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Effect of Diaminopropionic acid (Dap) on the Biophysical Properties of a Modified Synthetic Channel-Forming Peptide

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Abstract

Channel replacement therapy, based on synthetic channel-forming peptides (CFPs) with the ability to supersede defective endogenous ion channels, is a novel treatment modality that may augment existing interventions against multiple diseases. Previously, we derived CFPs from the second transmembrane segment of the α -subunit of the glycine receptor, M2GlyR, which forms chloride-selective channels in its native form. The best candidate, NK₄-M2GlyR T19R, S22W (p22-T19R, S22W), was water-soluble, incorporated into cell membranes and was non-immunogenic, but lacked the structural properties for high conductance and anion selectivity when assembled into a pore. Further studies suggested that the threonine residues at positions 13, 17 and 20 line the pore of assembled p22-T19R, S22W, and here we used 2, 3-diaminopropionic acid (Dap) substitutions to introduce positive charges to the pore-lining interface of the predicted p22-T19R, S22W channel. Dap-substituted p22-T19R, S22W peptides retained the α -helical secondary structure characteristic of their parent peptide, and induced short-circuit transepithelial currents when exposed to the apical membrane of Madin-Darby canine kidney (MDCK) cells; the sequences containing multiple Dap-substituted residues induced larger currents than the peptides with single or no Dap-substitutions. To gain further insights into the effects of Dap residues on the properties of the putative pore, we performed two-electrode voltage clamp electrophysiology on *Xenopus* oocytes exposed to p22-T19R, S22W or its Dap-modified analogs. We observed that Dap-substituted peptides also induced significantly larger voltage-dependent currents than the parent compound, but there was no apparent change in reversal potential upon replacement of external Na⁺, Cl⁻ or K⁺, indicating that these currents remained non-selective. These results suggest that the introduction of positively charged side chains in predicted pore-lining residues does not improve anion-to-cation selectivity, but results in higher conductance, perhaps due to higher oligomerization numbers.

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Keywords

Synthetic channel-forming peptide; ion permeation; ion selectivity; glycine receptor alpha subunit; anion channel; chloride channel; diaminopropionic acid; Dap; channel replacement therapy; cystic fibrosis

Introduction

Cystic fibrosis (CF) is a chronic, autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein, an anion-selective channel with essential roles in ion exchange in epithelial tissues. Impaired CFTR channel activity leads to the abnormal accumulation of mucus that underlies the clinical manifestations of the disease, such as airway inflammation and infection.¹ A major goal of CF treatment strategies, particularly gene therapy, has been the restoration of CFTR channel activity, but none of the methods developed so far have been successful in treating all the genotypes.²

A potential alternative may be the use of synthetic channel-forming peptides (CFPs), engineered for high anion selectivity and permeation, in target tissues and organs. CFPs, either designed *de novo* or based on pore-forming transmembrane segments of natural proteins, have been used to generate channel activity in lipid bilayers and cell membranes. Though these CFPs have been shown to assemble into ion-permeable pores, a common limitation has been the lack of combined high conductance and strong selectivity.^{3–8}

In our studies, the anion-selective pores of native channels, primarily the glycine receptor (GlyR), have been used as templates to synthesize and reconstitute CFPs that form functional synthetic channels in a variety of cell and membrane systems.^{9–14} GlyR, a member of the cys-loop super family of ligand-gated channels, is a heteropentameric anion channel formed by three α subunits and two β subunits.¹⁵ The α subunits alone can assemble into homopentameric anionic channels,¹⁶ and just the second transmembrane domain (M2) of the α subunit forms ion channels in lipid bilayers and single cells, which may facilitate ion transport across epithelial monolayers.^{14,17} Our founding CFP, M2GlyR, derived from the wild-type α -subunit M2 sequence PARVGLGITTTLTMTTQSSGSRA, conferred net transepithelial ion transport comparable to the parent GlyR channel pore,¹⁴ but had poor membrane insertion efficiency and a tendency to aggregate in aqueous solutions.^{14,17} In subsequent studies, modifications were introduced into the amino acid sequence of either the full-length peptide or a truncated version, including a tetralysyl tail at the N-terminus, and the replacement of C-terminal threonine and serine residues with arginine and tryptophan.^{18–21} The resulting synthetic peptide KKKKPARVGLGITTTLTMRQTQW, termed NK₄-M2GlyR p22-T19R, S22W (p22-T19R, S22W), demonstrated increased helical content, reduced aggregation, improved efficiency of insertion into epithelial cell membranes, and increased net transepithelial ion conductance in Madin-Darby canine kidney (MDCK) cell monolayers.²² The p22-T19R, S22W sequence also proved to be non-antigenic in mice at proposed clinical dosages,²³ but the putative assembled pore still lacked physiologically relevant anion selectivity.²⁴

