Membrane Interaction and Self-Assembly within Phospholipid Membranes of Synthetic Segments Corresponding to the H-5 Region of the Shaker K^+ Channel†

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ABSTRACT: The voltage-activated K^+ channels are assumed to be formed by the coassembly of four polypeptide monomers. Each of these monomers is postulated to consist of six transmembrane segments (S1 to S6), and long N- and C-terminal domains. The highly conserved linker, H-5, between the fifth and the sixth transmembrane segments, is hypothesized to line the lumen of the K^+ channel formed by the bundle of the transmembrane segments of the monomers. Herein we utilize the spectrofluorometric approach and investigate the interaction with phospholipid membranes of fluorescently-labeled synthetic peptides, whose sequences are derived from the H-5 region. Binding experiments reveal that the peptides can strongly bind to phospholipid membranes with partition coefficients on the order of 10^4 M^{-1}. However, a truncated peptide without four amino acids within the most conserved region (amino acids 432–435) did not bind to the membranes at all. Moreover, the single substitution of a conserved tryptophan at position 435 to serine reduced the partition coefficient of the peptide ~5-fold, which may account for a mutated K^+ channel with this substitution not producing functional channels (Yool & Schwarz, 1991). Structural characterization using circular dichroism spectroscopy (CD) reveals that H-5 can partially adopt an α-helix structure in hydrophobic environments. Resonance energy transfer (RET) experiments reveal that the H-5-derived segments can self-assemble within the membrane but cannot coassemble with other unrelated membrane-bound peptides. The results herein support the hypothesis that H-5 segments are packed in close proximity and might participate in mediating the appropriate assembly of the core region of K^+ channel monomers.

Voltage-activated K^+ channels are a large and diverse group within the family of voltage-activated ion-conducting channels that also includes Na^+ and Ca^{2+} channels. These K^+ channels share the common feature of a specific K^+-selective pore, and despite differences in their voltage-dependent gating, their ion conduction properties are similar. The K^+ channels are assumed to be formed by the coassembly of four polypeptide monomers of about 70 kDa each (Catterall, 1988; MacKinnon, 1991; Liman et al., 1992). Hydropathic analysis of these monomers suggests that each of them consists of six putative transmembrane segments, termed S1 to S6, and two long N- and C-terminal domains (Catterall, 1988). Although heteromultimeric functional channels might be formed by assembly of monomers from different members of the same subfamily of K^+ channels (Covarrubias et al., 1991; Christie et al., 1990; Isacoff et al., 1990; McCormack et al., 1990; Ruppersberg et al., 1990), combinations of monomers from different subfamilies do not yield functional channels (Covarrubias et al., 1991). Yet, it is not clear what structural components are involved in recognition and assembly of the monomers to form functional channels. Li et al. (1992) have proposed lately that the N-terminal domains of the monomers as well as the core region, which include the transmembrane domains, are involved in mediating the appropriate assembly of the channels. The existence of sites for monomer recognition within the core region was also proposed by McCormack et al. (1990).

Experimental approaches, used to examine the structural architecture of the K^+ core region, indicated that H-5, a highly conserved sequence that connects the S5 and S6 segments of K^+ channels, lines the lumen of the channel formed by the bundle of the transmembrane segments (MacKinnon & Miller, 1989; MacKinnon & Yellen, 1990; MacKinnon et al., 1990; Yellen et al., 1991; Hartmann et al., 1991; Yool & Schwarz, 1991; Heginbotham & MacKinnon, 1992). Modeling the channel also suggests that the amphipathic H-5 segments, although they do not necessarily span the entire membrane, may form the narrow part of a bundle of transmembrane segments, which together form the lumen of the channel (Guy & Seetharamulu, 1986; Guy & Conti, 1990; Durell & Guy, 1992).

Using a spectrofluorometric approach, herein we report that fluorescently-labeled synthetic peptides comprising the H-5 region can strongly bind to phospholipid membranes and self-associate there within. However, they cannot coassemble with other unrelated membrane-bound peptides. These findings support the hypothesis that H-5 segments are in close proximity in the bundle of transmembrane segments that form the lumen of the K^+ channel. Furthermore, they support the possible involvement of H-5 in the appropriate assembly of the channel. These results also support direct evidence that intramembranal segments might participate in specific interactions which involve in some cases sufficient energy to drive folding or oligomerization.

MATERIALS AND METHODS

Materials. BOC-amino acids-PAM[1] [(phenylacetamido)methyl] resins were purchased from Applied Biosystems (Foster City, CA), and BOC-amino acids were obtained from Peninsula Laboratories (Belmont, CA). Other reagents for peptide synthesis included trifluoroacetic acid (TFA) (Sigma), *N,N*-diisopropylethylamine (DIEA) (Aldrich, distilled over

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ninhydrin), dicyclohexylcarbodiimide (DCC) (Fluka), 1-hydroxybenzotriazole (HOBT) (Pierce), methylene chloride, and dimethyl formamide (DMF) (Bio-lab). Egg phosphatidylcholine (PC) was purchased from Lipid Products (South Nutfield, U.K.). Cholesterol (extra pure) was supplied by Merck (Darmstadt, Germany) and recrystallized twice from ethanol. 5(and 6)-Carboxyfluorescein succinimidyl ester and 5(and 6)-carboxytetramethylrhodamine succinimidyl ester were obtained from Molecular Probes (Eugene, OR). NBD (4-fluoro-7-nitrobenz-2-oxa-1,3-diazole) was obtained from Sigma. All other reagents were of analytical grade. Buffers were prepared using double-glass-distilled water.

