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Expression of an Artificial Cl^- Channel in Microperfused Renal Proximal Tubules

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Abstract. To better understand the process of fluid movement driven by Cl^- conductance, a Cl^- channel-forming peptide was delivered to the luminal membrane of microperfused rabbit renal proximal tubules. When the peptide (NK_4-M2GlyR) was perfused, a significant new conductance was observed within 3 min and stabilized at 10 min. Alteration of the ion composition revealed it to be a Cl^--specific conductance. Reabsorption of Cl^- (J_{Cl}) was increased by NK_4-M2GlyR, but not by a scramble NK_4-M2GlyR sequence, suggesting that the active peptide formed *de novo* Cl^- channels in the luminal membrane of the perfused tubules. In the presence of the peptide, reabsorption of fluid (J_v) was dramatically increased and J_{Na} and J_{Ca} were concomitantly increased. We propose that introduction of the new Cl^- conductance in the luminal membrane leads to a coordinated efflux of water across the membrane and an increase in cation translocation via the paracellular pathway, resulting in an increase in J_v. This novel method could prove useful in characterizing mechanisms of fluid transport driven by Cl^- gradients.

Key words: Cl^- conductance — Fluid transport — Kidney — NK_4-M2GlyR peptide

Introduction

Recent advances in molecular biology enable us to construct molecules expressed in living cells. However, molecular expression using in vitro microperfused tubules is made difficult because exogenous cDNA requires a long incubation time for expression. On the other hand, anion-selective channel-forming peptides can be designed, constructed and delivered to form functional ion channel pores in a variety of cell types. A 23-residue peptide based in the second transmembrane segment (M2) of the α1-subunit of the spinal cord glycine receptor (GlyR) forms a Cl^--permeable pathway in lipid bilayers [10]. A family of M2GlyR peptides (K_x-M2GlyR) was created by adding lysine residues to the N- or C-terminus of the M2GlyR sequence and their channel-forming activities were assessed [3]. Owing to the presence of the hydrophilic lysine residues, the peptide is highly soluble in water. The highly soluble peptides were still able to self-associate in the lipid bilayers to form homo-oligomeric associations [16], with tetramers or pentamers being the most prevalent assemblies [10]. Addition of the lysine-modified peptides to the apical membrane of Madin-Darby canine kidney (MDCK) and T84 cell monolayers was associated with both Cl^- and water secretion [17] as well as increased glutathione secretion in cultured human cystic fibrosis cells [6].

In contrast to cultured monolayers, renal proximal tubules reabsorb rather than secrete Cl^- and water. Wang et al. (1995) [18] reported that in the microperfused rat proximal tubule, the fluid absorptive rate was increased upon addition of cAMP. They further showed that this effect occurred only when there was a significant transepithelial Cl^- gradient and that the addition of Cl^- channel blockers negated the stimulatory effect of cAMP. This model proposed the existence of cAMP-dependent Cl^- channels in both the apical and basolateral membranes that would allow transepithelial movement of Cl^- along the Cl^- gradient. However, a controversy remained concerning the proposed Cl^- conductance [7] and none of the physiologic consequences were demonstrated by activation of luminal Cl^- conductance. Thus we decided to examine fluid transport by using a known anion-selective channel-forming peptide in perfused proximal tubules.

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Materials & Methods

Peptide Synthesis

The peptide synthesis was previously described [3]. The peptide was synthesized on a peptide synthesizer (model ABI 431A; Applied Biosystems, Foster City, CA) using Fmoc chemistries and purified by reverse-phase HPLC and characterized by matrix-assisted laser desorption time of flight mass spectroscopy. The sequence of the NK_4-M2GlyR is KKKKPARVGLGITTVLTMTTQSSGSRA. The scramble-peptide sequence is KKKKILASTRSQTGRMALGTTTPGVV.

