Nonselective cation transport in native esophageal epithelia

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The mechanisms mediating Na\(^+\) absorption in electrically tight epithelia have been extensively studied since the time of Ussing and Zerahn’s (41) publication regarding the nature of Na\(^+\) transport in frog skin. Ussing and Zerahn’s data and, subsequently, those of other investigators have elucidated that Na\(^+\) transport in these epithelia, with resistances reaching a few thousand ohms per centimeter squared, is mediated via an apical channel with distinct biophysical and pharmacological properties. These properties are hallmarks of this transport and include high Na\(^+\) -to-K\(^+\) selectivity and high sensitivity to the diuretic amiloride. Indeed, such epithelia exhibit \(K_i\) values for amiloride in the range of 100–500 nM.

Other Na\(^+\) channels have been functionally characterized; however, such channels are not normally found in tight epithelia and are moreover predominantly restricted to cultured cells grown under nonpolarized conditions (2, 13, 24, 28, 35). These other channels exhibit reduced amiloride sensitivity and altered Na\(^+\) -to-K\(^+\) selectivity, making the channel, in some instances, poorly amiloride sensitive and equally permeable to those monovalent cations. The biophysical properties of these other low or intermediate Na\(^+\) selectivity channels are also markedly different from those of the high-selectivity channel, which is sometimes referred to as the “classic” epithelial Na\(^+\) channel (ENaC). Indeed, the single-channel conductance of the classic channel is on the order of 5 pS, whereas those of the other channels vary from 20 pS and higher. This has led investigators to conclude that distinct membrane proteins likely form these channels.

The identity of the classic channel remained unknown until 1993 and 1994 when Canessa and colleagues (8, 9) identified and cloned three subunits that were necessary to reconstitute channel properties. These cloned subunits were referred to as α-, β-, and γ-ENaC. They are now known to produce a highly Na\(^+\) -selective, amiloride-sensitive channel, with α-ENaC as its core or structural subunit. This channel is found in all electrically tight Na\(^+\) -absorbing epithelia, and, when studied in the *Xenopus* oocyte expression system, it exhibited essentially identical properties to those found in native high-resistance epithelia. The relationship of these subunits to other low or intermediate Na\(^+\) selectivity channels remains unclear.

An initial report by Kizer et al. (19) has indicated that the expression of α-ENaC alone in fibroblasts leads to the appearance of a nonselective cation channel (NSCC). This channel was biophysically distinct from that present in tight epithelia. However, the interpretation of those data was complicated by the fact that these properties were also distinct from those observed with the expression of α-ENaC in *Xenopus* oocytes. Nonetheless, additional evidence supporting that different combinations of ENaC subunits can lead to subtle changes of the channel’s biophysical properties came from the data of Fyfe and Canessa (12). These authors found that the single-channel conductance, open probability, and, to some extent, selectivity are altered by selecting the α/β or α/γ combination for expression in *Xenopus* oocytes. These differences were rather small, and, moreover, it was unclear whether a small amount of oocyte endogenous ENaC subunits could complement the exogenously expressed ones and affect data interpretation. Nonetheless, the data of Kizer et al. (19) and Fyfe and Canessa (12) indicated that different combinations of ENaC subunits could lead to altered channel properties.

Recent reports from Jain and colleagues (16, 17) also indicated that deletion of α-ENaC expression in cultured lung alveolar type II cells, which normally express all three subunits, led to a marked decrease in the frequency of observing a nonselective 21-pS cation channel. The observation of this characteristic channel suggests that the endogenous α-ENaC subunit is important for the formation of the classic ENaC channel.
nonselective channel could also be switched to that of the classic ENaC by culturing cells on an air/water interface. These experiments are complementary to those above and together support the hypothesis of a modular Na⁺ channel coded for by one or more ENaC subunits. However, to our knowledge, there are no data from native epithelia supporting such a hypothesis.

