ELSEVIER

Biochimica et Biophysica Acta 1466 (2000) 47–60

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

A synthetic peptide based on a glycine-gated chloride channel induces a novel chloride conductance in isolated epithelial cells

Kathy E. Mitchell [a], Takeo Iwamoto [b], John Tomich [b], Lisa C. Freeman [a,*]

[a] *Department of Anatomy and Physiology, Kansas State University, 1600 Denison Avenue, VMSB 228, Manhattan, KS 66506-5602, USA*
[b] *Department of Biochemistry, Kansas State University, Manhattan, KS 66506, USA*

Received 12 July 1999; received in revised form 7 February 2000; accepted 17 February 2000

Abstract

CK_4-M2GlyR, an aqueous soluble peptide derived from the transmembrane M2 segment of the glycine-gated Cl^- channel found in postsynaptic membranes of the central nervous system, has previously been shown to increase transepithelial Cl^- and fluid secretion of epithelial monolayers. The goal of this study was to determine whether CK_4-M2GlyR exerts these effects via formation of a novel chloride conductance pathway, modulation of endogenous chloride channel activity, or a combination of these effects. Ionic currents were recorded from isolated epithelial cells before and after treatment with the peptide using the whole-cell configuration of the patch-clamp technique. CK_4-M2GlyR increased whole-cell Cl^- currents in all epithelial cell lines that were studied, including: Madin–Darby canine kidney cells, a human colonic epithelial cell line (T84), and airway epithelial cells derived from a human cystic fibrosis patient (IB3-1). No evidence was found for modulation of endogenous Cl^- channels by CK_4-M2GlyR based on both the electrophysiological properties of the observed currents and the pharmacological profile of the CK_4-M2GlyR-induced current. These results suggest that CK_4-M2GlyR increases Cl^- permeability in epithelial cells directly, by forming a distinct conduction pathway in cell membranes.

Keywords: Channel-forming peptide; Chloride transport; Cystic fibrosis transmembrane conductance regulator; Epithelial cell

1. Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) protein is located in the apical membranes of Cl^- secreting epithelia, and functions as a cAMP activated Cl^- channel [1–3]. Mutations in the CFTR gene result in abnormal chloride transport across epithelial cells [4]. There are several lines of evidence that suggest a relationship between insufficient Cl^- transport and the pathology of cystic fibrosis (CF). For example, residual Cl^- secretion in CF patients, presumably not mediated by CFTR, correlates with preserved pancreatic function and delayed presentation of the disease [5]. The CFTR knockout

Abbreviations: CaM kinase, Ca^{2+}- and calmodulin-dependent protein kinase; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; I_{SC}, short-circuit current; KN-62, 1-(*N*,*O*-bis[5-Isoquinolinesulfonyl]-*N*-methyl-L-tyrosyl)-4-ohenylpiperazine; M2GlyR, M2 transmembrane segment of the α-subunit of the brain glycine receptor; MDCK, Madin–Darby canine kidney; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; TFE, trifluoroethanol; VSOAC, volume-sensitive organic osmolute and anion channel

* Corresponding author. Fax: +1-785-532-4557;
E-mail: freeman@vet.ksu.edu

0005-2736/00/$ – see front matter
PII: S0005-2736(00)00170-X

mouse does not develop lung disease due to the presence of a non-CFTR Cl^- channel expressed in murine lung [6], although congenic CFTR knockout mice which lack this non-CFTR Cl^- channel, develop severe lung disease [8]. In addition, the severity of disease in the CFTR knockout mouse varies in different organs and exhibits an inverse correlation with the presence of this non-CFTR Cl^- conductance [7]. Collectively, these findings suggest that alternate pathways for Cl^- transport in epithelial cells can compensate for the loss of CFTR-mediated Cl^- transport. Synthetic channel-forming peptides shown to increase chloride conductance in epithelial cells could thus be useful for therapeutic intervention in CF [9,10], particularly if the novel conductance pathways formed respond to the altered driving force associated with deficient chloride transport.

CK_4-M2GlyR (PARVGLGITTVLTMTTQSSGSRAKKKK) is an aqueous soluble peptide derived from the transmembrane M2 segment of the glycine-gated Cl^- channel found in postsynaptic membranes of the central nervous system, and a potential therapeutic agent for cystic fibrosis [11]. Addition of this lysine-modified peptide to the apical surface of monolayers of Madin–Darby canine kidney (MDCK) cells significantly increased short-circuit current (I_{SC}), hyperpolarized transepithelial potential difference, and induced fluid secretion; removal of extracellular Cl^- inhibited these effects of the peptide [11]. In contrast, the non-modified, native M2GlyR sequence, which was shown previously to incorporate into lipid bilayers and self-assemble into anion-selective channels [12,13], had little effect on I_{SC} when applied to MDCK monolayers. While this previous work shows convincingly that CK_4-M2GlyR increases apical chloride permeation across MDCK and T84 monolayers, it does not address the underlying mechanism by which the lysine-modified peptide exerts its effects on transepithelial ion transport. In light of observations that polycationic peptides such as protamine and poly-lysine can activate endogenous chloride channel activity in some cell types [14,15] and that the unmodified M2GlyR sequence has minimal effects on I_{SC} in epithelial monolayers, it is possible that CK_4-M2GlyR may act, at least in part, by an indirect mechanism rather than channel formation.

The goal of this study was to determine whether CK_4-M2GlyR increases apical chloride permeation via formation of a novel chloride conductance pathway, modulation of endogenous chloride channel activity, or a combination of these effects. The ability of CK_4-M2GlyR to increase chloride current in isolated epithelial cells was assessed and compared to that of a scrambled permutation of the peptide. In addition, we looked for modulation by CK_4-M2GlyR of other Cl^- channels expressed in secretory epithelia. Experiments were designed specifically to test for effects of CK_4-M2GlyR on: CFTR, the cAMP-activated Cl^- channel; ClC-2, the inwardly rectifying, voltage-gated Cl^- channel; the volume-sensitive organic osmolute and anion channel (VSOAC); and the Ca^{2+}-dependent Cl^- channel regulated by the Ca^{2+}- and calmodulin-dependent protein kinase (CaM kinase) [16,17]. Preliminary reports of these studies have been published in abstract form [18,19].

2. Materials and methods

2.1. Peptide synthesis and purification

Peptides were synthesized and purified as described previously [10]. Aqueous stock solutions of the synthetic peptides (5 mM) were dialyzed against 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4, in a dialysis cassette with a 2000 molecular weight cut off. Protein concentrations of the resulting stock solutions were determined by the micro BCA assay (Pierce, Rockford, IL, USA). Stock solutions were stored in small volumes at −20°C, thawed and added to solutions for immediate use (same day).

2.2. Cell culture

MDCK cells and T84 cells were obtained from the American type culture collection (ATCC), and maintained in culture as described previously in detail [20]. Briefly, MDCK and T84 cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's media and Ham's F-12 (DMEM/F12) supplemented with 5% fetal bovine serum (FBS), 100 IU/ml penicillin G and 0.1 mg/ml streptomycin (P/S).

IB3-1 cells [21] and IB3-1 cells stably transfected

with antisense hClC-2 cDNA [22] were obtained from Dr. Garry R. Cutting (Johns Hopkins University School of Medicine, Baltimore, MD), and maintained in culture as previously described [21]. Briefly, IB3-1 cells were cultured in LHC-8 serum-free medium supplemented with endothelial growth supplement (15 μg/ml), imipenem (200 μg/ml), and tobramycin (80 μg/ml). IB3-1 cells stably transfected with antisense hCLC-2 cDNA were maintained in the presence of G418 (180 μg/ml). Tissue culture flasks were treated with Vitrogen (1:15 diluted in H_2O).

All cell types were maintained as subconfluent monolayers in a humidified atmosphere containing 5% CO_2 at 37 °C. For patch clamp analysis, cells were detached from culture dishes 48 h after plating (60–80% confluency) using a trypsin (0.5 mg/ml) containing physiological salt solution (137 mM NaCl; 2.7 mM KCl; 2.8 mM D-glucose; 3.4 mM $NaHCO_3$; 0.27 mM EDTA, sodium salt), pelleted by centrifugation, and resuspended in the external bath solution (see below).