To address these limitations towards the construction of anion-selective synthetic CFPs with therapeutic potential, we tested the possibility that increasing the net positive charge of the pore environment would improve permeation and anion selectivity of the predicted p22-T19R, S22W channels. Previous *in silico* structural analysis suggested that threonine residues at positions 13, 17 and 20 line the pore of hypothetical homopentameric p22-T19R, S22W channels; in particular, T13 would reside in the widest part of the permeation

pathway, whereas T17 and T20 would be located in the narrowest pathway segment.^{20,25} Here, we synthesized analogues of p22-T19R, S22W with 2, 3-diaminopropionic acid (Dap) substitutions at T13, T17 and T20 (Table 1), and tested for effects on ion permeation and selectivity, by means of electrophysiological methods in MDCK cells and *Xenopus* oocytes.

Experimental Section

Chemistry

Peptides were prepared by solid phase synthesis (Applied Biosystems Model 431A; Foster City, CA), using 9-fluorenylmethoxycarbonyl (F-moc) chemistry as described previously,^{21,25} the peptide sequences are shown in Table 1. CLEAR amide resin (0.3 mmol/g; Peptides International, Louisville, KY) and N^ε-F-moc amino acids (Anaspec Inc., San Jose, CA) were used. All peptides were purified by HPLC (System Gold HPLC; Beckman Instruments, Inc., Fullerton, CA) with a reversed-phase C-18 column (Phenomenex; Torrance, CA), and eluted using a linear gradient of 10–90% acetonitrile containing 0.1% trifluoroacetic acid at 1 mL/min.²⁶ Molecular mass was determined by matrix-assisted-laser desorption time-of-flight (MALDI-TOF/TOF) mass spectroscopy (Ultraflex II spectrometer; Bruker Daltronics, Billerica, MA). After characterization, peptides were lyophilized and stored as dry powders. Work solutions were prepared immediately before use and peptide concentrations were determined by measuring the absorbance at 278 nm, using the tryptophan extinction coefficient of 5579/M/cm. The secondary structures of peptides were determined by circular dichroism (CD) at 100 μM in 40% water/trifluoroethanol (TFE) with a spectropolarimeter (J-720, Jasco, Tokyo, Japan), as described previously.^{20,25} Under the conditions tested, all peptides were in solution as monomers.²⁴

Transepithelial Electrical Measurements

MDCK cells were generously provided by Lawrence Sullivan (Kansas University Medical Center, Kansas City, KS). The cells were cultured in a 1:1 blend of Dulbecco's Modified Eagle Medium and Ham's F-12 Nutrient Mixture (Life Technologies, Carlsbad, CA), supplemented with 5% fetal bovine serum (BioWhittaker, Walkersville, MD), and 10⁵ units/L penicillin and 100 mg/L streptomycin (Life Technologies), and prepared as described.^{17,25} MDCK monolayers were mounted vertically in modified Ussing-style flux chambers (DCV9; Navicte, San Diego, CA), and the apical and basolateral surfaces were bathed symmetrically with a modified Ringer's solution containing (mM) 120 NaCl, 25 NaHCO₃, 3.3 KH₂PO₄, 0.8 K₂HPO₄, 1.2 MgCl₂, and 1.2 CaCl₂, kept at 37 °C and bubbled with 5% CO₂ to maintain the pH at 7.5. Monolayers with resistances of at least 800 S cm² were clamped to zero mV (Model 558C; University of Iowa Department of Engineering, Iowa City, IA) and exposed on the apical side to increasing concentrations (10–300 μM) of test peptide. Transepithelial short-circuit current (*I*_{sc}) was recorded at 1 Hz using an MP100-CE interface, and AcqKnowledge software (BIOPAC Systems, Santa Barbara, CA) was used for data acquisition. *I*_{sc} data were fitted by Equation 1, for which *I*_{max} is the derived maximum current, *P* is the peptide concentration, *K*_{0.5} is the peptide concentration at which *I*_{sc} is half-maximal, and *n*_H is the Hill coefficient:

$$I_{sc} = I_{max} P^{n_H} / (K_{0.5}^{n_H} + P^{n_H}) \quad (1)$$

Two-Electrode Voltage-Clamp Electrophysiology

Mature female *Xenopus laevis* were purchased from Nasco (Fort Atkinson, WI). All animal protocols followed guidelines approved by Washington University and the National Institutes of Health. Frogs were anaesthetized with 0.1% tricaine buffered with 0.1%

