Aqueous Solubilization of Transmembrane Peptide Sequences with Retention of Membrane Insertion and Function

John M. Tomich,* Darren Wallace,# Kyle Henderson,# Kathy E. Mitchell,* Gary Radke,* Robert Brandt,* Carrie A. Ambler,* April J. Scott,* Jared Grantham,§ Lawrence Sullivan,# and Takeo Iwamoto*

*Department of Biochemistry, Kansas State University, Manhattan, Kansas 66506, and #Department of Physiology and §Departments of Medicine and Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, Kansas 66160 USA

ABSTRACT We recently reported that the peptide C-K_4-M2GlyR mimics the action of chloride channels when incorporated into the apical membrane of cultured renal epithelial monolayers. C-K_4-M2GlyR is one of a series of peptides that were prepared by the addition of lysine residues to the N- or C-terminus of the M2 transmembrane sequence of the brain glycine receptor. This study addresses how such modifications affect physical properties such as aqueous solubility, aggregation, and secondary structure, as well as the ability of the modified peptides to form channels in epithelial monolayers. A graded improvement in solubility with a concomitant decrease in aggregation in aqueous media was observed for the M2GlyR transmembrane sequences. Increases in short-circuit current (I_{SC}) of epithelial monolayers were observed after treatment with some but not all of the peptides. The bioactivity was higher for the more soluble, less aggregated M2GlyR peptides. As described in our previous communication, sensitivity of channel activity to diphenylamine-2-carboxylate, a chloride channel blocker, and bumetanide, an inhibitor of the Na/K/2Cl cotransporter, was used to assess changes in chloride selectivity for the different assembled channel-forming peptides. The unmodified M2GlyR sequence and the modified peptides with less positive charge are more sensitive to these agents than are the more highly charged forms. This study shows that relatively insoluble transmembrane sequences can be modified such that they are easier to purify and deliver in the absence of organic solvents with retention of membrane association, insertion, and assembly.

INTRODUCTION

Recently we documented the effect of a C-terminally adducted sequence C-K_4-M2GlyR (e.g., four lysine residues added at the C-terminus) on cultured monolayers of epithelial cells (Wallace et al., 1997). The aqueous soluble peptide, derived from the transmembrane M2 segment brain of the glycine receptor protein (Reddy et al., 1993), was applied to the apical surface of monolayers. The peptide has been modeled as inserting into the apical membrane, then associating to form ion-conducting pores that are composed of four and five peptide helix bundles in a parallel orientation (Montal, 1995; Marsh, 1996). Anion fluxing channels were produced, and transepithelial chloride secretion ensued. These channels were inhibited by the anionic channel blockers niflumic acid, 5-nitro-2(3-phenylpropylamino)-benzoate (NPPB), glibenclamide, and diphenylamine-2-carboxylate (DPC), and by omitting chloride from the media. The effect of these peptides on Cl^- transport was not due to an elevation in cellular cAMP content. The peptide-induced chloride secretion causes a net increase in the rate of transepithelial water secretion. The ability of epithelial tissues to secrete chloride and water is compromised in cystic fibrosis (CF) patients. Restoration of this activity through the addition of an easily synthesized and purified peptide might have clinical applications in treating patients with cystic fibrosis.

The C-K_4-M2GlyR sequence was identified during the study described herein. Our in vitro studies began with the unmodified sequence but proved difficult to deliver reliably: application of the peptide to the cells did not always result in measurable channel activity. Each time the peptide solutions were prepared, we observed that the degree of aggregation of the peptide solution was variable and impossible to control or predict. In the case of artificial bilayers, which we employed in earlier studies (Reddy et al., 1993; Montal et al., 1994), we overcame this problem by adding the peptide to the lipids in organic solvents, dried the mixture, and then formed bilayers with the peptides already associated with the lipids (Grove et al., 1991). Inclusion of organic solvents to increase the solubility of the peptide was not an option when studying living cells or tissues.

In this structure/activity study the effect of adding increasing positive charge to either peptide terminus was explored. Melittin, an unrelated membrane-active peptide, was the paradigm used for the designs employed. Melittin is best characterized as a detergent-like amphipath because of its 20-residue transmembrane segment followed by a C-terminal cationic hexapeptide tail with four positively charged residues. The transmembrane segment is composed of two helical regions divided by a central proline hinge. Increased solubility is due directly to the presence of the unstructured C-terminal hexapeptide that contains two arginine, two lysine, and two glutamine residues. Removal of the hydrophilic residues results in a sequence that is only 33% as effective in inserting into membranes and nearly

Received for publication 9 July 1997 and in final form 25 September 1997.

Address reprint requests to Prof. John M. Tomich, Department of Biochemistry, Willard Hall, Kansas State University, Manhattan, KS 66506. Tel.: 785-532-5956; Fax: 785-532-6297; E-mail: jtomich@ksu.edu.

0006-3495/98/01/256/12 $2.00

insoluble in water (Otada et al., 1992). Variants have been synthesized in which the charged residues that occur at the C-terminus have been transposed to the N-terminus (King et al., 1994), or the net charge has been switched from positive to negative (Ramalingam et al., 1992). All of these modifications yielded active peptides. Clearly, the presence of a highly charged terminus does not interfere with the lipid-associating properties of the hydrophobic portion of the molecule.

Furthermore, in a recent report (Englebretsen and Alewood, 1996) a "solubilizing tail" strategy for rendering hydrophobic peptides soluble in aqueous media was described. The tail consisted of $(Gly\text{-}Arg)_4$-Gly and was placed at the C-terminus of a hydrophobic peptide by using a cleavable glycoamide ester bond. The authors felt that the method was general and that the attachment of this segment to any hydrophobic sequence should render it more soluble and easier to purify by standard high-performance liquid chromatography (HPLC) protocols.

Although it may appear intuitive that adding charge to a hydrophobic sequence will enhance its aqueous solubility, this report represents the first study, to our knowledge, that examines the biophysical and biological consequences of the stepwise addition of positive charge to either terminus of a membrane-spanning, channel-forming, amphipathic helix. The solubility, degree of aggregation, channel activity, and sensitivity to certain drugs were monitored for each successive modification in net charge at the C- and N-termini. From these observations we draw hypotheses about the modes of solubilization afforded by the added lysines.

EXPERIMENTAL

Peptide synthesis

All peptides were prepared using fluoren-9-ylmethoxy carbonyl (Fmoc) chemistries on an Applied Biosystems model 431 peptide synthesizer (Perkin-Elmer, Norwalk, CT) at a synthesis scale of 0.1–0.2 mmol, using *p*-hydroxymethylphenoxymethyl polystyrene (HMP) resin preloaded with the first amino acid, and with the degree of substitution calculated. For the peptide C-K_4-M2GlyR-CO-NH_2, the resin 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl polystyrene (Fmoc-amide resin) was used with the degree of substitution calculated (0.64 mmol/g) (Perkin-Elmer). All solvents were reagent grade unless otherwise indicated, and the protected amino acids were purchased from one or more of the following vendors: Perkin-Elmer, Bachem (Torrance, CA), Peninsula Laboratories (Belmont, CA), and Peptides International (Louisville, KY). For the sequential N-terminal modifications (N-K_1–K_6), aliquots were removed from a common synthesis of the M2GlyR and M1CFTR sequences after each lysine residue was condensed. For the C-terminal modifications, separate syntheses had to be performed. All peptides, unless otherwise noted, were first HPLC purified and then characterized through automated Edman degradation and matrix-assisted laser desorption/ionization, time-of-flight mass spectral analyses (MALDI-TOF-MS).

Solubility studies

Saturated solutions of the crude peptides in Ringer's solution (pH 7.4: OSM 305; 2.5 mM K_2HPO_4; 2.0 mM $CaCl_2$; 1.2 mM $MgSO_4$; 5 mM glucose; 5.0 mM Na-acetate; 6 mM L-alanine; 1.0 mM Na_3-citrate; 115 mM NaCl; 4.0 mM Na-lactate; 0.5 mM *n*-butyric acid; 20 mM $NaHCO_3$; 14.1 mM raffinose) were prepared by dissolving increasing amounts of peptide directly in 0.1 ml of the above solution until no more went into solution. Samples were centrifuged for 3 min at 15,000 × *g*, and the supernatant was analyzed for protein concentration with the Pierce BCA protein assay. With the higher lysine analogs, K_4–K_6, a clear gel often formed at saturating concentrations. In those cases, just enough fluid was added to these solutions to break the gels.