The native rabbit esophagus is a well-characterized epithelium that is believed to transport Na⁺ in the luminal-to-serosal direction in a manner similar to that described by Ussing et al. (41) and Harvey (14) for frog skin and other electrically tight epithelia. Under short-circuit conditions, this epithelium exhibits a current ($I_{sc}$) consistent with active cationic absorption. This current is thought to be blocked by amiloride; however, the time course of this inhibition is delayed and, moreover, requires high (mM) concentrations of amiloride (39). Given our present findings, we believe that this delayed response is likely attributed to nonspecific and/or basolateral effects of high-amiloride concentrations in chambers that were not designed to minimize edge damage.

We utilized ENaC subunit-specific antibodies in search of molecular candidates that mediate transport in this epithelium. We found evidence for protein level expression of all three subunits in native esophageal epithelia and in HET1A, a cultured immortalized human esophageal cell line. Moreover, no significant differences were found between the amino acid sequence of rabbit esophageal alpha-ENaC (α-rbENaC) vs. that cloned from kidney (21). These findings led us to examine the properties of the native apical channel in more detail, taking advantage of the ability to permeabilize the basolateral membrane. We report that the previously postulated Na⁺-selective apical channel (5, 18, 39, 40) is, in fact, nonselective for monovalent cations (Na⁺, Li⁺, and K⁺) and is also amiloride insensitive. Despite the presence of α-, β-, and γ-ENaC, this native channel did not exhibit any of the properties of the classic Na⁺ channel. These findings indicate that ENaC may participate in the formation of a NSCC in a native epithelium.

**MATERIALS AND METHODS**

**Tissue isolation and $I_{sc}$ experiments.** New Zealand White male rabbits weighing between 8 and 9 lbs were killed by an intravenous administration of an overdose of pentobarbital (60 mg/ml). The esophagus was excised, opened, and stripped of its muscle layers in ice-cold oxygenated HEPES-buffered Ringer solution of the following composition (in mM): 140 Na⁺, 130 Cl⁻, 4.8 K⁺, 25 HEPES, 1.2 Ca²⁺, 1.2 Mg²⁺, 2.4 HPO₄²⁻, and 0.4 H₂PO₄, pH 7.4, 288 mol/kgH₂O osmolality. Solutions and tissues were maintained at 37°C and were gassed with 100% O₂. Mucosal and serosal solutions were connected to calomel half cells and Ag-AgCl electrodes with Ringer agar bridges for measurements of transepithelial voltage ($V_T$, open circuit) and automatic short-circuiting (0 mV) for measurement of the transepithelial $I_{sc}$. Transepithelial resistance ($R_T$) was calculated by using Ohm’s law from the values of $V_T$ and $I_{sc}$ or from the current deflection to an imposed 5-mV pulse.

**Nystatin permeabilization.** Baseline values were recorded from tissues equilibrated in symmetrical HEPES-buffered Na⁺ Ringer solution. The serosal solution was then replaced with one containing 0.1 mM nystatin. This was accompanied by a decline of the $I_{sc}$ toward zero (see RESULTS). Creation of a luminal-to-serosal Na⁺ gradient was then established by decreasing serosal Na⁺ 10-fold by isomolar substitution with the large impermeant cation N-methyl-d-glucamine (NMDG). In ion substitution experiments, currents were measured with Na⁺, K⁺, or Li⁺ gradients in buffered Ringer with equimolar concentrations of these ions. Addition of apical nystatin was without effects.

**HET1A cells.** Cells were a generous gift from Gary Stoner (Ohio State University, Columbus, OH). This SV-40 immortalized human esophageal epithelial cell line was cultured as previously described (27). Cells were grown in nonpermeable T75 flasks or tissue culture-permeable inserts (Costar Transwell Clear, Corning). Cells became confluent on either matrix; however, they did not develop any major changes of $V_T$ or $R_T$ on permeable supports. Therefore, experiments on these cells were limited to Western blot analyses.

