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A synthetic channel-forming peptide induces Cl^- secretion: modulation by Ca^{2+}-dependent K^+ channels

Darren P. Wallace [a,*], John M. Tomich [c], Jason W. Eppler [a], Takeo Iwamoto [c], Jared J. Grantham [b], Lawrence P. Sullivan [a]

[a] *Department of Molecular and Integrative Physiology, University of Kansas Medical Center, 3901 Rainbow Blvd. Kansas City, KS 66160-7401, USA*

[b] *Department of Medicine, and Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS 66160, USA*

[c] *Department of Biochemistry, Kansas State University, Manhattan, KS 66506, USA*

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Abstract

A synthetic Cl^- channel-forming peptide, C-K4-M2GlyR, applied to the apical membrane of human epithelial cell monolayers induces transepithelial Cl^- and fluid secretion. The sequence of the core peptide, M2GlyR, corresponds to the second membrane-spanning region of the glycine receptor, a domain thought to line the pore of the ligand-gated Cl^- channel. Using a pharmacological approach, we show that the flux of Cl^- through the artificial Cl^- channel can be regulated by modulating basolateral K^+ efflux through Ca^{2+}-dependent K^+ channels. Application of C-K4-M2GlyR to the apical surface of monolayers composed of human colonic cells of the T84 cell line generated a sustained increase in short-circuit current (I_{SC}) and caused net fluid secretion. The current was inhibited by the application of clotrimazole, a non-specific inhibitor of K^+ channels, and charybdotoxin, a potent inhibitor of Ca^{2+}-dependent K^+ channels. Direct activation of these channels with 1-ethyl-2-benzimidazolinone (1-EBIO) greatly amplified the Cl^- secretory current induced by C-K4-M2GlyR. The effect of the combination of C-K4-M2GlyR and 1-EBIO on I_{SC} was significantly greater than the sum of the individual effects of the two compounds and was independent of cAMP. Treatment with 1-EBIO also increased the magnitude of fluid secretion induced by the peptide. The cooperative action of C-K4-M2GlyR and 1-EBIO on I_{SC} was attenuated by Cl^- transport inhibitors, by removing Cl^- from the bathing solution and by basolateral treatment with K^+ channel blockers. These results indicate that apical membrane insertion of Cl^- channel-forming peptides such as C-K4-M2GlyR and direct activation of basolateral K^+ channels with benzimidazolones may coordinate the apical Cl^- conductance and the basolateral K^+ conductance, thereby providing a pharmacological approach to modulating Cl^- and fluid secretion by human epithelia deficient in cystic fibrosis transmembrane conductance regulator Cl^- channels.

Keywords: Chloride channel; Cystic fibrosis; Calcium-activated potassium channel

1. Introduction

Chloride and fluid secretion is a fundamental process for many epithelia including those lining the airway and intestine. In the hereditary disease cystic

* Corresponding author. Fax: +1-913-588-7430; E-mail: dwallace@kumc.edu

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fibrosis (CF), a mutation in the gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel diminishes the apical Cl^- conductance of transporting cells, thereby impairing Cl^- and fluid secretion by many of the secretory epithelia [1–5]. We have devised a novel approach to ameliorate the disease by restoring the Cl^- conductance through the insertion into the membrane of transporting cells a synthetic Cl^- channel-forming peptide derived from the pore region of a non-epithelial Cl^- channel.

M2GlyR, a synthetic peptide, mimics the 23 amino acid sequence of the second membrane-spanning region of the α-subunit of the glycine receptor found in the post-synaptic membranes of neurons in the brain and spinal cord. Reddy et al. reported that this peptide inserts into synthetic lipid bilayers and associates to form a four or five peptide multimer with Cl^- conductances of 25 and 49 pS; conductances similar to the native glycine receptor [6]. We have modified their original sequence by adding a 'solubilizing tail', consisting of four lysine residues, to the carboxy-terminus to form C-K4-M2GlyR. These positively charged residues improved the peptide's water solubility and increased the bioactivity of the peptide [7]. Application of the peptide to the apical surface of Madin–Darby canine kidney (MDCK) cell monolayers induced a sustained increase in short-circuit current (I_{SC}) and fluid secretion that was independent of the activation of the adenosine 3′,5′-cyclic monophosphate (cAMP) pathway [8]. The application of the peptide is associated with the appearance of an anion conductance with novel characteristics that differentiate it from endogenous conductances such as CFTR, Ca^{2+}-dependent Cl^- channels, ClC-2 channels and volume-sensitive organic osmolyte and anion channels [9]. The effects of the peptide are specific since the application of a scrambled sequence of the M2GlyR peptide containing the C-terminal lysine residues had no effect on whole cell current [9], Cl^- secretory current or net fluid movement [8].

The Cl^- current induced by C-K4-M2GlyR was accompanied by net secretion of fluid. This secretory current was inhibited by a variety of Cl^- channel blockers, bumetanide and by removing external Cl^- [8]. Thus, the anion conductance generated by the membrane insertion of C-K4-M2GlyR appears to functionally mimic the effect of activating endogenous Cl^- conductances, such as CFTR.

In the current study, we examined the effect of C-K4-M2GlyR on the human colonic cell line, T84, and determined if Cl^- secretion generated by the peptide could be modulated by pharmacological treatment of a basolateral K^+ channel opener, 1-ethyl-2-benzimidazolinone (1-EBIO). We chose to investigate this process in the T84 cell line since these cells originated from a human epithelium that is commonly affected in CF and is a classical cell line for the investigation of endogenous Cl^- channels involved in transepithelial Cl^- secretion. The transport properties of these cells have been extensively examined and found to have many of the mechanisms involved in salt and fluid secretion in the airway and intestine [3,10–14]. A clear advantage of the T84 cells over the MDCK cell line is that, in T84 cells, the secretion of Cl^- through activated apical Cl^- channels can be modulated by directly activating the basolateral K^+ conductance [12,15,16].

2. Materials and methods

2.1. Cell culture protocol

T84 cells were originally obtained from the American Type Culture Collection and grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (DME/F12; JRH Biosciences, Lenexa, KS) supplemented with 5% fetal bovine serum (FBS; HyClone, Logan, UT), 100 IU/ml penicillin G and 0.1 mg/ml streptomycin (P/S). Cells were maintained as a subconfluent monolayer on plastic in a humidified atmosphere containing 5% CO_2/95% room air at 37°C until harvested by trypsinization.

2.2. Electrical measurements

T84 cells (2.5×10^5) were plated on individual permeable supports (Snapwell, 12 mm diameter; Costar, Cambridge, MA) and incubated in DME/F12+5% FBS. After 5–8 days of growth, the Snapwell supports containing the confluent cell monolayers were mounted in Ussing chambers (Navicyte Inc., Sparks, NV), and bathed in a Ringer's solution containing (in mM): 147 Na^+, 119 Cl^-, 20 HCO_3^-, 6 alanine,

5 K^+, 5 acetate, 5 glucose, 4 lactate, 2.5 HPO_4^-, 1.2 Mg^{2+}, 1.2 SO_4^{2-}, 1 citrate, 0.5 butyric acid and 14 raffinose equilibrated with 5% CO_2/95% O_2. In experiments in which we removed Cl^- from the bathing solution, Cl^- was replaced by the molar equivalent of cyclamate. The transepithelial potential (V_{te}), short-circuit current (I_{SC}; the current necessary to clamp the voltage to zero) and transepithelial resistance (R_{te}) were measured at 5 min intervals with a dual epithelial voltage clamp apparatus (Warner Instrument, Hamden, CT) as previously described in detail [8,17]. T84 cell monolayers developed resistances from 0.5 to 10 kΩ·cm^2. The tissue was continuously short-circuited except for measurement of V_{te} and R_{te}.