In vitro Microperfusion of Rabbit Renal Proximal Tubules

In vitro microperfusion was performed according to the method of Burg and Green with modifications [4]. Kidneys were obtained from female New Zealand White rabbits weighing 1.5–2.5 kg and maintained on standard laboratory chow with free access to tap water. While anaesthetized with sodium pentobarbital (50 mg/kg, i.v.), the left kidney was removed, and coronal slices were made. Slices were then transferred to a chilled dish containing modified Collins' solution of the following composition (in mM): 14 KH_2PO_4, 44 K_2HPO_4, 15 KCl, 9 $NaHCO_3$, and 160 sucrose, with pH adjusted to 7.4. Segments of proximal tubule (S2) of lengths ranging from 0.7–1.2 mm, were isolated from the medullary ray of renal cortex by fine forceps under a stereomicroscope. Isolated tubules were transferred to the perfusion chamber, which was mounted on an inverted microscope (IMT-2; Olympus, Tokyo, Japan) and perfused in vitro at 37°C. Tubules were connected to the holding pipette and a single-barreled perfusion pipette was inserted into the tubular lumen. Triple-barreled polyethylene tubing was inserted into the perfusion pipette to allow rapid exchange of perfusion fluid. The perfusion rate was controlled at 10 to 20 nl/min by adjusting the height of the fluid reservoir, which was connected to the back end of the perfusion pipette. A system using a flow-through bath was used to permit rapid exchange within 2 sec. The composition of the basal control solution used in this study was as follows (in mM): 110 NaCl, 25 $NaHCO_3$, 5 KCl, 0.8 Na_2HPO_4, 0.2 NaH_2PO_4, 10 Na-acetate, 1.8 $CaCl_2$, 1 $MgSO_4$, 8.3 D-glucose and 5 L-alanine, and it was gassed with 95% O_2/5% CO_2, yielding a pH of 7.4 at 37°C. Replacing 110 NaCl with 1.5 NaCl and 108.5 Na-cyclamate constituted low-Cl^- (12 mM Cl^-) solution. Replacing 110 NaCl and 25 $NaHCO_3$ with 110 choline-Cl and choline-HCO_3 constituted low-Na^+ (12 mM Na^+) solution. When flux was measured, the bathing solution usually was comprised of control solution plus neutral dextran (2 g/dL, molecular mass 70,000, Sigma). A peristaltic pump operated at a rate of 14 mL/hr to maintain constant solute concentration in a continuously exchanging bath solution.

Electrophysiological Studies

Transepithelial voltage (V_T) was measured using the perfusion pipette as an electrode. The voltage difference between calomel cells, connected via 3 M KCl agar bridges between the perfusing and bathing solutions was measured using a high-impedance electrometer. Basolateral membrane voltage (V_b) was measured by intracellular impalement of the epithelia of the perfused segments with a conventional microelectrode. Apical membrane voltage (V_a) was calculated as: $V_a = V_T - V_b$.

Fluid Transport

The perfusate-collecting end was sealed into a holding pipette using Sylgard 184 (Dow Corning, Midland, MI). The length of each segment was measured using an eyepiece micrometer. [^{14}C] inulin was added to the perfusate at 10 μCi/mL, yielding ~30 counts/min/nL. Samples (68 nL) were collected under water-saturated mineral oil by timed filling of a calibrated volumetric pipette. Collections were obtained in triplicate, placed in 1 mL of water plus 6 mL of scintillation solution containing 4 mg Omnifluor (Packard Bioscience, Groningen, The Netherlands) per milliliter in toluene-Triton X-100 (2:1 vol/vol), and the beta emissions of ^{14}C were counted (Beckman LSC-3500; Aloka, Tokyo, Japan). The perfusion rate was generally 6–12 nL/min. Fluid absorption rate (J_v) was calculated as: $J_v = V_o (C_o/C_i - 1)/L$ where V_o is collection rate (nL/min), L is tubular length (mm), and C_o and C_i are concentrations of inulin at the outlet and at the inlet of the renal tubule of collected and perfused fluid, respectively.