Peptide Synthesis and Fluorescent Labeling. The peptides were synthesized by the solid-phase method on amino acid-PAM resins (0.075 mequiv) (Merrifield et al., 1982). At the end of the synthesis, the resin-bound peptides were treated for 3 min with 20% piperidine in DMF to remove the formyl protecting group from the tryptophan, then cleaved from the resins by HF, and finally extracted with ether after HF evaporation. All the peptides contained one major peak (as revealed by RP-HPLC), which was shown to be >70% pure peptide by weight. The synthesized peptides were purified by RP-HPLC on a C_4 reversed-phase Vydac column (300-Å pore size). The column was eluted in 40 min, at a flow rate of 0.6 mL/min, using a linear gradient of 25–80% acetonitrile in water in the presence of 0.1% TFA (v/v). The purified peptides were shown to be homogeneous (~99%) by analytical HPLC. The peptides were subjected to amino acid analysis in order to confirm their composition.

Labeling of the N-terminus of the peptides with fluorescent probes was achieved via labeling of resin-bound peptides as previously described (Rapaport & Shai, 1991, 1992). Resin-bound peptides (30–40 mg, 10–25 μmol) were treated with TFA (50% v/v in methylene chloride) to remove the BOC protecting group from the N-terminal amino group of the attached peptides. The resin-bound peptides were then reacted with (i) 5(and 6)-carboxyfluorescein succinimidyl ester (Flu-Suc), (ii) 5(and 6)-carboxytetramethylrhodamine succinimidyl ester (Rho-Suc) (5–7 equiv) in dry DMF (both reactions containing 2.5% v/v *N,N*-diisopropylethylamine), or (iii) NBD-F (4-fluoro-7-nitrobenz-2-oxa-1,3-diazole) in dry DMF. These three separate reactions led to the formation of resin-bound N^1-Flu-peptides, N^1-Rho-peptides or N^1-NBD-peptides, respectively. After 24 h, the mixtures were washed thoroughly with methylene chloride. The peptides were then cleaved from the resins by HF and purified by reversed-phase HPLC as described above.

Preparation of Small Unilamellar Vesicles. Small unilamellar vesicles (SUV) were prepared by sonication of egg phosphatidylcholine (PC) as previously described (Rapaport & Shai, 1991). Briefly, dry lipid and cholesterol (10:1 w/w) were dissolved in a $CHCl_3$/MeOH mixture (2:1 v/v). The solvents were then evaporated under a stream of nitrogen, and the lipids (at a concentration of 7.2 mg/mL) were put under vacuum for 1 h, and then resuspended in the appropriate buffer, via vortex mixing. The resultant lipid dispersion was then sonicated for 10–30 min in a bath-type sonicator (G1125SP1 sonicator, Laboratory Supplies Co. Inc., New York) until clear. The lipid concentration of the supernatant was determined by phosphorus analysis (Bartlett et al., 1959). Vesicles were visualized using a JEOL JEM 100B electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan). Vesicles prepared in this fashion are unilamellar, with an average diameter of 20–50 nm (Papahadjopoulos & Miller, 1967).

CD Spectroscopy. CD spectra were measured at room temperature using a Jasco J-500A spectropolarimeter after calibrating the instrument with (+)-10-camphorsulfonic acid. A cylindrical fused quartz optical cell of 0.5-mm path length was used. Spectra were obtained at wavelengths of 250–200 nm. Seven scans were taken at a scan rate of 20 nm/min. The peptides were scanned at a concentration of 1.6×10^{-5} M, in 40% trifluoroethanol (TFE) in water. Fractional helicities (Wu et al., 1981) were calculated as follows:

$$f_h = ([\theta]_{222} - [\theta]^0_{222})/[\theta]^{100}_{222}$$

where $[\theta]_{222}$ is the experimentally observed mean residue ellipticity at 222 nm, and values for $[\theta]^0_{222}$ and $[\theta]^{100}_{222}$, corresponding to 0% and 100% helix content at 222 nm, are estimated at 2000 and 30 000 (deg·cm²)/dmol, respectively (Chen et al., 1974; Wu et al., 1981).

Binding Experiments. The environmentally sensitive NBD fluorophore has been utilized previously for polarity and binding studies (Kenner & Aboderin, 1971; Frey & Tamm, 1990; Rapaport & Shai, 1991; Pouny & Shai, 1992; Ben-Efraim et al., 1993). Briefly, PC SUV were added successively to 0.1 μM NBD-labeled peptides at 24 °C. Fluorescence intensities were measured as a function of the lipid:peptide molar ratio on a Perkin-Elmer LS-5 spectrofluorometer, with excitation set at 470 nm, using a 10-nm slit, and emission set at 530 nm, using a 5-nm slit, in three separate experiments. To determine the extent of the lipids' contribution to any given signal, the readings observed when unlabeled peptides were titrated with lipid vesicles were subtracted as background from the recorded fluorescence intensities. The binding isotherms were analyzed as partition equilibrium (Schwarz et al., 1986, 1987; Rizzo et al., 1987; Beschiaschvili & Seelig, 1990; Rapaport & Shai, 1991), using the following formula:

$$X_b^* = K_p^* C_f$$

where X_b^* is defined as the molar ratio of bound peptide per 60% of the total lipid, assuming that the peptides were initially partitioned only over the outer leaflet of the SUV, as had been previously suggested (Beschiaschvili & Seelig, 1990), K_p^* corresponds to the partition coefficient, and C_f represents the equilibrium concentration of free peptide in the solution. To calculate X_b, F_∞ (the fluorescence signal obtained when all the peptide is bound to lipid) was extrapolated from a double reciprocal plot of F (total peptide fluorescence) versus C_L (total concentration of lipids) (Schwarz et al., 1986). Knowing the fluorescence intensities of unbound peptide, F_0, as well as bound peptide, F, the fraction of membrane-bound peptide, f_b, could be calculated using the formula

$$f_b = (F - F_0)/(F_\infty - F_0)$$

Having calculated the value of f_b, it is then possible to calculate C_f, as well as the extent of peptide binding, X_b^*. The curves resulting from plotting X_b^* versus the concentration of free peptides, C_f, are referred to as the conventional binding isotherms.

