Presence of the nucleic acid channel in renal brush-border membranes: allosteric modulation by extracellular calcium

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Leal-Pinto, Edgar, Avelino Teixeira, Baohuong Tran, Basil Hanss, and Paul E. Klotman. Presence of the nucleic acid channel in renal brush-border membranes: allosteric modulation by extracellular calcium. Am J Physiol Renal Physiol 289: F97–F106, 2005. First published February 22, 2005; doi:10.1152/ajprenal.00196.2004.—We have previously described a cell surface channel complex that is highly selective for nucleic acid (6, 7). The channel complex was purified to homogeneity by solubilizing renal brush-border membranes (BBM) with CHAPS and separation by liquid chromatography. It was characterized by reconstitution in planar lipid bilayers. The channel consists of a pore-forming subunit that is blocked by heparan sulfate and a regulatory subunit that is blocked by L-malate (7). The current studies were performed to compare the characteristics of the nucleic acid-conducting channel in native BBM with the characteristics that have been determined for the complex reconstituted from purified proteins. BBM were purified by differential centrifugation and reconstituted in lipid bilayers. Current was not observed until oligodeoxynucleotide (ODN) was added. Conductance was 9.1 ± 0.9 pS; rectification and voltage dependence were not observed. Reversal potential (Erev) shifted to +14 ± 0.1 mV by a 10-fold gradient for ODN but was not altered when gradients were created for any other ion. Open probability increased significantly with an increase in Ca2+ on the trans chamber of the bilayer apparatus. Changes in cis Ca2+ were without effect. Addition of L-malate to the cis chamber or heparan sulfate to the trans chamber significantly reduced the open probability of the channel. These data demonstrate that the nucleic acid channel in BBM is electrophysiologically and pharmacologically identical to that previously reported for purified protein and demonstrate that a nucleic acid-conducting channel is a component of renal BBM.

nucleic acid transport; oligonucleotide; calcium regulation; transport; kidney; epithelium

We have recently described a selective nucleic acid-conducting channel (NACH) purified from rat kidney apical membranes (6, 7). NACH is a complex of at least two proteins that migrate at 45 kDa (p45) and 36 kDa (p36) on SDS-PAGE (7). The purified complex, when reconstituted in planar lipid bilayers, forms a channel that is active only in the presence of nucleic acids. Shifts in reversal potential were observed only when concentration gradients were established for single stranded oligodeoxynucleic acids (ODN) and not when gradients were established for any other ion present in the buffered solution, suggesting that the channel is selective for ODN. Nucleic acid transfer across the bilayer was confirmed by radio-isotopic tracer studies which showed translocation of full length ODN only when channel activity was present (6). Channel conductance is 10 pS and there is no evidence of voltage dependence or rectification under the conditions of reconstitution (6). Channel open probability is influenced significantly by calcium concentration. The channel is essentially closed in the absence of calcium and open probability increases with increasing calcium concentration (15).

We have not found any evidence to support the translocation of other ions through the channel. Reversal potential shifts were not observed when concentration gradients were established for sodium, potassium, calcium, chloride, or gluconate. Furthermore, systematic elimination of these ions from the buffered solution did not alter channel activity. These data indicate that the channel is selective for ODN. We cannot, however, completely rule out the possibility that a small amount of another ion is moving through the channel with the DNA. This stems from our observation that ODN current is not Nernstian (6). There are several possible explanations for this observation. One such explanation is that another ion translocates with the ODN, which has been reported in other systems (1, 2).

NACH consists of two functional subunits, a pore-forming subunit and a regulatory subunit. When p45 is reconstituted alone in lipid bilayers, it forms a pore that conducts a number of ions including chloride, potassium, and oligonucleotides, is somewhat more selective for anions than cations, and is blocked by heparan sulfate (7). The regulatory subunit, p36, interacts with p45 to convert the conductive pathway to a nucleic acid specific channel. Several experiments indicate that the regulatory subunit is cytosolic malate dehydrogenase (cMDH). These include the following: 1) p36 contains cMDH as determined by peptide sequence; 2) the regulatory subunit function can be replaced by purified cMDH (Sigma, St. Louis, MO); 3) the enzyme substrate, L-malate, blocks NACH activity; and 4) antibodies that specifically recognize cMDH block channel activity (7). While cMDH has been described as a cytosolic protein, recent immunofluorescence studies in our laboratory localized cMDH to plasma membranes of the porcine renal epithelial cell line LLC-PK1 (7). Together, these data demonstrate a complex of proteins forms a nucleic acid-selective channel when reconstituted in planar lipid bilayers. Furthermore, they demonstrate that the channel is composed of at least two functional subunits, a pore-forming subunit represented by p45 and cMDH, which determines channel selectivity.