HPLC studies

Reverse-phase HPLC (PLRP-S300; 50 × 7.5 mm I.D.), with a linear 10-min gradient from 20% to 70% acetonitrile in water containing 0.1% trifluoroacetic acid, was used to check for aggregation in the soluble peptide fractions (Figs. 2 and 3) prepared above. Ten microliters of the soluble layer from the solubility studies above was injected. The concentrations of those peptides differed in each case and are indicated in Table 1. Elution from the column was monitored at 215 nm. HPLC-purified samples were used for the in vitro assays and biophysical studies. The crude samples were dissolved in water containing 50% acetonitrile at concentrations of ~5 mg/ml. Under these conditions the peptides did not aggregate to any great extent. These purified samples were taken to dryness under vacuum. NMR and circular dichroism (CD) samples were dissolved in water or D_2O as required.

Bioactivity of modified peptides

We investigated the channel-forming activity of M2GlyR and the lysine analogs (N- and C-termini) on monolayers of subtype of Madin-Darby canine kidney (MDCK) cells (Grantham et al., 1989) grown on permeable supports (Snapwell, 12-mm diameter; Costar Corp) (Mangoo-Karim et al., 1995). MDCK monolayers were placed in Ussing chambers containing 5 ml of Ringer's medium in the apical and basolateral compartments. The short-circuit current (I_{SC}) was measured 30 min before and up to 1 h after the addition of the peptide. The unmodified M2GlyR and the N-K1-M2GlyR were dissolved in dimethyl sulfoxide (DMSO) because of the low solubility of these peptides in aqueous solutions. The maximum final concentration of DMSO (1%) was without effect on I_{SC}. More soluble forms of M2GlyR were prepared as 2.5 mM stocks in Ringer's medium. In inhibitor experiments, either basolateral bumetanide (100 μM) or apical DPC (3 mM) was added after I_{SC} reached a steady state (usually 40 min) in the presence of the peptide. As an example (Fig. 1), bar graphs for the effect of C-K_4 M2GlyR (100 mM) on the MDCK monolayers are shown, as is the relative inhibition afforded by the addition of the two inhibitors, bumetanide and DCPC.

Circular dichroism and NMR studies

CD was used to monitor the secondary structure of N-K_4-M2GlyR and C-K_4 M2GlyR in water at 25°C (with and without trifluoroethanol (TFE) 10–40%). Spectra were recorded on a Jasco J-720 spectropolarimeter with a Neslab RTE-111M circulator using a cylindrical, water-jacketed, quartz cuvette with a 0.1-mm pathlength. The plots are an average of 8 scans, recorded at a rate of 20 nm/min. Peptide concentrations of 0.1–1.5 mM were employed. Protein concentrations were determined using the Pierce BCA micro-protein assay, with bovine albumin used as the standard. The data were analyzed with software provided by the manufacturer. A simple unfolding experiment was also performed: spectra for N-K_4-M2GlyR and C-K_4 M2GlyR were recorded at 25, 30, 37, 45, 50, 60, and 70°C. After the spectra were taken for both peptides at a given temperature, the water bath temperature was raised to the next higher temperature and then allowed to equilibrate for 30 min. Spectra using 8 scans were recorded, and then the temperature elevation and equilibration process was repeated.

NMR was used to examine aggregation. TOCSY spectra were generated in water containing 10% D_2O and 30% deuterated TFE for the N-K_0-K_6 M2GlyR series, C-K_4-M2GlyR, and C-K_4-M2GlyR-CO-NH_2. Peptide concentrations of 3.0 mM were used to generate all spectra. Two-dimensional

TABLE 1 Solubility of modified membrane sequences

M2GlyR modification	Δ charge	mg/ml	mM	MW
Wild type: PARVGLGITTVLTMTTQSSGSRA	0	3.3	1.4	2305
N-Ac-	−1	3.4	1.4	2353
N-Ac-DAP	0	4.1	1.68	2439
N-DAP	1	4.1	1.7	2391
N-Lys-DAP	2	4.3	1.7	2519
N-K_1	1	4.1	1.7	2433
N-K_2	2	6.9	2.3	2561
N-K_3	3	29.8	11.1	2689
N-K_4	4	37.8	13.4	2817
N-K_5	5	46.4	15.8	2946
N-K_6	6	64.9	21.1	3074
C-K_3	3	27.2	10.1	2689
C-K_4	4	77.8	27.5	2817
C-K_4-CO-NH_2	5	158	56.1	2815
C-K_5	5	147	49.9	2946
Scrambled: ILASTRSQTGRMALSGTTTPGVV	0	152	66	2305
Scrambled C-K_4	4	248.9	88	2817
M1CFTR modification				
Wild type: RFMFYGIFLYLGEVTKAVQPLLLG	0	0.69	0.26	2620
N-K1	1	0.73	0.27	2748
N-K2	2	0.73	0.25	2876
N-K3	3	0.92	0.31	3004
N-K4	4	1.0	0.32	3132
N-K5	5	38.8	11.9	3260
N-K6	6	72.1	21.3	3389

spectra were performed with a 11.5-T Varian Utilityplus spectrometer operating at 499.96 MHz for ^{1}H, with a 5-mm triple-resonance inverse detection probe. NMR data sets were collected at 30°C. A total of 256 increments of 2K data points were recorded with 100 ms mixing time. All data sets were collected in hypercomplex phase-sensitive mode and were processed and analyzed using Varian NMR software VNMR 5.2b on a Silicon Graphics workstation. Before processing, the t_1 dimensions of data sets of all experiments were zero-filled to 2K. When necessary, spectral resolution was enhanced by Lorentzian-Gaussian apodization.

Viscosity measurements

Peptide samples were dissolved in Ringer's solution (pH 7.4) or water at the indicated concentrations to a final volume of 4.0 ml. Samples were vortexed for 15 s, centrifuged at 4000 × *g* for 1 min, transferred to an Ostwald 100 viscometer immersed in a 37°C circulating water bath, and allowed to come up to temperature. The first time point was usually recorded at ~10 min. At each incubation point, three readings were taken and an average value (t) was determined. The viscosity (η) was calculated as the product of the time (t), the density (ρ) of the sample solution, and the viscometer constant (A). In those experiments in which shear was observed, only the first time point taken was used for the viscosity calculation.

RESULTS

Several families of peptides based on the M2 transmembrane segment of the brain glycine receptor (M2GlyR) and the M1 transmembrane segment of the human cystic fibrosis transmembrane conductance regulator protein (M1CFTR) sequences (Table 1) were prepared. The table includes the sequences of the peptides, along with the net increase or decrease in overall charge of the peptide and the solubility in Ringer's buffer (given in both mg/ml and mM as the molecular weights of the adducted peptides). The M2GlyR sequence is classified as a channel-forming amphipathic helical segment, whereas the M1CFTR sequence, used as a control, is composed almost exclusively of hydrophobic residues, which is considered to be more typical of a transmembrane sequence. The M1CFTR peptide associates with membranes but does not appear to form an ionic conducting channel like M2GlyR (Montal et al., 1994). The low intrinsic solubilities of the two unmodified transmembrane segments, 1.4 mM for M2GlyR and 0.26 mM for M1CFTR in Ringer's solution, reflect their membrane origin. The amphipathic sequence, M2GlyR, has a higher hydrophobic moment and is somewhat more soluble than M1CFTR. As one of the controls, the M2GlyR sequence was scrambled. In designing this scrambled sequence, we minimized any amphipathic structure or β-inducing structure.

