine stock solutions was from 2×10^{-3} to 1×10^{-2} M. Samples were placed in the dark and not left more than 2–3 h before analysis.

Solutions of 3-methylindole at 1×10^{-2} M in 1-octanol and decanol were prepared. To these, free base dibucaine was added to make solutions of the following concentrations: 2–12 mM.

Liposomes. Fig. 1 contains a schematic representation of the gramicidin-AC2/DOPC/dibucaine system. DOPC 25 mg/ml in chloroform (Sigma, Ontario) was used without further purification. Purified gramicidin-AC2, HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-D-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-CONHCH$_2$CH$_2$OCONH-$(CH_2)_2NH_3^+$ (gram-AC2) was a generous gift of Professor Woolley (University of Toronto). It has been demonstrated [8] that gram-AC2 is more easily incorporated into DOPC

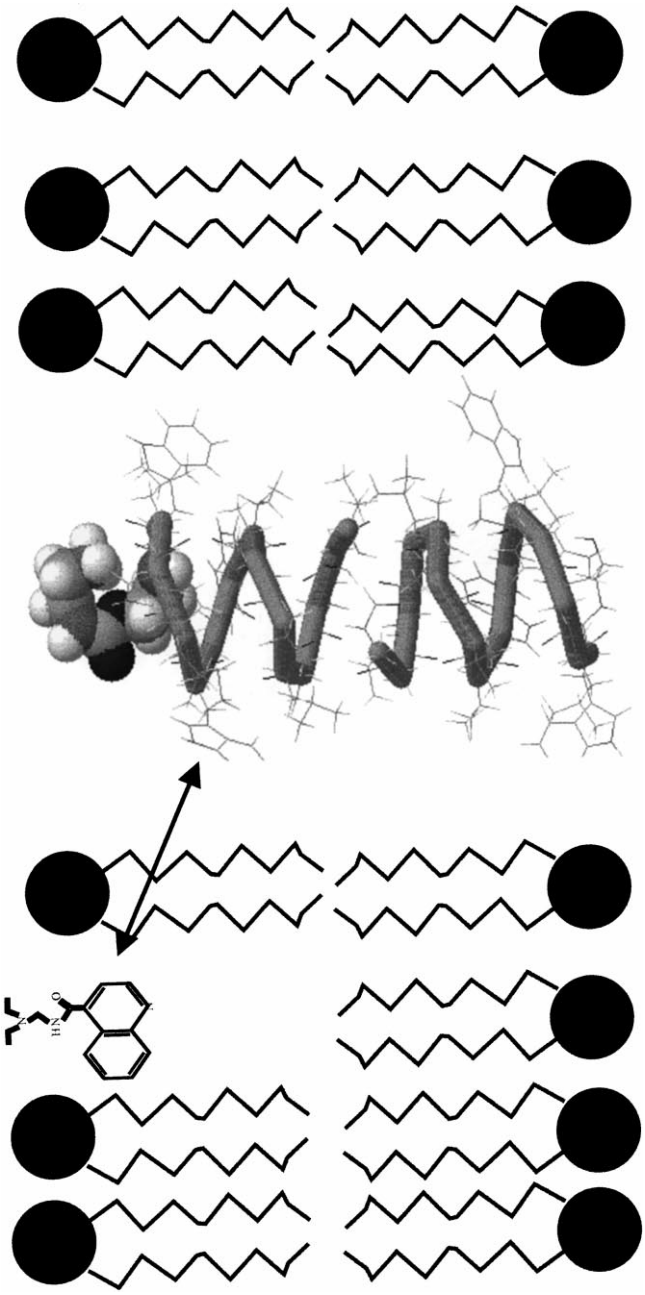

Fig. 1. A schematic representation of gramicidin-AC2 (left helix) and dibucaine situated in a DOPC bilayer.

vesicles than gramicidin A. Acetate buffer, 0.01 M, pH 6.5 was freshly prepared and used in the generation of all vesicles.

The procedure for liposome preparation was as follows. An appropriate volume of DOPC in chloroform (diluted to 4 mg/ml) was deposited in a 100 ml volumetric flask. The chloroform was then volatilized using a stream of dry nitrogen gas. A volume of gramicidin-AC2 in methanol (4 mg/ml) was then added to the test tube and the methanol volatilized as above. The flask was then filled to the mark with pH 6.5 acetate buffer. The solution was stirred for 2 h, after which it appeared homogeneously cloudy. The solution was then sonicated for a 10 min duration followed by a 30 min interval of resting and cooling. This procedure was repeated until the solution was clear. Gramicidin-free vesicles were prepared in the same way, omitting the gramicidin-AC2 addition. Dibucaine was added to the vesicle solution from a stock acetate buffer solution.

