Spectroscopic and Functional Characterization of the Putative Transmembrane Segment of the minK Potassium Channel†

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ABSTRACT: MinK (I_{sk}) is a voltage-dependent K^+ channel whose gene has been recently cloned and which consists of 130 amino acids [Takumi, T., Ohkubo, H., & Nakanishi, S. (1988) *Science 242*, 1042–1045]. The protein contains one putative transmembrane segment by hydropathy analysis. Whether this putative transmembrane segment is involved in the function of the protein was studied. A 32 amino acid peptide (residues 41–72) with the sequence SKLEALYILMVLGFFGFFTLGIMLSYIRSKKL, containing the hypothesized transmembrane domain, designated TM-minK, was synthesized and fluorescently labeled. The α-helical content of TM-minK, assessed in methanol using circular dichroism (CD), was 57%. The fluorescent emission spectrum of 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-labeled TM-minK displayed a blue shift upon binding to small unilamellar vesicles (SUV), reflecting a relocation of the fluorescent probe to an environment of increased apolarity, i.e., within the lipid bilayer. The increase in NBD's fluorescence upon mixing NBD-labeled TM-minK with small unilamellar vesicles (SUV) was used to generate a binding isotherm, from which was derived a surface partition coefficient of 5.5×10^4 M^{-1}. Fluorescence energy transfer measurements between carboxyfluoresceine-labeled and rhodamine-labeled analogues suggest that TM-minK aggregates within membranes. In addition, single-channel experiments revealed that TM-minK can form single channels in planar lipid membranes only when a trans negative potential is applied. The findings herein experimentally support a role of the transmembrane segment of minK both in the assembly and as a constituent of the pore formed by the protein.

Potassium (K^+) ion channels form a diverse group of membrane-spanning proteins which play a central role in the generation of electric signals in excitable membranes (Hille, 1992). These resulting potassium-selective channels can be opened or closed rapidly by changes in membrane potential. Two types of voltage-dependent K^+ channels have been distinguished on the basis of molecular characteristics. The first group includes K^+ channels expressed by the *shaker*, *shab*, and *shaw* loci of *Drosophila* (Schwarz et al., 1988; Kamb et al., 1988; Butler et al., 1989), as well as their homologues from rodent brain (Stuhmer et al., 1989; Frech et al., 1989). These *shaker*-type K^+ channels span the membrane at least six times and form functional channels through tetrameric associations (MacKinnon, 1991). Recently, several groups cloned a gene believed to encode for a voltage-dependent K^+ channel in mammalian kidneys, uterus, and heart (Takumi et al., 1988; Folander et al., 1990; Pragnell et al., 1990). The product of this gene, consisting of 130 amino acids, is a protein referred to as "minK" (or as I_{sk}). To elucidate the molecular nature of the K^+ currents elicited by the minK polypeptide, a synthetic gene encoding for minK was designed. In studies aimed at elucidating the molecular nature of the K^+ currents elicited by minK, Hausdorf et al. (1991) observed functional properties of voltage dependent gating, ion selectivity, and specific blockage, similar to those seen in other K^+ channels. Hydrophobicity analysis of minK predicts a single putative transmembrane α-helical domain (Takumi et al. 1988). Point mutations in this domain lead to subtle changes in functional attributes of the conduction pathway in minK-induced channels (Goldstein et al., 1991). By combining site-directed and deletion mutations with electrophysiological measurements of the resultant mutant proteins, in an oocyte expression system, Takumi and co-workers (1991) identified a sequence of 63 amino acid residues (1–9 and 40–93) that elicited a voltage-dependent K^+ channel activity indistinguishable from that of wild type minK. The ion channel activity of the protein and its truncated mutants might be explained by a model in which transmembrane α-helices are involved in the formation of ion channels. According to such a model, transmembrane amphiphilic α-helices form bundles in which their outwardly directed hydrophobic surfaces interact either with other hydrophobic transmembrane segments or with the lipid core of the membrane, while their inwardly facing hydrophilic surfaces produce a hollow conducting pore (Inouye, 1974; Guy & Steerhamulu 1986; Greenblatt et al., 1985).

In this article, the synthesis, fluorescent labeling (with 7-nitrobenz-2-oxa-1,3-diazol-4-yl [NBD],[1] carboxyflurescein or rhodamine), membrane association, and functional characterization of a 32 amino acid residue peptide (designated TM-minK), with a sequence identical to the hypothesized transmembrane domain and a few flanking amino acids of minK, is described. The secondary structure, membrane interaction, monomeric association, and channel formation

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[1] Abbreviations: TM-minK, transmembrane minK; NBD-Cl, 4-chloro-7-nitrobenz-2-oxa-1,3-diazole; CD, circular dichroism; PLM, planar lipid membrane; Flu-Su, 5-(and 6-)carboxyflurescein succinimidyl ester; Rh-Su, 5-(and 6-)carboxytetramethylrhodamine succinimidyl ester; BOC, butyloxycarbonyl; Pam, (phenylacetamido)methyl; HOBT, hydroxybenzotriazole; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; DMF, *N,N*-dimethylformamide; HF, hydrogen fluoride; SUV, small unilamellar vesicles; PC, egg phosphatidylcholine; diph-PC, diphytanoylphosphatidylcholine; FET, fluorescence resonance energy transfer.