The characteristics of NACH described above were determined using purified proteins reconstituted in a lipid bilayer. The presence of an active channel in the cell membrane in vivo has not been proven, however. We initiated the current studies...
to compare the characteristics of NAc in native membranes with those of the channel complex reconstituted from purified proteins. We designed a series of experiments in which brush-border membranes (BBM) were isolated from rat kidney and fused directly with planar lipid bilayers. The channel activity of these brush-border membranes was then examined using standard electrophysiological techniques, and the presence of NAc was evaluated using electrophysiological and pharmacological criteria.

MATERIALS AND METHODS

Isolation of BBM. Renal BBM were purified as previously described (6, 14). All animal protocols were approved by the Institutional Animal Care and Use Committee of the Mt. Sinai School of Medicine. Male Sprague-Dawley rats were anesthetized with inactin, and both kidneys were removed and placed in an ice-cold buffered solution consisting of 250 mM sucrose, 10 mM triethanolamine, pH 7.4, with HCl. Renal cortical tissue was collected with a Stadie Riggs microtome and the minced with a rotary homogenizer. The cell membranes were then disrupted by homogenization with three strokes of a glass-Teflon homogenizer (clearance 0.15–0.23 mm; 1,500 rpm). Nuclei and mitochondria were pelleted by isopycnic centrifugation two times at 700 g. Following each step of centrifugation, the supernatant and the upper white-fluffy layer of the pellet was collected and the remaining pellet layers were discarded. The collected sample was then centrifuged at 15,000 g for 20 min. The supernatant and upper layer of the pellet was again collected and homogenized with a glass-glass homogenizer. The homogenized sample was centrifuged again at 15,000 g for 20 min, and the upper layer of the pellet was collected; the supernatant and remaining pellet layers were discarded. The sample was homogenized again in a glass-glass homogenizer and centrifuged once again at 21,500 g for 20 min. The upper pellet layer was resuspended by homogenization with a glass-glass homogenizer in 4 ml of a buffered solution consisting of 300 mM sucrose, 10 mM Tris/HEPES, pH 7.4. The suspension was then mixed with a 15% Percoll gradient (in 280 mM sucrose, 100 mM Tris/HEPES, pH 7.4) and spun at 23,000 g for 30 min in a vertical rotor (SV-600) in a Sorval 5B centrifuge providing rate control. One-milliliter fractions were collected from the column. Each fraction was assayed for protein concentration, alkaline phosphate activity, and Na-K-ATPase activity (21). Alkaline phosphate activity and Na-K-ATPase were standard markers for brush-border and basolateral membranes, respectively (12, 13, 31). The Na-K-ATPase assay was adapted from Jorgensen (10) and the Na-K-ATPase activity in fractions collected from a Percoll gradient. One-milliliter fractions were collected from a Percoll gradient and analyzed for protein concentration (OD280), alkaline phosphate activity (●), and Na+-K+-ATPase activity (○). Fractions 2–10 showed a peak in protein concentration, which corresponded with increased alkaline phosphate activity and low-Na+-K+-ATPase activity. This profile is consistent with the apical membrane of renal epithelium. Fractions 25–30 similarly showed a peak of protein concentration; however, this peak is associated with high-Na+-K+-ATPase activity and low alkaline phosphate activity, suggesting that this peak contains basolateral membrane.

Lipid bilayer experiments. Reconstitution experiments were performed as previously described (6, 7, 15) except that instead of reconstituting purified proteins, as was done in previous publications, BBM were fused with the preformed planar lipid bilayer. Briefly, phosphatidylethanolamine and phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) were mixed 1:1, dried under nitrogen, and dissolved in decane to a final concentration of 50 mg/ml. This mixture was used to form a planar lipid bilayer over a 10- to 50-μm hole in Teflon film. The Teflon film separated two solution chambers, both of which were filled with 1 ml of a buffered solution consisting of 200 mM CsCl, 1 mM CaCl₂, 2 mM BaCl₂, 1 mM GdCl₃, and 10 mM HEPES, pH 7.4. Gadolinium and barium were present in the solution to block cationic channels present in rat kidney BBM (29).