2.2. Spectroscopy

Fluorescence spectra were gathered using a Photon Technologies International (Canada) spectrofluorometer. Either a right angle or a front face fluorescence collection geometry was used depending on the optical density of the sample. For the FRET studies, excitation wavelengths were chosen to provide the best contrast (i.e. largest $A_{trp}/A_{dibucaine}$) between tryptophan absorption and dibucaine absorption. For excitation at 280 nm the decadic molar extinction coefficients ($M^{-1}\,cm^{-1}$) for tryptophan and dibucaine are $\varepsilon_{280} = 5950$ and $\varepsilon_{280} = 3890$, respectively.

3. Theoretical approach

3.1. FRET

The efficiency FRET is represented in the following equation [9].

$$\text{Eff} = \frac{R_0^6}{R_0^6 + r^6} \tag{1}$$

where R_0 is the Förster distance for 50% quenching. R_0 was calculated using:

$$R_0 = 9.79 \times 10^3 (\kappa^2 n^{-4} \phi_d^2 J)^{1/6} \tag{2}$$

where κ^2 is the orientation factor between the two transition dipoles ($\kappa^2 = \frac{2}{3}$ for random orientation), n the refractive index of the medium (taken here as the average, 1.40, between water and lipid), ϕ_d the fluorescence quantum yield for the donor (here taken as 0.07) [10] and J is the spectral overlap integral:

$$J = \int_0^\infty F_d(\nu)\varepsilon_a(\nu)\nu^{-4}\,d\nu \tag{3}$$

where $F_d(\nu)$ is the normalized fluorescence spectrum of tryptophan, $\varepsilon_a(\nu)$ the decadic molar extinction coefficient for dibucaine and ν is in wavenumber units.

3.2. Spectral deconvolution

A set of three fluorescence spectra are recorded; one of the donor, one of the acceptor and one of the mixture. The changes in the fluorescence yields of donor and acceptor

in the mixture solution can be assessed using the following equation [11]:

$$I_{\text{mixture}}(\lambda) = aI_{\text{donor}}(\lambda) + bI_{\text{acceptor}}(\lambda) \quad (4)$$

Using non-linear least-squares, the constants a and b were allowed to vary until the best fit to the observed spectrum was obtained. Thus, the value of a in the presence of acceptor indicates the amount of energy transfer taking place.

3.3. High concentration interactions using front-face fluorescence detection

In order to model our ion channel local anesthetic system in the most elementary way, we have chosen to isolate the donor and acceptor chromophores in close proximity and determine their energy transfer characteristics. This was accomplished using high concentrations (i.e. $>10^{-3}$ M of tryptophan and dibucaine in acetate buffer, and 3-methyl indole and dibucaine in octanol or decanol). The steady-state fluorescence spectra were all collected using front-face geometry to avoid inner filter effects. Nevertheless, using front-face geometry, one must be wary of fluorescence yield reduction simply due the absorption of light by the acceptor molecule. This is the case for our tryptophan–dibucaine system using 280 nm excitation. The change in fluorescence yield of tryptophan is corrected for the light absorbed by dibucaine using [12]:

$$I_{\text{FF}} = G\phi R_A I_{\text{ex}} \quad (5)$$

where I_{FF} is the fluorescence intensity, G is a geometrical factor which accounts for the fluorometer geometry and collection efficiency, ϕ the fluorescence quantum yield, R_A the ratio of the absorbance of the fluorophore to the total absorbance, i.e. $A_{\text{trp}}/A_{\text{total}}$, And I_{ex} is the incident light intensity. Since we know the decadic absorption coefficients for tryptophan and dibucaine, we can calculate the ratio, R_A. The FRET efficiency can then be extracted from the corrected ratio of I_{FF} in the presence (primes) and absence of dibucaine.