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of this synthetic peptide were examined by biophysical and biochemical methods. Our findings experimentally support the hypothesis that the putative transmembrane segment of the minK channel assumes an α-helix structure and has a role both in the assembly and as a constituent of the pore formed by the protein.

EXPERIMENTAL PROCEDURES

Materials. BOC-Leu(*O*-benzyl)Pam resin was purchased from Applied Biosystems (Foster City, CA). BOC-amino acids were obtained from Peninsula Laboratories (Belmont, CA). Other reagents for peptide synthesis were obtained from Sigma. Egg phosphatidylcholine (PC) was purchased from Lipid Products (South Nutfield, U.K.). Cholesterol (extra pure), purchased from Merck (Darmstadt, Germany), was recrystallized twice from ethanol. 5-(and 6-)carboxyflurescein succinimidyl ester (Flu-Su) and 5-(and 6-)carboxytetramethylrhodamine succinimidyl ester (Rh-Su) were obtained from Molecular Probes (Eugene, OR). NBD-Cl (4-chloro-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, Fluorescent Labeling, and Purification. The peptide was synthesized by a solid phase method on Pam-amino acid resin (0.15 mequiv) (Merrifield et al., 1982), as previously described (Shai et al., 1990). Coupling was carried out with freshly prepared hydroxybenzotriazole (HOBT) active esters of BOC amino acids. The synthetic peptide was purified to a chromatographic homogeneity of >98% by reverse-phase HPLC on an analytical C_4 column using a linear gradient of 25–80% acetonitrile in 0.1% TFA, for 40 min. The peptide was subjected to amino acid analysis in order to confirm its composition.

Labeling of the peptide's N-terminus was achieved via labeling of resin-bound peptide as follows; Resin-bound peptide (30–70 mg, 10–25 μmol) was treated with TFA [50% (v/v) in methylene chloride] to remove the BOC protecting group from the N-terminal amino group of the attached peptide (Rapaport & Shai, 1991). The resin-bound peptide was then reacted with either (i) 5-(and 6-)carboxyflurescein succinimidyl ester (Flu-Su), (ii) 5-(and 6-)carboxytetramethylrhodamine succinimidyl ester (Rh-Su) (5–7 equiv), or (iii) NBD-Cl (4-chloro-7-nitrobenz-2-oxa-1,3-diazole) in DMF [containing 3% (v/v) triethylamine]. These three separate reactions led to the formation of resin-bound N^1-Flu-TM-minK, N^1-Rh-TM-minK, or N^1-NBD-TM-minK, respectively. After 24 h, the mixtures were washed thoroughly with methylene chloride. The peptides were then cleaved from the resin by HF, which was then removed by evaporation, precipitated with ether, and finally extracted with 50% (v/v) acetonitrile/water. Final purification was achieved by using reverse-phase HPLC as described above.

Preparation of Small Unilamellar Vesicles. Small unilamellar vesicles (SUV) were prepared from PC by sonication. Briefly, dry lipid and cholesterol were dissolved in $CHCl_3$/MeOH (2:1 v/v), such that the mixture contained 10% (w/w) cholesterol. The solvents were removed by evaporation under a stream of nitrogen, and the lipids were resuspended (at a concentration of 7.2 mg/mL) in buffer by vortex mixing. The resulting lipid dispersion was sonicated (10–30 min) in a bath-type sonicator (G1125SP1 sonicator, Laboratory Supplies Company Inc., New York) until the turbidity cleared. The lipid concentration of the solution was then determined by phosphorus analysis (Bartlet et al., 1959). Vesicles were visualized by depositing a drop containing vesicles onto a carbon-coated grid and negatively staining them with uranyl acetate. The resulting grids were examined 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 20–40 nm (Papahadjopoulos & Miller, 1967).

CD Spectroscopy. The CD spectrum of TM-minK was measured using a Jasco J-500A spectropolarimeter, after calibrating the instrument with (+)-10-camphorsulfonic acid. The spectrum was scanned, at 23 °C, in a capped quartz optical cell with a 0.5-mm path length. Spectra were obtained at wavelengths of 250 to 200–190 nm. Three scans were performed at a scan rate of 20 nm/min, with a sampling interval of 0.2 nm and at a peptide concentration of 1.1×10^{-5} M.

Fractional helicities (Wu et al., 1981) were calculated as follows:

$$f_h = \frac{([\theta]_{222} - [\theta]^0_{222})}{[\theta]^{100}_{222}} \quad (1)$$

where $[\theta]_{222}$ is the experimentally observed mean residue ellipticity at 222 nm, and the values for $[\theta]^0_{222}$ and $[\theta]^{100}_{222}$, corresponding to 0% and 100% helix content at 222 nm, were estimated to be 2000 and 30 000 deg·cm^2/dmol, respectively (Chen et al., 1974; Wu et al., 1981).