The solution chambers were connected to a patch-clamp amplifier through a head stage with a 10-GΩ feedback resistor and frequency band width of 10 kHz. The cis chamber is defined as the solution chamber connected to the voltage-holding electrode, and all voltages are referenced to the trans (ground) side. Current output of the patch clamp was filtered at 1 kHz through an eight-pole Bessel filter, digitized at 0.05 or 0.25 ms/point, and analyzed with commercial software (pCLAMP version 8.0.2). Once the bilayer was formed and allowed to stabilize for a minimum of 1 h, it was tested for the presence of channel-like activity or leak current by ramping holding potential from −200 to +200 mV. If the membrane was stable without signs of leak current and a resistance of more than 100 GΩ, 5 μl of isolated BBM were added to the trans chamber and stirred for ~5 min. The membrane was then allowed to stabilize again for ~2 h. If there was no evidence of channel activity, ODN (20 base homomultimer of thymidine, d(T₂₀) was added to both chambers to a final concentration of 5 μM and nucleic acid-dependent channel activity was assessed.

Channel characterization. In previous studies, it has been shown that NAc selectively conducts single-stranded nucleic acids, is dependent on calcium, and is blocked by heparan sulfate or 1-malate (6, 7, 14). To determine whether NAc has the same characteristics in native BBM, these kinetic and pharmacological criteria were investigated.

To assess the basal kinetics of the channel in native BBM, channel activity was analyzed under conditions in which both bilayer solution chambers were filled with the buffered solution described above. The holding potential was ramped from −150 to +150 mV, and the current output was collected and analyzed as described.

To examine channel selectivity, studies were performed in the presence of either symmetrical ODN or a 10-fold ODN gradient (10:1:cis:trans), holding potential was ramped from −150 to +150 mV, and current output was collected. Current-voltage relationships were determined and the relative selectivity of the channel was calculated according to the Goldman equation:
\[
E_{\text{rev}} = \frac{RT}{z} \log_\circ \left( \frac{P_{\text{ODN}/P_{\text{Cl}}} \alpha_{\text{ODN-cl}}} {P_{\text{ODN}/P_{\text{Cl}}} \alpha_{\text{ODN-trans}} + \alpha_{\text{Cl}}_{\text{trans}}} \right)
\]

where \( E_{\text{rev}} \) = reversal potential, \( RT/z \) have their usual meaning, \( P_{\text{ODN}/P_{\text{Cl}}} \) is the ODN-to-chloride permeability ratio, \( \alpha_{\text{ODN-cl}} \) and \( \alpha_{\text{ODN-trans}} \) represent the ODN concentration in the cis and trans chamber, respectively, and \( \alpha \) is the product of \( \gamma \) and the given ion concentration (\( \gamma \) equals the activity coefficient of the corresponding ion and in this case is assumed to be 1 due to complete dissociation of sulfate, or L-malate were added to the solution chamber.

To assess the pharmacological characteristics of the BBM channel, BBM were reconstituted as described above, baseline channel activity was assessed, and then increasing amounts of calcium, heparan sulfate, or L-malate were added to the cis or trans solution chamber. Channel activity was then reassessed. We also examined the effect of oxaloacetate, the product of the reaction between L-malate and NAD, on channel activity.

**RESULTS**

The first step in purifying NACH was the isolation of apical (brush border) membranes from rat kidneys. Apical membranes were isolated and enriched using differential centrifugation to separate the plasma membrane from other cell components and then separation of apical and basolateral membranes by density gradient centrifugation through a Percoll gradient. One-milliliter fractions were collected from the Percoll gradient and were analyzed for marker enzymes to identify fractions containing BBM or basolateral membrane. The enzymes used were alkaline phosphatase, a BBM marker, and Na\(^+\)-K\(^+\)-ATPase, which is present in the basolateral membrane (12, 13, 31). Figure 1 depicts protein content as determined by optical density (OD) at 280 nm (○), alkaline phosphatase activity (●), and Na\(^+\)-K\(^+\)-ATPase activity (○) in each fraction from a single experiment. In these experiments, fractions 2–7 showed high alkaline phosphatase activity and low-Na\(^+\)-K\(^+\)-ATPase activity; therefore, they were combined and designated as the BBM sample. Fractions 24–29 had high-Na\(^+\)-K\(^+\)-ATPase activity with little or no detectable alkaline phosphatase activity, indicating that these fractions contained basolateral membranes.