$$\frac{\phi'}{\phi} = \frac{I'_{\text{FF}}}{I_{\text{FF}} R'_A} \quad (6)$$

It follows that the ratio of the quantum yields ϕ'/ϕ represents the donor fluorescence quenching and thus $1-\phi'/\phi$ represents the FRET efficiency. Since $I'_{\text{FF}}/I_{\text{FF}}$ is equal to our fitted parameter, a, Eq. (6) becomes

$$\frac{\phi'}{\phi} = \frac{a}{R'_A} \quad (7)$$

The change of the reciprocal of this ratio as a function of acceptor concentration can then modeled using Stern–Volmer kinetics where:

$$\frac{F_0}{F} = \frac{\phi}{\phi'} = 1 + K_{\text{SV}}[Q] \quad (8)$$

Here the corrected fluorescence yield, F_0/F is equal to the ratio of the quantum yields from the previous equation, $[Q]$ the concentration of dibucaine and K_{SV} is the static interaction constant.

4. Results and discussion

4.1. Spectrum and position of dibucaine in the DOPC bilayer membrane

The fluorescence spectrum of dibucaine is known to be sensitive to the pH and polarity of the solvent [13]. In micelle solution the dibucaine·HCl fluorescence spectral maximum shifts to the blue by 3–8 nm compared with a neutral pH buffer [14]. This is suggestive of either a deprotonation of dibucaine upon entry to the micelle or a solvatochromic shift owing to the less polar micelle interior. As is displayed in Fig. 2, we observe a similar blue shift in fluorescence maximum from 412 nm in acetate buffer (pH = 6.5) to 406 nm in DOPC liposome solution. Since there is no broadening of the spectrum in DOPC solution, we assume that a large fraction of dibucaine has been incorporated into the DOPC liposome. Moreover, because the spectral shift is smaller than that observed for micelle incorporation, dibucaine may partition near to the surface of the lipid bilayer. This is in agreement with the study by Louro et al. [14] on dibucaine incorporation into sarcoplasmic reticulum vesicles, also with Cavalli et al. [15] who examined dibucaine interactions with mixed Langmuir monolayers and with Kuroda and Fujiwara [16] who examined dibucaine-egg yolk PC vesicle interactions. In Fig. 3, we present the

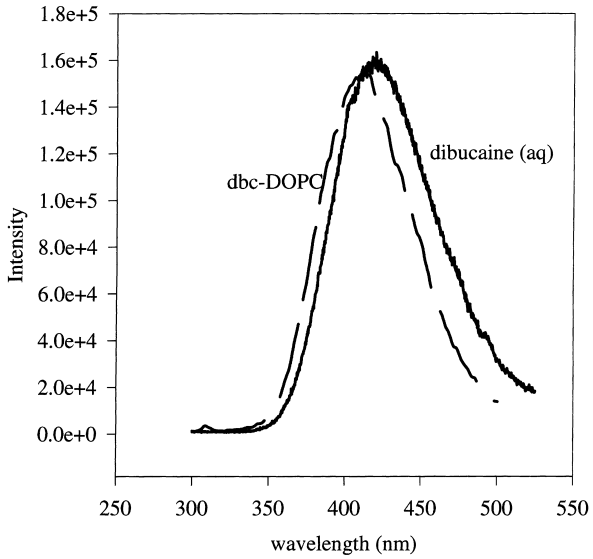

Fig. 2. Fluorescence spectra of dibucaine·HCl in acetate buffer (solid line) and in DOPC liposome solution (dashed line). Note the blue shift upon incorporation into the phospholipid bilayer. The excitation wavelength was 280 nm in both cases.

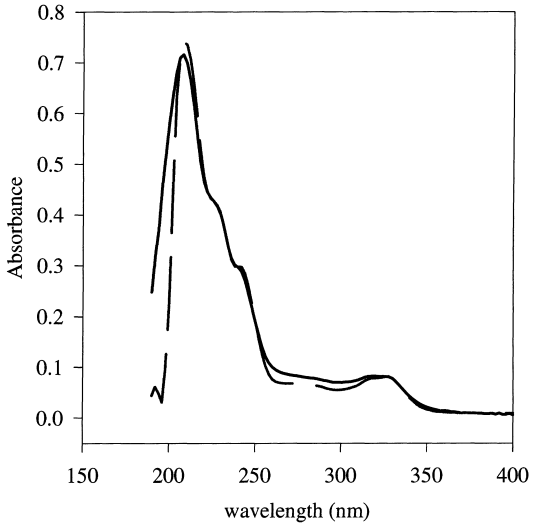

Fig. 3. Absorption spectra of dibucaine·HCl in acetate buffer (solid line) and in DOPC liposome solution (dashed line). The concentration of dibucaine·HCl was 400 mM.