Intrinsic Fluorescence Measurements. NBD-labeled TM-minK (0.2 nmol) was added to 2 mL of buffer (50 mM Na_2SO_4, 25 mM HEPES-SO_4^{2-}, pH 6.8) containing 75 μL (550 μg) of PC SUV to establish a lipid/peptide ratio (2700:1) in which all the peptide is bound to lipid. After a 2-min incubation, the emission spectrum of the NBD group was recorded (in three separate experiments) using a SLM-8000 spectrofluorometer (SLM Instruments, Urbana, IL), with excitation set at 468 nm (4-nm slit).

Binding Experiments. The degree of NBD-TM-minK association with phospholipid vesicles was assessed by a modification of a previously described experimental protocol (Rapaport & Shai, 1991). Briefly, to increasing concentrations of PC SUV in separate Eppendorf tubes was added 0.2 nmol of NBD-TM-minK, which was then diluted to 2 mL with 50 mM Na_2SO_4 and 25 mM HEPES-SO_4^{2-}, pH 6.8. The fluorescence intensities of these mixtures with increasing lipid/peptide molar ratios were measured at room temperature (in three separate experiments) with a Perkin-Elmer LS-5 spectrofluorometer (excitation set at 468 nm, using a 10-nm slit, and emission set at 530 nm, using a 5 nm slit). To determine the extent of the lipids' contribution to any given signal, background readings obtained when unlabeled TM-minK was mixed with lipid vesicles at each lipid/peptide molar ratio were subtracted from the recorded fluorescence intensity.

Resonance Energy Transfer Experiments. Peptides were incorporated into PC vesicles by either one of the following procedures: (i) Samples of dry lipid (200 μg) and cholesterol (20 μg) were dissolved in $CHCl_3$/MeOH (2:1 v/v). To the resulting mixtures were added methanolic solutions containing either 0.04 nmol of Flu-TM-minK (donor), a mixture of 0.04 nmol of Flu-TM-minK and 0.04 nmol of Rh-TM-minK (acceptor), or a mixture of 0.04 nmol of Flu-TM-minK and 0.12 nmol of Rh-TM-minK. The solvents were then removed by evaporation under a stream of nitrogen, and the films formed by the lipids and peptides were lyophilized for 1 h. The resulting films were dispersed in buffer (50 mM Na_2SO_4, 25 mM HEPES-SO_4^{2-}, pH 6.8) and sonicated (10–30 min) in a bath-type sonicator (G1125SP1 sonicator, Laboratory Supplies Company Inc., New York) until turbidity had cleared.

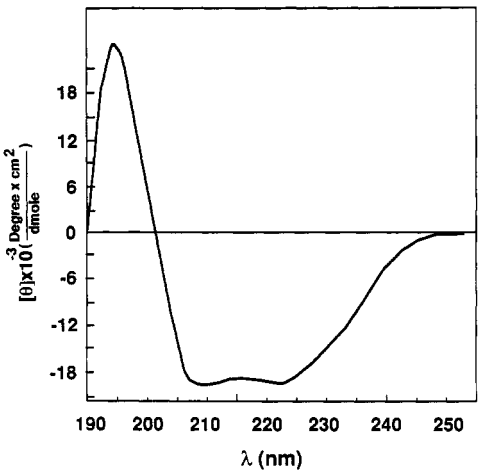

FIGURE 1: CD spectrum of TM-minK in methanol. The spectrum was taken as described under Experimental Procedures at a peptide concentration of 1.1×10^{-5} M.

(ii) To a solution of PC SUV (220 μg in 50 μL of buffer, 10:1 lipid/cholesterol) in separate Eppendorf tubes were added methanolic solutions containing 0.04 nmol of Flu-TM-minK (donor) and 0.04 nmol of Flu-TM-minK followed by the addition of 0.04 nmol of Rh-TM-minK (acceptor) or 0.04 nmol of Flu-TM-minK followed by the addition of 0.12 nmol of Rh-TM-minK. The solutions were then diluted to 2 mL with 50 mM Na_2SO_4, 25 mM HEPES-SO_4^{2-}, pH 6.8. 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 quartz cuvette in a final reaction volume of 2 mL. Although the excitation maximum for fluorescein is 490 nm, a lower wavelength was chosen to minimize the excitation of tetramethylrhodamine (Harris et al., 1991; Rapaport & Shai, 1992).

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 donor's emission wavelength, after correcting for membrane light scattering and the contribution of acceptor's emission. The value of E in percent was calculated by

$$E = (1 - I_{da}/I_d)100 \quad (2)$$

Correction for light scattering was achieved by subtracting the signal obtained when unlabeled analogue was incorporated into vesicles containing the donor molecules. Correction for the contribution of the acceptor's emission was made by subtracting the signal produced by the acceptor-labeled analogue alone.