2.3. Measurement of fluid transport

T84 cells were plated onto permeable cell culture supports (Transwell-Col, 24.5 mm diameter; Costar, Cambridge, MA) at a seeding density of 8×10^5 cells/Transwell and grown in DME/F12 containing 1% FBS for 5–7 days. The procedure for measuring fluid transport across epithelial cell monolayers has been previously described [17,18]. Briefly, the fluid bathing the apical surface of the monolayers (upper chamber of the Transwell) was removed and 200 μl of fresh DME/F12 containing 1% FBS was placed on the apical surface. The apical fluid was covered with 1.5 ml of sterile, water-saturated mineral oil to prevent fluid evaporation. The Transwells were placed in 6-well culture plates containing 2.5 ml of basolateral medium. After 12 h, the fluid and mineral oil on the apical surface were collected and the monolayers were rinsed with fresh mineral oil which was recollected to remove any residual fluid. The fluid and oil mixture was centrifuged to separate the two layers, and the fluid droplet at the bottom of the test tube was measured using calibrated microcapillary tubes (Drummond, Broomall, PA). The rate of fluid transport was calculated from the change in apical fluid volume during the experimental period and expressed in nl/h/cm^2. In order to eliminate possible Na^+ absorption via Na^+ channels, amiloride (10 μM) was added to the apical media in all fluid transport experiments. C-K4-M2GlyR was added only to the apical medium and 1-EBIO was added only to the basolateral medium.

2.4. Peptide synthesis

The synthesis of C-K4-M2GlyR has been previously described [7,8]. Briefly, the peptide was synthesized using solid-phase peptide synthesis employing an automated base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) strategy with a *p*-hydroxymethylphenoxymethyl polystyrene-lysine resin (Applied Biosystems model 431A peptide synthesizer; Perkin-Elmer, Norwalk, CT). The peptide was assembled by the successive step-wise repetition of deprotection of the amino acid, activation by a condensing agent, and coupling the amino acid to the resin. Once the entire sequence was assembled, the peptide was cleaved from the resin, extracted and dried by lyophilization. The peptide was purified by reverse-phase HPLC and the correct sequence was confirmed using a protein sequencer (Applied Biosystems; Perkin-Elmer). The amino acid sequence of C-K4-M2GlyR is PARVGLGITTVLTMTTQSSGSRAKKKK.

2.5. cAMP measurements

Confluent T84 cell monolayers were incubated in defined media or media containing either 500 μM apical C-K4-M2GlyR, 600 μM basolateral 1-EBIO or a combination of C-K4-M2GlyR and 1-EBIO for 1 h. Monolayers were rinsed in Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline. Trichloroacetic acid (TCA) was used to deactivate the endogenous phosphodiesterases. TCA was removed by extraction with a mixture of water and ether. cAMP was collected in the water phase and concentrations were determined by radioimmunoassay (Biomedical Technology, Stoughton, MA).

2.6. Pharmacological agents

In electrophysiological studies, clotrimazole, 1-EBIO (Aldrich Chemical Co., Milwaukee, WI) and amiloride were dissolved in dimethyl sulfoxide (DMSO) as 1000-fold stock solutions. Forskolin, A23187 and niflumic acid were dissolved in ethanol as 1000-fold stock solutions. The maximal final concentration of DMSO (0.1%) or ethanol (0.1%) was without effect on I_{SC}. Charybdotoxin (CTX; Alomone, Jerusulem, Israel) was made up as a 10 μM

stock in bathing solution containing 0.1% bovine serum albumin, to prevent non-specific binding to the glassware [19]. All medium additives and drugs were purchased from Sigma Chemical Co. (St. Louis, MO), unless noted otherwise.

2.7. Statistical analysis

Data are presented as means ± S.E.M. Where appropriate, Student's *t*-test was used to determine statistical significance. Sequential measurements of the I_{SC}, V_{te}, and R_{te} were compared by repeated measures analysis of variance (ANOVA) and the Student–Newman–Keuls (S-N-K) multiple comparison post test. Measurements of fluid transport and intracellular cAMP were compared by ordinary ANOVA and the S-N-K post test. Significant effects or changes were considered to have occurred when $P < 0.05$.

3. Results

3.1. Effect of C-K4-M2GlyR on electrical measurements

A typical effect of C-K4-M2GlyR on the electrical properties of a T84 monolayer is illustrated in Fig. 1. The application of 100 μM C-K4-M2GlyR to the apical surface of the monolayer increased positive I_{SC} (apical to basolateral) during a 40 min exposure. I_{SC} began to increase 10 min after the addition of the peptide and by 30 min I_{SC} reached a steady level that was 3.9 μA/cm^2 above the control current. The apically negative V_{te} hyperpolarized by −7.2 mV, following the same time course as the change in I_{SC}. The resistance across the monolayer (R_{te}) decreased to 50% of the control value with the addition of the peptide. The results of 29 such experiments are summarized in Table 1. The addition of 100 μM C-K4-M2GlyR to the medium bathing the apical surface of the monolayers increased I_{SC} five-fold, while V_{te} rose by 136% and R_{te} decreased 54%. Prior treatment of five T84 monolayers with 10 μM amiloride, a Na^+ channel blocker, did not significantly alter the baseline I_{SC} and had no effect on the magnitude of the secretory response induced by C-K4-M2GlyR.

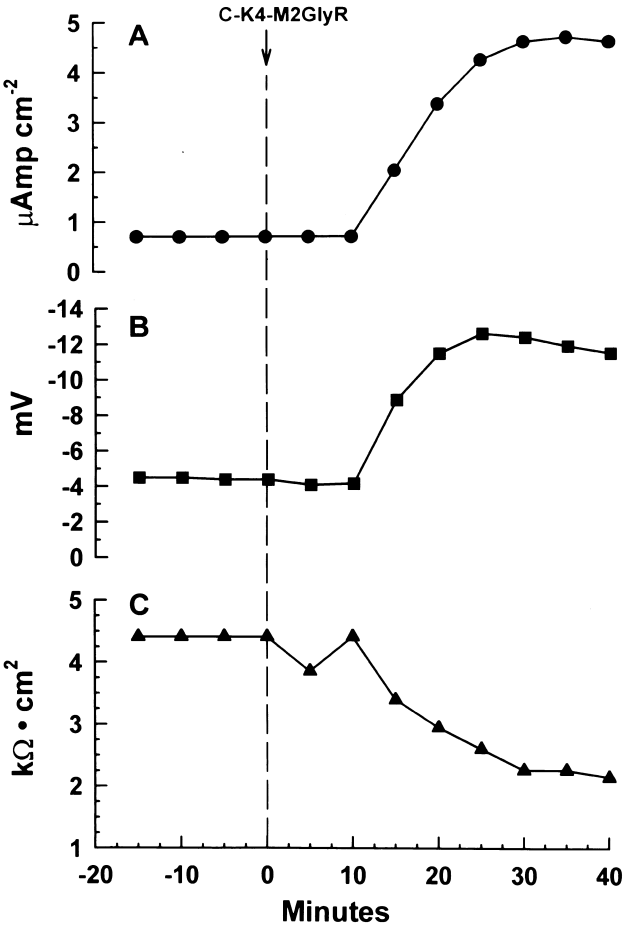

Fig. 1. Effect of 100 μM C-K4-M2GlyR added to the apical medium on the electrical properties of a T84 cell monolayer. (A) I_{SC}. (B) V_{te}. (C) R_{te}. A summary of the effects of C-K4-M2GlyR is presented in Table 1.