Sodium, Chloride, and Calcium Transport

The concentrations of sodium and calcium in perfusate and collected fluid were measured in a continuous flow microfluorimeter (Nanoflo; WPI, Sarasota, FL). Cl^- was measured with isotopically labeled compound (^{56}Cl). Three 68-nL collections were made per period and stored under water-saturated mineral oil. Perfusion rate was generally 6–12 nL/min. To measure lumen-to-bath X (X = sodium, calcium or chloride), flux (J_x, pmol/min/mm) was calculated as: $J_x = (V_i[X]_i - V_o[X]_o)/L$ where V_i and V_o are perfused and collection rate (nL/min) and $[X]_i$ and $[X]_o$ are concentrations of X in the perfusate and collected fluid, respectively. Positive values of J_x indicate net absorption of X.

Statistics

Data are presented as means ± SE. Paired comparison for each tubule was analyzed by a paired t-test using statistical software (Stat View). Significance was posited when $P < 0.05$.

Results

Peptide Concentrations and Incubation Periods

To determine functional peptide concentrations and suitable incubation periods, the lumen of proximal straight tubule (PST) was initially perfused with NK_4-MZGlyR for 20 min. V_T was significantly depolarized and stabilized after 10 min at a concentration of 10.6 μM (30 μg/mL). The concentration of NK_4-M2GlyR was subsequently reduced ten-fold to 0.3 μg/mL without significantly affecting V_T, suggesting that the lower concentration, 3 μg/mL (1.06 μM), could be employed for these studies. Thus we set the concentration of NK_4-M2GlyR as 3 μg/mL and obtained values after 15 min pre-incubation for all subsequent studies. We then compared the effects of NK_4-MZGlyR and its scramble form (NK_4-scramble) on V_T. The scramble did not significantly depolarize V_T (Fig. 1, $n = 6$, $P < 0.05$).

Effects of Luminal NK_4-M2GlyR on Na^+ and Cl^- Conductance

Alteration of V_T can be achieved by an increase in either anion (Cl^-) or cation (Na^+) conductance. To

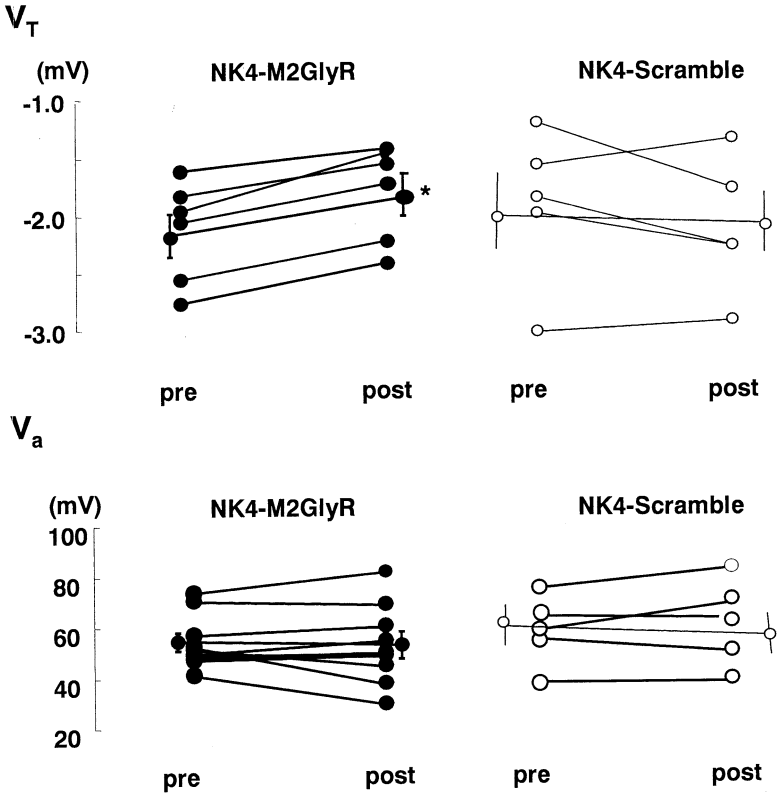

Fig. 1. Effect of luminal NK_4-M2GlyR on $V_T \cdot V_T$ (*upper panel*) and V_a (*lower panel*) were measured with microperfused PST before (*pre*) and after (*post*) addition of NK_4-M2GlyR (*left*) or its scramble peptide (*right*) at a concentration of 30 μg/ml to the lumen. $^*P < 0.05$