Single Channel Measurements on Planar Lipid Membranes (PLM). Planar bilayers were formed on the tip of glass pipettes using the method of Coronado and Latorre (1983). Micropipettes were pulled from borosilicate glass capillaries (A-M Systems, Inc., Everett, WA) on a Kopf model 700C puller (Tujunga, CA). A calomel electrode served as reference electrode. Bilayers, formed from mixtures of diphytanoyl lecithin (Avantipolar Lipid, Alabaster, AL) and cholesterol (50% w/w), were prepared by dissolving the lipids in *n*-hexane at a concentration of 1.0 mg/mL. One to two microliters of the lipid solution was introduced onto the surface (0.5 cm^2) of 300–600 μL of a salt solution (0.5 M KCl buffered with 5×10^{-3} M HEPES buffer, pH 7.4) in a glass dish with the micropipette tip placed in the solution. After evaporation of the organic solvent, the pipette was passed through the resulting interface, allowing formation of a bilayer. After formation of the bilayer (electrical resistance measured as 5–20 GΩ), the bilayer's stability was monitored for about 10 min at the highest potential chosen for subsequent single-channel recordings. When a satisfactory degree of stability was achieved, i.e., no intrinsic conductance fluctuations were observed, a few microliters of the peptide solution [freshly prepared in isopropyl alcohol/water (1:1 v/v)] was added to one side of the bilayer (virtual ground). Experiments were repeated seven times. Electrical measurements were performed on an Axopatch-1D patch clamp apparatus (Axon Instruments Inc., Foster City, CA). Current flowing through the bilayers was recorded on FM magnetic tape, Hewlett Packard Model 3694A. The experimental data obtained were analyzed off-line by replaying the tape recording data through a low-pass filter (Frequency Devices, Series 902, Haverhill, MA) into an IBM PC AT computer and analyzed with the Axotape and p clamp (version 5.5.1) programs (Axon Instruments).

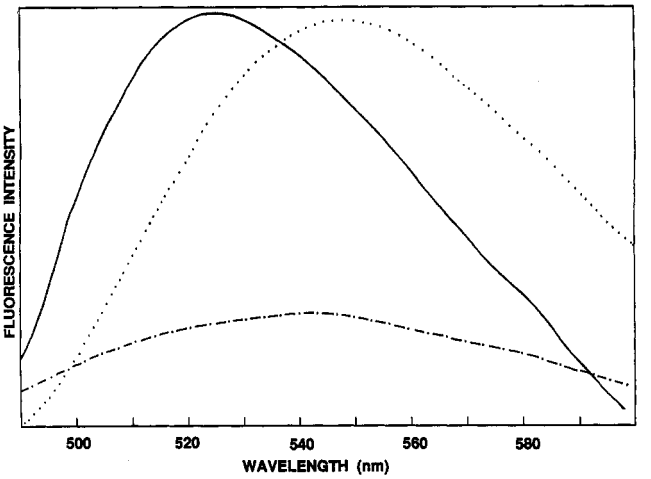

FIGURE 2: Fluorescence emission spectra of 0.1 μM NBD-TM-minK molecule in buffer composed of 50 mM Na_2SO_4, and 25 mM HEPES-SO_4^{2-} pH 6.8. The excitation wavelength was set at 468 nm. Emission was scanned from 490 to 600 nm. NBD-TM-minK in the presence of 270 μM PC vesicles (—); NBD-TM-minK in buffer (– · –); and NBD-aminoethanol (1 μM), (···).

RESULTS

To evaluate the functional role of the transmembrane domain of the minK protein, a 32 amino acid residue peptide containing the minK's hypothesized putative transmembrane segment and 4–5 amino acid residues in the flanking regions was synthesized. The sequence of this peptide, designated as TM-minK, is identical to residues 41–72 of the minK protein. Its sequence is

SKLEALYILMVLGFFGFFTLGIMLSYIRSKKL

Three fluorescently labeled analogues were prepared by modifying the synthesized peptide selectively at its N-terminal amino acid with either NBD (NBD-TM-minK), carboxyfluorescein (Flu-TM-minK, an energy donor), or with tetramethylrhodamine (Rh-TM-minK, an energy acceptor).