[1] Abbreviations: BOC, *tert*-butyloxycarbonyl; CD, circular dichroism; DCC, dicyclohexylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; DMF, dimethyl formamide; Flu, fluorescein; HF, hydrogen fluoride; HOBT, 1-hydroxybenzotriazole; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; PAM, (phenylacetamido)methyl; PC, egg phosphatidylcholine; Rho, tetramethylrhodamine; RP-HPLC, reversed-phase high-performance liquid chromatography; SUV, small unilamellar vesicles; TFA, trifluoroacetic acid.

Table I: Amino Acid Sequences and Designations

no.	designation	modification	sequence[a]
			424 446
1	H-5-N	X = acetyl	X- SFFKSIPDAFWWAVVTMTTVGYG
2	NBD-H-5-N	X = NBD	
3	Flu-H-5-N	X = Flu	
4	Rho-H-5-N	X = Rho	
5	NBD-H-5	X = NBD	X- AFWWAVVTMTTVGYGDMTPVG
6	Rho-H-5	X = Rho	
7	NBD-(S)H-5	X = NBD	X- AFWSAVVTMTTVGYGDMTPVG
8	Rho-(S)H-5	X = Rho	
9	H-5-C	X = H	X- AVVTMTTVGYGDMTPVG
10	NBD-H-5-C	X = NBD	
11	Rho-H-5-C	X = Rho	
12	NBD-Na-S-4[b]	X = NBD	X- RTFRVLRALKTITIFPGLKTIVRA
13	Rho-Na-S-4	X = Rho	
14	NBD-Par[c]	X = NBD	X-GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE
15	Rho-Par	X = Rho	

[a] Amino acid numbering was taken from shaker K^+ channel. [b] Taken from Rapaport et al. (1992). [c] Taken from Rapaport & Shai (1991).

Resonance Energy Transfer Measurements. Resonance energy transfer experiments (RET) were conducted as previously described (Rapaport & Shai, 1992). Fluorescence spectra were obtained at room temperature in a SLM-8000 spectrofluorometer, with the excitation monochromator set at 470 nm with a 4-nm slit width. Measurements were performed in a 1-cm-path-length glass cuvette in a final reaction volume of 2 mL. Although the excitation maximum for fluorescein is 490 nm, a lower wavelength was chosen in order to minimize the excitation of tetramethylrhodamine (Harris et al., 1991).

The efficiency of energy transfer (E) was determined by the decrease in the quantum yield of the donor as a result of the addition of acceptor. E was determined experimentally from the ratio of the fluorescence intensities of the donor in the presence (I_{da}) and in the absence (I_d) of the acceptor at the emission wavelength of the donor, after correcting for membrane light scattering and the contribution of the emission of the acceptor. The percentage value of E is given in the following equation:

$$E = (1 - I_{da}/I_d) \times 100$$

The correction for light scattering was made by subtracting the signal obtained when unlabeled analogues at concentrations which are equal to the sum of the donor and the acceptor were added to vesicles. Correction for the contribution of acceptor emission was made by subtracting the signal produced by the acceptor-labeled analogue alone.

RESULTS

Synthesis and Fluorescence Labeling of Peptides. We synthesized and fluorescently labeled peptides with sequences identical to those of (i) the H-5 region of the shaker K^+ channel and its truncated forms, (ii) the S-4 segment of the first internal repeat of the eel sodium channel, and (iii) a naturally occurring membrane-permeating toxin, the shark repellent pardaxin (Table I). Fluorescently-labeled peptide analogues were prepared by selectively attaching to the N-terminal amino acid of the peptides the fluorophore NBD, Flu, or Rho. Previous studies revealed that such modifications do not alter the activities of various peptides (Rapaport & Shai, 1991, 1992; Pouny et al., 1992).

Secondary Structure of H-5. To determine the secondary structure of H-5 segments in a hydrophobic environment, CD measurements of H-5-N, the N-terminal part of H-5, and H-5-C, the C-terminal part of H-5, were performed in 40% TFE in water (Figure 1). H-5-N exhibited a mean residual

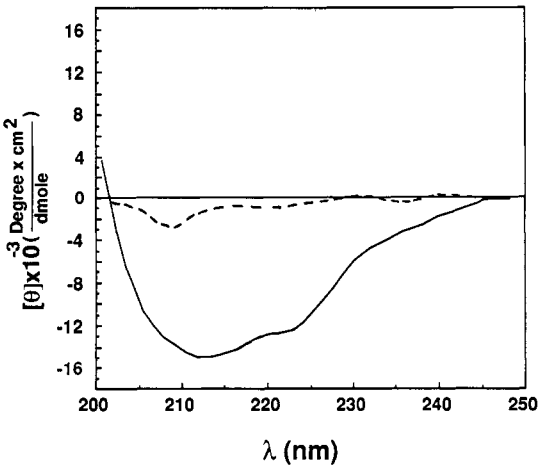

FIGURE 1: CD spectra of H-5-N (continuous line) and H-5-C (dashed line) in 40% TFE in water. Spectra were taken as described in the Materials and Methods at a peptide concentration of 1.6×10^{-5} M.

ellipticity $[\theta]_{222}$ of 13 550 (deg·cm^2)/dmol, which corresponds to a significant fractional helicity value of 0.38 (Wu et al., 1981). However, the CD spectrum of H-5-C did not show any significant signal, and therefore the results could not be interpreted to a defined structure.