NaHCO₃ prior to removal of a portion of the ovary, and were sacrificed by an overdose of tricaine. Stage V–VI oocytes were selected and maintained at 18 °C in modified Barth's solution containing (mM) 88 NaCl, 2.4 NaHCO₃, 1 KCl, 0.3 Ca(NO₃)₂, 0.4 CaCl₂, 0.8 MgSO₄ and 10 Hepes/Tris (pH 7.5), and supplemented with 50 mg/L gentamicin, 6 mg/L ciprofloxacin, and 100 mg/L streptomycin sulphate/100,000 units/L penicillin G sodium (Life Technologies, Carlsbad, CA). Two-electrode voltage-clamp (TEVC) was used to determine the response of oocytes to exposure to the peptide in different ionic environments. The standard extracellular solution, ND96, was (mM) 96 NaCl, 1 KCl, 1 MgCl₂, 1.8 CaCl₂, and 5 Hepes/Tris (pH 7.5). Typically, an oocyte was mounted in the TEVC setup, superfused in ND96 and held at −30 mV (V_h). In pilot trials, oocytes were superfused with wild-type or Dap-substituted peptides, and records were taken at 1, 2, 5, 10 and 20 min. In all cases, response was maximal at 5 min, and remained stable for at least 20 min. To allow for repeated measurements while ensuring the viability of the oocytes, peptide exposure was subsequently limited to 5 min. In basic experiments, the oocyte was exposed to ND96, first in the absence, then in the presence of synthetic peptide (50 μM), and then washed with peptide-free ND96 for at least 10 min and until the initial conditions were restored. In further experiments, the oocyte was first stabilized in ND96, subsequently superfused with the appropriate ion-substituted buffer, first in absence and then in presence of the peptide; after exposure, the oocyte was washed, first with the alternate solution, and then with ND96, to verify the reversibility of bath composition-induced changes. The ion-substituted buffers contained (mM) 96 KCl, 1 NaCl (KD96); 92.3 Na-gluconate, 9.6 NaCl, 1 KCl (Na-Gluc); or 96 K-gluconate, 1 Na-gluconate (K-Gluc); and 5 Hepes/Tris (pH 7.5), plus the appropriate amounts of CaCl₂ and MgCl₂ to attain constant free Ca²⁺ and Mg²⁺ concentrations, as calculated by means of the CaBuf program (<ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/cabuf.zip>). At the end of each exposure period, a pulse protocol was applied in which membrane potential (V_m) of oocytes was stepped from V_h to a test value for 1000 ms before returning to V_h . The test potential varied from +60 to −120 mV in 20 mV increments. Steady-state or pseudo steady-state currents were measured at the end of 1000 ms. The reversal potential (V_{rev}) of the current was estimated as the intercept of the linear current-voltage (I – V) relationships between +20 and −60 mV. pClamp and Axoscope software (Molecular Devices, Union City, CA) were used for pulse protocol application and data acquisition. All experiments were carried out at room temperature. Unless otherwise noted, experiments were performed in at least three oocytes from different donor frogs.

Data Analysis

Results were analyzed using Excel (Microsoft, Redmond, WA) and Clampfit 10.1 (Molecular Devices). SigmaPlot 10.0 (Systat Software, San Jose, CA) and CorelDRAW X3 13.0 (Corel Corporation, Mountain View, CA) were used for curve fitting, statistics, and figure preparation. Unless otherwise indicated, results are presented as mean ± SE (standard error of the mean). Where indicated, unpaired Student's *t* test was applied to evaluate statistical differences between groups.

Results and Discussion

Overall Secondary Structure Characteristics

Our model for the NK₄-M2GlyR p22-T19R, S22W (p22-T19R, S22W) pentameric channel suggests that the pore interface is lined by three stacked rings of five threonines, respectively in positions 13, 17 and 20; the T17 and T20 rings form the narrowest part of the pore, whereas the T13 ring defines the widest opening.^{20,25} In an attempt to modify the pore electrostatics to increase the conductance and improve the anion-to-cation selectivity of the assembled channel, we designed and synthesized a series of novel p22-T19R, S22W analogues, where T13, T17 or T20 were replaced with positively charged 2, 3-

diaminopropionic acid (Dap), a non-natural amino acid that will introduce a methylamine side chain (Table 1). Circular dichroism (CD) analysis showed that the introduction of single or multiple Dap substitutions did not significantly alter the secondary structure of the analogues (Figure 1). The CD spectra measured for the WT and Dap-substituted analogues indicated similar helical content, with characteristic α -helix double minima at 208 and 222 nm, and maximum at $\sim$ 192 nm. These results indicate that the replacement of T13, T17 or T20 with the cationic Dap residue does not affect the propensity of the peptides to adopt a helical conformation in a hydrophobic environment, in agreement with secondary structures determined in previous studies.^{20,22,25}