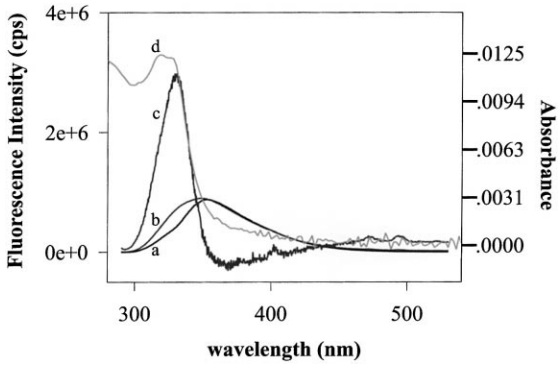

Fig. 4. Fluorescence spectrum of: (a) 3-methyl indole and free base dibucaine, both at 12 mM, in octanol; (b) the best theoretical fit of the spectrum found using Eq. (7) ($a = 0.65$, $b = 0.21$); (c) the difference between (a) and (b); (d) the absorption spectrum of free base dibucaine in octanol. The difference spectrum has been multiplied by a factor of 10 for clarity.

difference in absorption spectra of dibucaine in buffer versus DOPC liposome solution. There is minimal difference in the spectra. This suggests that the liposome-incorporated dibucaine is still in its protonated form [17].

4.2. 3MI–dibucaine in octanol and decanol

The solutions of high concentration 3MI and dibucaine in octanol or decanol were assessed for FRET in the following way. A set of three fluorescence spectra were recorded with equal concentrations; pure 3MI (donor), pure dibucaine (free base) (acceptor), and a 1:1 mole ratio of 3MI:dibucaine. The fluorescence spectrum of the mixture was modeled using Eq. (4).

In all cases, *a* and *b* were less than 1 because of the increased optical density of the mixture solution. This was accounted for using Eq. (7). The result was that *a* remains close to unity regardless of the donor and acceptor concentration. This suggests that no non-radiative energy transfer takes place under these conditions.

Upon inspection of the spectra used for the 3MI–dibucaine solutions (see Fig. 4), one notes that the fitted and observed spectra do not match perfectly. The blue edge of the observed FRET spectra appears to have red-shifted with respect to the tryptophan fluorescence spectrum used to model it. If one subtracts the observed from the model spectra, the difference bears a striking resemblance to the red-edge absorption spectrum of dibucaine. In Fig. 4, we present the observed combination spectrum, the best model fit using Eq. (4), the difference between model and FRET and the absorption spectrum of dibucaine. The excellent overlap of the red-edge of the difference spectrum and the dibucaine absorption spectrum indicates that any energy transfer results from radiative absorption of the 3-methyl indole fluorescence by dibucaine. This is known as direct radiative (or trivial) energy transfer (RET). Since these spectra were recorded from reasonably high (i.e. $\sim 10^{-2}$–10^{-3} M) concentration solutions, it should not be surprising to observe radiative energy transfer. Exactly the same RET behavior was observed for 3MI/dibucaine in decanol, suggesting that this behavior is likely not solvent-dependent for lipophilic solutions. These results must be taken into account for our following models if one is to use FRET quantitatively.

4.3. Tryptophan–dibucaine in acetate buffer

The same procedure was used to assess for FRET in the aqueous model system as in the non-aqueous model. In this case, some evidence of non-radiative energy transfer was apparent. After accounting for the front-face effect, tryptophan was found to be quenched by dibucaine. Therefore, a standard Stern–Volmer approach could be employed. A series of fluorescence spectra were recorded holding tryptophan concentration constant (10 mM) and varying dibucaine concentrations (0–10 mM). The resulting spectra are presented in Fig. 5. The quenched fluorescence yield was modeled using Eq. (4) and then corrected using Eq. (7). The resulting Stern–Volmer plot is displayed in Fig. 6a. The fit produced a Stern–Volmer constant, $K_{SV} = 46\,M^{-1}$.