examine luminal Na^+ and Cl^- conductance, luminal Na^+ or Cl^- were replaced rapidly with low-Na^+ or low-Cl^- solutions and V_b and V_T were monitored. Representative traces of V_b and V_T by low-Cl^- substitution are shown in Fig. 2. When the luminal fluid was changed rapidly to low Cl^- solution, V_b and V_T hyperpolarized immediately and V_a depolarized. The depolarization in V_a following the luminal Cl^--concentration-decrease increased significantly after application of NK_4-M2GlyR (Fig. 2, $n = 5$, $P < 0.05$). When the luminal fluid was replaced rapidly with the low-Na^+ solution, V_b hyperpolarized, V_T depolarized and V_a depolarized immediately. The depolarization in V_a following luminal Na^+ reduction was not significantly different after NK_4-M2GlyR application. Thus, the NK_4-M2GlyR formed a Cl^--conducting path on the luminal membrane of the proximal tubules.

Effects of Luminal NK_4-M2GlyR on J_V and J_{Cl}

We examined six proximal straight tubules averaging 0.7 ± 0.1 mm in length for the effect of NK_4-M2GlyR on J_V and J_{Cl} using isotopically labeled compound. J_V and J_{Cl} were measured concomitantly. NK_4-M2GlyR increased J_V and J_{Cl} significantly, while the scramble peptide had no influence (Fig. 3; $n = 6$; $P < 0.01$). Therefore, NK_4-M2GlyR will form Cl^--conducting pores in the luminal membrane of perfused PST, resulting in an increase in J_V and J_{Cl}.

Effects of Luminal NK_4-M2GlyR on Na^+ and Ca^{2+} transport

To investigate solute transport followed by an opening of the peptide Cl^- channel in the luminal membrane, we measured J_V, J_{Na} and J_{Ca} concomitantly. The baseline data using a 2 g/dL dextran bath revealed a J_V of 0.78 ± 0.01 nL/min/mm. After the addition of NK_4-M2GlyR, J_V increased to 2.59 ± 0.22 nL/min/mm. The baseline data showed a Na^+ absorption rate (J_{Na}) of 110.6 ± 3.1 pmol/min/mm ($n = 6$) and a Ca^{2+} absorption rate (J_{Ca}) of 6.69 ± 0.3 pmol/min/mm ($n = 4$). After NK_4-M2GlyR was added to lumen, the absorption rates increased to 161.2 ± 7.3 (Fig. 4, $n = 6$, $P < 0.05$) and 13.7 ± 1.0 for Na^+ and Ca^{2+}, respectively (Fig. 4, $n = 4$, $P < 0.05$). Thus, an increase in Cl^- conductance results in fluid and cation reabsorption in the PST.

Discussion

We have designed a system to investigate the resultant alteration of fluid transport arising from a change in luminal Cl^- conductance in the presence of an anion-selective channel-forming peptide. On the

