The amiloride-sensitive epithelial sodium channels (ENaC) mediate Na\(^+\) reabsorption in epithelial tissues including distal nephron, colon, lung, and secretory glands and plays a critical role in pathophysiology of hypertension and cystic fibrosis. The ENaC is a multimeric protein composed of \(\alpha\)-ENaC, \(\beta\)-ENaC, and \(\gamma\)-ENaC subunits. To study the biochemical properties of the channel, the subunit cDNAs of rat colon ENaC (rENaC) were subcloned into baculoviruses, and the corresponding proteins were expressed in Sf9 insect cells. The functional characteristics of the expressed rENaC were studied in planar lipid bilayers. The results show that expression of \(\alpha\)-rENaC and \(\alpha\)\(\beta\)\(\gamma\)-rENaC in Sf9 insect cells result in the generation of cation-selective large conductance channels. Although the large conductance channels observed in the \(\alpha\)\(\beta\)\(\gamma\)-rENaC-containing membranes were unaffected by amiloride, the large conductance channels found in \(\alpha\)\(\beta\)\(\gamma\)-rENaC complex-containing membranes exhibited voltage-dependent flickering in the presence of micromolar amiloride. Possible implications of these observations are discussed.

The highly selective Na\(^+\) channels (ENaC)\(^1\) located in the apical membranes of epithelial cells of renal tubules, distal colon, lung, and several exocrine glands mediate controlled entry of Na\(^+\) ions into cells from the luminal or mucosal fluids and exhibit high sensitivity to pyrazine-based K\(^+\)-sparring diuretics such as amiloride (1–3). Abnormal function of ENaC has been demonstrated in human diseases including hereditary hypertension (Liddle’s syndrome) \(4\), salt-sensitive hypertension \(5, 6\), and cystic fibrosis (CF) \(7, 8\), indicating the importance of these channels in normal and pathophysiology. In addition, ENaC belongs to newly emerged superfamily of ion channels that include degenerins \(9, 10\), Rossier and co-workers \(11, 12\) have first isolated three cDNAs coding for the rat colon ENaC (rENaC) subunits, \(\alpha\)-rENaC, \(\beta\)-rENaC, and \(\gamma\)-rENaC, and demonstrated that \(\alpha\)-ENaC is the channel-forming subunit and that \(\beta\)-ENaC and \(\gamma\)-ENaC subunits together greatly increase the channel activity of the \(\alpha\)-ENaC subunit. Subsequently, highly homologous ENaC subunits from other species were sequenced in several laboratories and established firmly that ENaC is a complex protein formed by the association of these three subunits \(13, 14\). Structurally, all of these subunits contain two transmembrane segments, a large extracellular region and cytoplasmically located relatively short NH\(_2\) and COOH termini, and share nearly 35\% amino acid sequence homology \(10–12, 15\). However, the number of these three subunits in a fully functional ENaC remains unclear. For example, Firsov et al. \(16\) reported that the ENaC is a complex of two \(\alpha\)-ENaC, one \(\beta\)-ENaC, and one \(\gamma\)-ENaC subunits. Snyder et al. \(51\) have shown that ENaC is a much larger complex formed by three each of the three subunits of ENaC. Based on the kinetic analysis, Berdiev et al. \(50\) have concluded that four \(\alpha\)-ENaC subunits together form the conduction pore.

It has been shown in the case of CF disease that the ion transport across the airway epithelia is abnormal and is characterized by decreased Cl\(^-\) secretion and increased Na\(^+\) absorption \(7, 8, 17, 18\). With the recognition that CFTR, a Cl\(^-\) channel, is defectively trafficked to the plasma membrane of CF epithelia, a hypothesis that CFTR down-regulates ENaC function was proposed to provide an explanation for the increased Na\(^+\) absorption and decreased Cl\(^-\) secretion in these epithelia \(19\). Subsequently, several laboratories have provided evidence for the functional interdependence and physical interactions between these two ion channels \(20–25\), supporting the above hypothesis. In addition, a variety of proteins including actin and other regulatory agents have been shown to interact with ENaC and regulate its channel kinetics \(26–31\). These studies collectively indicated that the function of ENaC is finely regulated.