We will now consider the extent to which radiative energy transfer is involved in our elementary aqueous model system. Since we observe a depletion of intensity for the entire tryptophan spectrum plus a small amount of radiative energy transfer, we argue that a significant portion of the tryptophan quenching arises from a non-radiative interaction with dibucaine. In fact, only about a 4% loss is due to radiative energy transfer compared with the general loss of 32%, at the higher dibucaine concentration. The 4% radiative loss is determined by first calculating the difference of the model spectra and the observed spectra and then computing the area of the difference. The difference spectrum

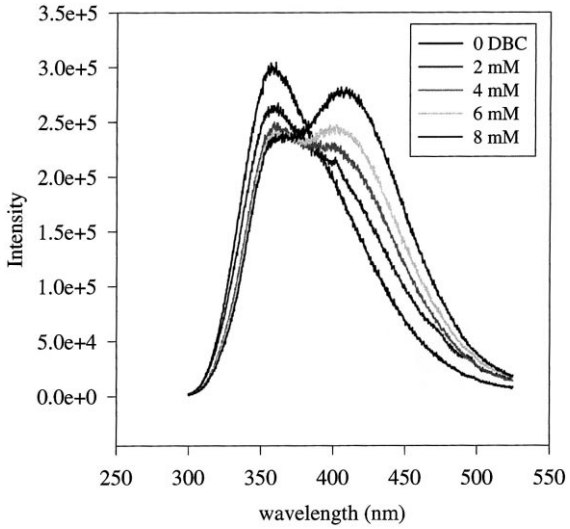

Fig. 5. Fluorescence spectra of tryptophan (10 mM) and a range of concentrations of dibucaine·HCl in acetate buffer. The excitation wavelength was 280 nm.

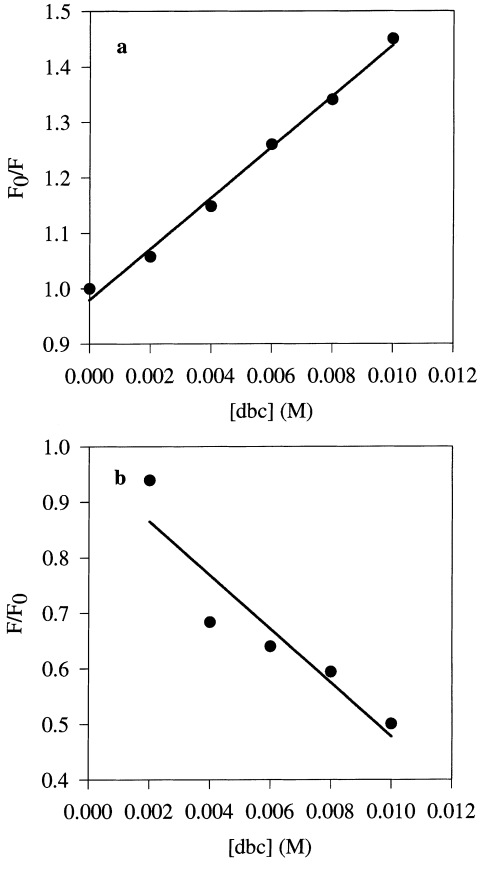

Fig. 6. (a) Stern–Volmer plot of the fluorescence yield of tryptophan in the presence of dibucaine. Data are taken from the fits to the spectra in Fig. 5 and can also be found in Table 1. (b) Plot of the fluorescence yield of various concentrations of dibucaine in the presence of tryptophan. Data are taken from the fits to the spectra in Fig. 5 and can also be found in Table 1.

Table 1
Fluorescence quenching data for tryptophan (10 mM) and dibucaine in an acetate buffer solution

Dibucaine (mM)	ϕ/ϕ' tryptophan	F/F_0 dibucaine
0.00	1.0000	–
2.00	1.0576	0.9391
4.00	1.1484	0.6837
6.00	1.2598	0.6399
8.00	1.3405	0.5939
10.00	1.4505	0.5004

appears as a bite out of the blue edge of the tryptophan spectrum similar to that shown in Fig. 4. The difference area is compared with the total area of the tryptophan contribution to the fluorescence spectrum. The 32% general loss is determined directly from the corrected quenching of tryptophan fluorescence found in Table 1 and represents a decrease in fluorescence intensity over the entire spectrum.

If the quenching mechanism results in FRET, then a concomitant increase in dibucaine fluorescence yield should be observed. After correcting the front-face effect, the yield of dibucaine fluorescence was found to decrease in the presence of tryptophan. These data are presented in Table 1 and Fig. 6b. Since the absorption spectrum of tryptophan and the fluorescence spectrum of dibucaine do not overlap, tryptophan cannot be quenching dibucaine via dipole–dipole interactions. It is likely that dibucaine self-quenches via aggregation at these high concentrations. Furthermore, it is possible that aggregation of dibucaine is promoted by tryptophan.