Binding of H-5 Peptides to Phospholipid Membranes. To facilitate the determination of the binding properties of the synthesized peptides to phospholipid membranes, the peptides were labeled selectively at their N-terminal amino acids with the environmentally sensitive fluorophore NBD, whose fluorescence intensity increases in a hydrophobic environment, as previously described (Kenner & Aboderin, 1971; Frey & Tamm 1990; Rapaport & Shai, 1991). In the peptide binding experiments, zwitterionic PC was used to avoid contributions of phospholipid head group charges to binding processes. To calculate the surface partition coefficients of the peptides, samples of NBD-labeled peptides were mixed with increasing amounts of PC SUV at pH 6.8. The resulting increases in the fluorescence intensities of the NBD-labeled peptides were plotted as a function of the lipid:peptide molar ratio to generate binding curves (see Figure 2A for results obtained with NBD-H-5-N). In these experiments the concentrations of the NBD-labeled peptides in the mixtures were low; therefore, it was assumed that the peptides did not disrupt the bilayer structure. When unlabeled peptides were titrated with lipids up to the maximal concentration used with NBD-labeled peptides, the fluorescence intensities of these solutions, after subtracting the contribution of the vesicles, remained unchanged.

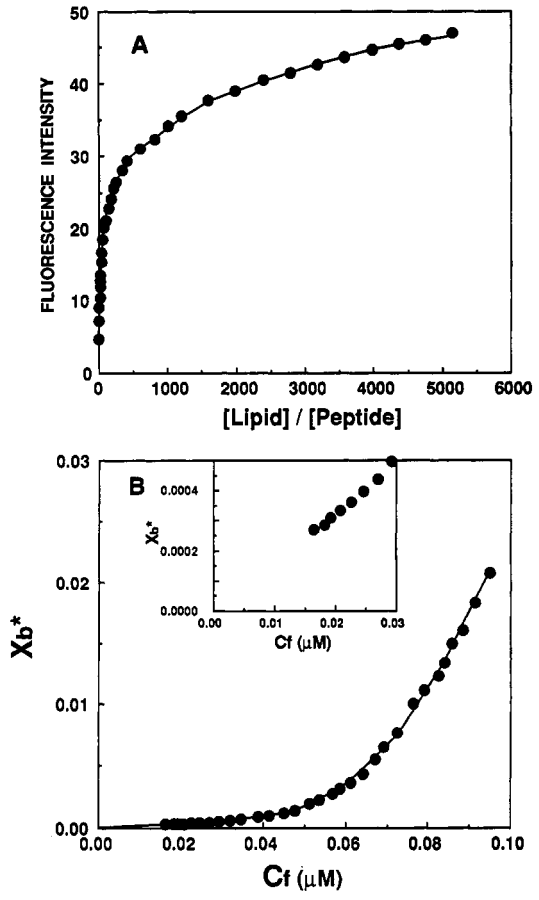

FIGURE 2: (A) Increase in the fluorescence of NBD-H-5-N upon titration with PC vesicles. The peptide (0.1 μM total concentration) was titrated with PC vesicles with excitation monitored at 470 nm and emission recorded at 530 nm. The experiment was performed at room temperature in 50 mM Na_2SO_4 and 25 mM HEPES–SO_4^{2-}, pH 6.8. (B) Binding isotherm derived from (A) by plotting X_b* (molar ratio of bound peptide per 60% lipid) versus C_f (equilibrium concentration of free peptide in the solution). The inset shows the low concentration range of the main figure.

Table II: Partition Coefficients of the Peptides As Determined from the Initial Slopes in the Binding Isotherms and Enthalpies of Insersion As Calculated According to Reynold et al. (1974)

peptide	partition coefficient (M^{-1})	ΔG_{inser} (kcal/mol)
NBD-H-5-N	$(3.5 \pm 1.6) \times 10^4$ (4)[a]	8.6
NBD-H-5	$(3.5 \pm 0.6) \times 10^4$ (3)	8.6
NBD-(S)H-5	$(7.1 \pm 1.7) \times 10^3$ (3)	7.7
NBD-H-5-C	not bound	
NBD-Na-S-4[b]	$(6.2 \pm 1.0) \times 10^4$ (3)	8.9
NBD-Par[c]	$(3.3 \pm 0.4) \times 10^4$ (5)	8.5

[a] Number of experiments is given in parentheses. [b] Taken from Rapaport et al. (1992). [c] Taken from Rapaport & Shai (1991).