2.3. Whole-cell patch clamp recording

The whole-cell patch clamp technique [23] was used to determine the effects of the CK_4-M2GlyR peptide on whole-cell currents in isolated MDCK, T84 cells, IB3-1 cells, and IB3-1 cells stably transfected with antisense hCLC-2 cDNA [22]. The MDCK and T84 cells were of the lineage used in the epithelial monolayer studies [11]. Internal and external solutions were selected to measure changes in whole-cell chloride permeability and to minimize cell swelling [24]. Internal recording solution (cytosolic) for the patch-clamp analysis contained 145 mM Tris–HCl, 5 mM MgATP, 5 mM HEPES, pH 7.45. The external bath solution consisted of 145 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 60 mM sucrose, 5 mM glucose, 5 mM HEPES, pH 7.45. Chloride is the only permeant ion in this external bath solution. To determine whether the CK_4-M2GlyR-induced current was sensitive to changes in external chloride in the presence of physiological external sodium, Tris–HCl was replaced with sodium chloride and/or sodium gluconate in the external bath solution. Current–voltage relationships obtained using the standard recording solutions were not corrected for liquid junction potentials (<1 mV). Current–voltage relationships obtained using the low chloride extracellular solutions were corrected for liquid junction potentials (−18.9 mV) that were calculated with pClamp 7.0 (Axon Instruments, San Jose, CA, USA), and verified under our recording conditions. Theoretical predicted reversal potentials were calculated using the Nernst equation.

2.4. Planar lipid bilayer experiments

Planar lipid bilayer experiments were conducted as described previously in detail [25]. Lipid bilayers were formed across a 100 μm aperture in the wall of a Delrin cup. The bilayer was formed with a solution containing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (22.5 weight %), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) (10 weight %) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) (67.5 weight %) in decane at a total lipid concentration of 50 mg/ml. Currents were measured in KCl, with concentrations as indicated in 10 mM HEPES, pH 7.4. The grounded side (equivalent to extracellular) of the bilayer chamber is designated *trans* while the side to which voltage is applied (intracellular) is the designated as the *cis* chamber. Peptide solutions were introduced into the grounded side (*trans*) of the bilayer chamber. Bilayer recordings were made with a GeneClamp 500 and CV-5B-100GU headstage from Axon Instruments (San Jose, CA). Data were acquired at 1 kHz and low-pass filtered at 200 Hz. Microcal Origin (Northhampton, MA) and either pCLAMP 6.0 or pCLAMP 7.0 (Axon Instruments, San Jose, CA) were used for data analysis.

2.5. Circular dichroism (CD) analysis

CD spectra of the peptides in 40% trifluoroethanol (TFE) were measured as described previously using a Jasco J-720 spectropolarimeter [10].

2.6. Data analyses

Statistical analyses were performed using MicroCal Origin Version 5.0 (Northhampton, MA) and Statistix for Windows Version 2.0. (Tallahasee, FL). ANOVA was used to test for effects of peptide treatment. Multiple comparisons were performed us-

ing Scheffe's procedure. Data were considered significantly different at $p < 0.05$.

2.7. Materials

Synthetic lipids were obtained from Avanti Polar Lipids (Alabaster, AL). All other reagents for the planar bilayer studies were purchased from Sigma (St. Louis, MO) and were of the highest purity available. DMEM/F12, P/S, FBS, and G418 were purchased from GIBCO Life Technologies (Rockville, MD). LHC-8 and FBS for IB3-1 cell lines were obtained from Biofluids (Rockville, MD). Endothelial growth supplement was obtained from Collaborative Biomedical Products (Bedford, MA). Vitrogen was obtained from Collagen Corporation (Palo Alto, CA). imipenem (Primaxin®) and tobramycin (Nebcin®) are human pharmaceuticals produced by Merck and Co. (West Point, PA) and Eli Lilly (Indianapolis, IN), respectively. Ionomycin, 1-(*N*,*O*-bis[5-Isoquinolinesulfonyl]-*N*-methyl-L-tyrosyl)-4-ohenylpiperazine (KN-62), tamoxifen, and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) were obtained from Sigma Chemical (St. Louis, MO).

3. Results

3.1. Does CK_4-M2GlyR induce a novel Cl^- current in isolated epithelial cells?

Initial work showing that apical treatment of monolayers of MDCK cells with CK_4-M2GlyR increased I_{SC} [10,11] suggested that this peptide should increase whole-cell currents. To confirm this, patch-clamp analysis of isolated MDCK cells was used to measure the effects of CK_4-M2GlyR on whole-cell chloride conductance. Currents were recorded during 20 mV voltage steps from −80 mV to +40 mV from a holding potential of −40 mV, before and after introducing peptide into the extracellular bath solution. Treatment of MDCK cells with CK_4-M2GlyR results in a gradual increase in whole-cell current leveling off after 20 min (Fig. 1). This is similar to the time-course observed for the effects of CK_4-M2GlyR on I_{SC} in MDCK monolayers [11]. Little if any change in whole-cell current was seen over a similar time period for MDCK cells that were not

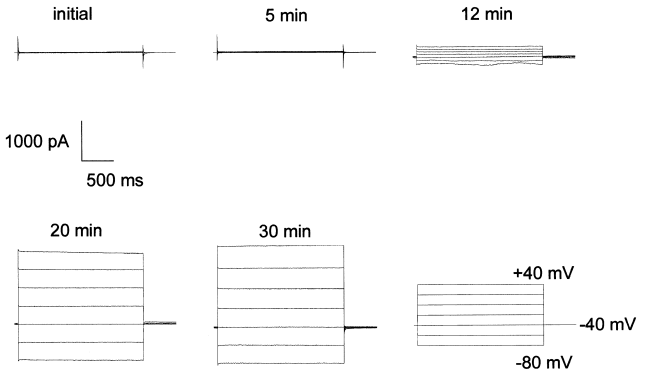

Fig. 1. Treatment of MDCK cells with CK_4-M2GlyR results in a gradual increase in whole-cell chloride current. Whole-cell currents were recorded during step changes in voltage from −80 to +40 mV (step 20) from a holding potential of −40 mV prior to and at the designated intervals after addition of 100 μM CK_4-M2GlyR to the external bath. The internal solution contained 145 mM Tris–HCl, 5 mM MgATP, 5 mM HEPES, pH 7.45 and the external solution contained 145 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 60 mM sucrose, 5 mM glucose, 5 mM HEPES, pH 7.45.

treated with peptide (Fig. 2A). Thus, the increase in whole-cell current observed in cells treated with CK_4-M2GlyR is unlikely to be merely the result of an increase in leak current over time.

To determine whether CK_4-M2GlyR was capable of increasing whole-cell currents against a different background of endogenous channels, the effect of CK_4-M2GlyR on T84 cells was measured under conditions identical to those used in the MDCK studies. Treatment of isolated T84 cells with 100 μM CK_4-M2GlyR (Fig. 2C) increased whole-cell currents with amplitudes similar to those observed in MDCK cells (Fig. 2B). In addition, the kinetic and voltage-dependent properties of the CK_4-M2GlyR-induced current were similar in both cell types. The CK_4-M2GlyR-induced currents (Fig. 2, difference) were obtained by subtracting the initial whole-cell recordings (Fig. 2, basal) from the currents recorded 20 min after the indicated treatments (Fig. 2, post-treatment). As shown in Fig. 2, the CK_4-M2GlyR-induced whole cell currents in both T84 and MDCK cells activated in a time-independent fashion, exhibited weak, if any, voltage-dependence, and showed no time-dependent inactivation at any test potential. The current–voltage relationship for the CK_4-M2GlyR-induced current in isolated T84 and MDCK cells is linear and reverses at 0 mV, as expected for currents re-