There is previous evidence of dibucaine self-association in aqueous solution. From Matsuki et al. [18], a critical micelle concentration (cmc) of 80 mM was determined for dibucaine·HCl in aqueous solution. Since we are well below the cmc, even at our highest concentration, it is unlikely that tryptophan is partitioning into a dibucaine micelle. It is possible, however, that tryptophan and dibucaine are forming pre-micellar aggregates of dimers, trimers, etc. These aggregates would result in energy transfer between tryptophan and dibucaine leading to quenching of the tryptophan fluorescence and a self-quenching of dibucaine fluorescence. Evidence of aggregation in our experiments can be found by examining the fluorescence intensity of dibucaine as a function concentration in buffer solution versus SDS micelle solution. From Fig. 7a and b it is clear that the maximum in fluorescence intensity occurs at 6 mM for aqueous solution, whereas in micelle solution the fluorescence intensity increases over the entire range of concentrations. In SDS (200 mM) solution dibucaine molecules are partitioned into the micelles in a 1:1 (dibucaine:micelle) mole ratio and would not interact appreciably.

4.4. Gramicidin–dibucaine FRET in liposomes

It is well-documented that gramicidin ion-channels span DOPC membranes by dimerization across the phospholipid

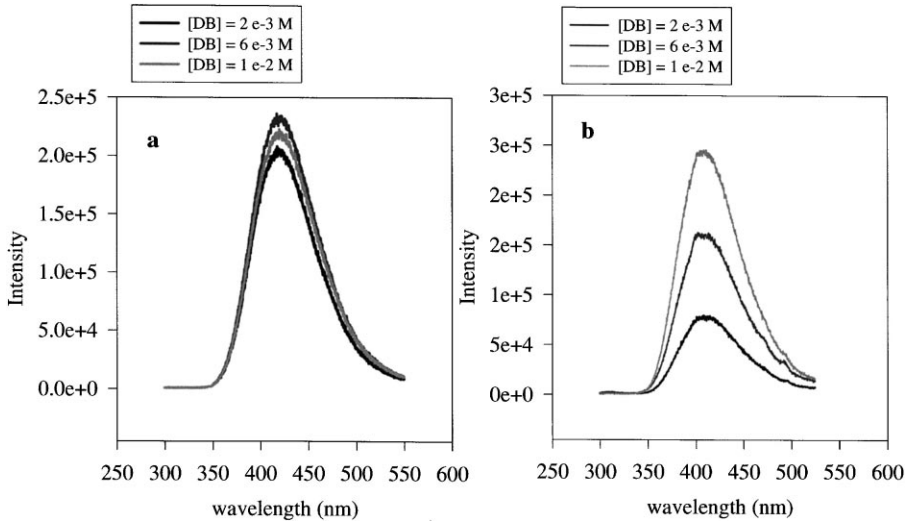

Fig. 7. (a) Dependence of the fluorescence spectrum of dibucaine on dibucaine·HCl concentration in acetate buffer. The excitation wavelength was 280 nm. (b) Dependence of the fluorescence spectrum of dibucaine on dibucaine concentration in an SDS micelle solution in acetate buffer. The excitation wavelength was 280 nm.

bilayer [19]. The evidence comes primarily from Na^+ conductivity measurements. It has also been established that positive iminium ions can be used as a block of the gramicidin channel [20]. It is therefore plausible that a positively charged dibucaine molecule will also block gramicidin. Results from the previous section indicate that dibucaine partitions strongly into non-aqueous media. This was determined from the solvato-chromic shift of dibucaine's fluorescence spectrum. Since there is a similar shift for liposome containing solution (see Fig. 2), it is a reasonable assumption that most of the dibucaine is partitioned into the phospholipid bilayer. In fact, previous work has shown that the phospholipid bilayer partition coefficient for dibucaine is 300 at pH = 7.4 for DMPG [21] and 32,000 for DMPC [22] (L_α phase) at pH = 5.4. Thus, short distance interactions between dibucaine and gramicidin could occur within the quasi two-dimensional DOPC membrane.