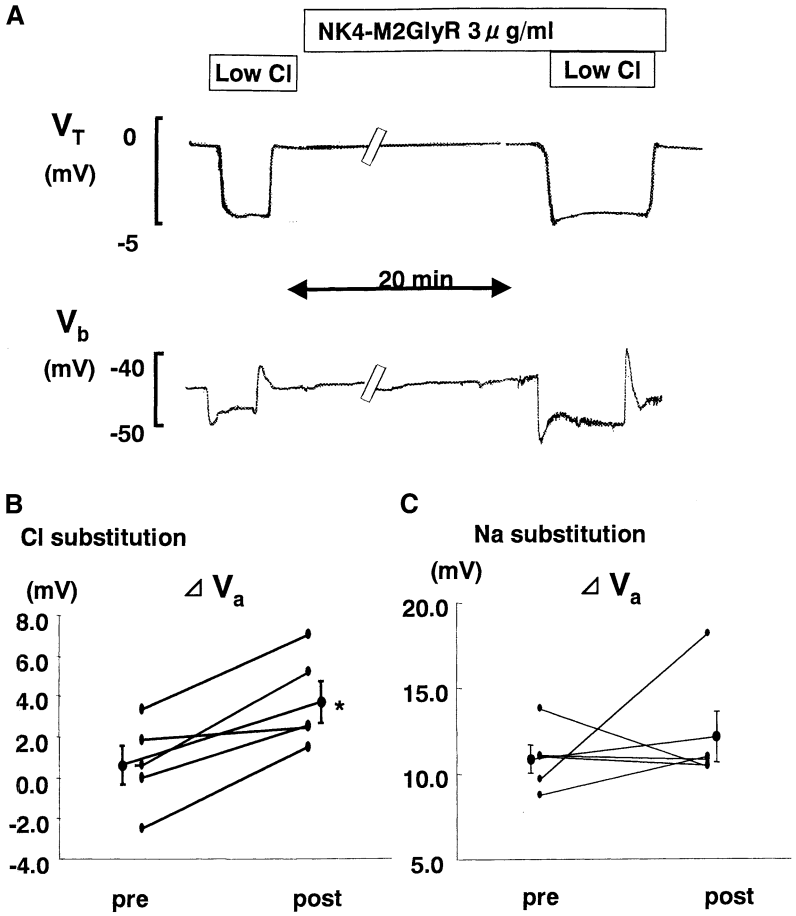

Fig. 2. V_T, V_b and ΔV_a in PST with NK_4-M2GlyR in response to low Cl^- luminal perfusate. (*A*) Representative traces for V_T (*upper panel*) and V_b (*lower panel*) were measured with microperfused PST 20 min after addition of NK_4-M2GlyR at a concentration of 30 μg/ml to lumen. Low-Cl^- solution was perfused in lumen of PST to estimate Cl^- conductance. V_T or V_b at a stabilized deflection during low Cl^- was determined by eye and ΔV_a was calculated. ΔV_a during low Cl^- (*B*) or low Na^+ (*C*). Means ± SE in control (*pre*) and in NK_4-M2GlyR (*post*) period were plotted with bars. $^*P < 0.05$.

basis of its channel-forming properties, pharmacology and high aqueous solubility (13.4 mM) [3, 16] NK_4-M2GlyR and related sequences are being pursued as potential therapeutics for the treatment of anion hyposecretory disorders [3]. Anion-selective pores form within a few minutes of exposure at concentrations of 100 nM or higher for the p27 peptide in lipid bilayers as well as in cultured monolayers. This is the first report that shows channel-forming activity in a primary tissue-based assay, microperfused renal tubules.

The effective concentration of NK_4-M2GlyR and interval for onset of channel activity are similar to results in MDCK monolayers [17]. During administration of the peptide, the pressure of the perfusate was increased to achieve a constant flow. This pressure increase was necessary to compensate for the narrowing of the lumen. This narrowing also occurs when fluid reabsorption is highly stimulated. This observation led us to determine Na^+ as well as Cl^- conductance levels. The resultant depolarization of V_a together with the Cl^- conductance indicated that NK_4-M2GlyR formed a Cl^--selective pore in the luminal membrane and Cl^- was driven from the lumen to the cell interior. The resting membrane potential (V_b) of the proximal cells was about −50 mV [2]. Supposing that intracellular Cl^- concentration was <30 mM, Cl^- would move into the cell from the luminal side. The value of the interior Cl^- concentration was slightly lower than that estimated by fluorescent dye [8].