The binding isotherms were analyzed as described in the Materials and Methods. The curves obtained by plotting X_b* (molar ratio of bound peptide per 60% of the total lipid assumed to be in the outer leaflet) versus C_f (the equilibrium concentration of free peptide in the solution) are referred to as the conventional binding isotherms (see Figure 2B for results obtained with NBD-H-5-N). The surface partition coefficients, K_p*, were estimated by extrapolating the initial slopes of the curves to zero C_f values. The estimated surface partition coefficients, K_p*, and the calculated free energies of insertion (ΔG_{inser}), calculated according to Reynolds et al. (1974), of the NBD-labeled peptides are listed in Table II. The K_p* and ΔG_{inser} values obtained are within the range of those of various signal peptides (Portlock et al., 1992), and of membrane-permeating bioactive peptides such as melittin and its derivatives (Stankowski & Schwarz, 1990), the *Staphylococcus* δ-toxin (Thiaudière et al., 1991), or pardaxin analogues (Rapaport & Shai, 1991). All these bioactive and signal peptides are known to form stable structures within membranes. NBD-H-5-N and NBD-H-5 have identical values of K_p* and of ΔG_{inser}, implying that the part of the sequence common to these two peptides (residues 432–446) is sufficient to mediate their membrane binding. NBD-H-5-C, which lacks a sequence containing four amino acids, a highly conserved sequence within K^+ channel subfamilies, is the only peptide tested herein that did not bind to phospholipid membranes, as revealed by the insensitivity of the fluorescence of its NBD group to the vesicular environment. The partition coefficient of NBD-(S)H-5 is ~5-fold lower than that of NBD-H-5, and as a result NBD-(S)H-5 is ~1 kcal/mol less stable than NBD-H-5 within the phospholipid membrane (Table II). The sequence of (S)H-5 is identical to that of the H-5 of a shaker K^+ channel mutant, in which serine replaces tryptophan at position 435 (Yool & Schwarz, 1991). Interestingly, the shaker K^+ channel mutant with this substitution did not form functional channels (Yool & Schwarz, 1991).

Self-Assembly of H-5 Peptides within Phospholipid Membranes. To evaluate whether the peptides could self-associate or form heteroaggregates within the membranes, they were labeled selectively at their N-terminal amino acids either with Flu (an energy donor) or with Rho (an energy acceptor) (Table I), and resonance energy transfer (RET) measurements were performed as described previously (Rapaport & Shai, 1992). Profiles of the energy transfer from Flu-H-5-N to Rho-H-5-N or Rho-Na-S-4 in the presence of PC phospholipid vesicles are depicted in parts A and B, respectively, Figure 3. Addition of Rho-H-5-N or Rho-Na-S-4 (final concentrations of 0.01–0.1 μM) to Flu-H-5-N (0.02 μM) with PC phospholipid vesicles (100 μM) quenched the donor's emission and increased the acceptor's emission, as consistent with energy transfer. These emission changes were marked with Rho-H-5-N, but not with Rho-Na-S-4. Similar experiments were performed with Flu-H-5-N as a donor and Rho-H-5, Rho-(S)H-5-N, Rho-H-5-C, or Rho-Par as an acceptor. The curves of the experimentally-derived percentage of energy transfer versus the total acceptor:lipid molar ratios are depicted in Figure 4A. To determine the actual percentage of energy transfer, the amounts of lipid-bound acceptors (Rho-peptides, termed "bound acceptor"), at the various acceptor peptide concentrations were calculated from the binding isotherms of the corresponding NBD-labeled peptides as previously described (Pouny et al., 1992). The curves of the experimentally-derived percentage of energy transfer versus the bound acceptor:lipid molar ratios are depicted in Figure 4B. A curve corresponding to random distribution of monomers (Fung & Stryer, 1978), assuming an R_0 of 45 Å, which was previously calculated for the Flu/Rho donor/acceptor pair (Rapaport & Shai, 1992) is also depicted. A high percentage of energy transfer was obtained with the Flu-H-5-N/Rho-H-5, Flu-H-5-N/Rho-H-5-N, or Flu-H-5-N/Rho-(S)H-5-N pair (Figure 4B). These values are markedly higher than those obtained assuming random distribution of monomers. Rho-H-5-C, which does not bind to vesicles (Table II), also does not affect the emission spectrum of Flu-H-5-N, and hence does not appear to associate with it. High-energy transfer between the Flu-H-5-N/Rho-(S)H-5 pair required higher total peptide:lipid molar ratios than with the Flu-H-5-N/Rho-H-5-N pair (Figure 4). For example, ~5-fold higher peptide:lipid molar ratios were required to reach 18%