We have examined FRET between dibucaine and gramicidin-AC2 at two different membrane protein concentrations. The first (8 mol% gram:DOPC) is where the average gramicidin–gramicidin lateral distance is ∼30 Å and the second (1 mol% gram:DOPC) is where the average gramicidin–gramicidin) lateral distance is ∼90 Å. In each case the concentration of dibucaine used was equal to the tryptophan concentration (i.e. [dibucaine] = 4[gramicidin–AC2]). Fig. 8 shows the fluorescence spectra of gramicidin and dibucaine in a DOPC liposome. By comparing the steady-state fluorescence yields of dibucaine and gramicidin with those found in liposomes containing exclusively gramicidin or dibucaine, it is clear that FRET is occurring. The occurrence of FRET is established in the following way. One can compare the spectra with both gramicidin and dibucaine present to that generated by a linear summation (i.e. holding $a = 1$ and $b = 1$) of the two spectra where in each only one of the species is present in the liposome. As shown in Fig. 8, there is a decrease in the intensity of the gramicidin emission and a concomitant increase in the intensity of the dibucaine emission when the two species are situated in the DOPC liposome. Note that at the lower concentrations employed here, there is little or no evidence of radiative energy transfer. By measuring the decrease in dibucaine fluorescence, we have calculated the efficiency of energy transfer. The FRET spectrum is modeled by the linear combination of the donor and acceptor fluorescence spectra, i.e. using Eq. (4). The multiplicative factor for the tryptophan spectrum from the fit of the FRET spectrum is used to determine FRET efficiency. For the

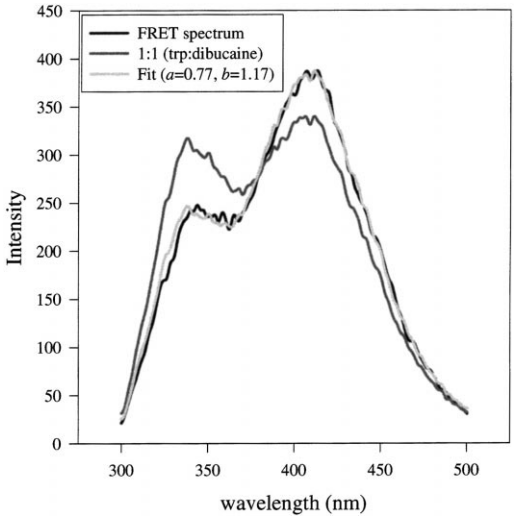

Fig. 8. Fluorescence spectrum of gramicidin-AC2 and dibucaine·HCl in a DOPC liposome at 8 mol% gramicidin:DOPC. The concentration of dibucaine was four times that of gramicidin. Included also are the best fit to the data and a spectrum created by a linear summation of equimolar tryptophan (in gramicidin) and dibucaine both alone in a DOPC liposome.

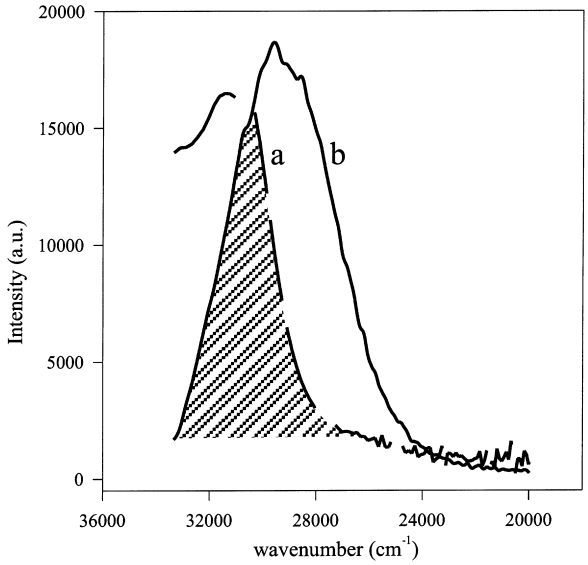

Fig. 9. The spectral overlap of: (a) dibucaine·HCl absorption (dashed line) and (b) gramicidin emission (solid line) used in the calculation of J (Eq. (3)) used to determine R_0.

higher mol% of gramicidin-AC2, the best fit gives a coefficient, $a = 0.77$. Therefore, the FRET efficiency for the higher mole-fraction gramicidin is 0.23 or 23%. The lower gain in the dibucaine fluorescence intensity, 17%, is likely due to self-quenching of dibucaine molecules in close proximity to each other. The efficiency of tryptophan quenching is used to estimate the average donor acceptor distance, r, using Eq. (1). Employing the overlap of the absorption spectrum of dibucaine with the emission spectrum of gramicidin-AC2, shown in Fig. 9, we calculate an overlap integral of, $J = 3.2 \times 10^{-16}$. Substituting this value into Eq. (3) yields $R_0 = 12$ Å. For 23% efficiency, this results in an average dibucaine–gramicidin distance, $r = 15$ Å.