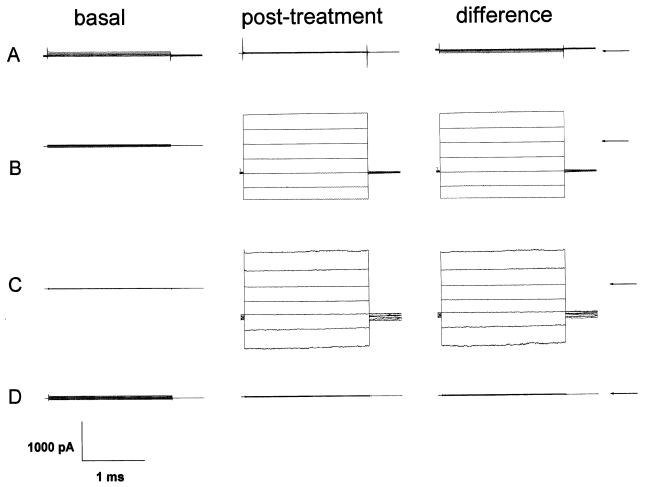

Fig. 2. CK_4-M2GlyR increases whole-cell chloride conductance in isolated epithelial cells. Whole-cell currents of epithelial cells with or without peptide treatment were recorded during step changes in voltage from −80 to +40 mV (step 20) from a holding potential of −40 mV. Basal whole-cell currents, recorded prior to treatment, are shown in the left column. Representative whole-cell recordings made 20 min after changing the extracellular solution are shown in the center column for: an MDCK cell treated with no peptide (A), an MDCK cell treated with 100 μM CK_4-M2GlyR (B), a T84 cell treated with 100 μM CK_4-M2GlyR (C) an MDCK cell treated with 100 μM scrambled CK_4-M2GlyR (D). Difference currents, obtained by subtracting the basal currents from the 20 min post-treatment currents, are shown in the right column. Arrows indicate the zero current level. The internal solution contained 145 mM Tris–HCl, 5 mM MgATP, 5 mM HEPES, pH 7.45 and the external solution contained 145 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 60 mM sucrose, 5 mM glucose, 5 mM HEPES, pH 7.45.

corded using virtually symmetrical chloride concentrations (Fig. 3); the reversal potential predicted by the Nernst equation was −0.69 mV.

To determine whether the CK_4-M2GlyR-induced current was sensitive to changes in external chloride in the presence of physiological external sodium, chloride was substituted in part by gluconate in the external bath solution. For an anion selective channel, the inward current should increase when the driving force for chloride efflux is increased by lowering the external chloride concentration. A representative experiment, in which the current–voltage relationship for CK_4-M2GlyR-induced currents in MDCK cells was measured in the presence of varying concentrations of external chloride, is shown in Fig. 4. After achieving whole cell configuration, 200 μM CK_4-M2GlyR was added to the bath. The current was monitored, and after 30 min the external bath solution containing 149 mM Cl^- was changed to a solution containing either 22.5 or 80.5 mM external chloride while the sodium concentration remained constant at 145 mM. The inward current (chloride efflux) recorded at −60 mV was larger at 80.5 and 22.5 mM external chloride compared to virtually symmetrical (145 mM internal vs 149 mM external) chloride (Fig. 4A). Current amplitude was significantly greater in the 22.5 mM external chloride solution than at 149 mM ($P < 0.05$) (Fig. 4A). Changing the external chloride from 149 mM to 22.5 mM was also associated with a +16 mV shift in reversal potential (Fig. 4B), less than the predicted change in reversal potential obtained from the Nernst relationship for an ideal chloride channel

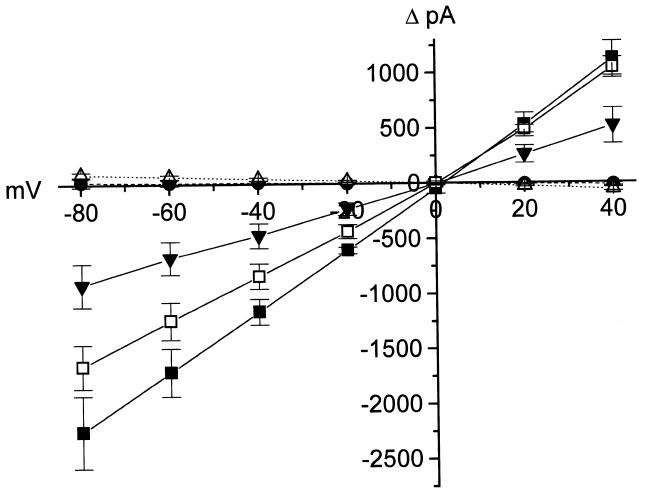

Fig. 3. CK_4-M2GlyR induces a novel current in isolated epithelial cells. *I-V* plots of mean difference currents for MDCK cells treated with: 50 μM CK_4-M2GlyR (down triangle, solid line) ($n = 11$), 100 μM scrambled CK_4-M2GlyR (circle, dotted line) ($n = 4$) without peptide treatment (up triangle, dotted lines) ($n = 5$), treated with 100 μM CK_4-M2GlyR (filled squares, solid line) ($n = 2$) and T84 cells treated with 100 μM CK_4-M2GlyR (open squares, solid line) ($n = 4$) are plotted at voltages from −80 to +40 mV. Error bars indicate the standard error of the mean. CK_4-M2GlyR induced currents differed significantly from the current changes observed in untreated and scrambled CK_4-M2GlyR-treated MDCK cells (at all voltages except 0 mV) using ANOVA. The internal solution contained 145 mM Tris–HCl, 5 mM MgATP, 5 mM HEPES, pH 7.45 and the external solution contained 145 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 60 mM sucrose, 5 mM glucose, 5 mM HEPES, pH 7.45.

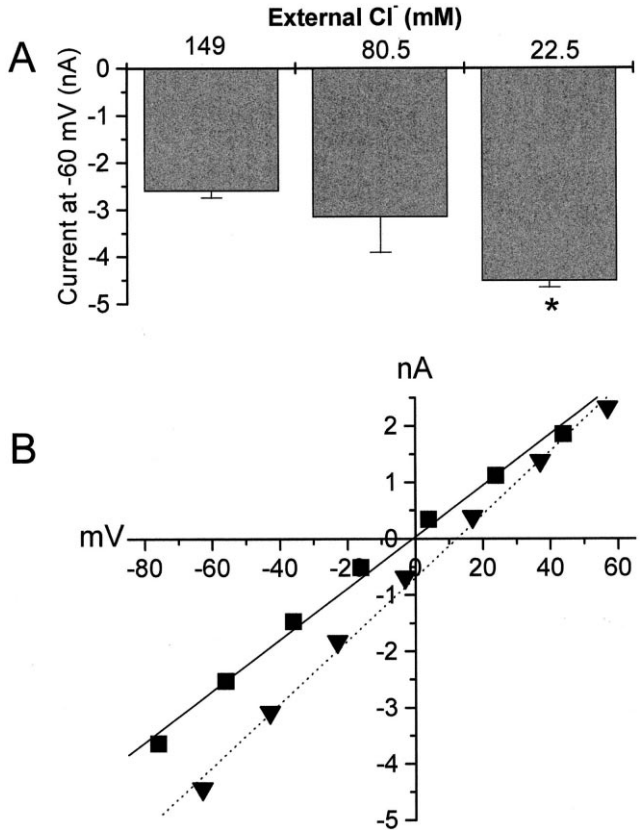

Fig. 4. CK_4-M2GlyR-induced current in MDCK cells is sensitive to changes in external chloride. Whole-cell currents were recorded from an isolated MDCK after treatment with 200 μM CK_4-M2GlyR at voltages from −80 mV to +40 mV. The internal solution contained 145 mM Tris–HCl, 5 mM MgATP, 5 mM HEPES, pH 7.45. The chloride concentration in the external solution (145 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 60 mM sucrose, 5 mM glucose, 5 mM HEPES, pH 7.45) was varied by substituting sodium gluconate for sodium chloride to obtain solutions with 149, 80.5 and 22.5 mM chloride. (A) The mean current amplitude measured at −60 mV for MDCK cells 30 min after treatment with CK_4-M2GlyR ($n=3$). The outward current is significantly larger at 22.5 mM external chloride than at 149 mM external chloride. (B) The current–voltage relations obtained at 149 mM external chloride (square, solid line) and 22.5 mM external chloride (down triangle, dotted line) show a +16 mV shift in reversal potential.