The amiloride-sensitive sodium channels are diverse and differ widely in conductance and ion selectivity \(2, 32\). However, the genes coding for these diverse channels are not known. On the other hand, the most distinguishable biophysical properties of the cloned ENaC are the low channel conductance of \(\sim 5\) pS, high selectivity for sodium, and slow gating with open and closed times on the order of seconds \(11, 12\). Although these features of ENaC expressed in mammalian and Xenopus oocytes were consistently reproduced by investigators using mainly the patch-clamp technique, Benos and co-workers \(33\) have observed that ENaC in planar lipid bilayers exhibits three open state conductance levels of 13, 26, and 40 pS with rapid gating. They have shown that these unusual biophysical properties of ENaC could be reversed to the expected upon interactions with actin in the planar lipid bilayers, suggesting that cytoskeletal proteins also modulate ENaC function \(34\). Importantly, this study firmly established that both patch clamping and planar lipid bilayer reconstitution procedures yield identical results, quelling doubts on the suitability of the latter technique in the ENaC analysis. Thus, it appears possible that ENaC can potentially exhibit diverse biophysical properties that could match the properties of other amiloride-sensitive
channels whose protein sequences are still unknown.

To investigate the biochemical properties of ENaC, we have established the Sf9 insect cell-baculovirus (BV) expression system to produce rENaC as a complex of α-rENaC, β-rENaC, and γ-rENaC subunits (35). Although the amiloride-sensitive sodium channels with conductance levels of 6 pS were observed, the membranes containing ENaC also exhibited large conductance cation-selective channels of >300 pS. The results presented in this paper suggest that these large conductance channels are exclusively found in membranes containing either α-rENaC subunit alone or in membranes containing αβγ-rENaC complex. The results also indicate that amiloride acts as a flickery block to the large conductance channels in membranes containing αβγ-rENaC complex, a characteristic of its action on ENaC.

EXPERIMENTAL PROCEDURES

Recombinant BVs—Construction of recombinant α-BV, β-BV, and γ-BV carrying the α-, β-, and γ-rENaC cDNAs, respectively, and the αβγ-BV harboring all of the three rENaC subunit cDNAs was reported previously (35). As controls, recombinant BVs carrying the MDR1 cDNA, breast cancer resistance protein (52) cDNA, and Escherichia coli β-galactosidase cDNA were used.

Sf9 Cell Culture, Infections, and Membrane Preparation—The Sf9 cells were maintained in Grace’s medium supplemented with 10% (v/v) fetal bovine serum at 27 °C as suspension in 250-ml spinner flask stirring at a rate of 70 rpm. For the production of rENaC, ~30 million cells were seeded into each T-175 cm² flask and infected with the recombinant BV. At 72 h post-infection, the cells were scraped into the medium and pellet by centrifugation at 1500 × g for 5 min. The cell pellet was washed once with an ice-cold buffer containing 300 mM mannitol, 50 mM Tris, 2 mM EDTA, pH 7.0, with HCl and then resuspended in a buffer containing 50 mM Tris-Cl, pH 7.0, 50 mM mannitol, 2 mM EDTA, 1 mM 2-mercaptoethanol. The cell suspension was homogenized in a glass Dounce homogenizer and centrifuged at 10000 × g for 10 min at 4 °C to remove the cell debris and unbroken cells. The supernatant was then centrifuged at 30000 × g for 30 min. The pelleted total membrane fraction was resuspended in 10 mM Tris-Cl buffer, pH 7.4, containing 1 M sucrose and was further fractionated by discontinuous sucrose gradient centrifugation procedure of Yang et al. (36). The ENaC-containing fraction was identified by Western analysis and used in these studies.