The average gramicidin–gramicidin distance can be estimated from the average DOPC phospholipid head group size, 72 Å^2, and the mole ratio of gramicidin-AC2:DOPC. In the high concentration regime (mole fraction 1:12), the average inter-gramicidin distance is approximately 30 Å. At this concentration there are 4.8 DOPC molecules in the first annulus and 7.9 DOPCs in the second annulus. Thus, a random distribution of dibucaine molecules would produce an average dibucaine:gram-AC2 distance of 15 Å. At lower relative concentrations of gramicidin, the average inter-gramicidin distance will be greater. At 1:100 mole fraction, the average inter-gramicidin distance is approximately 90 Å. At this distance, a random distribution of dibucaine molecules would result in an average gram/AC2-dibucaine distance of 50 Å. For our calculated R_0, the FRET efficiency would be 1.9×10^{-4}, for a random distribution of dibucaine in the membrane. Thus, we can use the FRET dependence on gramicidin—AC2/dibucaine concentration to determine whether or not specific drug ion channel interactions take place. This is because a lowering of the concentration (i.e. an increase in average donor–acceptor distance) should not affect FRET for site specific binding, whereas FRET will decrease if the proximity is due to a random distribution of donors and acceptors in the liposome.

At the lower gramicidin concentration, we observed no evidence of FRET and therefore we conclude that there are no specific binding interactions between dibucaine and gramicidin-AC2. This is consistent with the work of Bridal and Busath [7], where only a non-specific reduction in ion-channel function was observed by measuring changes in ion current. In their work, dissolution of gramicidin from the phospholipid bilayer, via solvation by local anesthetics, was cited as the cause of the reduced ion transport. We observed no evidence of this phenomenon in the present liposome studies. If dibucaine was solvating gramicidin and promoting its release into solution, then a very strong FRET signal should have been observed. However, it must be noted that the two studies were carried out in different bilayers. In the present study DOPC was used, whereas Bridal and Busath used glyceryl monoolein in hexadecane. Therefore, the free energy of gramicidin repartitioning from the bilayer into solution may be different. We submit that for DOPC bilayers, dibucaine would affect ion channel conductivity by causing an increase in bilayer viscosity similar in behavior to general anesthesia [23]. Indeed, this mechanism of interaction between other phosphatidylcholine membranes and dibucaine has been previously suggested. Using NOESY NMR spectroscopy Kuroda and Fujiwara [16] propose that because dibucaine sits near the PC head groups, it can serve as a head group clamp thus reducing the phospholipid mobility. Working on dipalmitoylphosphatidylcholine monolayers, Cavalli et al. [15] find a similar location of dibucaine and further suggest that the local anesthetic action of dibucaine can be attributed to blocking of the PC head groups, thus removing possible proton hopping sites. Finally, using fluorescence anisotropy Tanji et al. [24] observed a decrease in membrane fluidity in the membranes of *E. coli* induced by 2 mM dibucaine.

5. Conclusions

We have examined the interactions between the ion channel, gramicidin and the antiarrhythmic/local anesthetic, dibucaine, using fluorescence resonance energy transfer. Three model systems were employed. To examine aqueous environment mediated interactions, a high concentration solution of tryptophan and dibucaine was prepared. In this solution dibucaine was observed to quench tryptophan fluorescence, but without a concomitant increase in dibucaine fluorescence. This behavior can be attributed to a tryptophan-enhanced aggregation of dibucaine that leads to dibucaine self-quenching. Dibucaine interactions with gramicidin in a lipophilic environment were approximated using high concentration solutions of 3-methyl indole (the tryptophan chromophore) and free base dibucaine in octanol

or decanol. No direct interactions were observed, however a strong propensity for radiative energy transfer was perceived. Finally, the fluorescence spectra of gramicidin and dibucaine, incorporated into small unilamellar DOPC liposomes, were recorded. FRET was observed for liposomes containing relatively high concentrations of gramicidin and dibucaine, but not for lower concentrations. It can therefore be concluded that there is no specific association between gramicidin and dibucaine. The FRET observed can be attributed to the donors and acceptors being in close proximity due to the high relative concentration in the liposome.

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