Table 1
Effect of C-K4-M2GlyR on the electrical measurements across T84 monolayers

	I_{SC} (μA/cm^2)	V_{te} (mV)	R_{te} (kΩ·cm^2)
Control	1.0 ± 0.1	−5.2 ± 0.7	4.56 ± 0.47
100 μM C-K4-M2GlyR	4.6 ± 0.2*	−12.3 ± 1.2*	2.50 ± 0.21†

Values are means ± S.E.M.; $n = 29$. *Comparison to the control period, $P < 0.0001$; $^\dagger P < 0.05$. C-K4-M2GlyR (100 μM) was added to the apical media in all experiments.

The increase in I_{SC} caused by C-K4-M2GlyR was compared to the current generated by activating endogenous cAMP-dependent anion secretion. In ten monolayers, 10 μM forskolin, an adenylate cyclase agonist, increased I_{SC} by 4.0 ± 0.6 μA/cm^2, ($P < 0.0001$). Thus, the current induced by the application of the Cl^- channel-forming peptide, C-K4-M2GlyR, was approximately 90% of the forskolin-stimulated current in T84 cells. In other experiments ($n = 2$), I_{SC} was monitored for five h after the application of C-K4-M2GlyR. During the first hour, I_{SC} increased from 0.8 to 3.4 μA/cm^2. This current gradually decreased to 3.0 μA/cm^2 during the second h, then remained steady for the remaining three h at a value that was 70% of the current measured at one h. These data demonstrate that C-K4-M2GlyR induces changes in I_{SC}, V_{te}, and R_{te} that would be expected from insertion of an anion conductance into the apical membrane.

3.2. Inhibition of C-K4-M2GlyR-induced I_{SC} by K^+ channel blockers

Two classes of K^+ channels have been shown to participate in the secretory process in T84 cells. One K^+ conductance is activated by intracellular cAMP and is sensitive to Ba^{2+}. The other K^+ channel is activated by an elevation in intracellular Ca^{2+}, is insensitive to Ba^{2+}, and is potently inhibited by charybdotoxin (CTX) [14,16,20,21]. Clotrimazole, an antimycotic agent, has been shown to inhibit both populations of K^+ channels in T84 cells, but with different affinities. The apparent inhibition constant (K_i) for the Ca^{2+}-dependent K^+ current was 0.3 μM, whereas the apparent K_i for the cAMP-mediated K^+ conductance was 5.2 μM [12]. We first used clotrimazole to evaluate the K^+ conductive pathway involved in the secretory response mediated by the application of C-K4-M2GlyR (Fig. 2). Either 100 μM C-K4-M2GlyR was added to the apical surface or 10 μM forskolin was added to the basolateral surface of four pairs of T84 monolayers. When a steady-state response was achieved (~40 min), increasing concentrations of clotrimazole were added to the basolateral media and the degree of inhibition of the stimulated I_{SC} was determined (Fig. 2). The concentration of clotrimazole that inhibited 50% of the current (IC_{50}) induced by C-K4-M2GlyR was

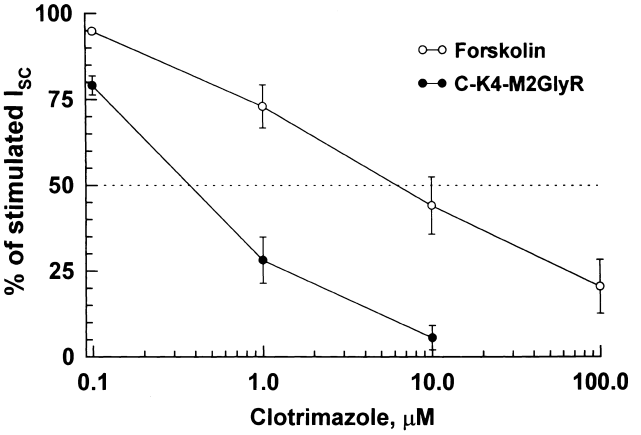

Fig. 2. Effect of varying concentrations of clotrimazole on I_{SC} in T84 cell monolayers stimulated with 10 μM basolateral forskolin (○) or 100 μM apical C-K4-M2GlyR (●). The increase in I_{SC} caused by either forskolin or the peptide was assigned to 100%. Concentration of clotrimazole added to the basolateral medium was sequentially increased after the response to the previous addition had reached a steady state; $n = 4$ pairs of monolayers. Data represent means ± S.E.M. Dashed line, 50% of the stimulated current. The concentration of clotrimazole required to inhibited 50% of the current generated by the peptide was lower (apparent $IC_{50} = 0.4$ μM) than the concentration required to inhibit 50% of the current stimulated by forskolin (apparent $IC_{50} = 6$ μM).

approximately 0.4 μM. A higher concentration of clotrimazole was required to inhibit the forskolin-stimulated current (approximate $IC_{50} = 6$ μM).

In other experiments ($n = 6$), we tested the effect of CTX on the I_{SC} response to C-K4-M2GlyR. In the control monolayers, the application of 100 μM C-K4-M2GlyR to the apical medium increased I_{SC} by 2.0 ± 0.1 μA/cm^2 ($P < 0.001$) (Fig. 3). In a paired group of monolayers treated with 50 nM CTX on the basolateral surface, the addition of the peptide increased I_{SC} by only 0.9 ± 0.2 μA/cm^2. Thus, CTX reduced the response of the peptide to 42% of that generated by the control group ($P < 0.001$). These data suggest that the Ca^{2+}-activated K^+ channel participates in the secretory response to C-K4-M2GlyR, apparently by maintaining the electrical gradient driving Cl^- efflux.

3.3. Effect of 1-EBIO

Recently a benzimidazolone, 1-EBIO, has been evaluated as a novel tool for inducing Cl^- secretion by T84 cells. Using cell monolayers, membrane

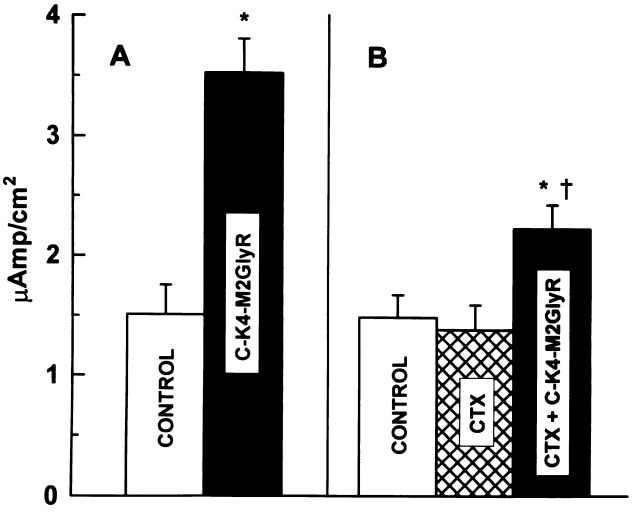

Fig. 3. Effect of basolateral application of charybdotoxin on I_{SC} induced by C-K4-M2GlyR and 1-EBIO. $n=6$ pairs of monolayers. (A) The effect of apical application of 100 μM C-K4-M2GlyR on I_{SC} in the control monolayers. (B) The effect of basolateral addition of 50 nM CTX and the subsequent application of C-K4-M2GlyR. *$P<0.001$ compared with the previous period; †$P<0.001$ compared with the C-K4-M2GlyR period in the control group.