Effects of N-terminal peptide modifications on solubility

Modifications that yielded net charges of $\leq +2$ at the N-terminus of wild-type M2GlyR sequence and $\leq +4$ at the N-terminus of M1CFTR did not significantly improve the aqueous solubility of the parent sequence. Nor did the

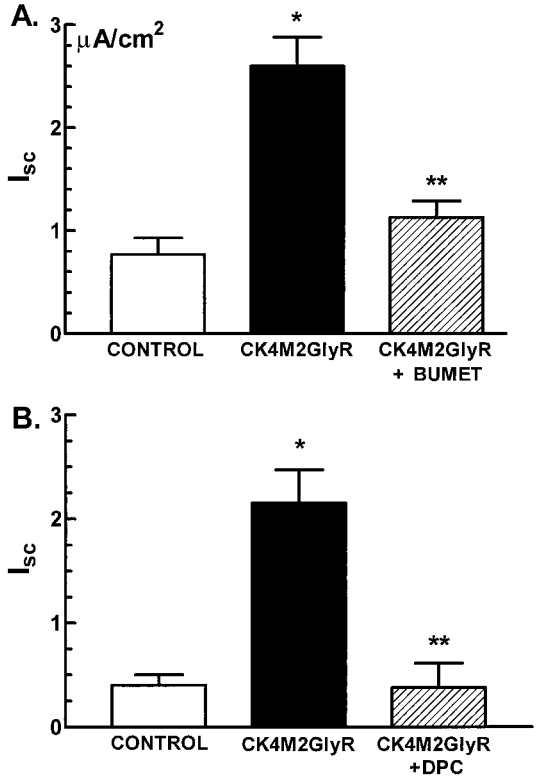

FIGURE 1 Effect of C-K_4-M2GlyR on I_{SC} and the subsequent effect of Cl^- transport inhibitors. C-K_4-M2GlyR (100 mM) was added to the apical surface of MDCK monolayers after a 30-min control period. Forty minutes later, either bumetanide (100 mM) was applied to the basolateral surface or DPC (3 mM) was added to the apical surface. (*A*) Effect of C-K_4-M2GlyR and subsequent effect of bumetanide, an inhibitor of the Na-K-2Cl transporter. The bar graph represents the average of 31 separate monolayers ($N = 31$). (*B*) Effect of C-K_4-M2GlyR and the subsequent effect of DPC, a Cl^- channel blocker ($N = 5$).

complete removal of charge from the N-terminus of M2GlyR alter the solubility. Reverse-phase HPLC and MALDI-TOF-MS confirmed that M2GlyR and M1CFTR form aggregates in Ringer's buffer. This buffer was used because of its requirement in the electrophysiological experiments on cultured kidney epithelium monolayers. The HPLC profiles for the M2GlyR and M1CFTR series are shown in the first panel of Figs. 2 and 3, respectively. Aggregated peptides appeared as multiple broad peaks that eluted slowly with increasing organic mobile phase. MALDI-TOF-MS analyses of samples, taken anywhere under the broad peaks, revealed only the mass of the monomers.

A graded simplification in the HPLC profiles was observed as the number of lysine residues added to M2GlyR was increased. With the addition of the third lysine, essentially a single peak eluted. Beyond the addition of the third lysine (net charge $\sim +3$ at neutral pH) to M2GlyR, solubility increased further (Table 1), although no additional changes were observed in the HPLC elution profile. It is somewhat surprising that the further addition of lysines did not alter the retention time of the apparent monomeric peptide, and this leads us to postulate that the bulk of the

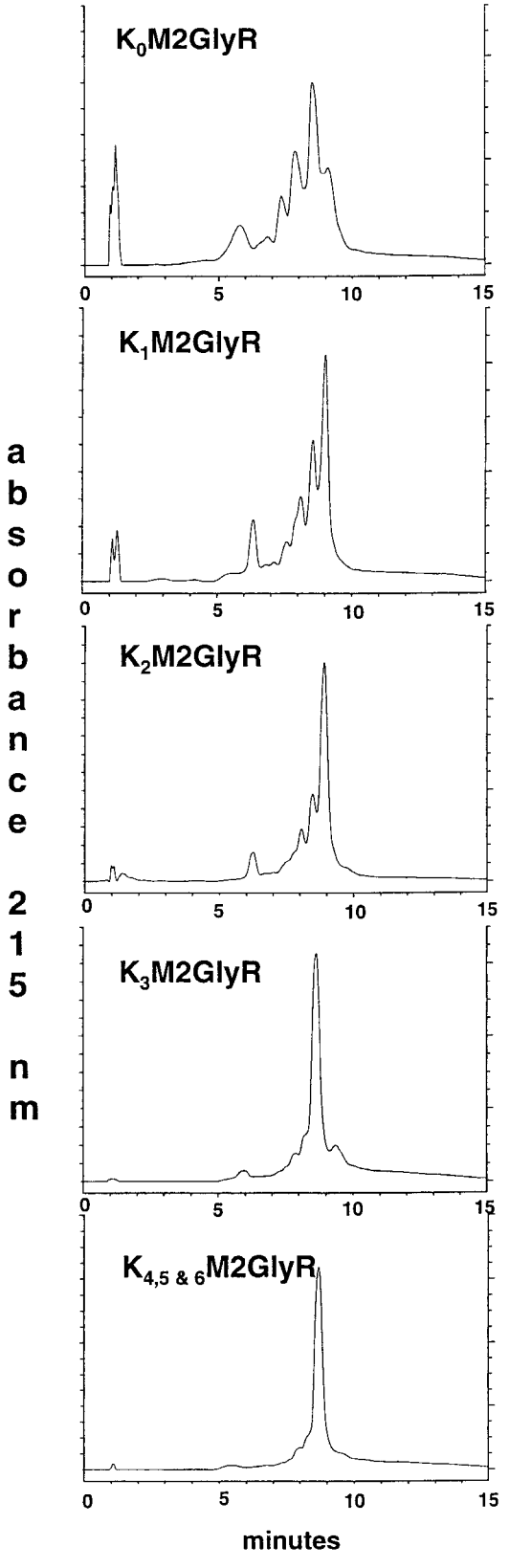

FIGURE 2 From a single chemical synthesis, six lysines were added in sequence to the N-terminus of M2GlyR. Aliquots were cleaved and checked for solubility in Ringer's solution, and the RP-HPLC pattern was determined. Increased solubility correlated well with the stepwise addition of lysine 3 and beyond. The HPLC pattern also simplified, indicating a gradual shift from aggregate toward monomer.