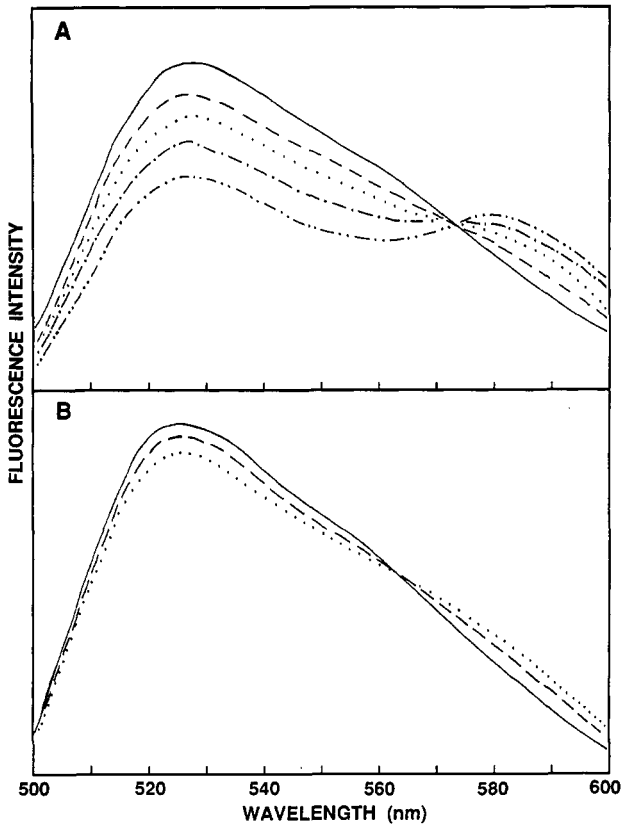

FIGURE 3: Fluorescence energy transfer dependence on Rho-peptide (acceptor) concentrations using PC vesicles. The spectrum of Flu-H-5-N (0.02 μM), the donor peptide, was determined in the presence or absence of various concentrations of the acceptor peptides Rho-H-5-N (panel A) and Rho-Na-S-4 (panel B). Each spectrum was recorded in the presence of PC vesicles (100 μM) in 50 mM Na_2SO_4 and 25 mM HEPES–SO_4^{2-}, at pH 6.8. The excitation wavelength was set at 470 nm; emission was scanned from 500 to 600 nm. The spectra of Rho-H-5-N and Rho-Na-S-4 in the presence of vesicles and unlabeled H-5-N were subtracted from the corresponding spectra. (A) Key: —, 0.02 μM Flu-H-5-N; - - -, a mixture of 0.02 μM Flu-H-5-N and 0.01 μM Rho-H-5-N; ···, a mixture of 0.02 μM Flu-H-5-N and 0.02 μM Rho-H-5-N; · - ·, a mixture of 0.02 μM Flu-H-5-N and 0.04 μM Rho-H-5-N; ·· - ··, a mixture of 0.02 μM Flu-H-5-N and 0.06 μM Rho-H-5-N. (B) Key: —, 0.02 μM Flu-H-5-N; - - -, a mixture of 0.02 μM Flu-H-5-N and 0.05 μM Rho-Na-S-4; ···, a mixture of 0.02 μM Flu-H-5-N and 0.10 μM Rho-Na-S-4.

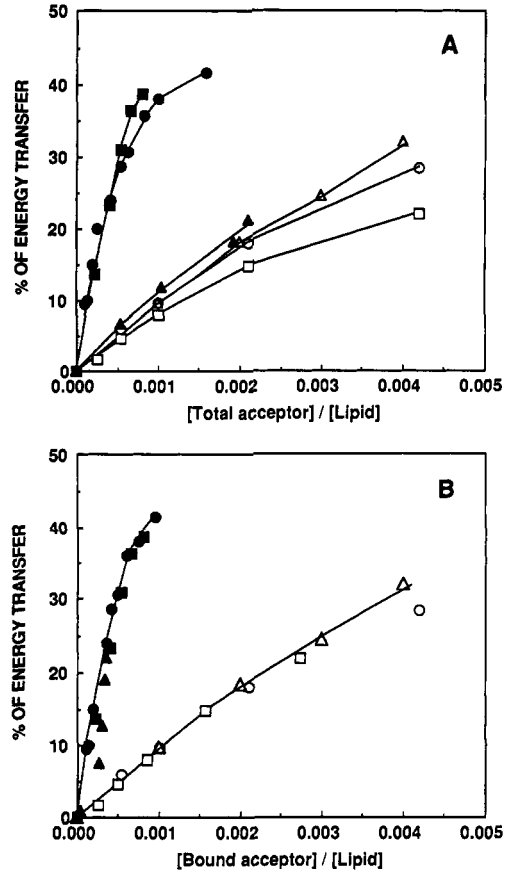

FIGURE 4: Theoretically and experimentally derived percentage of energy transfer. (A) Versus total acceptor:lipid molar ratio. The percentage of energy transfer was calculated as described in the Materials and Methods, and was plotted versus the molar ratio between the total concentration of the acceptor and the lipid concentration. (B) Versus bound acceptor:lipid molar ratio. The amount of lipid-bound acceptor (Rho peptides), C_b, at various acceptor concentrations was calculated from the binding isotherms. First, the fractions of bound acceptor, f_b, were calculated for the various peptide: lipid molar ratios from their binding isotherms (see Figure 1A) (Pouny et al., 1992). These values of f_b were used to calculate the amount of bound acceptor, c_b (c_b = (μM)f_b). Key: filled circles, Rho-H-5-N; filled squares, Rho-H-5; filled triangles, Rho-(S)-H-5-N; open squares, Rho-Na-S-4; open circles, Rho-Par; and open triangles, random distribution of the monomers (Fung & Stryer, 1978), assuming an R_0 of 45 Å.

energy transfer. Interestingly, efficiencies of energy transfer between Flu-H-5-N and the unrelated membrane-interacting peptides Rho-Na-S-4 and Rho-Par resemble those observed for random distribution. Therefore, they do not appear to coassemble with Flu-H-5-N. Thus, we concluded that the donor H-5-N and the acceptor H-5, H-5-N, or (S)H-5 are associated, rather than randomly distributed throughout the phospholipid membrane. The substitution of tryptophan with serine in position 435 does not influence the efficiency of energy transfer, although tryptophan is essential for the binding of the peptide to phospholipid membranes. Significant energy transfer (10%) was already detected with the Flu-H-5-N/Rho-H-5-N or with the Flu-H-5-N/Rho-H-5 pair at a lipid:peptide molar ratio of 4750 (the lowest ratio tested, Figure 4). Assuming that the peptides are equally distributed among vesicles, and that each SUV vesicle of 20–50-nm diameter is composed of 4000–10 000 lipid molecules, it appears that as few as 2–3 molecules within one vesicle can associate.

DISCUSSION

The pore lining region of the K^+ channels is postulated to contain a tetramer of H-5 segments. Whether H-5 is embedded within the membrane and spans all or only part of the membrane is a basic question in modeling the channel (Durell & Guy, 1992). Since H-5 appears to contain 17–21 amino acids, if it spans most of the membrane, long β-barrel structures would be favored (Yellen et al., 1991; Hartmann et al., 1991; Yool & Schwarz, 1991). However, if H-5 spans only the outer half of the membrane, short β-barrel structures or both short β-barrel and α-helix structures would be expected (Hartmann et al., 1991; Durell & Guy 1992; Kirsch et al., 1992). CD measurements (Figure 1) revealed a significant α-helical content in the H-5 region in a hydrophobic environment. This observed α-helicity is mostly due to the N-terminal part of H-5, since no significant CD signal was observed with the C-terminal part of H-5 (H-5-C, Figure 1). These results might support a model postulating that the secondary structure of H-5 consists partially of an α-helix at its N-terminal part (Guy & Conti, 1990).