vesicles, and excised inside-out patches of T84 cell membranes, Devor and associates demonstrated that 1-EBIO stimulates Cl^- secretion via the direct activation of the Ca^{2+}-dependent K^+ channels [16]. This K^+ conductance was potently inhibited by CTX and clotrimazole [12,16]. In the current study, the addition of 600 μM 1-EBIO to both the apical and basolateral media caused a rapid increase in I_{SC} from 1.3 ± 0.2 to 5.0 ± 0.8 μA/cm^2, ($P<0.05$) within 5 min. The effect remained constant during the 20 min period. V_{te} hyperpolarized from -4.2 ± 0.4 to -13.4 ± 1.7 mV, ($P<0.001$) and R_{te} decreased from 2.91 ± 0.17 to 2.39 ± 0.06 kΩ·cm^2 ($P<0.001$).

To determine if the direct activation of K^+ channels would alter the secretory response induced by the insertion of exogenous Cl^- channels, we treated the apical surface of six T84 cell monolayers with C-K4-M2GlyR, then added various concentrations of 1-EBIO to the basolateral medium only (Fig. 4). Each data point represents the increase in I_{SC} above the control value (ΔI_{SC}). The first data point (C) is the average change in I_{SC} obtained with apical application of C-K4-M2GlyR. I_{SC} recorded with subsequent additions of 1-EBIO (10 μM to 1 mM) are plotted against the log scale of the 1-EBIO concentration. The addition of 100 μM C-K4-M2GlyR increased I_{SC} by 4.1 ± 0.7 μA/cm^2. Subsequent additions of 1-EBIO increased I_{SC} above the effect of the peptide in a dose-dependent manner. A significant increase in peptide-induced I_{SC} was recorded for concentrations at and above 100 μM 1-EBIO.

The changes in the electrical properties of 16 monolayers exposed to 1-EBIO and C-K4-M2GlyR are summarized in Table 2. Basolateral addition of 600 μM 1-EBIO induced a small increase in I_{SC} and doubled V_{te}; only a nominal decrease in R_{te} was observed. The apical application of 100 μM C-K4-M2GlyR to the 1-EBIO-treated monolayers caused an eight-fold increase in I_{SC} from 2.1 ± 0.2 to 16.5 ± 0.9 μA/cm^2 ($P<0.001$), hyperpolarized V_{te} by -7.3 ± 0.5 ($P<0.001$) and reduced R_{te} by 1.53 ± 0.33 kΩ·cm^2 ($P<0.001$). In two of these experiments, the duration of the response of 1-EBIO and C-K4-M2GlyR was monitored for 5 h. I_{SC} remained relatively constant during the first 60 min; however, I_{SC} had decreased to 73% of this current at 2 h, 53% at 3 h, 41% at 4 h and 29% of the current at 5 h. In washout experiments ($n=2$), the current generated by the additions of 1-EBIO and C-K4-M2GlyR was monitored until the effect reached a steady state, then C-K4-M2GlyR was washed out

Table 2
Changes in electrical properties of T84 monolayers induced by 1-EBIO and C-K4-M2GlyR

	I_{SC} (μA/cm^2)	V_{te} (mV)	R_{te} (kΩ·cm^2)
Control	0.8 ± 0.1	-2.9 ± 0.6	2.68 ± 0.47
600 μM 1-EBIO	2.1 ± 0.2†	-6.2 ± 1.3*	2.27 ± 0.35†
100 μM C-K4-M2GlyR+1-EBIO	16.5 ± 0.9*	-13.5 ± 1.1*	0.74 ± 0.06*

Values are means ± S.E.M.; $n=16$. In all experiments, 1-EBIO was added to the basolateral medium and C-K4-M2GlyR was added to the apical medium. *Significant difference compared to previous period as determined by repeated measures ANOVA and S-N-K multiple comparison post test, $P<0.001$. †Not significant by ANOVA; however, in a paired Student t-test compared with the control value, $P<0.0001$.

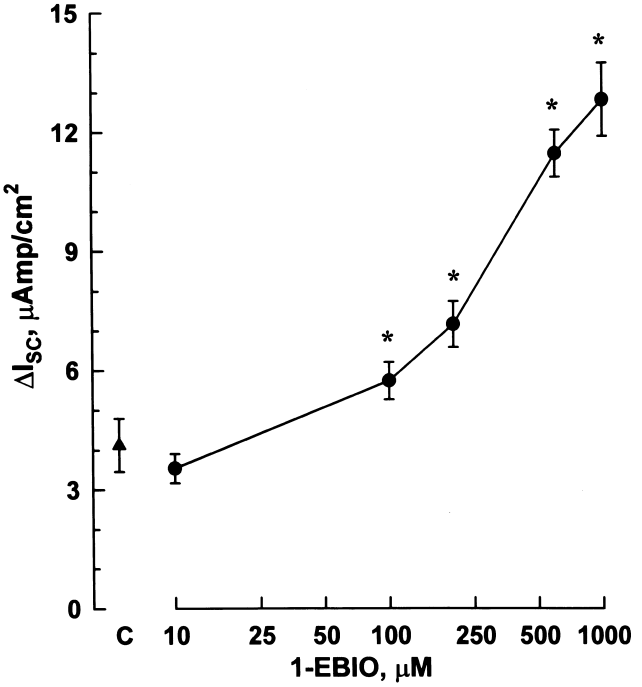

Fig. 4. Effect of varying concentrations of 1-EBIO on I_{SC} induced by C-K4-M2GlyR. Subsequent to the increase in I_{SC} generated by the addition of 100 μM C-K4-M2GlyR (C), increasing concentrations of 1-EBIO were added to the basolateral medium in a step-wise fashion following a stable response to the previous addition. Data represents mean ± S.E.M. $n=6$ monolayers. *Significant difference compared to the addition of C-K4-M2GlyR alone (C), determined by repeated measures ANOVA and S-N-K multiple comparison post test, $P<0.05$.

of the chamber. The removal of C-K4-M2GlyR from the medium decreased I_{SC} by 39%, whereas the subsequent removal of 1-EBIO decreased the current to control level.

3.4. Intracellular cAMP measurements

We determined whether the response of C-K4-M2GlyR and 1-EBIO on I_{SC} was mediated through the activation of endogenous mechanisms dependent on the generation of cAMP. Intracellular cAMP was measured by radioimmunoassay in groups of five T84 monolayers treated with either control media, 500 μM apical C-K4-M2GlyR, 600 μM 1-EBIO and C-K4-M2GlyR, or 10 μM forskolin for 1 h. Intracellular cAMP levels in the T84 monolayers treated with either C-K4-M2GlyR or the combination of 1-EBIO and C-K4-M2GlyR (10.6 ± 0.8 and 13.8 ± 1.1 pg/monolayer, respectively) were not significantly different from the level measured in the control monolayers (6.5 ± 0.6 pg/monolayer). The addition of 10 μM forskolin increased the level of cAMP to 80.5 ± 7.2 pg/monolayer ($P<0.001$). These data indicate that forskolin stimulates Cl^- secretion by T84 cells through the activation of a cAMP pathway, consistent with previous reports [10–13,15]. However, the current induced by the application of the peptide and 1-EBIO was independent of the cAMP-dependent pathway.