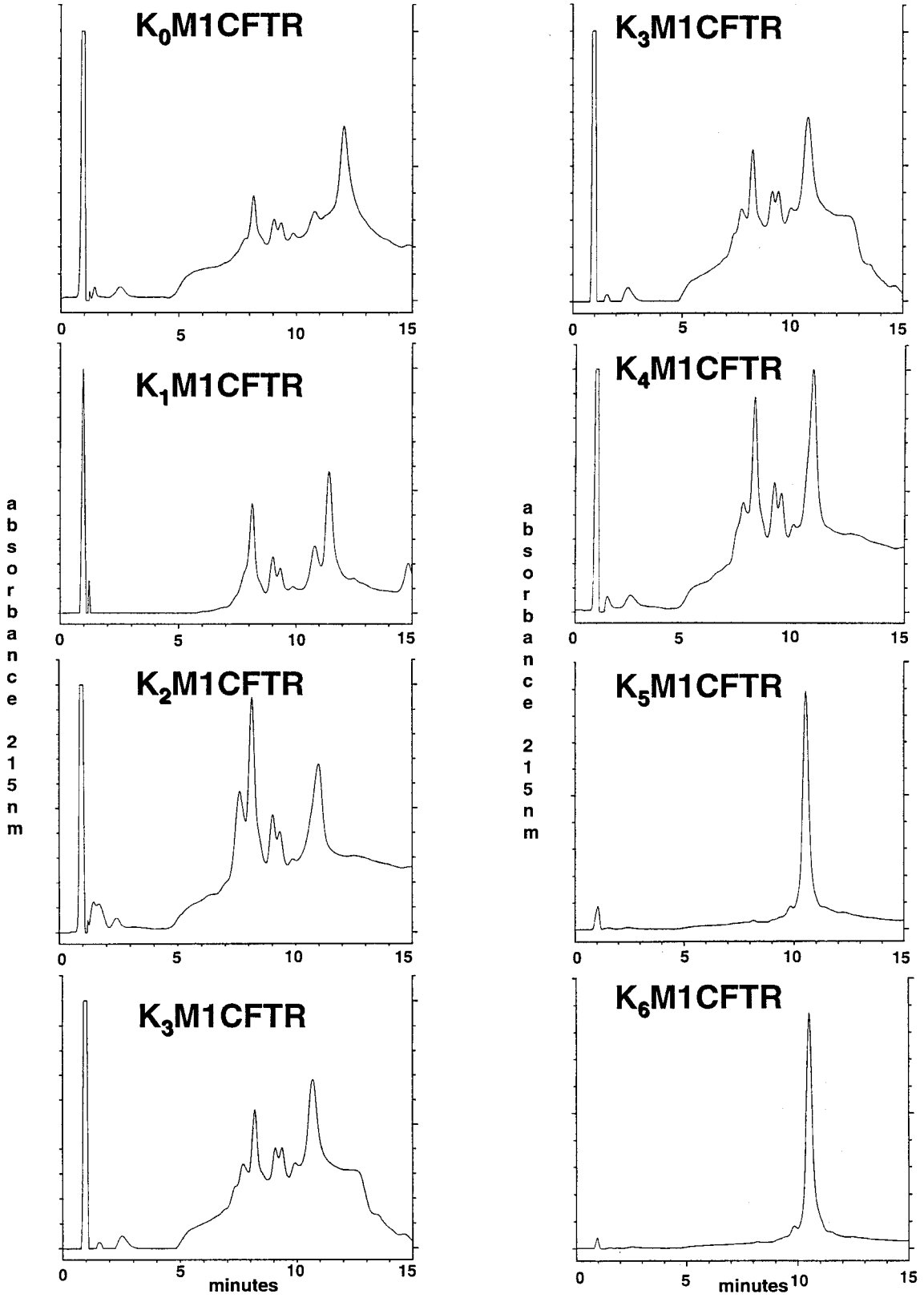

FIGURE 3 From a single chemical synthesis, six lysines were added to the N-terminus of M1CFTR. Aliquots were cleaved and checked for solubility in Ringer's solution as well as the RP-HPLC pattern. Increased solubility correlated well with the stepwise addition of lysine 5 and 6. Note that the transition toward monomer was much more abrupt for this sequence.

interaction with the RP-HPLC matrix was occurring via the hydrophobic portions of the molecule and not the positively charged lysines. However, the solubility of the nonamphipathic control sequence, M1CFTR, changed little through the addition of the fourth lysine, improving from 0.26 mM to only 0.32 mM. With the addition of the fifth lysine, a 37-fold increase in solubility was seen over that of the K_4 adduct, and the HPLC profile dramatically collapsed to essentially one peak. Increases in solubility due to the addition of the third lysine for M2GlyR (4.8-fold) and the fifth lysine for M1CFTR (34-fold) appeared to be cooperative in nature (Fig. 4 *A*). In addition to the increased solubility, the incorporation of lysines at the N-terminus of the two sequences apparently decreased the degree of aggregation, as judged by the HPLC profiles (Fig. 2).

To assess the degree of aggregation for the soluble peptides, TOCSY-NMR was used. Because NMR linewidths are related to the rotational correlation time of the peptide, monomers, being the smallest rotational unit, will have the sharpest linewidths, whereas aggregates, by virtue of their larger size and slower rotation, will have much broader linewidths. Other groups have used NMR effectively to examine aggregation (Hwang et al., 1988; Yang et al., 1994; Jayakumar et al., 1995; Altieri et al., 1995). Because of the complexity of the 1D proton spectra, we chose to use a 2D technique to assess the degree of aggregation. We generated the TOCSY spectra for all peptides (3.0 mM) in 30% deuterated TFE in water and examined the resolution of the CαH/NH connectivity signals. In these spectra, aggregated peptides generated signals that appeared smeared and overlapping, as opposed to monomeric peptides, which yielded discrete, well-defined signals. Based on the HPLC tracings, it seemed reasonable to expect that N-K_3 through N-K_6-M2GlyR would all be monomeric by virtue of their single sharp HPLC peaks. However, the TOCSY-NMR spectra for the sequences revealed qualitatively different levels of aggregation. N-K_{0-3} all appeared to be aggregated because of their many broad overlapping signals. N-K_4 appeared less aggregated, whereas N-K_5 and N-K_6 were judged to be essentially monomeric because of the presence of well-resolved, nonoverlapping signals. Representative spectra recorded using N-K_0-, N-K_3-, and N-K_5-M2GlyR are shown in Fig. 5. Although the sample preparation for the two systems, HPLC and TOCSY-NMR, are not identical, we believe that the comparison is still valid. HPLC elution profiles generated for the N-K_3 peptide that was dissolved in the absence of salts in 30% TFE/water showed an even better resolved profile (sharper and less broad) than that seen for peptide in Ringer's, as shown in Fig. 2. This observation suggests that TFE/water dissolved an even less aggregated preparation, yet when probed by the NMR technique, this sample reveals broadened signals characteristic of aggregated samples. The observation that N-K_3 appears monomeric by HPLC (conditions that yield a more aggregated mixture) yet aggregated by NMR (prepared using conditions that generate a less aggregated solution) suggests that NMR is clearly the more sensitive of the two techniques.

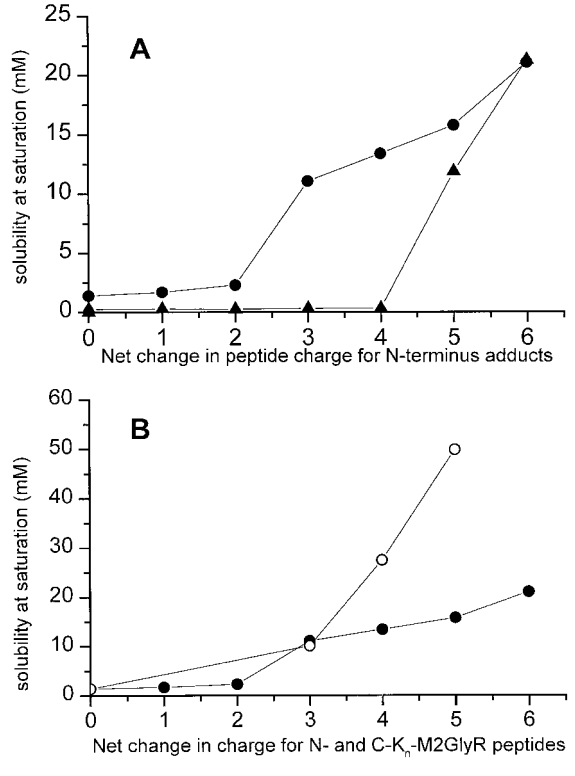

FIGURE 4 (*A*) The increase in solubility is plotted against the number of lysines added to the N-terminus of M2GlyR (●) and M1CFTR (▲). A single synthesis was carried out for each sequence, with aliquots taken after the addition of each new lysine. Both solubilizations appear to be cooperative. The amphipathic M2GlyR sequence was solubilized with less added lysines. (*B*) The increase in solubility is plotted against the number of maximum positive charges added to the N- or C-terminus of M2GlyR. A single synthesis was carried out for each sequence, with aliquots taken after the addition of each new lysine for the N-K series (●), and separate syntheses required for each of the C-K series (○).

Effects of C-terminal modifications on M2GlyR solubility

Selected modifications at the C-terminus were also studied (Table 1). This C-terminal series contained sequences with three to five positive charges added. The effect of adding positive charge at the C-terminus was much more dramatic than that observed for the N-terminus (Fig. 4 *B*). The wild-type sequence with its free C-terminus was used for the 0.0 net charge values for the C-terminal modification. The C-K_3 adduct's solubility was equivalent to that seen for N-K_3. However, placing four lysines at the C-terminus for C-K_4 peptide led to a solubility in excess of that seen for the N-terminal adduct, N-K_6. The C-terminal +5 species were generated as the C-K_5 analog or by synthesizing the C-K_4 peptide on a resin that placed the carboxamide at the C-terminus, thus neutralizing the negative charge. These peptides were extremely soluble, achieving levels of 147 mg/ml (49.9 mM) and 158 mg/ml (56.1 mM), respectively. This