CD Spectroscopy. The extent of α-helical secondary structure of TM-minK was estimated from its CD spectrum measured in methanol (Figure 1). The peptide exhibited a mean residual ellipticity $[\theta]_{222}$ of 19 000 deg·cm^2/dmol, corresponding to a relatively high fractional helicity value of 0.57 (Wu et al., 1981). Similar high values of mean residual ellipticity were reported for naturally occuring membrane-

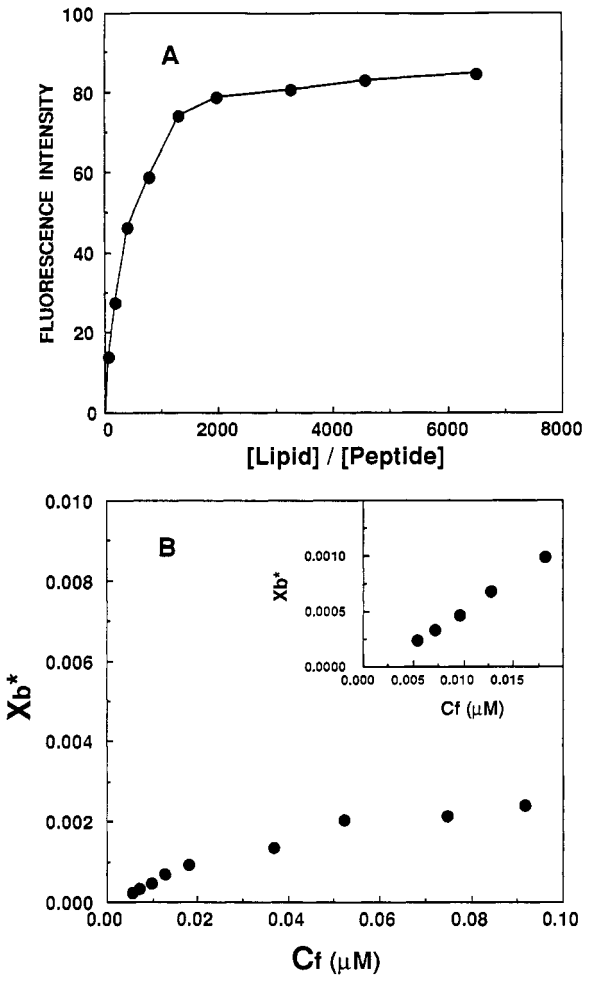

FIGURE 3: (A) Fluorescence of NBD-TM-minK upon incubation with PC vesicles. NBD-TM-minK (0.1 μM total concentration) was mixed with various concentrations of PC vesicles, at 24 °C (50 mM Na_2SO_4, 25 mM HEPES-SO_4^{2-}, pH 6.8), with its excitation set at 468 nm and emission monitored at 530 nm. (B) Binding isotherm of the NBD-TM-minK, derived from the binding curve in Figure 3, trace A, by plotting X_b* (molar ratio of bound peptide per 60% lipid) versus C_f (free peptide).

permeating peptides, such as melittin (Vogel, 1981), alamethicin (Rizzo et al., 1987), magainin (Chen et al., 1988), bombinin-like peptides (Gibson et al., 1991), cecropin (Andreu et al., 1985), and pardaxin and some of its analogues (Shai et al., 1990, 1991). This was also the case for peptides representing segments of protein channels, such as the M2-δ segment of the *Torpedo californica*'s acetylcholine receptor (Montal et al., 1990) and the M2 segment of the inhibitory glycine receptor (Langosch et al., 1991).

NBD Fluorescence Studies. The environmentally sensitive fluorophore NBD has been employed in polarity and binding studies (Kenner & Aboderin, 1971; Frey & Tamm, 1990; Baidin & Huang, 1990; Rapaport & Shai, 1991; Pouny & Shai, 1992). Herein, the fluorescence emission spectra of NBD-TM-minK and of NBD-aminoethanol, serving as a control, were measured in aqueous solutions and in the presence of PC vesicles. The labeled peptide and the control sample exhibited fluorescence emission maxima at 545 ± 1 nm and 549 ± 1 nm in buffer, respectively (Figure 2), in agreement with reported emission wavelength maxima for NBD derivatives placed in hydrophilic environments (Rajarathnam et al., 1989; Rapaport & Shai, 1991). However, when NBD-TM-minK or NBD-aminoethanol are added to a solution of PC vesicles (pH 6.8), the fluorescence emission maximum of NBD-TM-minK, but not of NBD-aminoethanol, exhibited a blue-shift (λ_{max} = 523 nm) and an enhanced increase in its fluorescence intensity. These changes reflect the relocation of the NBD group into a more hydrophobic environment. In these experiments, an elevated lipid/peptide molar ratio (2700:1) was consistently maintained, so that the spectral contributions of free peptide would be negligible.

To calculate the surface partition coefficient of NBD-TM-minK, samples of the labeled peptide (final concentration of 0.1 μM) were mixed with increasing amounts of PC SUV. The resulting increase in the fluorescence intensity of the NBD-labeled peptide at pH 6.8 was plotted as a function of the lipid/peptide molar ratio (Figure 3, trace A). SUV vesicles were used to minimize light scattering effects (Mao & Wallace, 1982). As a control, unlabeled TM-minK was mixed with lipids, up to the maximal concentration used in the assay. The fluorescence intensities of the resulting mixtures, after subtracting the contribution of the vesicles, remained unchanged.