When these BBM were fused with planar lipid bilayers, channel activity was observed before addition of oligonucleotide that was inhibited by gadolinium and barium (data not shown). When this barium/gadolinium-sensitive activity was eliminated, channel activity was not seen in the absence of oligonucleotide; however, on addition of oligonucleotide (5 μM dT20) to each chamber, channel activity was observed (Fig. 2). In the presence of dT20, clear transitions between the closed and open state are seen (Fig. 2A). The channel does not show any significant rectification or voltage dependence and has a tendency to spend most of the time in the open state (Fig. 2B). The mean slope conductance of this channel is 9.1 ± 0.9 pS (Fig. 2B, n = 10). There does, however, appear to be some decrease in current at holding potential above ±100 mV. The decrease in current likely represents saturation of the channel. Both the closed and open time histograms are best fit with single exponential functions (Fig. 2C), indicating that the channel resides in only a single open state and a single closed state. The time constants for these histograms are 1.3 and 21.9 ms for the open time and closed time, respectively. These characteristics of this channel are similar to what we have reported for NACH reconstituted from purified protein under similar experimental conditions (6).

To determine the ion selectivity of the channel, ion gradient and ion substitution experiments were performed and the reversal potential was measured. The reversal potential was not altered by either eliminating or creating gradients for chloride, cesium, calcium, gadolinium, or barium (data not shown).

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**Fig. 2.** Electrophysiological characterization of oligodeoxynucleotide (ODN)-dependent channel activity in brush-border membranes (BBM) fused with planar lipid bilayers. BBM vesicles were fused with planar lipid bilayers, both solution chambers were filled with a solution consisting of 200 mM CsCl2, 1 mM CaCl2, 2 mM BaCl2, 1 mM GdCl3, (pH 7.4) and 5 μM ODN, and channel activity was recorded. A: representative 2-min current traces of ODN-dependent channel activity were collected at the indicated holding potentials. The solid horizontal line represents 0 current. B: mean current-voltage relationship of the channel under conditions of symmetrical ionic distribution (n = 10). C: mean closed time (\( \tau_c \)) and mean open time (\( \tau_o \)) of the channel for the data shown in A.
Establishment of a 10-fold concentration gradient for dT_{20} resulted in a significant shift in reversal potential from 0 mV in symmetrical oligonucleotide to 14.0 ± 0.1 mV (n = 3). When the relative selectivity was calculated using the Goldman equation, the ratio P_{ODN}/P_{Cl} was 904, indicating that the channel is ~1,000 times more selective for ODN than Cl\(^{-}\). These data also suggest that cesium, gadolinium, barium and chloride do not contribute significantly to the observed current. These data, therefore, indicate that the current observed with BBM vesicles is indeed the consequence of ODN transport through the channel.

We previously reported that the NACh reconstituted from purified proteins is calcium dependent (15). Therefore, we performed experiments to determine whether NACh is also calcium sensitive in native membranes. In these experiments, BBM were fused with preformed bilayers as described above but in the absence of calcium. The holding potential was 100 mV, control data were collected, and 1 mM CaCl\(_2\) was then added. As demonstrated in Fig. 3, channel gating was rarely seen in the absence of calcium or when 1 mM CaCl\(_2\) was added to the cis side (Fig. 3, A and B, respectively). When 1 mM CaCl\(_2\) was subsequently added to the trans chamber, channel activity was seen and open probability increased to 44% (Fig. 3C). In the presence of Ca\(^{2+}\) at least two channels were present in this experiment, and transitions to zero were not seen during the data collection. These observations indicated that the channel is calcium dependent in native membranes and suggest that the calcium-binding site is accessible only from the trans side of the bilayer. Since we have shown that more than 90% of vesicles fuse with the bilayer “right-side-out” (14), the trans side of the bilayer represents the extracellular side of the membrane.

To further define the calcium dependence of the channel, dose-response curves were generated by adding increasing amounts of CaCl\(_2\) to either the trans or cis side of the bilayer (Fig. 4). Channel open probability was not changed when calcium concentration was increased in the cis solution from 0 to 1,000 \(\mu\)M (Fig. 4A, ○; n = 5). However, increases in calcium concentration in the trans solution (Fig. 4A, ●; n = 5) resulted in an increase in open probability that reached significance (\(P < 0.05\)) at 1,000 \(\mu\)M compared with zero calcium. The plot of percent open probability vs. Ca\(^{2+}\) concentration (Fig. 4A) likely has a sigmoidal shape suggesting an allosteric modulation of open probability by Ca\(^{2+}\) ions. When the experimental data were fit using the Hill equation (Fig. 4B, n = 5), the Hill number was equal to 3.72 ± 0.1. This suggests that there are at least three binding sites for Ca\(^{2+}\) on NACh. Since a few transitions were observed in the absence of Ca\(^{2+}\), it is unlikely that calcium mediates a transition from an inactive state to an active state. Instead, it appears that Ca\(^{2+}\) binding modulates open probability of the channel.