3.5. Effect of changes in cell calcium levels

To determine if an elevation in cell calcium is involved in the response of T84 cells to C-K4-M2GlyR and 1-EBIO, we examined the effect of increasing intracellular Ca^{2+} levels with the Ca^{2+} ionophore A23187 on steady-state current and the current generated by the peptide and 1-EBIO. The average baseline current for the four monolayers was 2.9 ± 0.1 μA/cm². Two of the monolayers (control group) were sequentially treated with C-K4-M2GlyR (100 μM) and 1-EBIO (600 μM) while the other two monolayers were treated with the Ca^{2+} ionophore A23187 (1 μM) for 20 min prior to the addition of the peptide and 1-EBIO. Treatment with A23187 did not alter steady-state I_{SC} (average increase was 0.1 μA/cm²) or the response to C-K4-M2GlyR (I_{SC} increased to an average of 4.2 versus 4.3 μA/cm² in the control group). However, the response to a subsequent addition of 1-EBIO was augmented by 76% by the prior treatment with A23187 (I_{SC} increased to an average of 28.7 versus 16.3 μA/cm² in the control group). Thus a rise in cell Ca^{2+} concentration failed to produce a sustained increase in I_{SC} by itself, indicating that the current generated by the peptide and C-K4-M2GlyR is not due to elevations in cell Ca^{2+}. A23187 treatment also failed to potentiate the secretory response to C-K4-M2GlyR but did increase the response to 1-EBIO.

3.6. Effect of K^+ channel blockers on I_{SC} induced by C-K4-M2GlyR and 1-EBIO

We next determined whether the potentiating effect of 1-EBIO on the current induced by C-K4-M2GlyR was dependent on basolateral K^+ channels. In six pairs of T84 monolayers, we evaluated the effect of

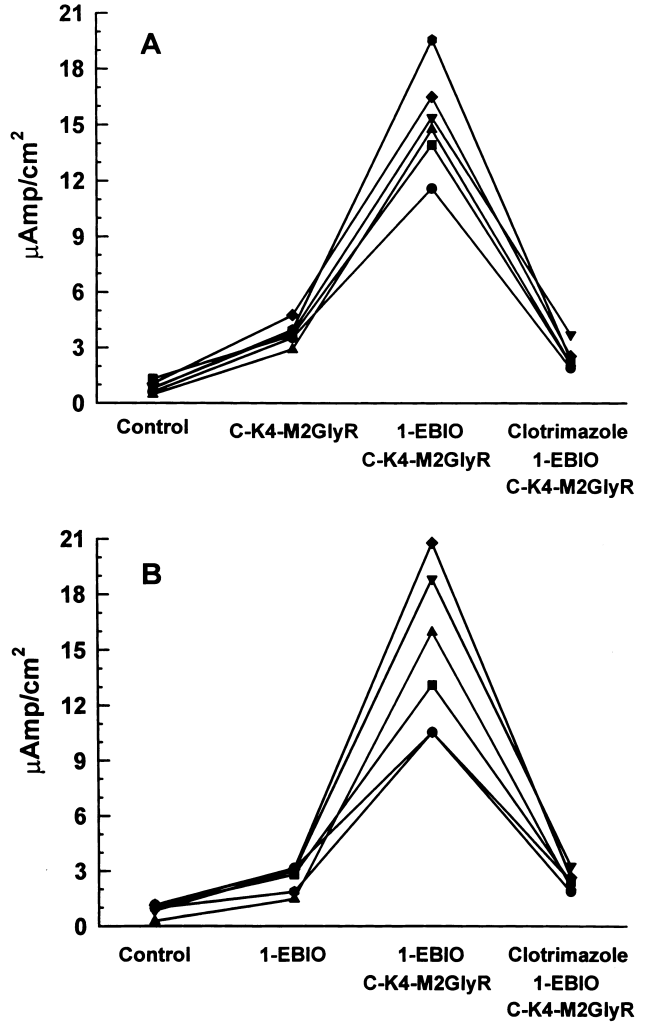

Fig. 5. Effect of basolateral clotrimazole on the I_{SC} generated by the combination of apical addition of 100 μM C-K4-M2GlyR and basolateral addition of 600 μM 1-EBIO. (A) C-K4-M2GlyR was added 40 min prior to the subsequent application of 1-EBIO. (B) 1-EBIO was added to the medium for 20 min before the apical application of the peptide. Clotrimazole (5 μM) was added to the basolateral medium.

basolateral application of clotrimazole on the current induced by the combination of C-K4-M2GlyR and 1-EBIO. One group of monolayers received C-K4-M2GlyR followed by the addition of basolateral 1-EBIO (Fig. 5A). The combined effect of the peptide and 1-EBIO increased I_{SC} 11.5 ± 1.0 μA/cm^2 above the control level ($P < 0.001$). The other group (Fig. 5B) received 1-EBIO prior to the addition of the peptide. The current increased by 12.4 μA/cm^2 with the combination of the two additions. In each set of experiments, the current induced by C-K4-M2GlyR and 1-EBIO was inhibited $89 \pm 1\%$ by the basolateral application of 5 μM clotrimazole.

In other experiments ($n = 4$), we tested the effect of CTX, a more specific inhibitor of the Ca^{2+}-dependent K^+ channels, on the current generated by the combination of C-K4-M2GlyR and 1-EBIO. In this study, the additions of 100 μM C-K4-M2GlyR and 600 μM 1-EBIO increased I_{SC} from 1.4 ± 0.2 to 6.8 ± 1.3 μA/cm^2 ($P < 0.01$). CTX (50 nM) applied to the basolateral surface of the monolayer reduced this current to 2.6 ± 0.2 μA/cm^2 ($P < 0.01$). These studies indicate that 1-EBIO potentiates the secretory response induced by C-K4-M2GlyR and that this effect is dependent on the activity of Ca^{2+}-dependent K^+ channels.

3.7. Effect of Cl^- transport inhibitors

We next examined the effects of the Cl^- transport inhibitors, bumetanide and niflumic acid (NFA) on the I_{SC} induced by the combination of 1-EBIO and C-K4-M2GlyR (Table 3). In four monolayers, blocking Cl^- entry with the basolateral addition of bumetanide (20 μM), an inhibitor of the Na^+-K^+-$2Cl^-$ cotransporter, decreased the current by $71 \pm 2\%$

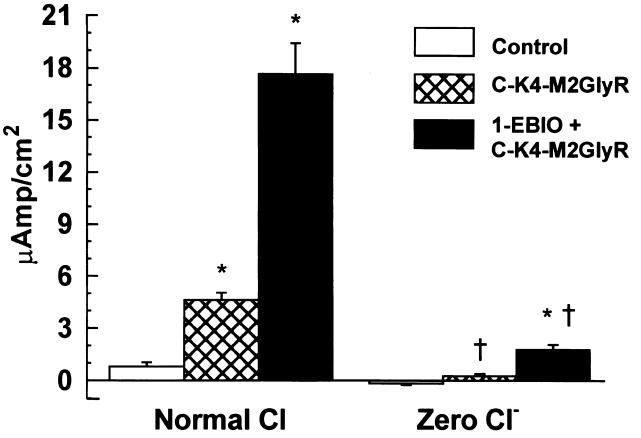

Fig. 6. Effect of C-K4-M2GlyrR in the presence and absence of extracellular Cl^-. Bars represent means ± S.E.M. $n = 4$ pairs of monolayers. Normal Cl^- concentration was 119 mM. In zero Cl^- medium, Cl^- was replaced with 119 mM cyclamate. C-K4-M2GlyR (100 μM) was added to the apical medium and 1-EBIO (600 μM) was added to the basolateral medium. *$P < 0.01$, comparison with previous period. †$P < 0.001$, comparison with normal Cl^- group.