Electrophysiological Measurements of Transepithelial Conductance

We tested the effect of the peptides on the electrical properties of MDCK cells mounted in Ussing chambers. Cell monolayers were clamped to 0 mV and exposed to 10–300 μ M of p22-T19R, S22W (WT), or analogues with one, two, or three Dap substitutions, and the transepithelial short-circuit current (I_{sc}) response was recorded (Figure 2). Concentration-dependence data were best fitted by eq 1 to estimate the kinetic parameters of peptide-induced transepithelial ion transport, and results are summarized in Table 2. For the WT, the apparent half-saturation constant ($K_{0.5}$) was $\sim$ 128 μ M, and for all the Dap-substituted analogues the $K_{0.5}$ values were within the same range (Table 2). For all test peptides, the Hill coefficient (n_H) was greater than 1 (Table 2), which indicates that a complex mechanism governs the concentration-dependence of the current response, and might reflect a functional cooperativity between the peptide monomers. In the presence of the WT peptide, the derived current maximum (I_{max}) was $\sim$ 38 μ A/cm², and it increased $\sim$ 3- to 4-fold in presence of the doubly- and triply-substituted analogues.

Voltage-Clamp Characterization of Peptide-Induced Currents

Next, we examined the voltage-dependence and the ion selectivity of the conductances activated by the different test peptides, by means of two-electrode voltage-clamp electrophysiology in *Xenopus* oocytes; results are summarized in Figures 3 and 4, and Table 3. Whole cell currents were recorded in response to 1-s voltage steps between +60 and $-$ 120 mV from a holding potential (V_h) of $-$ 30 mV (Figure 3A, *upper left panel*), with a 10-s interval between pulses. As exemplified in Figure 3A, oocytes superfused with standard ND96, high K⁺ (KD96) or low Cl[−] (Na-Gluc) solution showed small background currents (slope conductance, $\sim$ 1–2 pS), which reversed at $-$ 35 mV (ND96), $-$ 3 mV (KD96), and $-$ 39 mV (Na-Gluc), respectively (Figure 3B and Table 3). In low extracellular Na⁺ and Cl[−] (K-Gluc), the basal conductance increased ($\sim$ 15 pS), and the now clearly sigmoidal current-voltage (I - V) relationships reversed at $-$ 13 mV (Figure 3B and Table 3). When the oocytes were superfused in ND96 or KD96 in the presence of p22-T19R, S22W (WT), modest outward currents at depolarizing potentials above +20 mV, and larger, slowly-activating, slowly-deactivating inward currents at hyperpolarizing potentials below $-$ 80 mV, were evoked. These currents disappeared upon superfusion with peptide-free solution (Figure 3A). In Na-Gluc, exposure to WT led to the activation of fully reversible, outwardly rectifying currents (Figure 3A and 3C). In oocytes superfused with K-Gluc, the amplitude of the currents increased in presence of WT (Figure 3A), but the overall shape of the I - V curve remained unchanged (Figure 3C). In all experimental conditions, the WT peptide had a negligible effect on the V_{rev} (Figure 3C and Table 3), indicating that the peptide-activated currents were non-selective.

The substitution of Dap for putative pore-lining threonines led to an increase in conductance, but did not modify the ionic selectivity of the induced currents (Figure 4 and Table 3). As exemplified for ND96 (Figure 4, A and C) and Na-Gluc (Figure 4, A and B), the currents evoked by the Dap-substituted analogues in all solutions were generally larger

but showed similar voltage dependence than those induced by the WT peptide, and again no relevant changes in V_{rev} were observed (Table 3). As summarized in Figure 4D for the net peptide-induced currents measured at +60 mV (outward; *top*) and –100 mV (inward; *bottom*), the increase in current density with respect to the WT sequence was substantial (for example, up to 30-fold in presence of extracellular gluconate) but overall was similar for all Dap-substituted analogues, and there were no evident synergistic or antagonistic effects between the different substitutions.

M2GlyR-Derived Channel-Forming Peptides

As part of an ongoing effort to produce synthetic channel forming peptides (CFPs) as potential therapeutics for channel-based diseases, in previous studies we identified p22-T19R, S22W,^{22–24} a candidate single-spanning membrane protein based on the second transmembrane domain of the glycine receptor (GlyR) subunit, M2GlyR, which lines the pore of an anion-selective channel.^{14,17} p22-T19R, S22W showed desirable properties, such as optimal helical content, minimal aggregation in aqueous solutions, enhanced efficiency of membrane insertion and increased induction of ion transport across epithelial cell monolayers,²² and was not immunogenic.²³ However, the hypothetical assembled pore demonstrated only minimal selectivity for monovalent anions over monovalent cations, and relatively low conductance.²⁴ In a further study, we set out to identify relevant pore-lining residues that might be modified to rectify this limitation.²⁵ Structural analysis, computational modeling and functional assays in MDCK cells, all suggested that the narrowest opening of the putative pentameric assembly is defined by two stacked rings of threonines in positions 17 and 20, whereas the ring of threonines in position 13 forms the widest part.²⁵