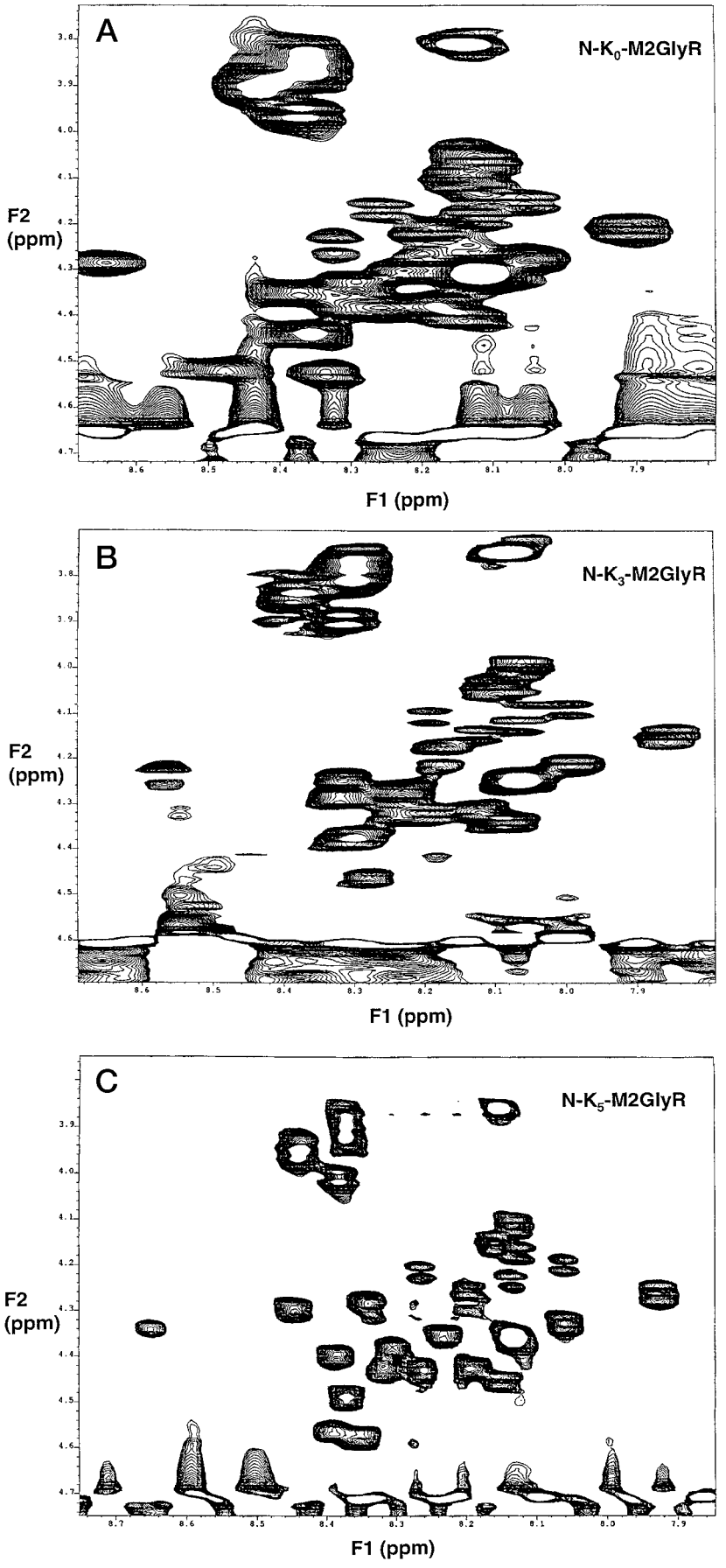

FIGURE 5 TOCSY NMR for selected sequences of the N-K series of M2GlyR at 3 mM in water containing 10% D_2O and 30% deuterated TFE. Only a portion of the TOCSY spectra ($C_\alpha H$/NH connectivity region) is shown, for greater detail. Note that only the K_5 adduct appears monomeric, based on the well-resolved cross-peaks.

represented about a 40-fold increase in solubility compared to the nonmodified peptide. The paradox here, in which similar increases in net charge (+4 and +5) at the C-terminus yield peptides that are twice as soluble as the analogous N-modified peptides, suggests that either modifications to the C-terminus yield more monomer per given charge or that the C-K_4 aggregate species have higher solubility.

TOCSY-NMR spectra suggested that the latter hypothesis is correct. NMR revealed that C-K_3 was aggregated, C-K_4 appeared to be a mixture of monomer and aggregate, whereas C-K_4-CO-NH_2 and C-K_5 were predominantly monomeric. The NMR data suggested that the C-K_n series behaves just like the N-K_n series with regard to aggregation. This finding indicates that although the relative solubility of the C-K_n series was much greater (Fig. 4 *B*), it was still apparently aggregated to the same extent. The aggregates formed by modifying the C-terminus were apparently more soluble than those produced with the N-K series.

Channel-forming activity of M2GlyR peptides

Channel-forming activity of the solubilized N-K_n-M2GlyR sequences in a subtype of MDCK cells was assessed. These cells were grown to confluent monolayers on permeable membranes. The monolayers were inserted into Ussing chambers, and the applied external current required to drive the negative transepithelial potential difference to zero (short-circuit current, I_{SC}) was measured (Wallace et al., 1997). The opening of native anion channels in the apical membrane of the cells or the insertion of foreign, anion channel-forming peptides results in an increase in positive current flow from the apical to the basolateral surface of these monolayers (Wallace et al., 1997; Mangoo-Karim et al., 1995).

Because of its low solubility in aqueous media, the unmodified M2GlyR sequence was dissolved in DMSO. This solution was added to the medium bathing the apical surface of the monolayers in amounts that achieved a final concentration of 100 μM peptide and 1% DMSO. Under these conditions, an increase in I_{SC} was recorded in only 65% of the monolayers tested. In these monolayers, an increase in I_{SC} of 1.0 $\pm$ 0.1 $\mu A/cm^2$ was observed (Table 2). $N = 24$ indicates the number of individual monolayer experiments that were averaged to generate this I_{SC} value and standard deviation. This stimulated I_{SC} was inhibited by the Na-K-2Cl cotransporter blocker, bumetanide, applied to the basolateral surface and by the chloride channel blocker DPC applied to the apical surface. These electrophysiological and pharmacological effects served as the standard for evaluating the modified sequences.

Peptides modified by the addition of one, two, or three lysines at the N-terminus underwent little enhancement of channel-forming activity. However, the addition of lysines 4, 5, and 6 improved the magnitude and reliability of the induced changes at each step (Table 2). The increases in I_{SC} for the N-K series ranged from 0.3 $\mu A/cm^2$ (for N-K_1) to 3.3 $\mu A/cm^2$ (N-K_6), compared to 1.0 $\mu A/cm^2$ for the unmodified M2GlyR. The frequency of producing chloride channel activity improved to a point that every experiment yielded increases in I_{SC}, increasing from a frequency from 65% for the unmodified sequence to 100% for N-K_4-, N-K_5-, C-K_4-, and C-K_4-carboxamide. The wild types, N-K_4–N-K_6, were tested for their response to bumetanide and DPC. As shown in Table 2, the N-K_4 completely retained the pharmacological characteristics demonstrated by the unmodified M2GlyR peptide. The N-K_4 channel activity was also inhibited by omitting chloride from the bathing media (data not shown). Although the highly soluble N-K_5- and N-K_6-M2GlyR yielded increased I_{SC}, the I_{SC} values were much less sensitive to the presence of the two inhibitors. The lack of response to bumetanide, which blocks chloride entry into the cell at the basolateral surface, suggested that chloride selectivity may have been lost.

Channel-forming activities for C-K_3-, C-K_4-, C-K_5-, and C-K_4-CO-NH_2 analogs were also investigated. Despite its increased solubility, TOCSY-NMR indicated that the C-K_3 analog was aggregated in solution. This peptide also showed little additional effect on the MDCK monolayers compared to the wild-type sequence. The C-K_4 analog, which was more soluble than the +7 charged N-K_6-M2GlyR sequence, exerted a much greater effect on I_{SC} than did N-K_4 and retained the pharmacological responses of the wild-type, unmodified sequence. The C-K_4-M2GlyR-CO-NH_2 and C-K_5-M2GlyR had the highest solubilities of any of the M2GlyR adducts, but paradoxically displayed