The binding isotherm of TM-minK was analyzed as a partition equilibrium (Schwarz et al., 1986, 1987; Rizzo et al., 1987; Beschiaschvili & Seelig, 1990; Rapaport & Shai, 1991), using the following formula:

$$X_b = K_p C_f \quad (3)$$

where X_b is the molar ratio of bound peptide per total lipid, K_p the partition coefficient, and C_f the equilibrium concentration of free peptide in the solution. Both X_b and C_f can be determined knowing f_b (fraction of bound peptide), which can be calculated by

$$f_b = (F - F_0)/(F_\infty - F_0) \quad (4)$$

where F_∞ is the fluorescence signal obtained when all the peptide is bound to lipid (Schwarz et al., 1986), F corresponds to the fraction of membrane bound peptide, and F_0 is the fluorescence intensity of unbound peptide. In our experiments, it was assumed that the TM-minK was initially partitioned only over the outer leaflet of the SUVs, which comprise 60% of the total lipid of SUV (Beschiaschvili & Seelig, 1990). Therefore, the values of X_b were corrected as follows:

$$X_b^* = X_b/0.6 \quad (5)$$

and eq 3 becomes

$$X_b^* = K_p^* C_f \quad (6)$$

An experimental binding isotherm (the plot of X_b* versus free peptide, C_f) was obtained from the interaction of NBD-TM-minK with PC SUV at pH 6.8 (Figure 3, trace B). The surface partition coefficient of TM-minK was estimated by extrapolating the initial slope of the curve to a C_f value of zero. The surface partition coefficient, K_p*, of NBD-TM-minK thus estimated was 5.5×10^4 M^{-1}. This K_p* value is within the range of those obtained for membrane-permeating peptides, such as melittin and its derivatives (Stankowski & Schwarz, 1990), the *Staphylococcus* δ-toxin (Thiaudière et al., 1991), pardaxin analogues (Rapaport & Shai, 1991), and the S-4 segment of the sodium channel (Rapaport et al., 1992).

Fluorescence Energy Transfer Studies. Intermolecular energy transfer studies were performed using TM-minK modified with fluorescein to serve as an energy donor or TM-minK modified with rhodamine to serve as a fluorescence acceptor. Energy transfer from Flu-TM-minK to Rh-TM-minK occurred within the PC membrane (Figure 4). Similar results were obtained when the peptides were incorporated into the vesicles by either one of the two methods described under Experimental Procedures. The emission spectrum of

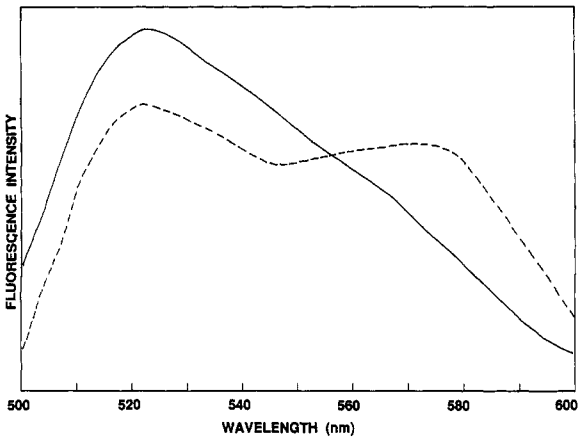

FIGURE 4: Fluorescence energy transfer. The fluorescence emission spectra (excited at 470 nm) of Flu-TM-minK (0.02 μM), incorporated into phospholipid vesicles (phospholipid concentration = 100 μM) in buffer, pH 6.8 (continous line), and after the incorporation of 0.06 μM Rh-TM-minK (dashed line) into the donor-vesicles mixture. The results are the average of three measurements with 2% uncertainty.

Flu-TM-minK (0.02 μM) alone, in the presence of PC vesicles (peptide/lipid molar ratio = 1:5000), at the fluorescein excitation wavelength (470 nm) is depicted by the continuous line. When Rh-TM-minK, at a final concentration of 0.06 μM (peptide/lipid molar ratio of 1:1666), was incorporated into PC vesicles containing Flu-TM-minK (0.02 μM), quenching of the donor's emission consistent with 21% energy transfer was observed (in three separate experiments; dashed line in Figure 4). In control experiments, no change in the emission spectrum of fluorescein was observed when the acceptor Rh-TM-minK was replaced by an equivalent amount of unlabeled TM-minK (data not shown). Furthermore, no decrease in emission at 520 nm was observed when the acceptor probe was attached to ethanolamine, which does not interact with the donor-labeled peptide, thus demonstrating that the decrease in donor emission at 520 nm was caused by a specific association of the donor- and the acceptor-labeled peptides. At the peptide/lipid molar ratio (1:1666) tested all of the acceptor-labeled peptide bound to the vesicles. Therefore, if a surface density of random distribution is assumed (Fung & Stryer, 1978), corresponding to an R_0 value of 45 Å, as previously calculated for the Flu/Rh donor/acceptor pair (Rapaport & Shai, 1992), an energy transfer of ~3% should be obtained. Further experiments were performed in which Rh-TM-minK, at a final concentration of 0.02 μM, was incorporated into PC vesicles containing Flu-TM-minK (0.02 μM). In these experiments (data not shown), 12% energy transfer was observed, not 0% of energy transfer expected for random distribution. Thus, the donor TM-minK and the acceptor TM-minK were not randomly distributed throughout the membrane but rather associated with each other within the membrane.