Table 3
Effect of chloride transport inhibitors on the changes in the electrical measurements induced by 1-EBIO and C-K4-M2GlyR in T84 monolayers

	I_{SC} (μA/cm^2)	V_{te} (mV)	R_{te} (kΩ·cm^2)
Bumetanide ($n=4$)			
Control	0.5 ± 0.1	−0.9 ± 0.1	1.39 ± 0.16
1-EBIO	1.3 ± 0.1	−1.9 ± 0.2*	1.36 ± 0.16
1-EBIO+C-K4-M2GlyR	16.0 ± 0.9*	−10.0 ± 0.2*	0.59 ± 0.05*
1-EBIO+C-K4-M2GlyR+bumetanide	5.0 ± 0.2*	−3.4 ± 0.3*	0.06 ± 0.04
Niflumic acid ($n=4$)			
Control	1.0 ± 0.1	−2.1 ± 0.6	1.67 ± 0.41
1-EBIO	1.9 ± 0.5	−3.8 ± 1.3	1.59 ± 0.36
1-EBIO+C-K4-M2GlyR	17.9 ± 1.5*	−12.9 ± 2.1*	0.64 ± 0.05†
1-EBIO+C-K4-M2GlyR+NFA	9.7 ± 1.6*	−8.4 ± 2.1†	0.80 ± 0.10

Values are means ± S.E.M.; $n=4$ for each series of experiments. In all experiments, 1-EBIO (600 μM) was added to the basolateral medium and C-K4-M2GlyR (100 μM) was added to the apical medium. Bumetanide (20 μM) was added to the basolateral medium. Niflumic acid (NFA, 200 μM) was added to the apical medium. *Significant difference compared to previous period as determined by repeated measures ANOVA and S-N-K multiple comparison post test, $P<0.001$. $^\dagger P<0.05$.

($P<0.001$). Bumetanide also caused a significant depolarization of V_{te} from −10.0 ± 0.2 to −3.4 ± 0.3 mV, $P<0.001$). R_{te} increased slightly, however, the effect was not significant. Previously, we showed that the Cl^- channel blocker, NFA, was an inhibitor of the C-K4-M2GlyR-generated I_{SC} in MDCK monolayers [8]. In four experiments, the addition of 200 μM NFA to the apical medium reduced the current generated by C-K4-M2GlyR and 1-EBIO by 49 ± 6% ($P<0.001$) and depolarized V_{te} by 4.5 ± 0.5 mV ($P<0.005$). In these experiments, the increase in R_{te} did not reach statistical significance.

3.8. Extracellular Cl^- removal

Fig. 6 summarizes the effect of removing external Cl^- on the I_{SC} response to C-K4-M2GlyR and 1-EBIO. Four pairs of T84 monolayers, grown under identical culture conditions, were mounted in Ussing chambers. The control monolayers were bathed in a normal Cl^- Ringer's (119 mM Cl^-). The other group was bathed symmetrically in a zero Cl^- Ringer's solution, containing the molar equivalent of cyclamate. In the control monolayers, I_{SC} increased from 0.8 ± 0.3 to 4.6 ± 0.4 μA/cm^2 ($P<0.05$) with apical addition of the peptide. The subsequent basolateral addition of 1-EBIO increased the current to 17.8 ± 1.8 μA/cm^2 ($P<0.001$). In the absence of Cl^-, the effect of C-K4-M2GlyR was abolished (−0.2 ± 0.1 to 0.3 ± 0.1 μA/cm^2, not significant). The successive addition of 1-EBIO produced a significant increase in I_{SC}; however, the magnitude of the response was greatly reduced (1.8 ± 0.3 μA/cm^2) and was blocked by the basolateral addition of 5 μM

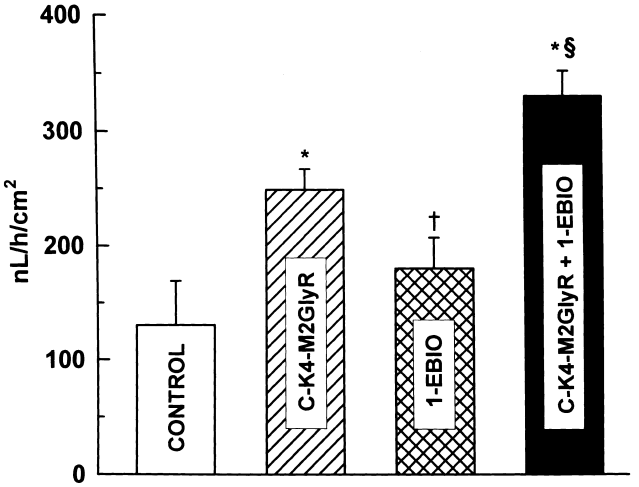

Fig. 7. Effect of C-K4-M2GlyR and 1-EBIO on fluid transport across T84 monolayers. $n=10$ monolayers were exposed to control medium, 500 μM apical C-K4-M2GlyR, 300 μM basolateral 1-EBIO, or a combination of C-K4-M2GlyR and 1-EBIO. *$P<0.001$ compared with the control group of monolayers; †not significantly different from control. $^\S P<0.05$ compared with the C-K4-M2GlyR and the 1-EBIO groups of monolayers. In another group of monolayers ($n=6$), 10 μM forskolin stimulated fluid secretion at a rate of 338 ± 40 nl/h/cm^2 compared to 10 ± 22 nl/h/cm^2 in the control monolayers ($P<0.001$).

clotrimazole (data not shown). These results demonstrate that the activation of basolateral K^+ channels with 1-EBIO potentiates the secretion of Cl^- through exogenous Cl^- channels generated by the membrane insertion of C-K4-M2GlyR.

3.9. Fluid secretion by T84 monolayers

We next determined whether the addition of 1-EBIO would increase the secretion of fluid induced by the apical application of C-K4-M2GlyR to T84 cell monolayers. For this study, T84 cells were grown as confluent monolayers on Transwell-Cols for measurement of fluid transport. In four groups of 10 monolayers, we measured the rate of fluid transport across the monolayers. Group I was incubated for 12 h in control medium, in group II the apical medium contained 500 μM C-K4-M2GlyR, in group III the basolateral medium contained 300 μM 1-EBIO and in group IV both C-K4-M2GlyR and 1-EBIO were used (Fig. 7). For these experiments, a lower concentration of 1-EBIO was selected to reduce possible activation of apical mechanisms during the 12 h incubation period. Monolayers incubated in control media secreted fluid at a rate of 130 ± 40 nl/h/cm^2 ($P < 0.01$). Incubating the monolayers in the presence of C-K4-M2GlyR significantly increased the rate of fluid secretion to 250 ± 20 nl/h/cm^2 ($P < 0.05$). Basolateral addition of 300 μM 1-EBIO in the absence of the peptide did not significantly alter the rate of fluid transport (180 ± 30 nl/h/cm^2, not significantly different from the control group). However, the combination of C-K4-M2GlyR and 1-EBIO increased the rate of fluid secretion to 330 ± 20 nl/h/cm^2 (a value that is significantly different from the other three groups, $P < 0.05$). These results demonstrate that anion secretion induced by the apical membrane insertion of C-K4-M2GlyR drives the secretion of fluid and that the direct activation of basolateral K^+ channels by 1-EBIO increases the rate of that secretion.