The fluid reabsorption was remarkably and significantly increased by NK_4-MZGlyR. It was assumed that chloride ions were reabsorbed entirely passively with sodium. More recent studies suggest that several distinct reabsorptive transport mechanisms operate in parallel [12]. Accordingly, a new model of proximal chloride transport has evolved that includes active, transcellular as well as passive, intercellular transport pathways. Transcellular chloride reabsorption involves anion exchange mechanisms in both the luminal and peritubular cell membranes, processes that also depend on sodium, hydrogen, and bicarbonate ions. The passive pathway model predicts that reabsorption of Na^+ establishes a new gradient between the luminal and basolateral space, which in turn forces Cl^- across the paracellular pathway to reestablish electroneutrality. Chloride transport is thus intimately related to sodium and fluid transport as well as to cell acid-base metabolism.

The Cl^- and water would exit through the basolateral or paracellular space via the Cl^- channels or

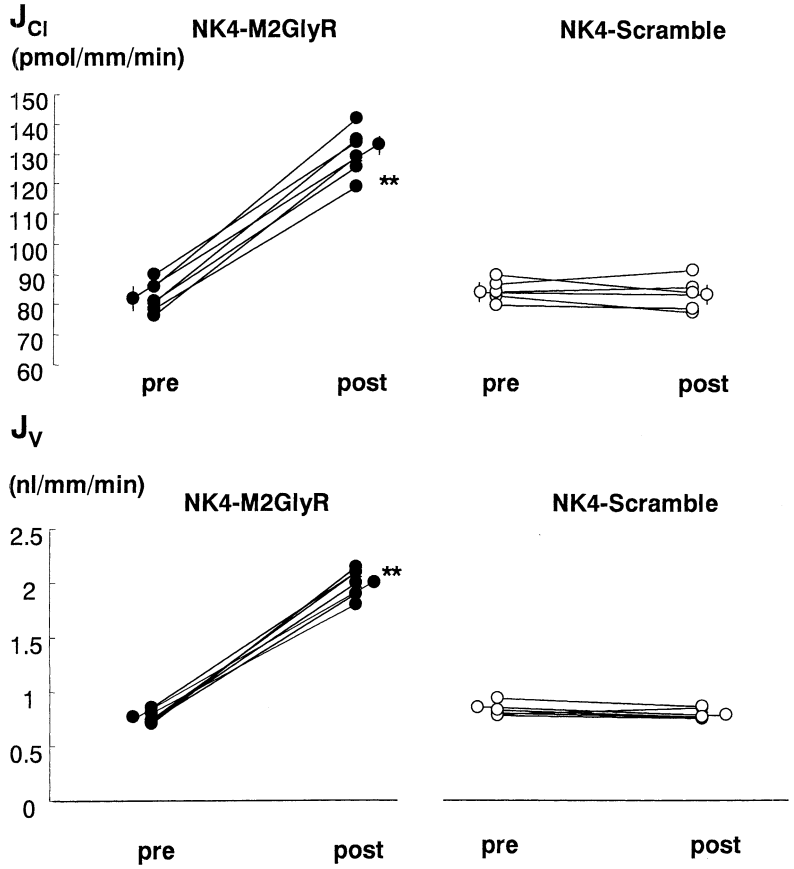

Fig. 3. Effect of NK_4-M2GlyR or its scramble peptide on fluid or Cl^- absorption rate in PST. J_v (*top*) or J_{Cl} (*bottom*) was concomitantly measured with the concentration of labeled inulin and Cl^-, respectively. NK_4-M2GlyR (*left*) or its scramble peptide (*right*) at a concentration of 3 μg/ml in luminal fluid, the former of which causes a significant increase in J_v and J_{Cl} $^{**}P < 0.01$.

transporters. Since V_b was not significantly altered, electroneutral transporters (K^+/Cl^-, anion exchanger, $Na^+/HCO_3^-/Cl^-$) may play a role in the exit of Cl^-. The remaining part of the fluid could be transferred via the paracellular pathway. Actually, flux of Na^+ was significantly increased without any change of ΔV_a. Thus Na^+ was transferred mainly via the paracellular pathway in response to the Cl^- flux or bulk solvent movement [1]. We attempted to establish the contribution of the paracellular pathway by using protamine to inhibit transiently this route [15]. However, this reagent produced inconsistent results under the conditions employed, even with long incubation times.