Because electrostatic interactions and the formation of hydrogen bonds within the channel cavity are key determinants of ionic selectivity,^{27,28} in the present work we replaced the predicted pore-lining residues T13, T17 and T20 of p22-T19R, S22W with the non-natural diaminopropionic acid (Dap), which carries a net positive charge at physiological pH and can potentially form strong hydrogen bonds with Cl^- ions. Preliminary computer modeling simulations, in which we employed the potential of mean force function²⁹ to estimate the energy required to move different ions across the simulated Dap-substituted channels, suggested that the introduction of Dap residues to form rings of positive charge in positions 13, 17 and 20 would yield enhanced Cl^- versus Na^+ selectivity. The resulting synthetic Dap-substituted analogues (Table 1) had the appropriate solubility and helical content (Figure 1), and were subsequently used in functional assays to determine their electrogenic properties (Figures 2, 3 and 4, and Tables 2 and 3).

Compared to the WT sequence, transepithelial ion transport rates increased up to 4-fold in MDCK cell monolayers exposed to the Dap-substituted analogues (Figure 2). In *Xenopus* oocytes, voltage-dependent currents induced by the Dap-substituted peptides (Figure 4) were also larger than those evoked by the WT peptide (Figures 3 and 4). However, replacement of extracellular Na^+ with K^+ , or Cl^- with gluconate, did not change the V_{rev} in any case (Table 3), suggesting that, in all cases, the conductance pathway activated by the peptides was non-selective. Nevertheless, it is intriguing, and important for the ultimate therapeutic potential of our CFPs, that the induced conductances consistently increased with the introduction of positive charges. This might reflect the Dap-substituted peptides associating in entities of a higher order than the pentameric assembly predicted for the parent sequence,^{20,24,25} consistent with reports of variable conductances associated to different aggregation states of channel subunits;³⁰ further charge substitutions should be considered for future modifications. Finally, though it is plausible that endogenous channels may be activated by interaction with p22-T19R, S22W and the different Dap-substituted analogues, currents of

similar nature are evoked by phospholemman, the Na⁺/K⁺-ATPase subunit and other single-transmembrane proteins of the FXYD family,^{31,32} all of which have been shown to have intrinsic channel activity in a variety of cell and membrane systems,^{33,34} as have our M2GlyR synthetic peptides.^{9–14}

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References

1. Cohen TS, Prince A. Cystic fibrosis: a mucosal immunodeficiency syndrome. *Nat Med.* 2012; 18:509–19. [PubMed: 22481418]
2. Becq F. Cystic fibrosis transmembrane conductance regulator modulators for personalized drug treatment of cystic fibrosis: progress to date. *Drugs.* 2010; 70:241–59. [PubMed: 20166764]
3. Abel E, Maguire GEM, Meadows ES, Murillo O, Jin T, Gokel GW. Planar bilayer conductance and fluorescence studies confirm the function and location of a synthetic, sodium-ion-conducting channel in a phospholipid bilayer membrane. *J Am Chem Soc.* 1997; 119:9061–9062.
4. Agawa Y, Lee S, Ono S, Aoyagi H, Ohno M, Taniguchi T, Anzai K, Kirino Y. Interaction with phospholipid bilayers, ion channel formation, and antimicrobial activity of basic amphipathic alpha-helical model peptides of various chain lengths. *J Biol Chem.* 1991; 266:20218–22. [PubMed: 1718959]
5. Krittanai C, Panyim S. Structural design and characterization of a channel-forming peptide. *Journal of Biochemistry and Molecular Biology.* 2004; 37:460–465. [PubMed: 15469734]
6. Oiki S, Danho W, Montal M. Channel protein engineering: synthetic 22-mer peptide from the primary structure of the voltage-sensitive sodium channel forms ionic channels in lipid bilayers. *Proc Natl Acad Sci US A.* 1988; 85:2393–7.
7. Pajewski R, Garcia-Medina R, Brody SL, Leevy WM, Schlesinger PH, Gokel GW. A synthetic, chloride-selective channel that alters chloride transport in epithelial cells. *Chem Commun (Camb).* 2006:329–31. [PubMed: 16391750]
8. Tosteson MT, Auld DS, Tosteson DC. Voltage-gated channels formed in lipid bilayers by a positively charged segment of the Na-channel polypeptide. *Proc Natl Acad Sci USA.* 1989; 86:707–10. [PubMed: 2536173]
9. Iwamoto T, Grove A, Montal MO, Montal M, Tomich JM. Chemical synthesis and characterization of peptides and oligomeric proteins designed to form transmembrane ion channels. *Int J Pept Protein Res.* 1994; 43:597–607. [PubMed: 7523324]
10. Montal M, Montal MS, Tomich JM. Synporins--synthetic proteins that emulate the pore structure of biological ionic channels. *Proc Natl Acad Sci US A.* 1990; 87:6929–33.
11. Montal MO, Iwamoto T, Tomich JM, Montal M. Design, synthesis and functional characterization of a pentameric channel protein that mimics the presumed pore structure of the nicotinic cholinergic receptor. *FEBS letters.* 1993; 320:261–6. [PubMed: 7681786]
12. Oblatt-Montal M, Buhler LK, Iwamoto T, Tomich JM, Montal M. Synthetic peptides and four-helix bundle proteins as model systems for the pore-forming structure of channel proteins. I. Transmembrane segment M2 of the nicotinic cholinergic receptor channel is a key pore-lining structure. *J Biol Chem.* 1993; 268:14601–7. [PubMed: 7686900]
13. Oblatt-Montal M, Reddy GL, Iwamoto T, Tomich JM, Montal M. Identification of an ion channel-forming motif in the primary structure of CFTR, the cystic fibrosis chloride channel. *Proc Natl Acad Sci US A.* 1994; 91:1495–9.
14. Reddy GL, Iwamoto T, Tomich JM, Montal M. 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.* 1993; 268:14608–15. [PubMed: 7686901]