Planar Lipid Bilayer Reconstitution—Single channel analysis of rENaC was performed in planar lipid bilayers by following the general procedures adapted in Dr. Rosenberg’s laboratory (37) with slight modifications. Planar lipid bilayers were formed at room temperature from a 20 mg/ml n-decane solution of phospholipids, phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (w/w/w 5:3:2) by painting over a 0.9-mm diameter aperture separating cis and trans bath chambers. After a stable bilayer was formed in symmetrical solutions of 50 mM sodium aspartate, 25 mM HEPES, pH 7.4, with 1 M NaOH, as monitored by capacitance measurements, the experimental conditions were changed to asymmetric concentrations of sodium aspartate. The concentration of sodium aspartate in the cis chamber is higher than in the trans chamber, which was indicated in the individual experiments. Some experiments were carried out in symmetrical sodium aspartate concentration. Aliquots of membrane fractions were added to the cis chamber (final membrane protein concentration is ~5 μg/ml of buffer in the cis chamber) and mixed by a small magnetic stir bar placed in the cis chamber. The Ag/AgCl electrodes were connected to the chambers through 2 × KCl agar bridges. Generally, channels were incorporated into the lipid bilayer within 5 min and were detected as step-like increases in current. Channel currents were amplified (BC-525C; Warner Instruments, Hamden, CT), filtered by a low pass eight-pole Bessel Filter, digitized (Digidata 1200; Axon Instruments, Foster City, CA), and stored on a computer hard drive. Single channel currents collected at different voltage potentials and analyzed by using pCLAMP 7.0 software (Axon Instruments), QuB-Software for Single Channel Analysis (www.qub.buffalo.edu/Research). Foundation State University of New York was also used in the analysis and preparation of figures.

Other Methods—A description of anti-rENaC antibodies was presented previously (35). The polyclonal antibodies to the human ENaC subunits were raised in rabbits against the following epitopes: α-ENaC (residues 51–76), β-ENaC (residues 614–638), and γ-ENaC (residues 626–648). The rat and human ENaC antibodies were used to probe for the presence of endogenous ENaC subunits in Sf9 cells by Western blotting (35). RNA protection assay was performed according to the manufacturer-supplied protocols (Ambion, Austin, TX). Briefly, total RNA from Sf9 cells and from Sf9 cells infected with αβγ-BV were isolated using TriZol reagent (Invitrogen). The antisense probes ranging up to ~500 bases from the rat and human ENaC subunit cDNAs (subcloned into psPORT and pBluescript SK+.plasmids) were synthesized in the presence of [α-32P]UTP and hybridized with the total RNA according to the manufacturer’s instructions (Ambion). After hybridization, the mixture was digested with RNase A/T1, and the remaining isolated RNAs were analyzed by autoradiography after separation on a 8 % urea, 5% polyacrylamide gels. The protein content in the membrane fractions was determined by the modified Lowry method using bovine serum albumin as standard (38).

Materials—The Sf9 culture media were obtained from Invitrogen. The phospholipids were obtained from Avanti Polar Lipids. Amiloride, N-(phenylthranilic acid, 5-nitro-3-phenyl propylamine) benzoate, and glybenclamide were purchased from Sigma. [α-32P]UTP was obtained from ICN. MAXScript SP6/77 kit and RPA III kits were obtained from Ambion. The other reagents were of analytical grade.

RESULTS

Expression of rENaC Subunits in Sf9 Insect Cells—Because the Sf9 insect cell-BV expression system is well known for the production of high amount of proteins in functional form (39–46), we have explored this expression system to obtain rENaC. The Sf9 insect cells infected with the α-, β-, γ-, and αβγ-BVs express the ENaC subunits, which quantitatively represent ~3% of the total membrane protein (35). A significant portion of the expressed ENaC subunits is fully N-glycosylated, as expected (35). Because it is now recognized that ENaC is also found in nonepithelial cell lines (53), the possibility that Sf9 cells express ENaC homolog was tested by RNA protection assays using the antisense ENaC subunit probes prepared from rat and human ENaC cDNAs. No RNase-protected RNA fragments were found in the total RNA isolated from uninfected Sf9 insect cells, suggesting that these cells do not express ENaC-like proteins under the growth conditions adapted in the laboratory. In support of this conclusion, the antibodies to rat and human ENaC subunits did not react with proteins in the control Sf9 insect cell lysates (partially reported in Ref. 35). Thus, the large scale expression of fully N-glycosylated ENaC in Sf9 insect cells and lack of endogenous ENaC suggested that the Sf9 insect cell-BV expression system is an alternative expression system for ENaC.