A discrepancy remained between J_v and $J_{Na} \cdot J_v$ displayed an increase that ranged from 100–200%, while an increase in J_{Na} did not correlate directly with $J_v \cdot J_{Na}$ increased less than 100%. This is well explained if water reabsorbed more than Na^+. In this setting, water and Cl^- were absorbed via the transcellular route, a transporter excreted Cl^-, and exit of Cl^- forces Na^+ across to the basolateral side through the paracellular pathway. Thus Cl^- gradients when stimulated, such as those seen in response to the additions of the artificial channel, may play a major role as an osmotic driving force. Further study will be required to fully confirm this hypothesis.

Interestingly, Ca^{2+} reabsorption was also enhanced by NK_4-M2GlyR, probably via the paracellular pathway. This result suggests that Ca^{2+} reabsorption is altered by a change of Cl^- conductance in the proximal tubules.

Appearance of Cl^- conductance during signal transduction is known to occur. Evidence for the expression of cAMP-dependent Cl^- channels in proximal tubule has been obtained from single-channel recordings in the rat [5], rabbit [14] and *Ambystoma* [13]. However, numerous studies were unable to reproduce significant Cl^- conductance with whole-cell currents [7, 9, 11]. Emergence of Cl^- conductance as an effective driving force for fluid transport in the present study is remarkable. It should be noted that, while Cl^- conductance was artificially manipulated in this study by the addition of an anion-specific channel-forming peptide, other means within the cell have the potential to drive this process. Thus, appearance of Cl^- conductance, even on the single-channel level in the apical membrane, may play a role in fluid reabsorption and cation metabolism for longer time periods.

The channel-forming peptide used in this study shows high Cl^- conductance at reduced peptide concentrations [3] and shows promise as therapeutics for patients with cystic fibrosis. Peptides such as this

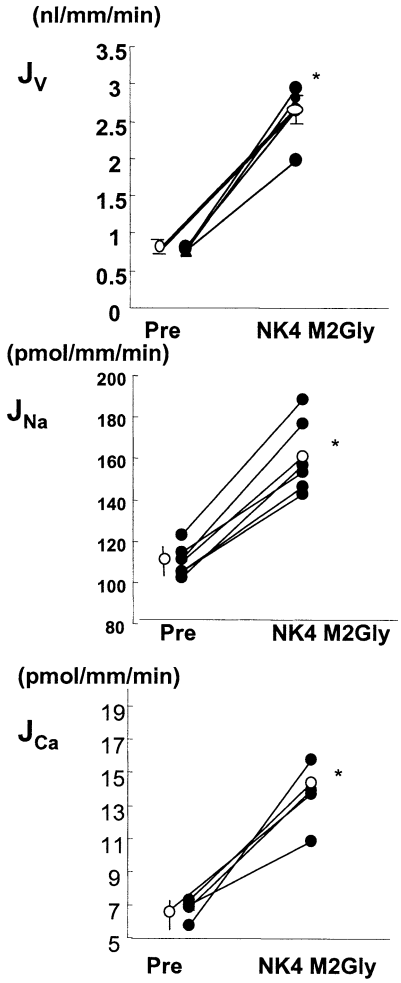

Fig. 4. Effect of luminal NK_4-M2GlyR and $HgCl_2$ on fluid, Na^+ and Ca^{2+} absorption rate (J_v, J_{Na}, J_{Ca}) in PST. J_v, J_{Na} or J_{Ca} was measured with the concentration of labeled compound before and after addition of 3 μg/ml NK_4-M2GlyR to the luminal perfusate. Empty circles with bar indicate mean ± SE for J_v (*top panel*), J_{Na} (*middle panel*) and J_{Ca} (*lower panel*). *$P < 0.05$. Addition of 0.3 mM $HgCl_2$ to luminal fluid suppressed the increase (*not shown*).

one might prove useful in providing specific conductance to other targeted membranes.

Cl^- gradient when magnified by the artificial channel may turn out to play a major role in water and calcium rather than fluid transport. This approach might find application in addressing the previously undetectable questions involving mechanisms of fluid transport.

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