The results also demonstrated that the H-5 region interacts with phospholipid membranes and is able to form stable structures within them (Table II). According to current K^+ models, although located within the membrane, H-5 is

surrounded by other transmembrane segments, and therefore is not in direct contact with the membrane lipids. The relevance of membrane interactions with H-5 segments can be comprehended if one considers a "two-stage" model for membranal protein folding and oligomerization (Popot et al., 1987; Popot & Engelman, 1990). In this two-stage model, the final structure in membranes results from the packing of smaller elements, each of which reaches thermodynamic equilibrium with the lipid and aqueous phases before packing. The model excludes structures incorporating transmembrane segments that are not individually stable. This model can explain the formation of an active molecule of bacteriorhodopsin by the assembly of two enzymatically cleaved single transmembrane α-helices, and a third segment composed of five transmembrane α-helices (Kahn & Engelman, 1992). That H-5 can interact strongly with phospholipid membranes, and that this interaction is stabilized by 8.6 kcal/mol, was demonstrated herein (Table II). Therefore, H-5 fulfills an important requirement for the two-stage model, i.e., stabilization within the lipid membrane. Although the partition coefficient of NBD-(S)H-5 in phospholipid membranes is significantly lower than that of NBD-H-5 (Table II), it can coassemble with Flu-H-5-N (Figure 4B). However, due to the lower partition coefficient of Rho-(S)-H-5, high concentrations are required to enable the detection of significant energy transfer. This might imply that the probability of mutated K^+ channel monomers, in which serine replaces tryptophan in position 435, to pack correctly to form a channel is lower than that of wild-type monomers. Since four monomers are required to form the lumen of the channel, the effect of the reduced partitioning within the membrane on the probability to form a functional channel is even more pronounced. This hypothesis might explain the observation that a K^+ channel mutant with this substitution did not form functional channels (Yool & Schwarz, 1991).

Herein, a self-assembly between fluorescently-labeled H-5 segments with variable lengths or a coassembly between fluorescently-labeled H-5 and (S)-H-5 was implied by the RET experiments (Figure 4). However, both Rho-Na-S-4, which is randomly distributed in its membrane-bound state (Rapaport et al., 1992), and Rho-Par, which self-aggregates within membranes (Rapaport & Shai, 1992), do not coassemble with Flu-H-5-N (Figure 4). This implies that the H-5-derived segments can specifically interact with each other, but not with unrelated membrane-bound polypeptides. Those results support rapidly accumulating data that intramembranal sequences of integral membrane proteins can participate in specific interactions that contribute to specific recognition, association, and oligomerization of those proteins within the lipid environment. A few examples of such participation of intramembranal sequences of proteins in peptide interactions are as follows: Synthetic peptides corresponding to the transmembrane domain of glycophorin A form a complex with the native protein (Bormann et al., 1989). A specific association between a pair of transmembrane segments was observed in the aspartate sensory receptor of *Escherichia coli* (Lynch & Koshland, 1991). In the T cell receptor complex (TCR), the transmembrane domain of the α chain can target the unassembled protein for degradation, and this domain also contains all the structural features required for association with the CD3δ chain (Bonifacino et al., 1990a,b; Manolios et al., 1990). It was suggested that a five amino acid motif in the transmembrane α-helices of several members of the tyrosine kinase family of growth factor receptors is responsible for the dimerization of these receptors, which is essential for their activation (Sternberg & Gullick, 1990). Synthetic peptides with sequences identical to those of the single transmembrane domain of the minK K^+ channel self-associate within phospholipid membranes, suggesting that the channel is formed via the assembly of a few monomers (Ben-Efraim et al., 1993). These examples and others [see review by Lemmon and Engelman (1992)] suggest that protein sequences within the membrane can contribute to specific recognition and assembly in other proteins as well.

The results herein support the hypothesis that H-5 segments are packed in close proximity in the tetrameric bundle of the K^+ channel. These closely packed H-5 segments may have a role as a structural element that participate in mediating the appropriate association of the hydrophobic core of K^+ channel monomers as previously proposed (Li et al., 1992; McCormack et al., 1990; Pak et al., 1991; VanDongen et al., 1990). This association of the hydrophobic region possibly occurs after the coassembly of the channel monomers by the interaction of their hydrophilic N-terminals (Li et al., 1992).

REFERENCES

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Ben-Efraim, I., Bach, D., & Shai, Y. (1993) *Biochemistry 32*, 2371–2377.

Beschiaschvili, G., & Seelig, J. (1990) *Biochemistry 29*, 52–58.

Bonifacino, J. S., Suzuki, C. K., & Klausner, R. D. (1990a) *Science 247*, 79–82.

Bonifacino, J. S., Cosson, P., & Klausner, R. D. (1990b) *Cell 63*, 503–513.

Bormann, B. J., Knowles, W. J., & Marchesi, V. T. (1989) *J. Biol. Chem. 264*, 4033–4037.

Catterall, W. A. (1988) *Science 242*, 50–61.

Chen, Y. H., Yang, J. T., & Chau, K. H. (1974) *Biochemistry 13*, 3350–3359.