**Western blotting.** All chemicals and reagents used in Western blot analyses were from Bio-Rad (Hercules, CA), Pierce (Rockford, IL), and Sigma (St. Louis, MO), unless noted otherwise. Western blotting was performed by using previously described procedures (4). In brief, HET1A cells were maintained in culture on permeable supports and in standard tissue culture flasks. Cells were grown to confluence and extracted with gentle agitation at 4°C for 1–2 h in RIPA lysis buffer (in mM): 150 NaCl, 10 NaPO₄, 1% deoxycholate, 1% Triton X-100, and 0.1% sodium dodecyl sulfate (pH 7.2) supplemented with protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, tosylphenylalanyl chloromethyl ketone, and 1-chloro-3-ctosylamido-7-aminomethane). Frozen rabbit esophagus (epithelia stripped of the underlying muscle and connective tissue; see above) and rat kidneys (1 mg/ml) were extracted with sonication in 0.25 M sucrose and 10 mM HEPES (pH 7.4) supplemented with protease inhibitors. Extracts were subsequently homogenized by using a dounce homogenizer. After cellular debris was cleared, standardization of total protein concentration (~1–2 µg/µl), and addition of sample buffer (0.05% bromphenol blue, 10% glycerol, 3% SDS, 1 mM EDTA, 77 mM HCl-TRis, and 20 mM dithiothreitol), cell and tissue homogenates were heated to 85°C for 10 min with 50- to 100-µg total protein (per lane), separated by standard SDS-PAGE (7.5%) and transferred to nitrocellulose membranes (0.2 µm). Blots were probed with previously described affinity-purified anti-α- and anti-β-rbENaC (22) and chicken anti-γ-ENaC (44) antibodies at 1/1,000 dilution (37). These antibodies have been successfully utilized to detect ENaC subunits in various preparations (4, 16, 17, 22, 24, 37, 44). For competition experiments, immunizing antigen was added in 10-fold excess of total cellular protein. Tween 20 (0.1%) and 5% dried milk were used as blocking reagents. All 2° horseradish peroxidase conjugates were purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD) and Kodak BioMax Light-1 film. Digital images of Western blots were generated by using a ScanJet4200C (Hewlett Packard, Houston, TX).

**Cloning of α-rbENaC.** Total RNA from rabbit esophagus was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA), according to the manufacturer’s instructions. One microgram of RNA was then used as a template for a reverse transcription reaction using the Smart Race DNA amplification kit (Clontech, Palo Alto, CA). cDNA was amplified using the following forward and reverse primers: 5’-CAGCCCACCCAAAGACCATGAAAG-3’ and 5’-TTGTTGGCTGTTGGTCGTCTCC-3’. A fragment of 2,230 bp was amplified and later cloned into a TA vector. The insert was sequenced using the vector primer. To sequence the rest of the insert, a deletion of 730 bp was realized. The sequence of α-rbENaC was deposited into the database (GenBank accession no. AY521461).

Except where noted, data are reported as means ± SE. Statistical significance was determined by using Student’s t-test at P < 0.05.

**RESULTS**

ENaC protein in esophageal epithelia. Northern blot analysis from previously published data (32) indicated that the rat esophagus expressed α-, β-, and γ-ENaC mRNA, inferring that esophageal epithelia express ENaC protein. To test whether
this results in detectable protein expression and whether a similar conclusion can be drawn in the rabbit, we performed Western blot analyses on homogenate from isolated rat and rabbit esophageal epithelia. We utilized subunit-specific antibodies previously described by others (see MATERIALS AND METHODS) to detect rat, human, and Xenopus ENaC. The α- and β-antibodies were generated in rabbit, whereas the γ-antibody was generated in chicken. Given the high background that we observed when probing rabbit material with the rabbit-generated α- and β-antibodies, we substituted rat material in those blots.