(+46 mV). The response of the CK_4-M2GlyR-induced current to changes in external chloride concentration is consistent with the moderate chloride selectivity typical of endogenous channels. For example, a 19-fold decrease in external chloride from 114 mM to 6 mM caused a +33.5 mV shift in reversal potential for the calcium activated chloride channel from bovine trachea under recording conditions where the shift in reversal potential predicted by the Nernst equation was approximately +75 mV [26].

3.2. *Do non-specific electrostatic interactions mediate the effects of CK_4-M2GlyR on whole-cell chloride conductance?*

After demonstrating that CK_4-M2GlyR was capable of increasing whole-cell chloride conductance in isolated epithelial cells, it was important to explore whether these effects were via formation of novel chloride permeation pathway, modulation of endogenous chloride channel activity, or a combination of these effects. If CK_4-M2GlyR modulates endogenous chloride channels as a result of nonspecific electrostatic effects such as those observed with protamine and poly-lysine in canine airway cells [14,15], a scrambled permutation of CK_4-M2GlyR should be capable of inducing increases in whole-cell chloride current similar to those observed in cells treated with the unscrambled peptide. To determine whether the polycationic nature of the lysine-modified sequence plays a role in modulating endogenous channel activity, a scrambled peptide (ILASTRSQTGR-

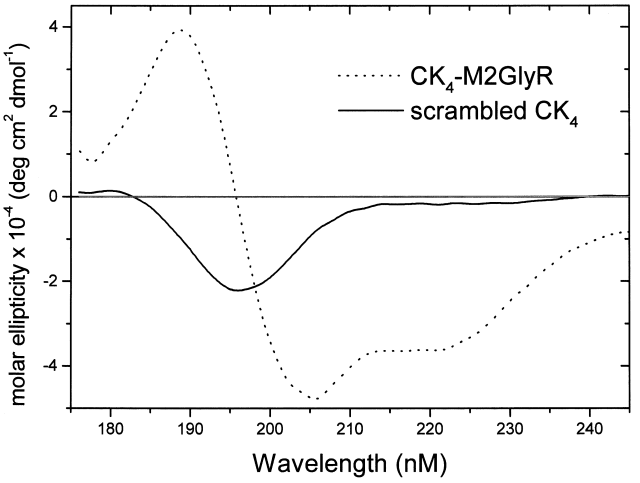

Fig. 5. Scrambled CK_4-M2GlyR does not form α-helical secondary structure. CD spectra of 200 μM CK_4-M2GlyR and a scrambled permutation of CK_4-M2GlyR were made using a Jasco 720 spectropolarimeter in 40% TFE at wavelengths from 175 to 245 nm. The plots are an average of eight scans recorded at a rate of 20 nm/min.

MALSGTTTPGVVKKKK) with the same amino acid composition as CK_4-M2GlyR was synthesized and used to treat isolated MDCK cells. CD analysis of the scrambled peptide indicated that this sequence does not form a helical secondary structure in 40% TFE (Fig. 5) and thus should not be capable of forming a helical transmembrane ion-conducting bundle. This was confirmed by planar lipid bilayer analysis of the scrambled peptide (data not shown). Treatment with the scrambled CK_4-M2GlyR sequence (Fig. 2D) did not significantly alter whole-cell chloride conductance in MDCK cells. The difference currents obtained for scrambled CK_4-M2GlyR-treated cells were minuscule in amplitude, and comparable to those of untreated control cells (Fig. 2D and A, respectively). In Fig. 3, the mean *I–V* relationships for difference currents obtained from MDCK cells that were untreated ($n=7$), treated with 50 μM CK_4-M2GlyR ($n=11$), and treated with 100 μM scrambled CK_4-M2GlyR ($n=4$) are shown. The mean changes in whole-cell current for the scrambled CK_4-M2GlyR-treated and the untreated control cells do not differ significantly from zero at any test potential (Fig. 3). In contrast, the amplitudes of whole-cell currents induced in the CK_4-M2GlyR treated MDCK cells differ significantly from both the untreated and scrambled peptide control treatments at all potentials except zero mV ($P<0.01$, ANOVA). These results indicate that CK_4-M2GlyR does not increase whole-cell chloride conductance in MDCK cells by modulation of endogenous chloride channels via a non-specific electrostatic mechanism.

3.3. Does CK_4-M2GlyR modulate the activity of endogenous epithelial chloride channels?

MDCK and T84 cells are used extensively as model systems for secretory epithelia and these cell lines are known to express constitutively: CFTR [27–29], ClC-2 [30,31], VSOAC [31–33], and Ca^{2+}-activated chloride channels [34–36]. While the data shown above suggest that CK_4-M2GlyR induces a novel chloride current in epithelial cells, they do not rule out modulation of endogenous chloride channels by CK_4-M2GlyR in a sequence-specific manner. Additional experiments were designed specifically to test for effects of CK_4-M2GlyR on CFTR, ClC-2, VSOAC, and the Ca^{2+}-dependent Cl^- channel regulated by CaM kinase.

Like the CK_4-M2GlyR-induced current, the cAMP-activated current associated with CFTR is time- and voltage-independent. Thus, it was critical to determine if CK_4-M2GlyR increased whole-cell chloride current by activating CFTR. It has been shown previously that CK_4-M2GlyR does not increase intracellular concentrations of cAMP [11]. To rule out CK_4-M2GlyR-activation of CFTR by other means, the currents induced by CK_4-M2GlyR in IB3-1 cells were compared to the currents induced in MDCK and T84 cells. IB3-1 cells are an immortalized cell line created from a primary culture of bronchial epithelia isolated from a human patient with CF; the CF phenotype is preserved in this cell line [21]. Currents induced by CK_4-M2GlyR in IB3-1

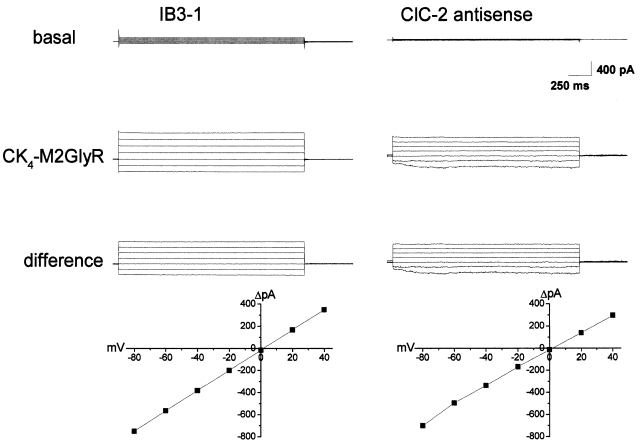

Fig. 6. CK_4-M2GlyR increases whole-cell conductance in IB3-1 and IB3-1 cells transfected with anti-sense hClC-2. Whole-cell currents were recorded during step changes in voltage from −80 to +40 mV (step 20) from a holding potential of −40 mV in IB3-1 cells (left panels), and IB3-1 cells stably transfected with anti-sense cDNA to hClC-2 (right panels). Current traces recorded prior to treatment, 30 min after treatment with 50 μM CK_4-M2GlyR, and difference currents, obtained by subtracting the basal currents from the post-treatment currents, are the in upper, center and bottom current traces, respectively. The *I-V* relations for the CK_4-M2GlyR-induced currents indicate that the properties of the currents induced in these two cell lines are similar not only to each other, but also to those observed in MDCK and T84 cells (Figs. 2 and 3). The internal solution contained 145 mM Tris–HCl, 5 mM MgATP, 5 mM HEPES, pH 7.45 and the external solution contained 145 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 60 mM sucrose, 5 mM glucose, 5 mM HEPES, pH 7.45.

cells were similar to those induced in T84 and MDCK cells with respect to amplitude, time-, and voltage-independence (Fig. 6, left panels). The ability of CK_4-M2GlyR to consistently increase whole-cell chloride conductance in a cell line lacking functional CFTR argues against stimulation of CFTR as the dominant mechanism of CK_4-M2GlyR action. Furthermore, the similar amplitudes of CK_4-M2GlyR-induced currents in IB3-1, MDCK, and T84 cells suggest that peptide stimulation of CFTR is insignificant.