Single Channel Formation by TM-minK in Planar Bilayers (PLM). The ability of TM-minK to induce single channels in PLM composed of diphythanoyl-PC was assessed. Channel activity was induced by the addition of TM-minK on one side of a preformed bilayer (virtual ground). Figure 5, panels A and B, shows representative single-channel traces for TM-minK at potential of –30 mV, and Figure 5C shows the traces obtained when a trans positive potential was applied. Single channels appeared only when a trans negative potential was applied. When potentials were reversed to positive values, up to +100 mV (in seven experiments), the channel activity declined with time (0.5–4 min) and eventually disappeared. However, when the potential was reversed back to a negative

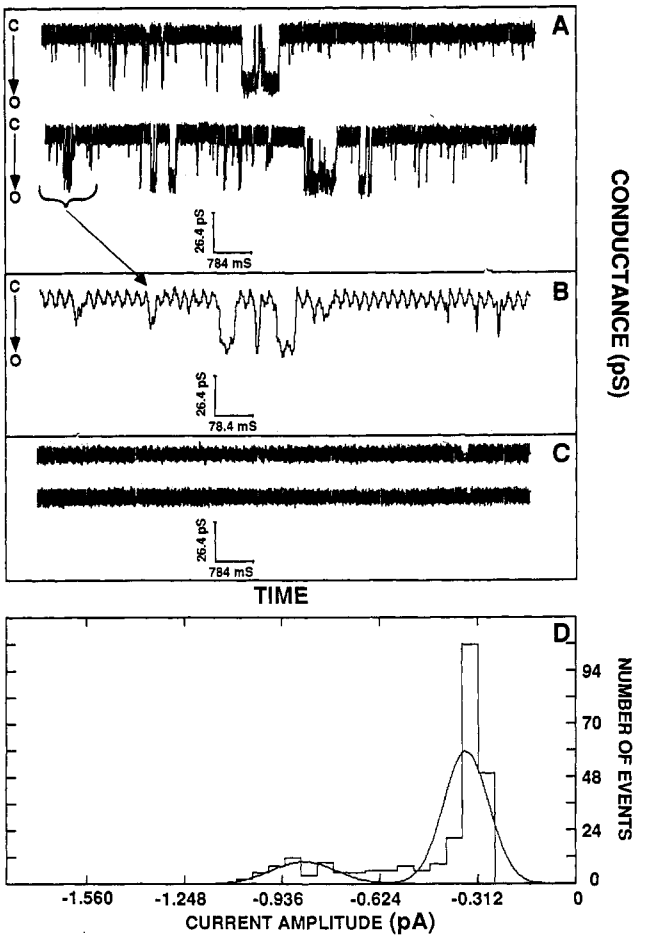

FIGURE 5: (A) Single-channel traces for TM-minK at an applied potential of –30 mV and filtration at 100 Hz. (B) An expanded single-channel trace derived from trace A. (C) Recorded current traces at positive potential. Upper line, +30 mV; lower line, +100 mV. (D) Histograms of the channels' current. The histograms were constructed from traces similar to those in panel A.

value, channels could be detected again. From single-channel traces, a histogram of current amplitude was constructed (Figure 5D). The size of the current fluctuations and the open time of the single channels is variable (Figure 5A,B). The current amplitude of the channels is also clearly variable (Figure 5D, two major conductance levels, γ, of 11.7 and 29 pS). These phenomena may be explained by the formation of aggregates composed of variable numbers of TM-minK monomers, associating and dissociating at various rates.

DISCUSSION

Recently, studies with synthetic peptides corresponding to portions of channel-forming proteins suggested that specific transmembrane segments of these proteins are constituents of the channel pores. These proteins include the Na^+ channel of *Electrophorus* electric organ (Oiki et al., 1988; Tosteson et al., 1989; Rapaport et al., 1992), the acetyl choline receptor (Montal et al., 1990; Ghosh & Stroud, 1991), the glycine receptor (Langosh et al., 1991), and the DPH-sensitive Ca^{2+} channel (Grove et al., 1991).

The minK protein differs from these channel-forming proteins in its small size (130 amino acids) and the presence of a single putative transmembrane α-helical segment, as detected by hydropathy analysis (Takumi et al., 1988). The minK protein has not yet been isolated and functionally reconstituted, although its gene has been cloned. Although there is no homology between the minK protein and other

known protein channels, upon expression in *Xenopus* oocytes the minK gene product shows properties characteristic of K^+ channels, including voltage-dependent gating, ion selectivity, and blocker pharmacology (Takumi et al., 1988; Murai et al., 1989; Folander et al., 1990; Pragnell et al., 1990; Hausdorff et al., 1991; Goldstein & Miller, 1991). If the minK protein can indeed form ion channels, it might form a bundle of transmembrane amphiphilic α-helices which line the channel pore as has been hypothesized for other ion channels (Inouye, 1974; Guy & Steerhamulu, 1986; Greenblatt et al., 1985; Lear et al., 1988). This possibility was examined in the present study.