TABLE 2 Physiological effects of selected N- and C-modified M2GlyR peptides

Peptide	Frequency of activity (%)	I_{SC} increase ($\mu A/cm^2$)	Bumetanide (% inhibition)	DPC (% inhibition)
M2GlyR	65 ($N = 37$)	1.0 $\pm$ 0.1 ($N = 24$)	100 ($N = 5$)	100 ($N = 5$)
N-K_1-M2GlyR	42 ($N = 12$)	0.3 $\pm$ 0.0 ($N = 5$)	100 ($N = 3$)	100 ($N = 2$)
N-K_2-M2GlyR	67 ($N = 12$)	0.9 $\pm$ 0.2 ($N = 8$)	96 ($N = 4$)	99 ($N = 4$)
N-K_3-M2GlyR	80 ($N = 10$)	0.6 $\pm$ 0.1 ($N = 8$)	55 ($N = 4$)	87 ($N = 4$)
N-K_4-M2GlyR	97 ($N = 58$)	1.2 $\pm$ 0.1 ($N = 56$)	69 ($N = 29$)	97 ($N = 5$)
N-K_5-M2GlyR	100 ($N = 13$)	2.8 $\pm$ 0.4 ($N = 13$)	37 ($N = 5$)	22 ($N = 6$)
N-K_6-M2GlyR	100 ($N = 10$)	3.3 $\pm$ 0.6 ($N = 10$)	10 ($N = 5$)	22 ($N = 5$)
C-K_3-M2GlyR	91 ($N = 11$)	1.1 $\pm$ 0.3 ($N = 10$)	99 ($N = 4$)	61 ($N = 6$)
C-K_4-M2GlyR	100 ($N = 94$)	2.7 $\pm$ 0.2 ($N = 94$)	77 ($N = 31$)	93 ($N = 5$)
C-K_4-M2GlyR-carboxamide	100 ($N = 14$)	1.2 $\pm$ 0.2 ($N = 14$)	99 ($N = 6$)	77 ($N = 5$)
C-K_5-M2GlyR	83 ($N = 12$)	0.8 $\pm$ 0.1 ($N = 10$)	58 ($N = 5$)	58 ($N = 5$)

lower channel-forming activities. This result suggested that these two forms have decreased affinity for the membrane. Of the modified peptides listed in Table 2, C-K_4-M2GlyR gave the greatest increase in I_{SC} while retaining a near-normal response to bumetanide and DPC. C-K_1 and C-K_2 were not prepared, because of the expense of generating two peptides, which we felt would be uninteresting because of the relative activity of C-K_3-M2GlyR. The C-K_6 derivative of M2GlyR was not prepared because of the reduced I_{SC} observed with both C-K_4-M2GlyR-CO-NH_2 and C-K_5-M2GlyR.

For the in vitro assay activity controls, a scrambled C-K_4-M2GlyR sequence was generated (Table 1). The scrambled wild-type sequence yielded a 47-fold increase in solubility over the native amphipathic sequence. The addition of the four lysines increases its solubility by 63-fold over the native M2GlyR sequence but by only 3.2-fold more than the C-K_4 adduct. The C-K_4 scrambled sequence, however, failed to produce channel activity in MDCK cells. Addition of the five lysines to M1CFTR, a sequence that previously was shown to be unable to form channels (Montal et al., 1994), resulted in a modest rise in I_{SC} (0.4 $\mu A/cm^2$; $N = 2$). Although this activity appears as good as several of the M2GlyR adducts, we have no knowledge of the ion selectivity, single channel conductance, or pharmacology. This result simply eliminated this peptide as a suitable control sequence.

The sequences C-K_4-M2GlyR and N-K_4-M2GlyR were tested for an effect on fluid secretion in experiments in which monolayers were incubated for 24 h with 500 μM peptide. The monolayers exposed to C-K_4-M2GlyR secreted fluid at a rate of 0.15 ± 0.02 $\mu l/h/cm^2$ ($N = 8$) (the rate for paired control monolayers was -0.01 ± 0.02 $\mu l/h/cm^2$; $p < 0.01$). The rate of fluid transport by monolayers incubated with N-K4 did not differ from the control rate (0.0 ± 0.02 $\mu l/h/cm^2$, $N = 8$).

Circular dichroism analysis of peptide secondary structure

CD studies were initiated to identify any structural differences between two best sequences, C-K_4-M2GlyR and N-K_4-M2GlyR. CD spectra of N-K_4 and C-K_4 were recorded at varying concentrations in water (100 mM, 500 mM, 1 mM, and 1.5 mM). The spectra were all remarkably similar, each showing one strong minimum at ~197 nm. However, because the spectra were not superimposable, we suspect that there are slight differences in the degree of random coil or degree of aggregation. No concentration-dependent changes in the CD spectrum were observed over the concentration range tested (data not shown). A gradual transition from random coil was observed as the percentage of trifluoroethanol (TFE) was increased. The peptide displayed high helical content at 30% TFE in water, with a slight increase in helicity when the TFE concentration was increased to 40%. In Fig. 6, the CD spectrum for the 0.5 mM concentrations of N-K_4 and C-K_4 are shown in the presence and absence of 40% TFE in water. At this TFE concentration (Fig. 6) the CD spectra for the two peptides are essentially identical and indicate that they both form about the same amount of helix, estimated to be 60% based mean-residue ellipticity at 222 nm (Chen et al., 1974). CD was also employed for a temperature study in 40% TFE. Spectra were taken from 25°C to 70°C in hopes of seeing which of the N- or C-K_4 sequences unfolded at a lower temperature. Both adducts, however, retained their full structure up to and including the highest temperature tested.

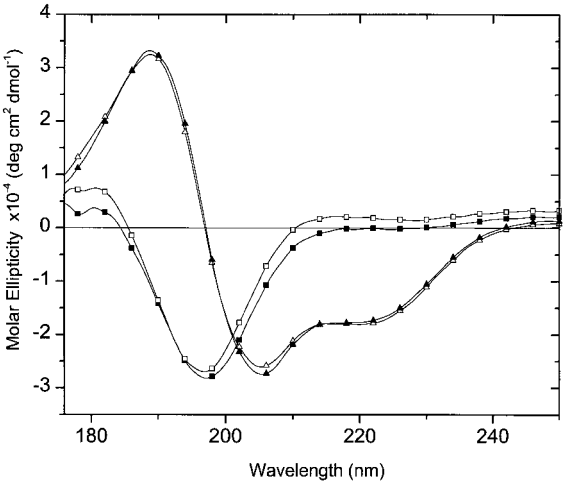

FIGURE 6 CD spectra for 0.5 mM solutions for selected M2GlyR sequences: C-K_4 in water (□), C-K_4 in water containing 40% TFE (△), N-K_4 in water (■), and N-K_4 in water containing 40% TFE (▲).

Viscosity measurements of peptide aggregation rates

The effects of salt on aggregation were tested by monitoring changes in the viscosity of C-K_4-M2GlyR and N-K_4-M2GlyR solutions. Relatively high concentrations of peptide were employed to yield measurable rates. From NMR experiments conducted in the absence of salt, we know that these samples at 3 mM are already partially aggregated. The time dependence of aggregation in Ringer's buffer (at pH 7.4) and water is shown (Fig. 7). In water, no increase in viscosity was observed for either C-K_4-M2GlyR or N-K_4-M2GlyR. However, in the presence of Ringer's, further aggregation was promoted. Different modes and rates of aggregation were seen for 4.67 mM C-K_4-M2GlyR and 4.67 mM N-K_4-M2GlyR in Ringer's solution. The kinetics of C-K_4-M2GlyR aggregation were best described as a first-order reaction, whereas those of N-K_4-M2GlyR were second order. At 2.33 mM, N-K_4-M2GlyR aggregated at a fourfold slower rate, thus confirming the second-order process for the N-K_4 peptide. The reasonable fits for the theoretical curves and the data further support this claim. In the bioassays performed using concentrations of peptide in the

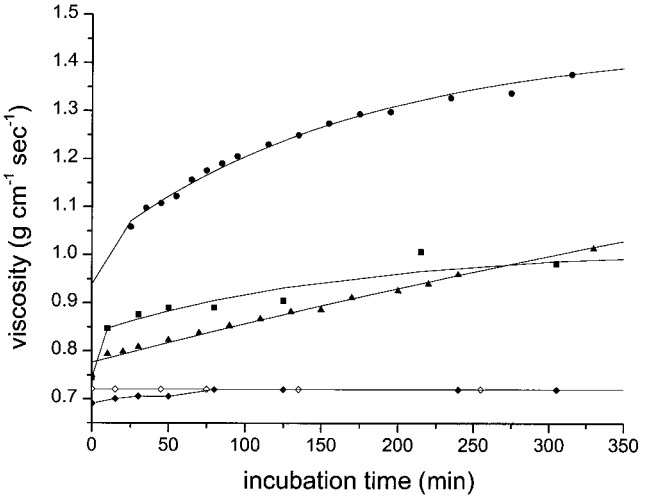

FIGURE 7 The changes in viscosity with time for the N-K_4 and C-K_4-M2GlyR peptides were followed in Ringer's (with salt) and water (without salt). Samples were as follows: N-K_4-M2GlyR in Ringer's, 4.67 mM (●), 2.34 mM (■); N-K_4-M2GlyR in water, 4.67 mM (♦); C-K_4-M2GlyR in Ringer's, 4.67 mM (▲); C-K_4-M2GlyR in water, 4.67 mM (◇). The actual data points are shown, and the curves represent the theoretical plots for first- and second-order processes.