ClC-2 channels are ubiquitously expressed in secretory epithelia; expression of ClC-2 in T84 and IB3-1 cells is well documented [17,22,30]. ClC-2 channels conduct hyperpolarization-activated, pH- and volume-sensitive chloride currents which exhibit inward rectification and time-dependent activation at voltages more negative than −20 mV [17,22,30,37]. The activation kinetics and voltage-dependence of the currents induced by CK_4-M2GlyR (Fig. 2) are inconsistent with CK_4-M2GlyR-stimulation of ClC-2 as a mechanism of CK_4-M2GlyR action. In addition, currents induced by CK_4-M2GlyR in IB3-1 cells permanently transfected with antisense ClC-2 cDNA [22] (Fig. 6, right panels) were indistinguishable from those induced in the IB3-1 cells (Fig. 6, left panels), and quite comparable to those induced in MDCK and T84 cells (Fig. 2). These data suggest that CK_4-M2GlyR stimulation of ClC-2 is insignificant.

Ca^{2+}-sensitive chloride channels are involved in fluid and electrolyte movement in Cl^- secreting epithelia [17]. The Ca^{2+}-dependent Cl^- currents in T84 cells have been well described. The steady-state current voltage relation is strongly outwardly rectifying, and the activation kinetics are both Ca^{2+} and voltage-dependent [35]. In T84 cells, Ca^{2+}-dependent Cl^- channels can be activated by ionomycin; the induced currents are blocked not only by DIDS, but also by the CaM kinase inhibitor KN-62 [36]. A series of experiments was conducted to determine if activation of calcium-dependent Cl^- channels contributes to the CK_4-M2GlyR-induced current in T84 cells. First, the DIDS sensitivity of the CK_4-M2GlyR-induced current was determined, and compared to the DIDS sensitivity of ionomycin-induced current. As expected, a substantial fraction of the ionomycin-induced current was blocked by DIDS (Fig. 7). In contrast, the DIDS-sensitivity of the CK_4-M2GlyR-induced current was negligible (Fig. 8). Moreover, CK_4-M2GlyR-treatment further increased whole-cell current in T84 cells previously exposed to ionomycin and DIDS. Likewise, ionomycin treatment after initial exposure to CK_4-M2GlyR resulted in an additional increase in whole-cell chloride conductance (data not shown). Previously, it was demonstrated by Arreola et al. [36], that pretreatment with the CaM kinase inhibitor, KN-62, for 30 min, markedly inhibits the ionomycin-induced current in T84 cells. However, pretreatment with KN-62 did not diminish the CK_4-M2GlyR-induced current in T84 cells (Fig. 9). These pharmacological data strongly suggest that the CK_4-M2GlyR-induced current is distinct from the calcium-dependent Cl^- channel in T84 cells.

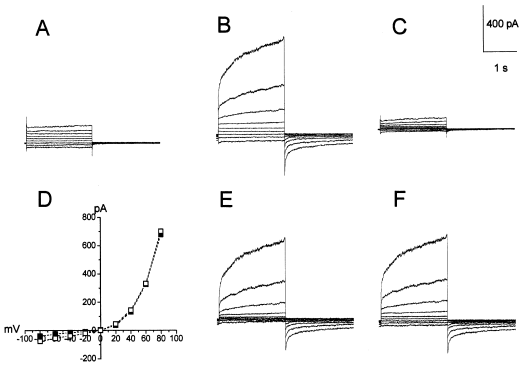

Fig. 7. DIDS-sensitivity of endogenous chloride currents in T84 cells. Traces recorded before (A) and after application of 10 μM ionomycin (B), followed by treatment with 200 μM DIDS (C). The ionomycin-induced (E) and DIDS-sensitive (F) currents were obtained by subtracting the previously measured current from the current measured in the presence of the compound of interest. (D) Current–voltage relations of the ionomycin-induced (filled squares, solid line) and DIDS-sensitive currents (open squares, dashed line). Whole-cell currents were recorded during step changes in voltage from −80 to +80 mV (step 20) from a holding potential of −40 mV. The internal solution contained 145 mM Tris–HCl, 5 mM MgATP, 5 mM HEPES, pH 7.45 and the external solution contained 145 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 60 mM sucrose, 5 mM glucose, 5 mM HEPES, pH 7.45.

The electrophysiological properties of the CK_4-M2GlyR-induced current are also different from those of the calcium-dependent Cl^- current in T84 cells. The current–voltage relationships of the CK_4-M2GlyR-induced current and ionomycin-induced currents were compared. As previously described

[16,36], the ionomycin-induced current exhibited outward-rectification (Fig. 7). In contrast, the CK_4-M2GlyR-induced current had a linear current–voltage relationship (Fig. 8). Furthermore, the ionomycin-induced current clearly showed time- and voltage-dependent activation at positive test potentials, whereas the CK_4-M2GlyR-induced current did not (Figs. 7 and 8, respectively). These data along with the pharmacological evidence strongly support the contention that CK_4-M2GlyR does not activate the calcium-dependent Cl^- current.

VSOACs are expressed in MDCK and T84 cells [31–33]. These volume-sensitive channels are outwardly rectifying, and at positive potentials the swelling-activated whole-cell current inactivates [17]. The activation kinetics and voltage-independence of the currents induced by CK_4-M2GlyR (Figs. 2–5) are inconsistent with CK_4-M2GlyR-stimulation of VSOAC as a mechanism of CK_4-M2GlyR action. Furthermore, tamoxifen, an agent that completely

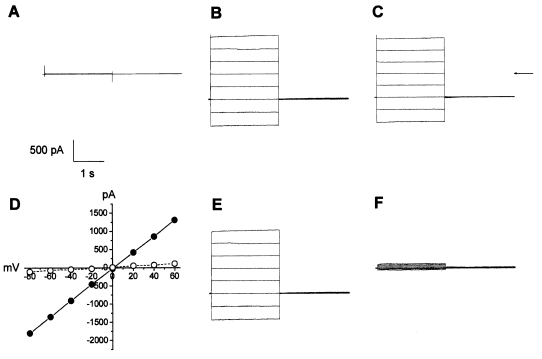

Fig. 8. DIDS-sensitivity of CK_4-M2GlyR-induced chloride current in T84 cells. Traces recorded before (A) and 20 min after treatment with 200 μM CK_4-M2GlyR (B) followed by treatment with 200 μM DIDS (C). The CK_4-M2GlyR-induced current (E) was obtained by subtracting the current measured prior to peptide treatment from the current recorded 20 min after peptide treatment. The DIDS-sensitive current (F) was obtained by subtracting the current measured in the presence of DIDS from the current measured 20 min after peptide treatment. (D) Current–voltage relations of the CK_4-M2GlyR-induced (filled circles, solid line) and DIDS-sensitive currents (open circles, dashed line). Whole-cell currents were recorded during step changes in voltage from −80 to +80 mV (step 20) from a holding potential of −40 mV. The internal solution contained 145 mM Tris–HCl, 5 mM MgATP, 5 mM HEPES, pH 7.45 and the external solution contained 145 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 60 mM sucrose, 5 mM glucose, 5 mM HEPES, pH 7.45.