15. Cascio M. Structure and function of the glycine receptor and related nicotinic receptors. *J Biol Chem.* 2004; 279:19383–6. [PubMed: 15023997]
16. Schmieden V, Grenningloh G, Schofield PR, Betz H. Functional expression in *Xenopus* oocytes of the strychnine binding 48 kd subunit of the glycine receptor. *EMBO J.* 1989; 8:695–700. [PubMed: 2470588]
17. Wallace DP, Tomich JM, Iwamoto T, Henderson K, Grantham JJ, Sullivan LP. A synthetic peptide derived from glycine-gated Cl⁻ channel induces transepithelial Cl⁻ and fluid secretion. *Am J Physiol Cell Physiol.* 1997; 272:C1672–9.
18. Broughman JR, Mitchell KE, Sedlacek RL, Iwamoto T, Tomich JM, Schultz BD. NH(2)-terminal modification of a channel-forming peptide increases capacity for epithelial anion secretion. *American journal of physiology Cell physiology.* 2001; 280:C451–8. [PubMed: 11171563]
19. Broughman JR, Shank LP, Prakash O, Schultz BD, Iwamoto T, Tomich JM, Mitchell K. Structural implications of placing cationic residues at either the NH₂- or COOH-terminus in a pore-forming synthetic peptide. *J Membrane Biol.* 2002; 190:93–103. [PubMed: 12474074]
20. Cook GA, Prakash O, Zhang K, Shank LP, Takeguchi WA, Robbins A, Gong YX, Iwamoto T, Schultz BD, Tomich JM. Activity and structural comparisons of solution associating and monomeric channel-forming peptides derived from the glycine receptor m2 segment. *Biophys J.* 2004; 86:1424–35. [PubMed: 14990471]
21. Tomich JM, Wallace D, Henderson K, Mitchell KE, Radke G, Brandt R, Ambler CA, Scott AJ, Grantham J, Sullivan L, Iwamoto T. Aqueous solubilization of transmembrane peptide sequences with retention of membrane insertion and function. *Biophys J.* 1998; 74:256–67. [PubMed: 9449327]
22. Shank LP, Broughman JR, Takeguchi W, Cook G, Robbins AS, Hahn L, Radke G, Iwamoto T, Schultz BD, Tomich JM. Redesigning channel-forming peptides: amino acid substitutions that enhance rates of supramolecular self-assembly and raise ion transport activity. *Biophys J.* 2006; 90:2138–50. [PubMed: 16387776]
23. van Ginkel FW, Iwamoto T, Schultz BD, Tomich JM. Immunity to a self-derived, channel-forming peptide in the respiratory tract. *Clin Vaccine Immunol.* 2008; 15:260–6. [PubMed: 18094111]
24. Herrera AI, Al-Rawi A, Cook GA, Gao J, Iwamoto T, Prakash O, Tomich JM, Chen J. Structural characterization of two pore-forming peptides: consequences of introducing a C-terminal tryptophan. *Proteins.* 2010; 78:2238–50. [PubMed: 20544961]
25. Bukovnik U, Gao J, Cook GA, Shank LP, Seabra MB, Schultz BD, Iwamoto T, Chen J, Tomich JM. Structural and biophysical properties of a synthetic channel-forming peptide: Designing a clinically relevant anion selective pore. *Biochim Biophys Acta.* 2012; 1818:1039–48. [PubMed: 21835162]
26. Ivanov I, Cheng X, Sine SM, McCammon JA. Barriers to ion translocation in cationic and anionic receptors from the Cys-loop family. *J Am Chem Soc.* 2007; 129:8217–24. [PubMed: 17552523]
27. Bichet D, Grabe M, Jan YN, Jan LY. Electrostatic interactions in the channel cavity as an important determinant of potassium channel selectivity. *Proc Natl Acad Sci US A.* 2006; 103:14355–60.
28. Dutzler R, Campbell EB, MacKinnon R. Gating the selectivity filter in ClC chloride channels. *Science.* 2003; 300:108–12. [PubMed: 12649487]
29. Allen TW, Andersen OS, Roux B. Molecular dynamics - potential of mean force calculations as a tool for understanding ion permeation and selectivity in narrow channels. *Biophysical Chemistry.* 2006; 124:251–67. [PubMed: 16781050]
30. Lin MC, Kagan BL. Electrophysiologic properties of channels induced by Abeta25–35 in planar lipid bilayers. *Peptides.* 2002; 23:1215–28. [PubMed: 12128079]
31. Minor NT, Sha Q, Nichols CG, Mercer RW. The gamma subunit of the Na,K-ATPase induces cation channel activity. *Proc Natl Acad Sci US A.* 1998; 95:6521–25.
32. Sha Q, Pearson W, Burcea LC, Wigfall DA, Schlesinger PH, Nichols CG, Mercer RW. Human FXYP2 G41R mutation responsible for renal hypomagnesemia behaves as an inward-rectifying cation channel. *Am J Physiol Renal Physiol.* 2008; 295:F91–F99. [PubMed: 18448590]