Fig. 3. Effect of calcium on nucleic acid channel (NACh) activity. The channel was reconstituted as described, membrane potential was clamped at 100 mV, and data were collected under the following conditions: A: in the absence of Ca\(^{2+}\); B: after addition of 1 mM Ca\(^{2+}\) to the cis solution chamber; or C: after addition of 1 mM Ca\(^{2+}\) to the trans solution chamber. Data shown are representative 30-s current output traces (left), current histograms (middle), and percent open probability (%OP, right).
In these experiments, the increase in trans chamber Ca\(^{2+}\) concentration was associated with the appearance of additional current levels (Fig. 4C). In most cases, the different levels of current were multiples of the current level observed in the absence of trans Ca\(^{2+}\). This suggests that addition of calcium does not change single channel conductance but instead increases the number of open channels. This effect reached a plateau at 3 mM trans Ca\(^{2+}\) (data not shown). In most traces, transitions between the minimal level of current and the maximal level of current, without apparent transition to intermediate states, were seen for both channel opening and channel closing (Fig. 4C). This suggests some form of interaction between channels that allows for cooperative gating of a multichannel complex. The relationship between maximal conductance and calcium concentration was plotted and fit with a Hill equation; the Hill coefficient for this interaction was 2.6 ± 0.34 (Fig. 4D). It is unlikely that the increase in electrical activity results from the opening of other channels, for instance calcium channels. Added calcium in the absence of ODN did not result in any channel activity. In control experiments, Ca\(^{2+}\) at concentrations as high as 1 mM did not result in channel activity unless ODN was also present. Furthermore, ion gradient studies indicate that calcium does not permeate the membrane through NACH (data not shown).

We previously showed that cytosolic malate dehydrogenase is the regulatory subunit of the NACH and that increasing amounts of L-malate, a substrate for cMDH, and antibodies raised against cMDH block channel activity (7). Experiments were performed here to determine whether cMDH is also the regulatory subunit of the BBM channel. To accomplish this, we tested the effect of these compounds on NACH reconstituted from BBM vesicles. In the first series of experiments, BBM were reconstituted, control recordings of NACH activity were obtained, and either L-malate or D-malate was added to a concentration of 1 mM. The addition of n-malate had no effect on channel open probability (Fig. 5A), whereas open probability in the presence of L-malate was ~35% of control (P < 0.05). cMDH antibodies were also used to assess a role for this protein in channel activity. A cMDH monoclonal antibody that recognizes cMDH in Western blots (Fig. 5B, left) produced a dose-dependent block of BBM NACH (Fig. 5B, right). Dose-response profiles were also obtained for L-malate (Fig. 6). After control traces were collected, L-malate was added stepwise to the cis or trans solution chamber. Figure 6A depicts 30-s current traces (left), current histograms (middle), and percent open probability (%OP, right) of NACH when Ca\(^{2+}\) concentration on the trans side of the bilayer was as follows: a: 0 μM; b: 250 μM; c: 500 μM; d: 750 μM; and e: 1,000 μM. D: Hill plot of the relationship between channel conductance and Ca\(^{2+}\) concentration in the trans solution chamber (n = 5). The Hill coefficient for this relationship is 2.62 ± 0.3. All values are means ± SE.
stituted from BBM vesicles. Figure 8A shows 30-s current traces from a single experiment when heparan sulfate was added to either the cis or trans side of the bilayer. Figure 8B illustrates the mean open probability in the presence of 0, 1, or 20 μM heparan sulfate (n = 6). Channel gating, current, and open probability were not different from control when heparan sulfate was added to the cis side of the bilayer (Fig. 8, A and B). However, stepwise addition of heparan sulfate to the trans side of the bilayer resulted in a dose-dependent decrease in both current and open probability (Fig. 8, A and B).

DISCUSSION

We have previously described the characteristics of a purified nucleic acid-conducting channel complex reconstituted in planar lipid bilayers using purified proteins. The purpose of the current studies was to determine whether this channel could be detected in native cell membranes. To address this objective, channel activity was assessed in renal BBM fused with a planar lipid bilayer. The BBM were isolated using a standard differential centrifugation technique (14), which yields BBM vesicles that are enriched in alkaline phosphatase, a marker for BBM, and deenriched in Na+/H+-ATPase, a basolateral membrane marker (Fig. 1).