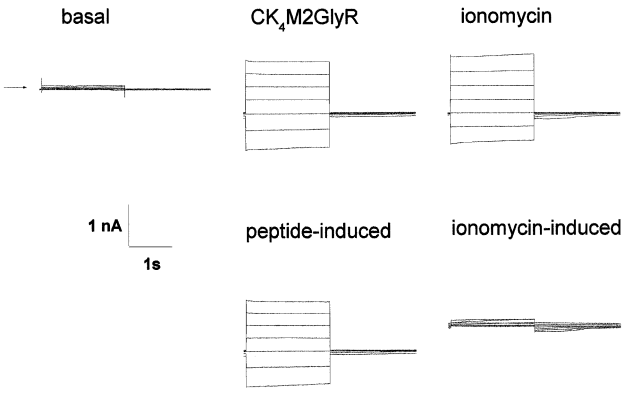

Fig. 9. Calmodulin kinase sensitivity of CK_4-M2GlyR-induced currents in T84 cells. T84 cells were incubated with 10 μM KN-62, a calmodulin kinase inhibitor, for 30 min in the dark. Subsequently, the ability of CK_4-M2GlyR to increase chloride conductance in these cells was assessed. Treatment with 200 μM CK_4-M2GlyR resulted in an increase in current amplitude similar to that observed in cells not treated with KN-62. Subsequent treatment with ionomycin did not result in a further increase in whole-cell current. The CK_4-M2GlyR and ionomycin-induced currents were obtained by subtracting the currents measured prior to treatment with the specified compound from those measured after treatment. Whole-cell currents were recorded during step changes in voltage from −80 to +60 mV (step 20) from a holding potential of −40 mV. The internal solution contained 145 mM Tris–HCl, 5 mM MgATP, 5 mM HEPES, pH 7.45 and the external solution contained 145 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 60 mM sucrose, 5 mM glucose, 5 mM HEPES, pH 7.45.

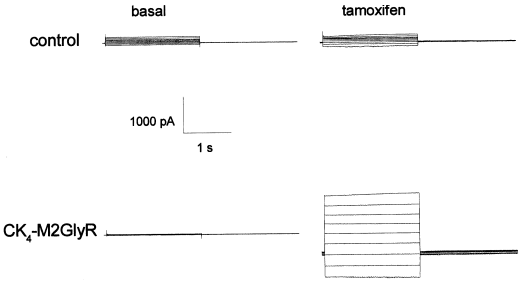

Fig. 10. Tamoxifen-sensitivity of CK_4-M2GlyR induced currents in T84 cells. Whole-cell currents of T84 cells were measured before and after treatment with 100 μM tamoxifen, an inhibitor of VSOAC, in an untreated cell (top panel) and a cell treated with 200 μM CK_4-M2GlyR (bottom panel). Tamoxifen had little or no effect on the endogenous basal current and did not prevent induction of the novel conductance induced 20 min after exposure to CK_4-M2GlyR. Whole-cell currents were recorded during step changes in voltage from −80 to +80 mV (step 20) from a holding potential of −40 mV. The internal solution contained 145 mM Tris–HCl, 5 mM MgATP, 5 mM HEPES, pH 7.45 and the external solution contained 145 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 60 mM sucrose, 5 mM glucose, 5 mM HEPES, pH 7.45.

blocks the swelling-activated, outwardly rectifying current in T84 cells at a concentration of 10 μM [31], did not prevent CK_4-M2GlyR from inducing a novel Cl^- current in T84 cells (Fig. 10). Taken together, these data strongly suggest that CK_4-M2GlyR stimulation of VSOAC in isolated epithelial cells is insignificant.

3.4. Can CK_4-M2GlyR form channels from an aqueous solution in the absence of endogenous channels?

The M2GlyR sequence, derived from the α-subunit of the brain glycine receptor, was originally shown to form anion channels when mixed directly with lipid prior to formation of the bilayers [13]. Based on these findings, the previously reported effects of CK_4-M2GlyR on epithelial monolayers [11] and its ability to induce a novel chloride conductance in isolated epithelial cells (described above) [10], the lysine-modified sequence was expected to form channels in planar lipid bilayers. In contrast to the parent compound, M2GlyR, the lysine-modified sequence, CK_4-M2GlyR, was expected to partition into the bilayer and form channels from an aqueous solution. In fact, channel activity was observed when CK_4-M2GlyR was introduced to the bilayer chamber at concentrations as low as 100 nM (Fig. 11). Importantly, channel activity in lipid bilayers was not observed with the scrambled permutation of the CK_4-M2GlyR sequence under a variety of ionic conditions and with a variety of lipid compositions ($n = 6$, data not shown). CK_4-M2GlyR forms channels that, like those formed by M2GlyR [13,14] and many channel-forming peptides, are 'noisy' (Fig. 11). In addition, the CK_4-M2GlyR current in planar lipid bilayers is time- and voltage-independent (Fig. 11), consistent with the properties of the CK_4-M2GlyR-induced current in isolated epithelial cells (Figs. 2 and 3). The ability of CK_4-M2GlyR to form channels in a synthetic lipid membrane supports the contention that this peptide is capable of spontaneous insertion into cell membranes where it assembles into channel-forming helical bundle structures thereby increasing whole-cell chloride conductance.

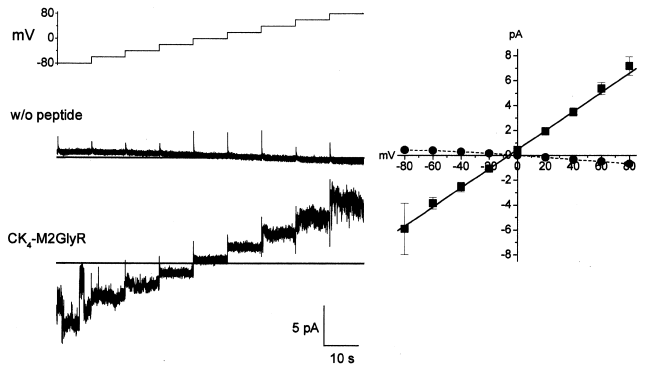

Fig. 11. CK_4-M2GlyR channel activity in planar lipid bilayers. Bilayer traces recorded for 10 s intervals during 20 mV steps from −80 mV to +80 mV in symmetric 0.1 M KCl. CK_4-M2GlyR was introduced to the *trans* (grounded) side of the bilayer chamber from an aqueous stock solution to obtain a final peptide concentration of 100 nM. The voltage protocol is shown in the upper panel. Bilayer recordings made in the presence and absence of CK_4-M2GlyR are shown in the lower and center panels, respectively. The solid line in these traces represents the zero current level. The *I-V* relation derived from the means and standard deviations of the bilayer currents recorded in the absence (circles, dashed line) and presence of CK_4-M2GlyR (square, solid line-obtained by linear regression) is shown on the right.

4. Discussion

Recent findings suggest not only that abnormal chloride transport across epithelial cells, which results from mutations in the CFTR protein, is implicated in the pathophysiology of CF, but also that the severity of CF disease is dictated, at least in part, by the level of residual chloride transport (non-CFTR-mediated) in affected tissues. For example, residual Cl^- secretion in CF patients was found to correlate with preserved pancreatic function and delayed presentation of the disease [5]. In addition, studies of the CFTR knockout mouse have revealed that the chloride transporters other than CFTR have a significant effect on the phenotype of these mice [6–8]. Restoration of chloride transport to tissues affected by the CF defect, particularly in the lung, may be a viable treatment for CF disease. This is the rationale for developing synthetic chloride-conducting channel-forming peptides as potential therapeutic agents.

Previously, it was demonstrated that synthetic peptides corresponding to the transmembrane M2 segment of the α-subunit of the brain glycine receptor

form anion-selective channels in phospholipid bilayers [12]. In addition, it was found that modifying the hydrophobic membrane-derived M2GlyR sequence by addition of four lysines to the C-terminus (CK_4-M2GlyR) results in a peptide with enhanced aqueous solubility that is capable of inducing transepithelial chloride and fluid secretion when added to the apical surface of MDCK monolayers [10]. Based on these findings [11], this peptide may be a candidate for introducing an alternative chloride permeation pathway in CF-affected tissues.

The goals of the present study were to determine whether CK_4-M2GlyR could exert effects on whole-cell chloride conductance in isolated epithelial cells, and if the observed effects were the result of formation of a novel chloride conductance pathway, modulation of endogenous chloride channel activity, or a combination of these effects. The major findings of this communication are that extracellular application of CK_4-M2GlyR to isolated MDCK, T84, and IB3-1 cells resulted in increased permeability of the cells to chloride. The experimental evidence suggests a direct mechanism of action.