100–200 mM range in Ringer's, the rate of aggregation would be negligible over the time course of the experiment.

One experiment was performed that included 40% TFE along with the Ringer's to test the rate of aggregation of helical segments as opposed to the random coils seen for Ringer's alone. Unfortunately, the rate of aggregation was too rapid to be measured accurately using our viscometer.

DISCUSSION

The lack of solubility of channel-forming amphipathic peptides is a problem that we previously had to endure (Reddy et al., 1993; Grove et al., 1991; Montal et al., 1994; Iwamoto et al., 1994). Not only is this class of peptides relatively insoluble, the limited amount of peptide that does dissolve does so in an aggregated state. Other channel-forming peptides, such as δ-toxin, have been shown to form aqueous aggregates comprising ~70 molecules (Kantor et al., 1972), whereas [Ala14]-melittin analog formed aggregates containing ~50 molecules (John and Jahnig, 1992). To overcome this intrinsic property, 16 separate modified M2GlyR peptides were designed, synthesized, and analyzed with regard to their aqueous solubility, degree of aggregation, and bioactivity. Peptides were modified by varying the net positive charge at either the N- or C-terminus of the peptide. The complete removal of charge from the N-terminus of M2GlyR did not alter the solubility, suggesting that the amphipathic character of the segment is driving its limited solubilization.

We postulated that by adding positive charge to either terminus, a water-soluble channel-forming sequence could be developed that ideally produces channels each time it is presented to membranes, while preserving the channel properties of the relatively insoluble unmodified sequence. Several criteria were used to evaluate the effect of the added charges. We first assayed the solubility at saturation of the different peptides in buffer at neutral pH. We then checked the aggregation state of the solubilized sequences by using HPLC and TOCSY-NMR. In addition, we measured electrophysiological and pharmacological parameters to try and correlate solubility and degree of aggregation to bioactivity. These experiments clearly demonstrated that adding more charge promoted increased solubilization. Addition of the third lysine to the N-terminus of M2GlyR led to a cooperative effect that greatly enhanced solubility. However, this enhanced solubility did not appear to correlate directly with bioactivity. Some lysine-modified sequences such as N-K_3-M2GlyR, which showed a dramatic increase in solubility over the unmodified sequence, had poor activity when tested in the MDCK monolayers. In contrast, N-K_4-M2GlyR, which was only slightly more soluble than N-K_3, showed good activity with the cultured cells. Thus solubilities alone do not account for the differences in channel activity for these two compounds.

Based on the HPLC profiles for the different peptides, increasing the number of charged residues at either terminus decreased the apparent amount of aggregation. For the unmodified and modified sequences of M2GlyR containing fewer than two lysines, the elution profile contained numerous broad and poorly resolved peaks. This profile suggested a mixed population of aggregates of varying sizes. The elution profiles for lysine adducts of M2GlyR containing three or more lysines were sharp, well resolved, and late eluting. The apparent transition from aggregated to less aggregated or monomeric states seen in the HPLC pattern correlated well with the cooperative step seen in the solubilization studies.

The TOCSY-NMR data suggested that the transition seen in the HPLC profiles most likely reflects a transition from a high aggregation to a reduced aggregation state. N-K_{0-2}-M2GlyR formed complex aggregates that were detectable by HPLC and NMR and gave poor results in the bioassays. The HPLC profile N-K_3 peptide appeared identical to that seen for the N-K_{4-6}-adducts, yet this fraction appeared more aggregated by TOCSY-NMR than the others and failed to reliably stimulate I_{SC} to the degree that the others did. The most logical conclusion is that N-K_3-M2GlyR forms small aggregates that are readily soluble in aqueous systems yet interact poorly with membranes, thus producing no functional channels. Peptides N-K_4 through N-K_6 were highly soluble, contained differing percentages of monomer in water containing 30% TFE (as judged by NMR), and always produced an increase in short-circuit current (I_{SC}).

In reconciling the solubility and activity data as well as the TOCSY-NMR spectra and HPLC profiles, three different states of association appear to exist for the peptide, depending on the amount of positive charge added to the sequence. In solutions of the wild type, N-K_1 and N-K_2-M2GlyR, larger aggregates predominate that are relatively

insoluble in aqueous buffers. We suspect that solutions containing these peptides contain few if any monomers. N-K_3-M2GlyR forms smaller aggregates that are relatively soluble (up to 11 mM). Yet these peptides still yield poorly resolved TOCSY-NMR spectra and are relatively inefficient in forming active channels in epithelial cells. The cooperative effect seen in solubility, jumping from 2.3 to 11.1 mM for N-K_3-M2GlyR, reflects the break-up of the large aggregates into smaller aggregates that have the same retention time as monomer on reverse-phase HPLC. In aqueous solutions of C- and N-K_4-M2GlyR, small aggregates are apparently mixed with monomers that almost always generate channel activity. This transition occurs over a small change in solubility, occurring somewhere between 11.1 and 13.4 mM for N-K_n-M2GlyR series. Monomer predominates when the fifth lysine is added. In terms of solubility, the mixed form gives way to monomer between 13.4 and 15.8 mM. The differences in solubility of the different aggregation states reflect the free energies required to not only solvate the peptides but also to overcome solute-solute hydrophobic interactions. We hypothesize that the cooperative step in going from large aggregates to small aggregates reflects breaking of the solute-solute hydrophobic interactions.

These results suggest that HPLC profiles are not sensitive for detecting the smaller aggregates seen in our system. In a comparison of these two techniques, we found increased sensitivity with multidimensional NMR. The results of the NMR work allowed us to formulate the hypothesis that an increase in monomers leads to high activity. By examining the resolution of the individual TOCSY-NMR resonances, one can better estimate aggregation compared to analyzing HPLC profiles.

The addition of positive charge to the C-terminus results in sequences that are considerably more soluble than their N-K-M2GlyR counterparts (Table 1). The nonequivalence of placing the same net charges at the C-terminus versus the N-terminus suggests that the peptides are asymmetrical in one or more properties. CD of the N-K_4- and C-K_4-M2GlyR shows them to be unstructured to a similar degree in water. However, N-K_4- and C-K_4-M2GlyR appear to adopt the same helical conformation in 40% TFE, and these structures appear to be stable to the same degree from 25°C to 70°C.

Based on the viscosity studies, electrostatic repulsion between the positively charged tails of the modified M2GlyR peptides was responsible for their increased solubility. Shielding of the added positive charge through the addition of mono- and divalent anions leads to increased aggregation. Ringer's buffer contains several divalent anions, such as phosphate, sulfate, and citrate, that might interact with one or more lysine residues. The addition of the divalent phosphate anion to melittin promotes aggregation (van-Veen et al., 1995). However, differences in aggregation rates of high concentrations of C- and N-K_4-M2GlyR in Ringer's and in water indicate that adding charge to the C- and N-termini yields modified peptides with different properties. The difference in the rate orders (first for C-K_4 and second for N-K_4) suggests that the assembly of aggregates is different for the two forms.

MDCK cells incubated with 100 μM C-K_4-M2GlyR reach maximum activation after 30–40 min, indicating that channel assembly is not spontaneous. After reaching maximum activity, if the medium containing the peptide is replaced with fresh peptide-free medium, activity begins to drop off immediately, and by 2 h no measurable increase in I_{SC} is seen. This result suggests that the peptide may establish an equilibrium between membrane-bound and free monomer in the extracellular buffer fractions. Thus the lower activity seen with the highly soluble C-K_5- and C-K_4-CO-NH_2-M2GlyR peptides may be due to such an equilibrium, which favors the free monomer in solution.

Once the monomeric peptide binds to the apical membrane of the MDCK cells, it must orient relative to the end of the peptide that is modified with the charged tail. Both the C $\rightarrow$ N and N $\rightarrow$ C orientations of the assembled peptides yielded functional channels that were inhibited by DPC. We believe that because of the high energy barrier that exists for translocating the positively charged tail across the membrane, it is unlikely that the peptide enters the cell. The observation that C-K_4 exerted a greater effect on I_{SC} than N-K_4 suggests that the C-terminal modification may yield a more favorable orientation.