A peptide (TM-minK) with a sequence identical to that of the predicted transmembrane segment of the minK protein, including extended regions at both termini, was synthesized. The ability of this peptide to interact with phospholipid membranes, to penetrate membranes, and to form aggregates, and, as a result, ion channels, was examined. For these studies, the synthetic peptide was fluorescently labeled with either NBD, Flu, or Rh.

These studies revealed two important properties of TM-minK. First, the transmembrane segment adopts a high value of α-helicity when placed in a hydrophobic surrounding like methanol (Figure 1), and second, it incorporates into phospholipid bilayers with a high water/membrane partition coefficient (Figure 3). Both properties are characteristic of known membrane-permeating polypetides, such as the antimicrobial peptide alamethicin, the bee venom melittin, and the neurotoxin pardaxin. Moreover, the environment encountered by an NBD group located at the N-terminus of the TM-minK was more hydrophobic (emission maximum of 523 nm; Figure 2) than that detected with an NBD probe located on the surface of the membrane (emission maximum of 533 nm) (Chattopadhyay & London, 1987; Rapaport & Shai, 1991) or than that detected with an NBD probe only slightly embedded within the membrane (emission maximum of 528 nm) (Rapaport et al., 1992; Pouny et al., 1992), thus suggesting that TM-minK becomes embedded within the lipid bilayers, which agrees with the results of electron microscopy studies (Sugimoto et al., 1990).

The FET studies (Figure 4) clearly showed that the percentage of energy transfer between membrane-embedded monomers (21%) is higher than that calculated for randomly distributed monomers ($\sim$3%), thus, suggesting that aggregation of TM-minK peptides occurs within membranes, in agreement with the hypothesis that a bundle of monomers can build a pore.

The shape of a peptide's binding isotherm indicates the organizational state of the peptide within the membrane (Schwarz et al., 1986, 1987; Rizzo et al., 1987; Rapaport & Shai, 1991; Rapaport et al., 1992; Pouny & Shai, 1992). Isotherms which contain an initial "lag", i.e., start flat but eventually rise sharply upon crossing a threshold concentration (C_f), represent peptides that can form large aggregates or large pores within membranes. The binding isotherm of NBD-TM-minK is almost a straight line, suggesting that, when embedded within the membrane, its transmembrane segment does not form aggregates at all or does not form aggregates containing a large number of monomers. However, since FET experiments with TM-minK support a nonrandom distribution of its monomers within the membrane, the isotherm may indicate that TM-minK can form aggregates with only a limited number of monomers. Similarly, shapes of binding isotherms observed with pardaxin analogues suggested that they formed small aggregates within membranes (Rapaport & Shai, 1991, 1992).

Experimental approaches have been generally concerned with the ability of a peptide to associate with lipid when added in the aqueous phase (McLean et al., 1991), because protein–protein interactions in the aqueous phase prior to lipid binding may reduce the rate of association of the peptide to the lipid bilayer. Since the TM-minK peptide is relatively hydrophobic (22 of its 32 amino acids are hydrophobic), the experiments described herein were conducted under conditions that limit self-aggregation of the peptide in the aqueous buffer solution. This was achieved by either incorporating the peptide into the liposome membrane or by mixing the peptide with a concentrated solution of liposomes prior to dilution to the desired concentration.

Preliminary data from single-channel recordings indicate that TM-minK can form single channels in lipid bilayers only when trans negative potential is applied. This is similar to the cis positive depolarization, characteristic of the minK protein expressed in oocytes. The heterogeneity of channel conductance observed with TM-minK can be explained by the association of different numbers of monomers in the peptide aggregate that forms the channel. In other words, the two conductances may reflect a difference in the multimeric state of the peptide forming the channel. Heterogeneity of channel conductance was also observed with MA-β peptide corresponding to a segment of the nicotinic acetylcholine receptor (Ghosh et al., 1991) and with the M2-δ segment of the nicotinic cholinergic receptor (Oiki et al., 1988). In this study, TM-mink did not discriminate between K^+ and Na^+ (data not shown), suggesting that this segment is not responsible for the selectivity properties of the minK channel. These results are in view of point mutation studies suggesting that the selectivity filter of the channel is located outside the transmembrane segment (Goldstein & Miller, 1991; Takumi et al., 1991). A detailed study on the ion channel activity of TM-minK is being performed in order to better understand its role in the channel formation properties of minK.

Overall, the results herein suggest that the transmembrane domain of minK has a role as a constituent of the pore formed by minK and in the assembly of minK monomers within membranes that result in the formation of a conducting pore.

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