BBM were allowed to fuse with a preformed planar lipid bilayer made with a mixture of phosphatidylserine and phosphatidylethanolamine. This is the same lipid composition used in our previous studies (6, 7, 15). The buffered solution used in these experiments contained Gd³⁺ and Ba²⁺, which were added to block cationic channel activity observed in preliminary experiments in the absence of ODN (29). The presence of cationic channels in renal BBM is well documented (32) and therefore not unexpected. In all experiments, complete pharmacological blockade of these cationic channels was achieved before investigation of NAc activity.

The electrophysiological and pharmacological characteristics of the ODN-dependent channel in native BBM indicate that it is not significantly different from the channel formed by reconstituting purified proteins. Evidence in support of this
support for the hypothesis that the NACCh is a native component of renal BBM.

Apical membranes of renal proximal tubule cells are not readily amenable to patch clamping because the microvilli make it difficult to achieve a proper seal with the patch pipette. Another issue complicating use of a patch-clamp technique on these membranes is that the number of NACChs present on each epithelial cell is estimated to be low and the distribution along the membrane is unknown. It is not unusual, though, for membrane proteins to cluster in the cell membrane (4, 28, 30), further decreasing the likelihood of success with a classic patch-clamp approach. To work around these technical limitations, we used an alternative approach where BBM vesicles were purified using standard techniques and reconstituted directly in planar lipid bilayers. This is an approached used by a number of other investigators to circumvent the limitations of patch clamping renal BBM (20 –23, 32) and results in insertion of essentially all BBM proteins into the preformed bilayer. As such, this approach allows for study of membrane proteins in more native states of conformation and protein-protein interactions.

Transport of macromolecules through aqueous pores has been well documented in different systems, both in prokaryotic and eukaryotic cells. Proteins derived from virus, bacteria, and even protozoa have been shown to form aqueous pores in the host cell membranes, which allow simple diffusion of high molecular mass compounds (sugar, proteins, nucleic acids). It is well established that these macromolecular channels are key structural elements in metabolite exchange between different cellular compartments and also between cells. Now, evidence mounts that transmembrane channels also play a central role in protein trafficking (3, 27). Moreover, a recent study suggests that particles as large as phage f1 (about 7 nm in diameter) can exit Escherichia coli host cells with the help of a phage-encoded channel protein (16).

Some of these macromolecular aqueous pores are single protein structures, for example α-hemolysin and maltoporins include 1) the conductance of the channel reconstituted from BBM (9.1 ± 0.9 pS) is not different from the conductance of the channel formed from purified protein (10.4 ± 0.4 pS) (6); 2) purified NACCh and NACCh in native BBM are more than 900-fold more selective for ODN than for any other ion tested; 3) neither purified NACCh nor NACCh in native BBM show significant rectification or voltage dependence when reconstituted in similar symmetrical solutions; 4) open probability of both purified NACCh and NACCh in native BBM increases with increasing calcium; and 5) heparan sulfate and l-malate result in dose-dependent blockade of both purified NACCh and NACCh in native BBM. These data indicate that the channel found in renal BBM vesicles is electrophysiologically and pharmaco-logically indistinguishable from the channel reconstituted using purified proteins. Furthermore, these data provide strong

Fig. 6. l-Malate concentration-dependent changes in NACCh activity. NACCh was reconstituted as described and channel activity was recorded during stepwise addition of l-malate to the cis or trans solution chamber. A: representative 30-s current traces (left), current histograms (middle), and percent open probability (right) of the channel obtained while clamping of membrane potential to 100 mV. l-Malate concentrations are as follows: a: control, 0 μM l-malate; b: 1,000 μM in the trans solution; c: 250 μM in the cis solution; d: 500 μM in the cis solution; and e: 750 μM in the cis solution. B: open probability, expressed as a percent of control when l-malate was added to the trans solution chamber (○) or to the cis solution chamber (●) for 11 experiments. The addition of l-malate to the trans side of the bilayer did not significantly alter channel open probability; however, significant dose-dependent changes were observed when l-malate was added to the cis side. All values are means ± SE.

Fig. 7. Effect of oxaloacetate on NACCh activity. The channel was reconstituted as described, membrane potential was clamped at 100 mV, and data were collected under control conditions and in the presence of 1 mM oxaloacetate in the cis solution chamber (n = 8). Oxaloacetate did not alter channel open probability under any experimental conditions.
addition of 10 or 20 nM heparan sulfate added to the left solution (filled bars). All values are means ± SE. *P < 0.05.