Our success in solubilizing the nonamphipathic M1CFTR sequence suggests that other transmembrane sequences can benefit from this approach. We suspect that most types of transmembrane sequences could be made soluble in aqueous buffers. A different group reported similar increases in solubility and activity when increased positive charge was added to peptide–magainin-2 (Bessalle et al., 1992). The major difference between the solubilization of M1CFTR and M2GlyR observed through the addition of lysine residues is the transition between insoluble and soluble forms. The solubility of M2GlyR gradually increases up to the addition of the third lysine, with a gradual decrease in the complexity of the HPLC elution pattern, whereas the M1CFTR appears essentially unchanged. We suspect that for some very hydrophobic peptides, solubilization could compromise bioactivity. We feel this is a distinct possibility, based on the altered activity observed when the fifth and sixth lysines were added to M2GlyR.

CONCLUSIONS

The desire to find a water-soluble, Cl^--specific, channel-forming peptide was the driving force behind this study. Using the structure of melittin as a model, we added sufficient positive charge to the N- or C-terminus of the M2 transmembrane segment of the glycine receptor, to increase its solubility in aqueous media through electrostatic repulsion with retention of its affinity toward membranes. When incubated with the apical membranes of MDCK cells, the hydrophobic terminus of the positively charged peptides apparently inserts into the membrane, assembles, and then

functions in much the same way that the unmodified M2GlyR did in artificial lipid membranes (Reddy et al., 1993). Although increased solubilization was attained with the addition of lysines to either terminus, many of the more soluble species were still aggregated and did not interact well with membranes. Only the C-K_4-M2GlyR adduct, which we believe formed a mixture of monomer and small aggregates in aqueous systems, was able to substantially increase short-circuit current and retain sensitivity to DPC and bumetanide. Peptides with higher solubilities showed decreased frequencies of channel formation, and those channels that did form appeared to lack the same ion selectivity and pharmacology of the unmodified sequence (which we tried to preserve). The ability to stimulate chloride and water secretion in epithelial cells by using a small water-soluble channel-forming peptide suggests a new method by which a more normal phenotype can be restored to epithelial cells in CF patients.

We thank Dr. Om Prakash for running the NMR-TOCSY on a 500-MHz instrument purchased with the assistance of NSF-EpSCoR funds, and Prof. Michael Doughty for assisting in the circular dichroism experiments. We also thank Drs. Sybille Rex and Thomas E. Roche for assisting in the preparation of the manuscript.

Support for this work was provided by grants from the National Institutes of Health (GM-43617 to JMT) and the National Institute of Diabetes and Digestive and Kidney Diseases (DK-45614 to JG) and in part by the Kansas Agricultural Experiment Station (KAES manuscript 97-305-J). Portions of this study were published in abstract form (*Protein Sci.* 5(Suppl. 1):150, 1996).

REFERENCES

Altieri, A. S., D. P. Hinton, and R. A. Bryd. 1995. Association of bimolecular systems via pulsed field gradient NMR self-diffusion measurements. *J. Am. Chem. Soc.* 117:7566–7567.

Bessalle, R., H. Haas, A. Goria, I. Shalit, and M. Fridkin. 1992. Augmentation of the antibacterial activity by positive-charge chain extension. *Antimicrob. Agents Chemother.* 36:313–317.

Chen, Y. H., J. T. Wang, and K. H. Chau. 1974. Determination of the helix and β form of proteins in aqueous solution by circular dichroism. *Biochemistry.* 13:3350–3359.

Englebretsen, D. R., and P. F. Alewood. 1996. Boc SPPS of two hydrophobic peptides using a "solubilizing tail" strategy: dodecaalanine and chemotactic protein 10^{42-55}. *Tetrahedron Lett.* 37:8431–8434.

Grantham, J. J., M. Uchic, E. J. Cragoe, J. Kornhaus, J. A. Grantham, V. Donoso, R. Mangoo-Karim, A. Evan, and J. McAteer. 1989. Chemical modification of cell proliferation and fluid secretion in renal cysts. *Kidney Int.* 35:1379–1389.

Grove, A., J. M. Tomich, and M. Montal. 1991. A molecular blueprint for the pore-forming structure of voltage gated calcium channels. *Proc. Natl. Acad. Sci. USA.* 88:6418–6422.

Hwang, L.-P., P.-L. Wang, and T. C. Wong. 1988. Theory of differential line broadening in coupled spin systems in macromolecules and molecular aggregates. Application to the study of micellar systems. *J. Phys. Chem.* 92:4753–4758.

Iwamoto, T., A. Grove, M. O. Montal, M. Montal, and J. M. Tomich. 1994. Chemical synthesis and characterization of peptides and oligomeric proteins designed to form transmembrane ion channels. *Int. J. Pept. Protein Res.* 43:597–607.

Jayakumar, R., C. Jayanthy, and C. Gomathy. 1995. Peptide aggregates: a novel model system to study self-assembly of peptides. *Int. J. Pept. Protein Res.* 45:129–137.

John, E., and F. Jähnig. 1992. A synthetic analogue of melittin aggregates in large oligomers. *Biophys. J.* 63:1536–1543.

Kantor, H. S., B. Temples, and W. V. Shaw. 1972. Staphylococcal delta hemolysin: purification and characterization. *Arch. Biochem. Biophys.* 151:142–156.

Kersh, G. J., J. M. Tomich, and M. Montal. 1989. The M2δ transmembrane domain of the nicotinic cholinergic receptor forms ion channels in human erythrocyte membranes. *Biochem. Biophys. Res. Commun.* 162: 352–356.

King, T. P., D. Wade, M. R. Coscia, S. Mitchell, L. Kochoumian, and R. B. Merrifield. 1994. Structure-immunogenicity relationship of melittin, its transposed analogs and D-melittin. *J. Immunol.* 153:1124–1131.

Mangoo-Karim, R., M. Ye, D. P. Wallace, J. J. Grantham, and L. P. Sullivan. 1995. Anion secretion drives fluid secretion by monolayers of cultured human polycystic cells. *Am. J. Physiol.* 269:F381–F388.

Marsh, D. 1996. Peptide models for membrane channels. *Biochem. J.* 315:345–361.

Montal, M. 1995. Molecular mimicry in channel-protein structure. *Curr. Opin. Struct. Biol.* 5:501–506.

Montal, M. O., G. L. Reddy, T. Iwamoto, J. M. Tomich, and M. Montal. 1994. Identification of an ion channel-forming motif in the primary structure of CFTR, the cystic fibrosis chloride channel. *Proc. Natl. Acad. Sci. USA.* 91:1495–1499.

Otada, K., S. Kimura, and Y. Imanishi. 1992. Interaction of melittin derivatives with lipid bilayer membrane. Role of the basic amino acids and their replacement with lactose. *Biochim. Biophys. Acta.* 1112:1–6.

Ramalingam, K., S. Aimoto, and J. Bello. 1992. Conformational studies of anionic melittin analogues: effect of peptide concentration, pH, ionic strength and temperature—models for protein folding and halophilic proteins. *Biopolymers.* 32:981–992.

Reddy, L. G., T. Iwamoto, J. M. Tomich, and M. Montal. 1993. Synthetic peptides and four-helix bundle proteins as model systems for the pore-forming structure of channel proteins. III. Transmembrane segment M2 of the brain glycine receptor channel is a plausible candidate for the pore-lining structure. *J. Biol. Chem.* 268:14608–14615.

Van-Veen, M., G. N. Georiou, A. F. Drake, and R. J. Cherry. 1995. Circular dichroism and fluorescence studies on melittin: effects of C-terminal modifications on tetramer formation and binding to phospholipid vesicles. *Biochem. J.* 305:785–790.

Wallace, D. P., J. M. Tomich, T. Iwamoto, K. Henderson, J. J. Grantham, and L. P. Sullivan. 1997. A synthetic peptide derived from the glycine-gated Cl^- channel generates Cl^- and fluid secretion by epithelial monolayers. *Am. J. Physiol* 272(*Cell Physiol.* 41):C1672–C1679.

Yang, Y., K. H. Mayo, T. J. Daly, J. K. Barry, and G. J. LaRosa. 1994. Subunit association and structural analysis of platelet basic protein and related proteins investigated by 1H NMR spectroscopy and circular dichroism. *J. Biol. Chem.* 269:20110–20118.