As shown in Fig. 1A, these subunit-specific antibodies recognized Xenopus ENaC in A6 epithelia, a well-characterized renal epithelial cell line model. A similar result was also observed with rat kidney, indicating that these antibodies are indeed capable of cross-species recognition, as previously described (see MATERIALS AND METHODS). It is clear that these antibodies also recognized proteins of appropriate mass in rat and rabbit esophagus and in HET1A, a human esophageal cell line. This finding was also confirmed in HET1A cells grown on nonpermeable (flasks) and permeable supports (tissue culture inserts).

![Diagram](image)

**Fig. 1.** Expression of epithelial Na\(^+\) channel (ENaC) subunits in esophageal epithelia. Western blot with ENaC subunit-specific antibodies was carried out as described in MATERIALS AND METHODS. A: blots were carried out on isolated native esophageal epithelia (rat or rabbit) and in cultured human esophageal cells (HET1A) on permeable (flasks) and nonpermeable supports (tissue culture inserts). A6 cell and rat kidney homogenates were used as a positive control. B: the observed specific bands with the rabbit-generated antibodies (anti-α and anti-β) were eliminated in the presence of excess immunizing peptide. This was observed in HET1A cells on flasks or inserts and in rat esophagus (E) or kidney (K) homogenates. Rat heart (H) and secondary alone were used as additional negative controls.

To provide further evidence of the specificity of the anti-ENaC antibodies used in the present study, additional blots were carried out on material probed in the presence of an excess immunizing peptide. These findings are shown in Fig. 1B and indicate specific detection of ENaC subunit proteins. Blots with secondary alone also ruled out nonspecific recognition of these proteins. This result was observed in both rat esophagus and kidney. Rat heart was used as an additional negative control. A similar competition was also observed with excess peptide in HET1A cells on flasks and tissue culture inserts.

An interesting finding was that the protein recognized by the γ-ENaC antibody in the native polarized rabbit cells and in human cells cultured on permeable supports exhibited a reduced molecular mass to that observed in rat kidney, A6 cells, and HET1A cells grown on flasks. This pattern is reminiscent of that observed with γ-ENaC in renal epithelia after treatment with aldosterone or restricted Na\(^+\) diet and presumed activation of channel-activating proteases (23). The significance of this finding remains unclear.

**α-ENaC sequence in rabbit esophagus.** As previously mentioned, the α-ENaC subunit is believed to be the structural subunit and is necessary for channel formation (8, 9). Data from Fig. 1 indicate clear expression of α-ENaC; however, it was unclear whether subtle differences existed in the amino acid sequence of this subunit in the native rabbit esophagus. To address this question, we cloned this isoform from native esophageal cells, as described in MATERIALS AND METHODS. The sequence obtained (GenBank accession no. AY521461) was found to code for a protein identical to rbENaC cloned from the colon with two substitutions (21). The first substitution was silent at the amino acid level and occurred 995 bp from the start site. The second substitution resulted in an F to S alteration at amino acid 265. It is unlikely that this change is significant, as Serine-265 is conserved in the rat and human isoforms (see Fig. 2).

**Fig. 2.** Partial amino acid sequence alignment of various α-ENaC from rabbit esophagus (E), colon (C), human lung, and rat colon. A and C: amiloride binding domains reported by Ismailov et al. (15) as well as Schild et al. (33) are preserved. B: region of divergence between α-ENaC cloned from rabbit esophagus (present report) and colon (21). Overall homology between the rabbit and human or rat α-ENaC was ~84%.

The α-subunit of ENaC has been characterized to contain two amiloride binding sites (15, 33). As shown in Fig. 2, the presumed binding sites in both rat and human are also conserved in the rabbit esophageal isoform. Despite this finding, the native esophageal channel is amiloride insensitive (see below), indicating additional yet-to-be characterized binding sites for amiloride or potential interactions with additional esophageal protein. This is consistent with the finding that different combinations of ENaC subunits exhibited differences in amiloride sensitivity (12).