33. Moorman JR, Ackerman SJ, Kowdley GC, Griffin MP, Mounsey JP, Chen Z, Cala SE, O'Brian JJ, Szabo G, Jones LR. Unitary anion currents through phospholemman channel molecules. *Nature*. 1995; 377:737–40. [PubMed: 7477264]
34. Chen ZH, Jones LR, Moorman JR. Ion currents through mutant phospholemman channel molecules. *Receptors Channels*. 1999; 6:435–47. [PubMed: 10635061]

Figure 1. Circular dichroism spectra of p22-T19R, S22W (WT) and Dap-substituted analogs
Peptides were reconstituted to 100 μ M in 40% water/TFE, and spectra were recorded at 25 $^{\circ}$ C (Neslab RTE-IIM circulator; Thermo Scientific, Waltham, MA). Data are the averages of five scans, after background subtraction.

Figure 2. Concentration-dependence of short-circuit currents (I_{sc}) induced by p22-T19R, S22W and Dap-substituted peptides in MDCK cells

Symbols, MDCK monolayers mounted in Ussing chambers were clamped to 0 mV, and transepithelial I_{sc} were measured in presence of increasing concentrations of p22-T19R, S22W (WT, *black*) or analog peptides with one (*dark gray*), two (*light gray*) or three (*white*) Dap-substituted residues. Data are means $\pm$ SE of 5–7 replicates. *Lines*, I_{sc} data were fitted by eq 1 to estimate the kinetic parameters of peptide-induced transepithelial ion transport; results are shown in Table 2.

Figure 3. Voltage dependence of currents measured in *Xenopus* oocytes

A, representative experiments. Oocytes were superfused with ND96 or the appropriate ion-substituted solution, first in absence (*control*), then for 5 min in presence of 50 μ M p22-T19R, S22W (WT), and again in the absence of peptide for a minimum of 10 min and until initial conditions were restored (*wash out*). Currents were recorded as the membrane potential was held at -30 mV and stepped to test values, as in the pulse protocol shown in the upper left panel. The dotted lines indicate zero current. **B–C**, current-voltage (I – V) relationships from the oocytes shown in **A**. For each experimental buffer, steady-state or pseudo steady-state currents induced in absence ($I_{control}$; **B**) or presence of peptide (I_{WT} ; **C**) were measured at the end of the 1000-ms voltage pulse.

Figure 4. Currents evoked by p22-T19R, S22W and Dap-substituted peptides in *Xenopus* oocytes Experiments were performed as in Figure 3. Oocytes were exposed to 50 μ M WT (*black*) or singly- (*dark gray*), doubly- (*light gray*) or triply- (*white*) Dap-substituted analogues, and the net peptide-induced currents (I_{net}) were calculated by subtracting the total currents from the currents measured in the absence of peptides. **A**, currents recorded in representative oocytes exposed to WT or selected analogues in ND96 or Na-Gluc buffer. **B–C**, I – V relationships from the experiments shown in **A**. **D**, summary of peptide-induced outward (+60 mV; *top*) and inward (–100 mV; *bottom*) currents measured in the different experimental solutions. The background currents were, at +60 mV (in nA), 245 ± 36 (KD96), 114 ± 10 (ND96), 152 ± 15 (Na-Gluc), and 484 ± 137 (K-Gluc); and at –100 mV (in nA), -257 ± 36 (KD96), -117 ± 16 (ND96), -110 ± 15 (Na-Gluc), and -416 ± 146 (K-Gluc). Results are means $\pm$ SE of 3–8 experiments. * $p < 0.05$ as compared to the WT sequence (unpaired Student's t test).