4. Discussion

4.1. Channel-forming peptides

Current research in CF has focused on strategies to restore the apical Cl^- conductance in the CFTR-deficient epithelia [5,22,23]. One possible treatment for CF is the incorporation of synthetic Cl^- channel-forming peptides into the apical membrane of the epithelial cells to assist in restoring Cl^- and fluid secretion. A variety of small amphipathic peptides have been demonstrated to form ion channels including a wide range of bacterial and insect toxins [24,25]. The bee venom toxin, melittin, is a well known channel-forming peptide. Melittin is an amphipathic, α-helical peptide composed of a 20 amino acid transmembrane segment and four positively charged residues on the C-terminus. The charged terminal amino acids have been shown to be essential for the aqueous solubility of the peptide. Synthetic peptides derived from the original sequence of melittin have been shown to form ion channels in lipid bilayers [24].

In mammals, defensive antimicrobial peptides called cryptdins are released into the crypts of the small intestine. Recently, Lencer et al. reported that two of the cryptdins that were examined increased anion secretion when applied to the apical surface of T84 monolayers [26].

Other channel-forming peptides have been synthesized from sequences representing putative channel-forming regions of endogenous ion channels. These synthetic peptides are relatively short, with a secondary structure that just spans a lipid bilayer [6,27]. M2GlyR, a synthetic peptide that mimics the 23 amino acid sequence in the second membrane-spanning region of the glycine receptor, can be incorporated into artificial lipid membranes and will self-assemble into Cl^- conductive channels [6]. We have modified this peptide by adding four lysine residues to the C-terminus (C-K4-M2GlyR), forming a peptide that is similar to the structure of melittin. This modification greatly improved the peptide's water solubility and increased the bioactivity of the peptide [7]. In lipid bilayer studies, the channel formed by the wild-type peptide, M2GlyR, was found to have conductance levels similar to those reported for the native glycine receptor [6,28]. M2GlyR may form a channel by the association of five monomers into a pentamer, analogous to the pore structure of GlyR [29]. The properties of the modified peptide, C-K4-M2GlyR are similar to those described for the parent sequence, M2GlyR [9].

4.2. T84 cell model

The T84 cells represent an extensively characterized human intestinal cell line that possesses many of the transporters common to secretory epithelia and for that reason has been used regularly in CF research. Chloride is transported into the cell above its electrochemical gradient by an electrically neutral Na^+-K^+-$2Cl^-$ cotransporter located in the basolateral membrane. Chloride channels provide an efflux pathway for Cl^- across the apical membrane and Cl^- efflux is driven by the transmembrane electrochemical gradient. Basolateral K^+ conductance is essential for maintaining a sufficient electrical gradient to drive Cl^- efflux across the apical membrane [16,19,21]. Activation of apical Cl^- channels without a comparable increase in the activity of K^+ channels would shift the cell membrane potential towards the Cl^- equilibrium potential and limit the electrochemical driving force for Cl^- efflux [21,30].

4.3. C-K4-M2GlyR induces Cl^- and fluid secretion by T84 cells

In the current study, we determined if the Cl^- channel-forming peptide, C-K4-M2GlyR, could insert into the apical membrane of a human intestinal cell line and induce Cl^- and fluid secretion. Addition of 100 μM C-K4-M2GlyR to the medium bathing the mucosal surface of T84 cell monolayers produced a sustained increase in I_{SC} (Figs. 1, 3–6 and Table 1). This response began approximately 10 min after the application of the peptide and may represent the time required for the insertion of the peptide and the self-assembly of the monomers in the membrane into a Cl^- conductive pore (Fig. 1). C-K4-M2GlyR also caused V_{te} to hyperpolarize and reduced R_{te}, responses thought to be due to an increase in the apical membrane Cl^- conductance (Fig. 1 and Table 1).

Basolateral application of bumetanide, an inhibitor of Na^+-K^+-$2Cl^-$ cotransport, and the apical application of the Cl^- channel blocker NFA significantly attenuated I_{SC} generated by the combination of C-K4-M2GlyR and 1-EBIO (Table 3). In experiments in which external Cl^- was replaced with cyclamate, the effect of the peptide was completely abolished; however, the subsequent addition of 1-EBIO produced a small increase in I_{SC}. This current may be attributed to HCO_3^- secretion through either the channel formed by C-K4-M2GlyR or through CFTR Cl^- channels [31].

We also found that the application of 20 nmol (500 μM) C-K4-M2GlyR to the apical surface of T84 monolayers nearly doubled the rate of fluid secretion compared to monolayers bathed in control media (Fig. 7), consistent with the effect on MDCK cell monolayers [8]. 1-EBIO increased fluid secretion and the effects of the combination of C-K4-M2GlyR and 1-EBIO on fluid secretion were additive. The incremental increase in fluid secretion did not match the magnitude of the change in I_{SC} and may reflect a limiting influence of the water permeability of the monolayers on the rate of fluid secretion. These data support the hypothesis that the synthetic peptide, C-K4-M2GlyR, is capable of producing a Cl^- secretory pathway that promotes the net secretion of solute and fluid in human intestinal epithelial cells.

4.4. C-K4-M2GlyR generates a novel chloride conductance

Evidence obtained in studies with planar lipid bilayers, individual MDCK and T84 cells and polarized cell monolayers have demonstrated that the insertion of C-K4-M2GlyR into membranes induces a novel channel with characteristics that do not resemble any known endogenous Cl^- conductance found in either MDCK or T84 cells. The characteristics and kinetics of the whole cell conductance generated by exposing isolated epithelial cells to C-K4-M2GlyR indicate that the conductance formed by the peptide is unlikely due to the activation of CFTR, Ca^{2+}-dependent Cl^- channels, ClC-2 channels or the volume-sensitive organic osmolyte and anion channels [9]. CFTR is abundantly expressed in the T84 cells and its channel is enhanced by agents that increase cellular cAMP and is inhibited by high concentrations of the chloride channel blocker, DPC. We have shown that the Cl^- secretory current induced by C-K4-M2GlyR in MDCK cells is more sensitive to DPC than that induced by forskolin. In addition, the peptide did not affect cAMP levels in either MDCK cells [8] or in the T84 cells. It may be argued that the current generated by C-K4-M2GlyR is the result of activation of endogenous CFTR Cl^- chan-

nel by the poly-lysine tail, an effect observed with protamine applied to airway epithelial cells [32]. However, a peptide in which the M2GlyR sequence was scrambled and to which the poly-lysine tail was attached did not alter I_{SC} or fluid transport [8]. In patch clamp experiments, C-K4-M2GlyR also increased Cl^- conductance in an airway epithelial cell line derived from a human patient with cystic fibrosis [9].