To determine the properties of this channel, we attempted patch-clamp analysis of esophageal cells. The native esophag-
A native epithelial apical NSCC

gus contains a layer of dead “cornified” luminal facing cells that do not contribute to $R_T$ as evident from impedance analysis (11), but, nonetheless, precluded the utilization of patch-clamp analysis. Moreover, neither primary cultures of native esophageal epithelium nor cultures of HET1A cells on permeable supports formed a polarized cell model, as assessed by their inability to develop $V_T$ values and increased resistances. Thus, whereas the cultured cells could be patch clamped, they suffered shortcomings similar to those observed when studying Na$^+$ channels in cells grown on nonpermeable supports. As the main emphasis of this work was the properties in native cells, we focused our attention on native epithelia with permeabilized basolateral membranes.

Basolateral permeabilization. The rabbit esophagus is a high-$R_T$ epithelium that is known to transport Na$^+$ in a luminal-to-basolateral direction via an apical channel and an overall scheme (30), similar to that described by Ussing and coworkers (20, 41) and others (see Refs. 13, 14) in Na$^+$-absorbing electrically tight epithelia. To understand the apical Na$^+$ channel properties, the electrical contribution of the basolateral membrane was eliminated. This was carried out by permeabilizing the basolateral membrane with the pore-forming antibiotic nystatin.

Shown in Fig. 3 are the effects of basolateral nystatin permeabilization on the electrical properties of short-circuited rabbit esophageal epithelia. Addition of nystatin to tissues bathed in symmetrical Ringer solution caused a decrease of the $I_{sc}$ to values close to zero (also see Figs. 4–8). This decrease is expected and consistent with elimination of the electrical and chemical gradients across the apical membrane subsequent to elimination of the electrical contribution of the basolateral membrane. This decrease could be restored to essentially control values after the establishment of a 10:1 apical-to-basolateral Na$^+$ concentration gradient (NMDG substitution for Na$^+$). Establishment of a gradient in the absence of basolateral nystatin was also without sustained effects on the $I_{sc}$ (see Fig. 3A time control, where a small transient is observed due to basolateral solution exchange). Addition of apical nystatin was also without effects (data not shown). These observations are consistent with the poor cationic permeability of the paracellular pathway in electrically tight Na$^+$-absorbing epithelia and indicate that the current formed after nystatin is due to transcellular ion transport through an intact apical membrane.

The decrease of $I_{sc}$ with nystatin was accompanied by $\sim$500 $\Omega \cdot \text{cm}^2$ decrease of $R_T$. This decrease is consistent with the electrical elimination of the basolateral membrane. The magnitude of this decrease is also similar to that expected from the value of basolateral membrane resistance based on circuit analysis and intracellular microelectrode data (38). These changes of $R_T$ were not observed in time control experiments.

Monovalent cation selectivity. As mentioned above, a hallmark of ENaC is high Na$^+$-to-K$^+$ selectivity. To assess the monovalent cation selectivity of the apical Na$^+$ channel in rabbit esophageal epithelia, we utilized Li$^+$ for Na$^+$ and K$^+$ for Na$^+$ substitutions. As shown in Fig. 4A, substitution of apical Na$^+$ with the large cation NMDG eliminated the current, indicating impermeability to large cations. However, a similar current was observed with apical Na$^+$-to-Li$^+$ (Fig. 4B) and Na$^+$-to-K$^+$ substitution (Fig. 4C). Baseline current was also essentially unchanged in nonpermeabilized epithelia when the apical solution alone was switched from the Na$^+$ Ringer to the K$^+$ Ringer (data not shown). These findings are in sharp contrast to native and cloned ENaC, which displays a selectivity sequence of Li$^+$ $>$ Na$^+$ $>$ K$^+$.