Table 1
Sequences of NK₄-M2GlyR p22-T19R, S22W (p22-T19R, S22W) and Dap-substituted peptides^a

Peptide ID	Sequence	g/mol
p22-T19R, S22W (WT) ²²	(K) ₄ PARVGLGTTVLTMRQTW	2512.1
p22-T19R, S22W, T13Dap (Dap13)	(K) ₄ PARVGLGIDapTVLTMRQTW	2497.1
p22-T19R, S22W, T17Dap (Dap17)	(K) ₄ PARVGLGTTVLTMRDapQTW	2497.1
p22-T19R, S22W, T20Dap (Dap20)	(K) ₄ PARVGLGTTVLTMRDapQW	2497.1
p22-T19R, S22W, T13Dap, T17Dap (Dap13,17)	(K) ₄ PARVGLGIDapTVLTMRDapQTW	2482.1
p22-T19R, S22W, T13Dap, T20Dap (Dap13,20)	(K) ₄ PARVGLGIDapTVLTMRDapQW	2482.1
p22-T19R, S22W, T17Dap, T20Dap (Dap17,20)	(K) ₄ PARVGLGTTVLTMRDapQW	2482.1
p22-T19R, S22W, T13Dap, T17Dap, T20Dap (Dap13,17,20)	(K) ₄ PARVGLGIDapTVLTMRDapQW	2467.1

^a Abbreviations used in the text are shown in parentheses.

Table 2

Kinetics of short-circuit currents in MDKC cells exposed to p22-T19R, S22W (WT) and Dap-substituted analogues^a

<i>Peptide ID</i>	<i>K_{0.5} (μM)</i>	<i>n_H</i>	<i>I_{max} (μA/cm²)</i>
WT	128 ± 29	2.2 ± 0.6	38 ± 6
Dap13	224 ± 273	2.2 ± 1.6	55 ± 59
Dap17	207 ± 156	2.6 ± 1.5	90 [†] ± 62
Dap20	326 ± 951	1.7 ± 1.6	90 [†] ± 233
Dap13,17	216 ± 277	2.1 ± 1.6	141 ± 152
Dap13,20	150 ± 62	3.1 ± 2.0	100 ± 28
Dap17,20	114 ± 21	3.3 ± 1.8	121 ± 15
Dap13,17,20	139 ± 71	2.9 ± 2.5	104 ± 35

^aConcentration-dependence I_{SC} data were fitted by eq 1 to estimate the maximum current (I_{max}), the peptide concentration for half-maximal I_{SC} ($K_{0.5}$), and the Hill coefficient (n_H) of peptide-induced transepithelial ion transport. All coefficients describe the solid lines that fit the data sets summarized in Figure 2.

[†]For Dap17 and Dap20, I_{max} was constrained to 90 μA/cm² for fitting purposes. The standard error of the fits is shown.

Table 3

Reversal potential (V_{rev}) of currents recorded in *Xenopus* oocytes superfused with the different experimental buffers, and changes in V_{rev} (ΔV_{rev}) induced by exposure to NK₄-M2GlyR p22-T19R, S22W (p22-T19R, S22W) or Dap-substituted peptides^a

	ND96	KD96	Na-Gluc	K-Gluc
<i>In the absence of peptide</i>				
	V_{rev} (mV)			
	-35 ± 3	-3 ± 5	-39 ± 4	-14 ± 2
<i>In the presence of peptide</i>				
	V_{rev} (mV)			
WT	4 ± 2	2 ± 1	4 ± 1	6 ± 1
Dap13	2 ± 3	8 ± 3	3 ± 2	6 ± 1
Dap17	5 ± 2	2 ± 2	6 ± 3	9 ± 3
Dap20	5 ± 3	6 ± 1	1 ± 1	6 ± 2
Dap13,17	5 ± 1	5 ± 3	5 ± 4	7 ± 3
Dap13,20	3 ± 2	6 ± 1	6 ± 1	3 ± 1
Dap17,20	5 ± 2	5 ± 3	7 ± 1	4 ± 1
Dap13,17,20	5 ± 4	4 ± 1	4 ± 2	5 ± 2

^aOocytes were held at -30 mV and stepped to test values ($+60$ to -120 mV, in 20 mV increments), and steady-state or pseudo steady-state currents were measured at the end of each 1000 -ms voltage step. V_{rev} was estimated as the intercept of linear I - V relationships between $+20$ and -60 mV. Data are from the same experiments as in Figures 3 and 4. Results are means $\pm$ SE of 3–8 experiments.