For example, the lack of increase in whole-cell current in MDCK cells treated with scrambled CK_4-M2GlyR suggests that the poly-lysine tail of CK_4-M2GlyR does not activate endogenous CFTR channel activity in a manner similar to that observed with protamine in airway epithelial cells [14]. One potential caveat to the scrambled CK_4-M2GlyR as a control for electrostatic effects of the peptide is that its partitioning and membrane localization are different than for the α-helical native sequence. It is feasible that a transmembrane sequence is required for the electrostatic effect of the poly-lysine tail to activate CFTR. However, an idealized amphipath based on the CK_4-M2GlyR peptide retains the secondary structure of the native M2GlyR sequence but does not form channels in planar lipid bilayers and has no effect on I_{SC} in MDCK monolayers [38]. Taken together, these findings suggest that a poly-lysine modified α-helical peptide is not sufficient to activate endogenous CFTR by an electrostatic mechanism.

In addition, the ability of CK_4-M2GlyR to induce a time- and voltage-independent current in the IB3-1 cell line, which lacks functional CFTR, suggests that CK_4-M2GlyR does not increase chloride current by activating CFTR. The time- and voltage-independence of the CK_4-M2GlyR-induced current, as well as the pharmacological profile of the induced current, strongly suggest that CK_4-M2GlyR does not activate other endogenous chloride currents in the isolated epithelial cells, specifically: the hyperpolarization activated current associated with ClC-2, the swelling-activated current associated with VSOAC, the Ca^{2+}-dependent Cl^- current activated by CaM Kinase II. Together, these results support the premise that the M2GlyR peptides increase I_{SC} in epithelial monolayers by forming a novel permeation pathway for chloride rather than by activation of endogenous chloride conductances. Recordings of CK_4-M2GlyR-induced single channel events in isolated epithelial cells are needed to determine fully the electrophysiological properties of the channels formed by CK_4-M2GlyR in biological membranes.

It is particularly noteworthy that treatment of the immortalized airway cell line that carries the CFTR mutation (IB3-1) with CK_4-M2GlyR resulted in enhanced whole-cell chloride conductance. In terms of the eventual goal to use an M2GlyR peptide as a therapeutic treatment for CF, it is important to consider how the activity of the peptide channel would be regulated. We assume that the CK_4-M2GlyR current would respond to changes in chloride driving force and that this would be the level at which control would occur. In whole-cell studies, the CK_4-M2GlyR-induced chloride current increases in the inward direction when the external chloride concentration is lowered. This is the expected result for a chloride-selective current when the driving force for efflux of chloride has been increased. Furthermore, we have shown that CK_4-M2GlyR acts synergistically with EBIO to enhance chloride secretion across T84 monolayers [39]. EBIO activates a basolateral potassium channel, which in turn increases the activity of the basolateral $Na^+K^+2Cl^-$ cotransporter resulting in an increase in the driving force for chloride secretion [39]. In the presence of CK_4-M2GlyR at the apical surface and EBIO at the basolateral surface of T84 monolayers, chloride secretion was dramatically increased above that of untreated monolayers or monolayers treated with either agent alone. Taken together, these findings suggest that CK_4-M2GlyR-mediated chloride transport is regulated by changes in chloride driving force. Further studies will be required to determine whether regulation by changes in

Cl^- driving force alone is sufficient for the chloride secretion produced by CK_4-M2GlyR to act as an adequate replacement for CFTR-mediated chloride transport.

As expected, CK_4-M2GlyR retains the ability of its parent sequence, M2GlyR, to form channels in planar lipid bilayers. In contrast to the M2GlyR peptide, which must be delivered to planar bilayers in lipid vesicles or mixed with lipid prior to bilayer formation [12,13], CK_4-M2GlyR forms channels in planar lipid bilayers or isolated cells when applied as an aqueous solution. These properties, aqueous solubility and lipid partitioning, of the CK_4-M2GlyR peptide may be useful in developing this compound as a therapeutic agent; they suggest that delivery and insertion of the peptide into affected tissues may be feasible with an aerosolized or injectable solution.

Significantly, treatment of cells with CK_4-M2GlyR during whole-cell patch clamp or Ussing chamber experiments does not result in either seal breakdown or loss of monolayer resistance, respectively [11] as would be expected if the peptide had acute adverse effects on membrane integrity or cell viability. These findings along with studies in which CK_4-M2GlyR was found to have an insignificant effect on release of lactate dehydrogenase (a measure of cytotoxicity) or cell growth rates at concentrations up to 1 mM during 24 h treatments of MDCK, T84 or IB3 monolayers [40] suggest that the utility of the CK_4-M2GlyR peptide as a therapeutic agent in CF will not be limited by acute cytotoxicity.

In summary, this investigation shows for the first time that a synthetic channel forming peptide based on the sequence of an endogenous ion channel can spontaneously insert into the plasma membrane of an isolated mammalian cell, and increase measured whole-cell currents by forming a novel permeation pathway, without either perturbing existing constitutive conductance pathways or compromising cell viability. The feasibility of developing CK_4-M2GlyR and/or other synthetic channel forming peptides as therapeutic agents for treatment of diseases associated with ion channel defects is as yet uncertain. Further investigation of this potential is warranted, especially for diseases like CF where existing treatments are not completely safe nor effective.

Acknowledgements

We would like to acknowledge Dr. Garry R. Cutting (Johns Hopkins University School of Medicine) for the IB3-1 and anti-sense transfected hClC-2 IB3-1 cells; Dr. Kathleen Giangiacomo (Temple University) and Dr. Michael Fill (Loyola University, Chicago, IL) for their assistance in setting up a bilayer apparatus; Dr. James Hall (U.C. Irvine) for his thoughtful contributions to the experimental design and data analysis; Gary Radke for peptide purification. The work was supported by NIH Grants NIGMS-RO143617 (J.T.), F32GM19332 (K.M.), the Cystic Fibrosis Foundation (J.T.), the American Heart Association, Kansas Affiliate (KS-95-GB-7, LF) and the Kansas Agricultural Experimental Station (KES manuscript 98-410-J).

References

[1] J.R. Riordan, J.M. Rommens, B. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J.L. Chou, Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA, Science 245 (1989) 1066–1073.

[2] H.A. Berger, M.P. Anderson, R.J. Gregory, S. Thompson, P.W. Howard, R.A. Maurer, R. Mulligan, A.E. Smith, M.J. Welsh, Identification and regulation of the cystic fibrosis transmembrane conductance regulator-generated chloride channel, J. Clin. Invest. 88 (1991) 1422–1431.

[3] G.M. Denning, L.S. Ostedgaard, S.H. Cheng, A.E. Smith, M.J. Welsh, Localization of cystic fibrosis transmembrane conductance regulator in chloride secretory epithelia, J. Clin. Invest. 89 (1992) 339–349.

[4] J.R. Riordan, The cystic fibrosis transmembrane conductance regulator, Annu. Rev. Physiol. 55 (1993) 609–630.

[5] H.J. Veeze, D.J. Halley, J. Bijman, J.C. de Jongste, H.R. De Jonge, M. Sinaasappel, Determinants of mild clinical symptoms in cystic fibrosis patients. Residual chloride secretion measured in rectal biopsies in relation to the genotype, J. Clin. Invest. 93 (1994) 461–466.

[6] R. Rozmahel, M. Wilschanski, A. Matin, S. Plyte, M. Oliver, W. Auerbach, A. Moore, J. Forstner, P. Durie, J. Nadeau, C. Bear, L.C. Tsui, Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor, Nat. Genet. 12 (1996) 280–287.