Fig. 8. Heparan sulfate concentration-dependent changes in NACH activity. NACH was reconstituted as described and channel activity was recorded during stepwise addition of heparan sulfate to the cis or trans solution chamber. A: representative 4-s current traces (left), current histograms (middle), and percent open probability of NACH at a holding potential of 100 mV (right). Heparan sulfate concentrations are as follows: a: control, 0 μM heparan sulfate; b: 20 μM added to the cis solution; c: 10 μM added to the trans solution; and d: 20 μM heparan sulfate added to the trans solution. B: effect of heparan sulfate on NACH open probability (n = 3) under control conditions (0 μM), following addition of 10 or 20 μM to the cis solution (hatched bars) and then to the trans solution (filled bars). All values are means ± SE. *P < 0.05.

(11), whereas others are multiprotein complexes that require the interaction of more than one protein to form and stabilize the channel in the membrane. Disruption of the complex leads to the disappearance of the pore as is seen, for instance, in VDAC (17).

One of the most intriguing aspects of NACH is its selectivity; NACH is at least 1,000-fold more selective for ODN than the other ions tested. Recent evidence is beginning to demonstrate higher levels of selectivity for macromolecular channels than was initially predicted. Emerging experimental evidence has shown that permeation through these structures can involve intimate molecular interactions between the substrate and the channel. For instance, metabolite-specific channels exhibit high affinity for their metabolites; permeating molecules do not just diffuse through the pore but feel strong attraction to the pore-lining residues. Recent examples include ATP interactions with VDAC (25) and penicillin antibiotic interactions with the general bacterial porin OmpF (19). These types of substrate-channel interactions provide a rational hypothesis to explain the degree of selectivity reported for some macromolecule-conducting channels. The selectivity of NACH may be explained by a similar mechanism.

With the data detailed above and from previous publications, we are beginning to develop a model of the NACH. We have shown previously that the channel is composed of at least two proteins, cMDH and p45 (7). The transmembrane pore is formed by p45 and channel selectivity is determined by cMDH. Channel activity is altered by Ca$^{2+}$, l-malate, and heparan sulfate. All of these agents interact with the channel from only one side of the bilayer. These observations indicate that the channel inserts into the bilayer in a specific orientation and that the orientation is consistently in the same direction. The “sidedness” of action of these agents combined with specific and consistent orientation of the channel in the bilayer allow us to make inferences about channel topology. l-Malate blocks the channel only when added to the cis solution chamber. Because cMDH is present inside the cell and is not known to be extracellular, the cis side of the bilayer has been defined as the intracellular side of the bilayer. The effects of heparan sulfate and calcium were observed only when these agents were added to the trans solution chamber, suggesting that the binding sites for Ca$^{2+}$ and heparan sulfate are located on region(s) of the channel that present themselves on the extracellular side of the bilayer.

Analysis of the kinetics of Ca$^{2+}$ activation of the channel has led us to develop a model of allosteric regulation of NACH by Ca$^{2+}$. Ca$^{2+}$ modulation of NACH is seen as a shift between a state in which channel activation is slow and more difficult (D) and a state in which activation is easier (E). Both D and E represent monomeric forms of the channel (i.e., a single channel consisting of cMDH and p45). In this model, D and E are intrinsic states of the channel and Ca$^{2+}$ modulates the equilibrium between these states. The rate-limiting step in the equilibrium is the association of Ca$^{2+}$ to its first binding site in the channel complex. Once Ca$^{2+}$ is bound to its first binding site, binding of Ca$^{2+}$ to other sites is rapid and facilitates transition through the multimeric forms of the complex (i.e., sequential addition of monomers forming E2, E3, and E4). Activation of NACH by Ca$^{2+}$ and formation of the multimeric forms is described by the following model.

In developing this model we have made the following assumptions. 1) A single functional channel unit consists of p45 and cMDH. The stoichiometric relationship of p45 and cMDH is currently unknown and under investigation. The single functional unit will be referred to as a monomeric channel and is represented by E (the form with bound Ca$^{2+}$) or D (the Ca$^{2+}$ free form). 2) Each monomer has only one functional Ca$^{2+}$ binding site. 3) The presence of Ca$^{2+}$ stabilizes the channel as a multimeric complex consisting of several channel monomers that tend to gate as a single unit. The electrophysiological data presented above demonstrates the presence of cooperative channel gating (Fig. 4C), which supports the oligomerization hypothesis. 4) The model shows a complex no larger than four subunits (E4). This maximal size was set since analysis using the Hill equation suggests the presence of three or more calcium binding sites and, in high Ca$^{2+}$, the most stable level of current is four times larger than the unitary conductance. 5) The initial state of the stepwise model starts with the presence of a monomeric channel with a fixed number of calcium binding sites (N) and Ca$^{2+}$ ions. 6) Once the first Ca$^{2+}$ has bound to D (converting it to E), there...
is a sequential interaction of channel monomers based on a "rapid equilibrium." 7) The behavior is similar to that observed in allosteric enzymes, and the subsequent equilibrium constant includes interaction factors (a, b, c, etc).