When T84 cells are allowed to form a cell monolayer with tight junctions, these cells appear to down regulate the expression of the Ca^{2+}-dependent Cl^- channel [33]. Others have reported that increasing cellular Ca^{2+} in T84 monolayers failed to induce an apical Cl^- conductance [3,12]. We confirmed this result by testing the effect of the Ca^{2+} ionophore A23187 on I_{SC} across T84 monolayers. A23187 did not produce a sustained increase in I_{SC}. We have also shown that DIDS, an inhibitor of the Ca^{2+}-dependent Cl^- channel, did not affect I_{SC} generated by C-K4-M2GlyR in MDCK cell monolayers [8]. The data from whole cell recordings and from short-circuit current experiments of MDCK and T84 cell monolayers strongly suggest that the current generated by C-K4-M2GlyR is not the result of activating either CFTR or a Ca^{2+}-dependent Cl^- conductance.

To further examine the possibility that C-K4-M2GlyR may be activating endogenous Cl^- channels, we applied the peptide to the basolateral aspect of the cell. We found that basolateral application of C-K4-M2GlyR diminished an established cAMP-activated Cl^- secretory current in both MDCK monolayers [8] and in T84 monolayers (unpublished results). The decrease in current was evidently caused by the insertion of Cl^- channels in the basolateral membrane and the short-circuiting of Cl^- transport. Polycationic peptides, such as protamine, on the other hand activate endogenous conductances such as CFTR, therefore are effective only when applied to the apical surface. In the aggregate, these data support the hypothesis that C-K4-M2GlyR is associated with the insertion of a novel anion conductance.

4.5. The activity of basolateral K^+ channels modulates Cl^- secretion induced by C-K4-M2GlyR

The results of several studies indicate that basolateral K^+ channels play a role in Cl^- secretion [3,10,12,14,21]. Evidently, the hyperpolarization of the cell membrane increases the electrochemical driving force for Cl^- secretion across the apical membrane through constitutively active Cl^- channels [3]. In intestinal cells, Cl^- secretion is normally regulated by both cAMP and Ca^{2+} receptor-mediated agonists. Elevation of intracellular cAMP activates the apical membrane Cl^- conductance by stimulating protein kinase A phosphorylation of the CFTR Cl^- channel. Several studies have demonstrated that cAMP may also activate a cAMP-dependent K^+ channel; although this channel has not been characterized by patch clamp techniques [12,14,21]. The mechanism for Ca^{2+}-mediated Cl^- secretion in T84 cells and other intestinal cells is less well defined. In polarized T84 monolayers, factors that elevate intracellular Ca^{2+} levels have been shown to transiently increase the rate of Cl^- secretion by activating basolateral K^+ conductance without increasing an apical Cl^- conductance [3,19].

The antimycotic agent, clotrimazole, was shown to inhibit both the cAMP- and the Ca^{2+}-dependent K^+ channels; however, there is a several-fold higher affinity for clotrimazole inhibition of the Ca^{2+}-dependent K^+ conductance ($K_i = 0.3$ μM) compared to the cAMP-dependent K^+ conductance ($K_i = 5.2$ μM) [12]. In the current study, clotrimazole inhibited I_{SC} induced by C-K4-M2GlyR with an IC_{50} of 0.4 μM (Fig. 2), consistent with clotrimazole's affinity for the Ca^{2+}-dependent K^+ channel reported by Devor and associates [12]. Furthermore, prior treatment of the T84 monolayers with CTX significantly reduced the effect of C-K4-M2GlyR (Fig. 3). These data suggest that the Ca^{2+}-dependent K^+ conductance is a major K^+ efflux pathway during Cl^- secretion caused by membrane insertion of exogenous Cl^- channels into the apical membrane of T84 cells.

Recently, 1-EBIO was shown to directly activate the Ca^{2+}-dependent K^+ channels in T84 cells and potentiated the I_{SC} response induced by the activation of the CFTR Cl^- channels [16]. Cuthbert et al. showed that 1-EBIO has an EC_{50} close to 600 μM [34], the dose also used by Devor and associates [16]. In the present study, the application of 600 μM 1-EBIO to the T84 cells stimulated a small but sustained increase in I_{SC} and hyperpolarized V_{te}, changes that are consistent with Cl^- secretion (Table 2). The effect was greatest when 1-EBIO was applied

to both sides of the cell monolayer; however, the addition of 1-EBIO to either the basolateral or the apical medium was sufficient for a significant and sustained increase in I_{SC} (data on the apical application are not shown).

Chloride and fluid secretion induced by exposing the apical surface of the T84 monolayers to C-K4-M2GlyR was greatly increased by 1-EBIO addition to the basolateral media (Table 2, Fig. 7). The I_{SC} response to the combination of peptide and 1-EBIO was reduced by clotrimazole (Fig. 5) and by CTX. These data demonstrate that direct activation of the Ca^{+2}-dependent K^+ channels potentiates Cl^- secretion induced by C-K4-M2GlyR.

Increasing intracellular Ca^{2+} with A23187 failed to potentiate the secretory response of C-K4-M2GlyR. It has been shown that elevating intracellular Ca^{2+} produces only a transient increase in Cl^- secretion by T84 cells. It has been suggested that the transient response of Ca^{2+}-mediated Cl^- secretion is due to the activation of Ca^{2+}-dependent K^+ channels followed by a delayed inactivation of these channels through a protein kinase C (PKC)-dependent process [13]. In the current study, we speculate that PKC activation, following a rise in cell Ca^{2+} with A23187, may limit the activity of the Ca^{2+}-dependent K^+ channel, preventing a potentiating effect on Cl^- secretion induced by the peptide. A23187 did augment the response to direct activation of the K^+ channels with 1-EBIO in the presence of C-K4-M2GlyR, an effect that is consistent with the known pharmacology of 1-EBIO. This compound does not directly activate the Ca^{2+}-dependent K^+ channel, but rather increases the sensitivity of the K^+ channel to Ca^{2+} [16]. Therefore, the combination of 1-EBIO and increased cellular Ca^{2+} act synergistically to increase basolateral K^+ conductance through Ca^{2+}-activated K^+ channels.

We found that 1-EBIO alone did not significantly increase cAMP in T84 cells, data consistent with the lack of an effect of 1-EBIO on the cAMP-dependent K^+ channels in this cell line [16]. The increase in Cl^- and fluid secretion induced by combination of C-K4-M2GlyR and 1-EBIO was independent of intracellular cAMP levels since the addition of the two compounds did not significantly affect cAMP content (ΔcAMP was $<10\%$ of the forskolin response). This implies that C-K4-M2GlyR and 1-EBIO had minimal effect on CFTR Cl^- conductance or other cAMP-dependent processes. Therefore, we propose that the major action of 1-EBIO is to activate the Ca^{2+}-dependent K^+ channels and that the increase in the Cl^- secretory current is due to increasing the driving force for Cl^- efflux through synthetic Cl^- channels generated by the membrane insertion of C-K4-M2GlyR.

In summary, we have demonstrated that the synthetic Cl^- channel-forming peptide, C-K4-M2GlyR, induces Cl^- and fluid secretion by the human intestinal cell line, T84. The magnitude of this response can be modulated by activators and inhibitors of the basolateral Ca^{2+}-dependent K^+ conductance. We propose that the combination of the novel synthetic Cl^- channel-forming peptide, C-K4-M2GlyR, and agents like 1-EBIO may have pharmacological benefits for inducing and modulating transepithelial Cl^- and fluid secretion independent of the cAMP-dependent Cl^- secretion that is impaired in cystic fibrosis.

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