Christie, M. J., North, R. A., Osborne, P. B., Douglass, J., & Adelman, J. P. (1990) *Neuron 4*, 405–411.

Covarrubias, M., Wei, A. A., & Salkoff L. (1991) *Neuron 7*, 763–773.

Durell, S. R., & Guy, H. R. (1992) *Biophys. J. 62*, 238–250.

Fung, B. K. -K., & Stryer, L. (1978) *Biochemistry 17*, 5241–5248.

Frey, S., & Tamm, L. K. (1990) *Biochem. J. 272*, 713–719.

Guy, H. R., & Seetharamulu, P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 508–512.

Guy, H. R., & Conti, F. (1990) *Trends Neurosci. 13*, 201–206.

Harris, R. W., Sims, P. J., & Tweten, R. K. (1991) *J. Biol. Chem. 266*, 6936–6941.

Hartmann, H. A., Kirsch, G. E., Drewe, J. A., Taglialatela, M., Joho, R. H., & Brown, A. M. (1991) *Science 251*, 942–944.

Heginbotham, L., & MacKinnon, R. (1992) *Neuron 8*, 483–491.

Isacoff, E. Y., Jan, Y. N., & Jan, L. Y. (1990) *Nature 345*, 530–534.

Kahn, T. W., & Engelman, D. M. (1992) *Biochemistry 31*, 6144–6151.

Kenner, R., & Aboderin, A. (1971) *Biochemistry 10*, 4433–4440.

Kirsch, G. E., Drewe, J. A., Hartmann, H. A., Taglialatela, M., de Biasi, M., Brown, A. M., & Joho, R. H. (1992) *Neuron 8*, 499–505.

Lemmon, M. A., & Engelman, D. M. (1992) *Curr. Opin. Struct. Biol. 2*, 511–518.

Li, M., Jan, Y. N., & Jan, L. Y. (1992) *Science 257*, 1225–1230.

Liman, E. R., Tytgat, J., & Hess, P. (1992) *Neuron 9*, 861–871.

Lynch, B. A., & Koshland, D. E. Jr. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10402–10406.

MacKinnon, R., & Miller, C. (1989) *Science 245*, 1382–1385.

MacKinnon, R., & Yellen, G. (1990) *Science 250*, 276–279.

MacKinnon, R., Heginbotham, L., & Abramson, T. (1990) *Neuron 5*, 767–771.
MacKinnon, R. (1991) *Nature 350*, 232–235.
Manolios, N., Bonifacino, J. S., & Klausner, R. D. (1990) *Science 249*, 274–277.
McCormack, K., Lin, J. W., Iverson, L. E., & Rudy, B. (1990) *Biochem. Biophys. Res. Commun. 171*, 1361–1371.
Merrifield, R. B., Vizioli, L. D., & Boman, H. G. (1982) *Biochemistry 21*, 5020–5031.
Pak, M. D., Baker, K., Covarrubias, M., Butler, A., Ratcliffe, A., & Salkoff, L. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4386–4390.
Papahadjopoulos, D., & Miller, N. (1967) *Biochim. Biophys. Acta 135*, 624–638.
Popot, J.-L., Gerchman, S.-E., & Engelman, D. M. (1987) *J. Mol. Biol. 198*, 655–676.
Popot, J.-L., & Engelman, D. M. (1990) *Biochemistry 29*, 4031–4037.
Portlock, S. H., Lee, Y., Tomich, J. M., & Tamm, L. K. (1992) *J. Biol. Chem. 267*, 11017–11022.
Pouny, Y., Rapaport, D., & Shai, Y. (1992) *Biochemistry 31*, 12416–12423.
Rapaport, D., & Shai, Y. (1991) *J. Biol. Chem. 266*, 23769–23775.
Rapaport, D., & Shai, Y. (1992) *J. Biol. Chem. 267*, 6502–6509.
Rapaport, D., Danin, M., Gazit, E., & Shai, Y. (1992) *Biochemistry, 31*, 8868–8875.
Reynolds, J. A., Gilbert, D. B., & Tanford, C. (1974) *Proc. Nat. Acad. Sci. U.S.A. 71*, 2925–2927.
Rizzo, V., Stankowski, S., & Schwarz, G. (1987) *Biochemistry 26*, 2751–2759.
Ruppersberg, J. P., Schroter, K. H., Sakmann, B., Stocker, M., Sewing, S., & Pongs, O. (1990) *Nature 345*, 535–537.
Schwarz, G., Stankowski, S., & Rizzo, V. (1986) *Biochim. Biophys. Acta. 861*, 141–151.
Schwarz, G., Gerke, H., Rizzo, V., & Stankowski, S. (1987) *Biophys. J. 52*, 685–692.
Stankowski, S., & Schwarz, G. (1990) *Biochim. Biophys. Acta 1025*, 164–172.
Sternberg, M. J. E., & Gullick, W. J. (1990) *Protein Eng. 3*, 245–248.
Thiaudière, E., Siffert, O., Talbot, J. C., Bolard, J., Alouf, J. E., & Dufourcq, J. (1991) *Eur. J. Biochem. 195*, 203–213.
Vandongen, A. M. J., Frech, G. C., Drewe, J. A., Joho, R. H., & Brown, A. M. (1990) *Neuron 5*, 433–443.
Wu, C.-S. C., Ikeda, K., & Yang, J. T. (1981) *Biochemistry 20*, 566–570.
Yellen, G., Jurman, M. E., Abramson, T., & MacKinnon, R. (1991) *Science 251*, 939–942.
Yool, A. J., & Schwarz, T. L. (1991) *Nature 349*, 700–704.