\[ N + Ca^{2+} \rightarrow E \]

Where \( N \) = the number of calcium binding sites, and \( E \) = active monomeric channel

\[ \frac{ak_3}{bk_3} \quad \frac{ck_3}{E \rightarrow \frac{ak_4}{bk_4} \quad \frac{ck_4}{E} \]

Step 1 is slow and rate limiting, whereas the steps represented in equation 2 are fast and not limiting. Since step 1 is the rate-limiting step, one can define the velocity of the reaction (\( V \)) as:

\[ V = \frac{k_1 (Ca^{2+})(N)}{k_2 (E)} \]

Since \( N \) is constant, the rate for the process will be:

\[ v = K_1 \left( \frac{Ca^{2+}}{E} \right) \text{ Where, } K_1 = \frac{k_1}{k_2} \]

Due to the oligomerization process, at any given moment

\( (E)_i = (E) + (E_2) + (E_3) + (E_4) \)

Therefore,

\[ v = K_1 \frac{(Ca^{2+})}{(E) + (E_2) + (E_3) + (E_4)} \]

This can be rearranged to:

\[ v = \frac{K_1(Ca^{2+})}{E(1 + ak_3(E) + abk_3(E)^2 + abck_3(E)^3)} \]

Where \( v \) = rate constant, \( K_1 \) = equilibrium constant, and \( E \) = monomeric form of the complex.

This model is derived directly from the allosteric model of Monod et al. (18, 26) and is similar in form to described allosteric enzymes (9), allosteric modulation of Ca\(^{2+}\) channels (5, 8), neurotoxin activation of sodium channel, and block of sodium channels by local anesthetics (the "modulated receptor hypothesis") (5). The stoichiometry depicted in this model was derived from our electrophysiological and pharmacological data and has yet to be tested using other biophysical approaches.

In most of the experiments in which the Ca\(^{2+}\) concentration in the trans (external) side was modified, there was also a stepwise increased in the channel conductance that correlated with the Ca\(^{2+}\) concentration. The channel starts in a closed state (\( C \)) and then progresses "staircase" fashion through four distinguishable conducting states that we call S (small, \( \approx 7 \) pS mean conductance), ML (medium low, 15 pS), MH (medium high, 30 pS), and L (large, 70 pS). These states \( S, ML, MH, \) and \( L \) correspond to \( E_1, E_2, E_3, \) and \( E_4 \), respectively. This kind of distribution is consistent with the oligomerization of multiple subunits to form a complex that functions as a single unit. This complex is formed as a consequence of changes in conformation of the monomer due to agonist binding.

If the states we identified do indeed correspond to different numbers of bound agonist molecules, then the current amplitude histogram should change in an orderly way as the agonist concentration is increased. At the lowest agonist concentrations, the \( S \) state should predominate; at very high, agonist concentrations the channel should be in the \( L \) state most of the time, and the amplitude histogram should exhibit a mixture of states in between. This prediction is confirmed by the amplitude histograms shown in Fig. 4C for Ca\(^{2+}\) concentrations 0 to 1,000 \( \mu M \). In this particular case, the binding of Ca\(^{2+}\) to its binding site is also a requisite for the associations of different monomers of the complex and explains the different conductance states observed in most cases as has been demonstrated for glutamate receptor channel (24).

In summary, in this study we provided evidence that the nucleic acid-conducting channel is present in native membranes. These data support the hypothesis that NACH is indeed part of a mechanism for transporting macromolecules across epithelial cell plasma membranes. Additional experiments are necessary to determine whether ODN is the natural substrate for the channel or whether, in its native membrane, the channel is capable of transporting other molecules as well. The physiological role of a nucleic acid-conducting channel is also not clear at this time and additional studies are required to elucidate its role in cellular function. As we learn more about the function of this channel and begin to place this function within the context of the cell, the physiological meaning of the channel will emerge.

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