Pharmacological profile. Given that ENaC may somehow form or participate in the formation of this NSCC, it was of interest to examine the effects of amiloride and its analogs on this channel. This is especially important given that the esoph-
ageal α-ENaC isoform preserves the amiloride binding sites described by Ismailov and colleagues (15) and Schild and colleagues (33). Addition of apical amiloride at concentrations as high as 1 mM (Fig. 6A) was without effects on the $I_{sc}$. A similar lack of response was also observed with phenamil (Fig. 6B) and EIPA (Fig. 6C). These agents, especially EIPA, are known to have some inhibitory effects on low-selectivity Na$^{+}$/H$^{+}$ channels in some preparations. These findings indicate a lack of sensitivity of the NSCC to amiloride and two of its analogs at concentrations sufficient to produce complete or partial blockade of many ENaCs of varying selectivity.

It is important to point out that the absence of a blockade by amiloride and its analogs cannot be explained by a diffusion barrier across the apical membrane. This is surmised from many observations, including 1) readily observable effects of apical NMDG for Na$^{+}$ or K$^{+}$ substitution on the $I_{sc}$; 2) the presence of two membrane time constants, that of the apical and basolateral membranes, without a large series resistance in impedance analysis studies (11); and 3) inhibition by high doses of diltiazem (see below). Thus these observations indicate potential participation of other α-ENaC sites or other channel subunits in amiloride binding.

The experiments in Fig. 6 were also carried out in the presence of 1 mM ouabain to eliminate any residual contribution of the basolateral Na$^{+}$/K$^{+}$-ATPase to transepithelial transport. This concentration of ouabain is known to inhibit this

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**Fig. 4.** Monovalent cationic selectivity of the apical esophageal channel. A: the $I_{sc}$ observed after the Na$^{+}$ gradient is eliminated in NMDG. A similar current is observed with apical Na$^{+}$ to Li$^{+}$ (B) and Na$^{+}$ to K$^{+}$ (C) substitutions. Values are means ± SE; $n$ = 5.

**Fig. 5.** Absence of apical K$^{+}$-selective channels. Similar currents are observed in Na$^{+}$- or K$^{+}$-based Ringer solutions. Addition of Ba$^{2+}$ was also without effects on the $I_{sc}$ (see text for more details). Values are means ± SE; $n$ = 5.

**Fig. 6.** Insensitivity of transport to amiloride and its derivatives. A: $I_{sc}$ is unaffected by amiloride at concentrations as high as 1 mM. Similarly, phenamil (B) or EIPA (C) is also without effects on the $I_{sc}$. Time control data are shown in shaded circles. All experiments were pretreated with ouabain. This treatment was without significant effects on the $I_{sc}$, providing further evidence for the elimination of the basolateral membrane contribution to transepithelial transport. Values are means ± SE; $n$ = 4.
enzyme in rabbit esophageal epithelia (40), and its lack of action provides further evidence supporting the electrical elimination of the basolateral membrane.

To test whether this NSCC is related to the cGMP-gated channel described by Stanton and colleagues (26, 42) in the inner medullary collecting duct (IMCD), we tested the effects of cGMP on the $I_{sc}$. Cyclic GMP, at concentrations known to modulate the NSCC in the IMCD, was without effect on the $I_{sc}$ (Fig. 7A). The IMCD channel is also known to be blocked by amiloride, dihydrobenzamil, and diltiazem (26, 31, 42). As shown above, amiloride and benzamil were without effects. However, diltiazem, particularly at a high pharmacological concentration of 1 mM, caused an ~40% inhibition of the $I_{sc}$ (Fig. 7B). Given the compelling lack of effects of cGMP, amiloride, and benzamil, and the low affinity for diltiazem, we conclude that the channel described here is pharmacologically distinct from the cGMP-gated channel in the IMCD. This is consistent with the finding that ENaC is molecularly distinct from the IMCD channel, while it may be part of the channel described in the present paper.