[7] L.L. Clarke, B.R. Grubb, J.R. Yankaskas, C.U. Cotton, A. McKenzie, R.C. Boucher, Relationship of a non-cystic fibrosis transmembrane conductance regulator-mediated chloride

conductance to organ-level disease in Cftr(−/−) mice, Proc. Natl. Acad. Sci. USA 91 (1994) 479–483.
[8] G. Kent, R. Iles, C.E. Bear, L.J. Huan, U. Griesenbach, C. McKerlie, H. Frndova, C. Ackerley, D. Gosselin, D. Radzioch, H. O'Brodovich, L.C. Tsui, M. Buchwald, A.K. Tanswell, Lung disease in mice with cystic fibrosis, J. Clin. Invest. 100 (1997) 3060–3069.
[9] D.P. Wallace, J.J. Grantham, L.P. Sullivan, Chloride and fluid secretion by cultured human polycystic kidney cells, Kidney Int. 50 (1996) 1327–1336.
[10] J.M. Tomich, D. Wallace, K. Henderson, K.E. Mitchell, G. Radke, R. Brandt, C.A. Ambler, A.J. Scott, J. Grantham, L. Sullivan, T. Iwamoto, Aqueous solubilization of transmembrane peptide sequences with retention of membrane insertion and function (in process citation), Biophys. J. 74 (1998) 256–267.
[11] D.P. Wallace, J.M. Tomich, T. Iwamoto, K. Henderson, J.J. Grantham, L.P. Sullivan, A synthetic peptide derived from glycine-gated Cl- channel induces transepithelial Cl- and fluid secretion, Am. J. Physiol. 272 (1997) C1672–C1679.
[12] D. Langosch, K. Hartung, E. Grell, E. Bamberg, H. Betz, Ion channel formation by synthetic transmembrane segments of the inhibitory glycine receptor - a model study, Biochim. Biophys. Acta 1063 (1991) 36–44.
[13] G.L. Reddy, T. Iwamoto, J.M. Tomich, M. Montal, Synthetic peptides and four-helix bundle proteins as model systems for the pore-forming structure of channel proteins. II. Transmembrane segment M2 of the brain glycine receptor is a plausible candidate for the pore-lining structure, J. Biol. Chem. 268 (1993) 14608–14615.
[14] M.D. Smith, C.M. Penland, M.R. Van Scott, Differential effects of polycationic protein on Cl^- secretory and Na^+ absorptive airways, Am. J. Physiol. 272 (1997) L762–L771.
[15] M.R. Van Scott, M.D. Smith, C.A. Welch, C. Bentzel, W.J. Metzger, Bioelectric response of human nasal epithelial cells to polycationic protein, Am. J. Physiol. 271 (1996) L159–L165.
[16] S.A. Cunningham, M.S. Awayda, J.K. Bubien, I.I. Ismailov, M.P. Arrate, B.K. Berdiev, D.J. Benos, C.M. Fuller, Cloning of an epithelial chloride channel from bovine trachea, J. Biol. Chem. 270 (1995) 31016–31026.
[17] T. Begenisich, J.E. Melvin, Regulation of chloride channels in secretory epithelia, J. Membr. Biol. 163 (1998) 77–85.
[18] K.E. Mitchell, J.M. Tomich, T. Iwamoto, L.C. Freeman, Synthetic peptides derived from the brain glycine receptor form channels in planar lipid bilayers and increase chloride conductance in MDCK cells, Biophys. J. 74 (1998) A388.
[19] K.E. Mitchell, J. Broughman, L.C. Freeman, J.M. Tomich, Synthetic channel-forming peptides increase whole cell chloride conductance in IB3-1 cells, Biophys. J. 76 (1999) A445.
[20] J.J. Grantham, M. Uchic, E.J.J. Cragoe, J. Kornhaus, J.A. Grantham, V. Donoso, R. Mangoo-Karim, A. Evan, J. McAteer, Chemical modification of cell proliferation and fluid secretion in renal cysts, Kidney Int. 35 (1989) 1379–1389.
[21] P.L. Zeitlin, L. Lu, J. Rhim, G. Cutting, G. Stetten, K.A. Kieffer, R. Craig, W.B. Guggino, A cystic fibrosis bronchial epithelial cell line: immortalization by adeno-12-SV40 infection, Am. J. Respir. Cell Mol. Biol. 4 (1991) 313–319.
[22] E.M. Schwiebert, L.P. Cid-Soto, D. Stafford, M. Carter, C.J. Blaisdell, P.L. Zeitlin, W.B. Guggino, G.R. Cutting, Analysis of ClC-2 channels as an alternative pathway for chloride conduction in cystic fibrosis airway cells, Proc. Natl. Acad. Sci. USA 95 (1998) 3879–3884.
[23] O.P. Hamill, A. Marty, E. Neher, B. Sakmann, F.J. Sigworth, Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches, Pflug. Arch. 391 (1981) 85–100.
[24] E.M. Schwiebert, M.E. Egan, T.H. Hwang, S.B. Fulmer, S.S. Allen, G.R. Cutting, W.B. Guggino, CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP, Cell 81 (1995) 1063–1073.
[25] P. Mueller, D. Rudin, T.H. Tien, W.C. Westcott, Reconstitution of excitable membrane structures in vitro, Circulation 26 (1962) 1167–1171.
[26] H.L. Ji, M.D. DuVall, H.K. Patton, C.L. Satterfield, C.M. Fuller, D.J. Benos, Functional expression of a truncated Ca^{2+}-activated Cl^- channel and activation by phorbol ester, Am. J. Physiol. 274 (1998) C455–C464.
[27] M.P. Anderson, M.J. Welsh, Calcium and cAMP activate different chloride channels in the apical membrane of normal and cystic fibrosis epithelia, Proc. Natl. Acad. Sci. USA 88 (1991) 6003–6007.
[28] R.J. Gregory, S.H. Cheng, D.P. Rich, J. Marshall, S. Paul, K. Hehir, L. Ostedgaard, K.W. Klinger, M.J. Welsh, A.E. Smith, Expression and characterization of the cystic fibrosis transmembrane conductance regulator, Nature 347 (1990) 382–386.
[29] C.M. Fuller, D.J. Benos, CFTR!, Am. J. Physiol. 263 (1992) C267–C286.
[30] L.P. Cid, C. Montrose-Rafizadeh, D.I. Smith, W.B. Guggino, G.R. Cutting, Cloning of a putative human voltage-gated chloride channel (CIC-2) cDNA widely expressed in human tissues, Hum. Mol. Genet. 4 (1995) 407–413.
[31] T.D. Bond, C.F. Higgins, M.A. Valverde, P-glycoprotein and swelling-activated chloride channels, Methods Enzymol. 292 (1998) 359–370.
[32] G. Roy, U. Banderali, Channels for ions and amino acids in kidney cultured cells (MDCK) during volume regulation, J. Exp. Zool. 268 (1994) 121–126.
[33] F. Lang, M. Paulmichl, Properties and regulation of ion channels in MDCK cells, Kidney Int. 48 (1995) 1200–1205.
[34] A.P. Braun, H. Schulman, A non-selective cation current activated via the multifunctional Ca^{2+}-calmodulin-dependent protein kinase in human epithelial cells, J. Physiol. (Lond.) 488 (1995) 37–55.
[35] R.T. Worrell, R.A. Frizzell, CaMKII mediates stimulation of chloride conductance by calcium in T84 cells, Am. J. Physiol. 260 (1991) C877–C882.
[36] J. Arreola, J.E. Melvin, T. Begenisich, Differences in regulation of Ca^{2+}-activated Cl^- channels in colonic and parotid secretory cells, Am. J. Physiol. 274 (1998) C161–C166.

[37] K. Staley, R. Smith, J. Schaack, C. Wilcox, T.J. Jentsch, Alteration of GABAA receptor function following gene transfer of the CLC-2 chloride channel, Neuron 17 (1996) 543–551.

[38] Z. Tavakkol, K.E. Mitchell, C.A. Ambler, J. Stucky, T. Iwamoto, J.M. Tomich, Biophysical characterization of M2GlyR peptides in lipid and aqueous environments, Biophys. J. A74 (1998) 388.

[39] D.P. Wallace, J.M. Tomich, J. Eppler, T. Iwamoto, J. Grantham, L. Sullivan, A synthetic channel-forming peptide induces Cl- secretion: modulation by Ca^{2+}-dependent K^{+} channels. Biochim. Biophys. Acta, in press.

[40] B. Drotos, K.E. Mitchell, J.M. Tomich, L.C. Freeman, Cytotoxic effects of synthetic channel-forming peptides on MDCK, T84 and IB3 cell lines, Biophys. J. A76 (1999) 442.