To further compare the esophageal NSCC with ENaC, we examined the effects of cAMP stimulation of PKA. ENaC, in some native epithelia, responds to increases of cAMP and PKA stimulation with doubling or larger increases of the $I_{sc}$. As shown in Fig. 8, 5 μM forskolin was without effects on the $I_{sc}$.

These findings are consistent with early reports of lack of effects of ADH on transport in the esophagus (5). These findings are also consistent with the lack of activation of ENaC in the rat colon (6) and in Xenopus oocytes (1) by cAMP.

DISCUSSION

Epithelial Na$^+$ transport occurs via a rate-limiting apical Na$^+$ channel that has been extensively studied in a variety of preparations. It is well accepted that ENaC subunits form the molecular basis of this channel in native and cultured electrically tight epithelia. A link between ENaC and other ENaCs that display nonclassic properties has been emerging. However, these findings have been limited to cultured epithelia that may have altered channel properties due to culture conditions. In the current study, we present the first evidence, to our knowledge, of a potential link between ENaC and a NSCC in a native, intact epithelium. Our data are consistent with all these ENaC subunits participating in the formation of this NSCC. It remains to be seen whether the differences in channel properties occur via specific amino acid divergence between the different ENaC homologs, via interactions with other esophageal proteins that render the channel nonselective, or via an altered subunit stoichiometry or oligomerization.

Channel properties (relationship to ENaC). Before molecular cloning of the ENaC subunits, it was recognized that multiple types of ENaCs existed. With very few exceptions, like the IMCD and sheep omasum (34), low-selectivity channels (see below) were essentially restricted to cultured cells: either immortalized or primary cultures. ENaCs from these cells were empirically grouped according to two proposed classifications. The first method was proposed by Smith and Benos (35) and classified channels into an L- or H-type, based primarily on their amiloride sensitivity and Na$^+$-to-K$^+$ selectivity. The second classification was proposed by Palmer (28) and classified channels into Na(5), Na(9), and Na(28), according to their single-channel conductance. Although these classifications are not mutually exclusive, they both indicate the presence of a variety of Na$^+$ channels with varying biophysical and pharmacological properties. Adding credence to these classifications was the fact that studies of purified Na$^+$ channels reconstituted into lipid bilayers (see Ref. 35 for a review).

Fig. 8. Lack of effects of cAMP activation on transport. Addition of forskolin, a known activator of PKA, does not affect the $I_{sc}$. This is consistent with the native rat colon ENaC and Xenopus oocyte expressed ENaC, which are known to be insensitive to PKA activation (see text). Values are means ± SE; n = 5.

Fig. 7. Effects of cGMP and diltiazem on transport. A: no activation is observed after the addition of 2 mM cGMP. B: addition of l-cis-diltiazem, a known blocker of cGMP-gated channels (see text), caused minimal effects at 200 μM. At higher pharmacological doses, an inhibition is observed. Values are means ± SE; n = 7. *Significantly different from control data, P < 0.05.
resulted in the observation of channels with conductances ranging between 4 and 80 pS and selectivity that spanned the Smith and Benos (35) and Palmer (28) classifications.

The relationship between the channels observed in bilayer experiments and ENaC subunits has never been directly addressed. However, it is unequivocal that ENaC subunits are needed to form the Na(5) or H-type classic ENaC (see Ref. 13). Moreover, based on the recent data from Jain and colleagues (16, 17) among others (see Introduction), α-ENaC may be at the core of other lower selectivity Na+ channels. Consistent with this hypothesis, it is also interesting to note that ENaC reconstituted in lipid bilayers displays, under the majority of conditions, properties distinct from those of the classic channel in tight epithelia (1, 3).

Our present findings are also consistent with this premise. However, three unique findings allow us to further support the hypothesis that ENaC is needed for the formation of many Na+ channels. First, we utilized native epithelia, thus ruling out any culture condition artifacts. Second, we found biochemical evidence for all three ENaC subunits, implicating the entire complex rather than just the α-subunit. Third, we provided the molecular sequence of α-ENaC, the structural subunit, from the rabbit esophagus. These findings do not prove that ENaC is a component of the observed apical NSCC. However, it appears highly unlikely that easily detectable protein expression of all three subunits is not accompanied by comparable membrane expression of functional channels. This is especially true given that the sequence of α-ENaC does not grossly differ from that of other cloned isoforms (84% overall homology with the rat and human α isoforms). It remains to be seen whether these differences alone can account for some or all of the properties of the NSCC observed in the present report. As the native esophagus is not amenable to patch-clamp or conventional antisense technology, a further test of this hypothesis will require cloning of the other esophageal ENaC subunits and expression in a heterologous system.

ENaC interacting proteins. Another potential explanation for the divergence between the classic ENaC and the NSCC in the esophagus is the presence of interacting proteins. Early attempts of biochemical channel purification (see Ref. 35) have indicated the presence of multiple subunits. The relationship between these subunits and ENaC is unknown, but these purified proteins could very well represent interacting proteins that convey epithelial-specific properties onto the cloned ENaC subunits. This hypothesis is supported by the cloning of two known groups of channel-interacting subunits. The first was termed apical protein Xenopus (APX) (36). The presence of APX was necessary for the observation of amiloride-sensitive Na+ channels in Xenopus oocytes after the injection of A6 fractionated mRNA (36) or α-, β-, and γ-ENaC (45). APX alone did not form a Na+ channel and was also found to co-immunoprecipitate with ENaC in A6 cells.

Another group of interacting proteins are the neuronal ENaC subunits, including δ-ENaC, the acid-sensing ion channels, and brain Na+ channels. Whereas these form channels on their own, some are able to interact with the epithelial subunits. For example, the neuronal δ-subunit can interact with the epithelial subunits and, when co-expressed in Xenopus oocytes, can double the amount of amiloride-sensitive currents (43). It is unlikely that these subunits play a role in esophageal function, as the native esophagus is relatively insensitive to luminal acidic solutions down to pH 4 (18). Nonetheless, these findings provide additional evidence for the hypothesis proposed above.

Relationship to the IMCD channel. Given that the cGMP-gated channel found in the IMCD is also nonselective among monovalent cations, we tested its relationship with the esophageal NSCC. It is known that the IMCD cGMP-gated channel is molecularly distinct from ENaC (10, 26, 42). It is also well known that the activity of this channel is modulated by divalent cations (Ca2+ and Mg2+). Moreover, it is sensitive to amiloride, benzamil, and diltiazem. With the exception of pharmacological doses of diltiazem, none of these agents, including cGMP, had any effects on the channel in the esophagus. Indeed, we routinely alter the apical Ca2+ concentration as a pharmacological switch to decrease paracellular resistance and do not observe any consistent effects on the IC38. Combined with the findings of ENaC in this epithelium, these results argue against a relationship between the luminal NSCC described here and epithelial cGMP-gated channels.

Potential function in the esophagus. The physiological function of the luminal NSCC and/or transepithelial ion transport in esophageal epithelia is poorly determined. However, we speculate a function similar to ENaC’s role in keratinocytes. Recent work by Brouard et al. (7) and Mauro et al. (25) have implicated ENaC in cell differentiation and the formation of polarized keratinocytes from undifferentiated basal or germinative cells. It is interesting to note that the stratified squamous epithelial cells of the esophagus are similar in their architecture and function to those of the skin. They both form a barrier made of multilayer polarized cells that is continuously replenished by undifferentiated basal cells. In this regard, it is interesting that damage to esophageal epithelia is augmented by agents like H+, which, at pH < 4, can inhibit transport in this native epithelium (38). We speculate that such a blockade may result in eventual injury to the intact epithelium through its ability to inhibit differentiation and the replenishment of polarized barrier cells.

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