Channel Activity in Control Sf9 Cell Membranes—The membranes prepared from Sf9 cells infected with a variety of BV mentioned under “Experimental Procedures” contained a variety of cation-selective channels. Fig. 1A shows representative records of typical channels observed under sodium aspartate concentrations in trans and cis chambers of the bilayer setup (75 and 200 mM, respectively). Channels in the control membranes prepared from different batches were analyzed, and the channel activities obtained from more than 100 experiments could be grouped broadly into 7.5, 14, 22, 84, 120, and 145 pS channels based on their conductances. The open probabilities of most of these channels were >0.5; open and closed times were in the range of seconds and were not voltage-dependent as deduced from their current-voltage relationships (data not shown). Each time any channel activity was observed, the effects of amiloride were tested by adding up to a concentration of 1 mM in both cis and trans chambers. However, none of these channels exhibited any flickering or reduced open times. Similarly, the channel activities observed in membranes containing β-ENaC or γ-ENaC were also not sensitive to amiloride (not shown). A typical 7.5 pS channel whose gating was unaffected by the presence of 10 μM amiloride both in cis and trans chambers is shown in Fig. 1B. These data collectively suggested that Sf9 cell membranes do not contain any endogenous amiloride-
sensitive channels, although they contain large number of cation-selective channels, and few of them exhibit gating properties resembling that of the ENaC.

Large Conductance Cation-selective Channels in α-ENaC-containing Membranes—The membrane fractions prepared from Sf9 cells infected with α-BV were reconstituted into the planar lipid bilayers that were voltage clamped to measure the single channel current transitions. A very noticeable and regularly observed phenomenon upon channel incorporation was the appearance of multiple current levels, because of the activity of as many as three to five channels after an apparently single fusion event. Fig. 2A shows a representative selection of single channel recordings obtained in 200 mM sodium aspartate in the cis chamber and 75 mM sodium aspartate in the trans chamber at several membrane voltages and their relationship in the form of current-voltage plot. Nearly all of these channels have long open and closed times, which are on the order of seconds. Under these conditions, the reversal potential, $E_{rev}$, deduced from their linear current-voltage relationships (Fig. 2B), was $-15 \text{ mV}$, which is lower than the expected reversal for Na$^+$, indicating that this channel is cation-selective. The conductance of the largest channel is $\sim 300 \text{ pS}$. A prominent feature of these channels is the existence of several conductance substates, which were observed at all holding potentials in these experiments, precluding us in estimating the conductance of these channels/substates accurately. The appearance of these conductance levels was a consistent observation from experiment to experiment, from different batches of membranes either fresh or frozen. To further characterize this channel, various channel blockers, including N-phenylanthranilic acid, 5-nitro-(3-phenylpropylamino) benzoate, amiloride, benzamil, and glybenclamide, were added in both cis and trans chambers up to a final concentration of 500 $\mu$M. Neither the current amplitude nor the open probabilities were changed in the presence of these compounds (data not shown), suggesting that these are unique channels.

Channel Activity in αβγ-ENaC-containing Membranes—The αβγ-ENaC-containing membranes were added to the cis chamber of the bilayer setup, and channel recordings were measured in the presence of 200 and 75 mM sodium aspartate in the cis and trans chambers, respectively. Fusion of membrane vesicles with the bilayer was rapid, and channels were detected within minutes after the addition of membranes into the cis chamber. These membranes characteristically contained the small channel of $6 \text{ pS}$ that exhibits flickering in the presence of amiloride is shown in Fig. 3A. However, as in the case of α-rENaC containing membranes, multiple channels with large conductance were also observed in membranes containing the αβγ-rENaC complex. All of these channels exhibited long open times on the order of seconds. A prominent and consistent feature in these records was in the existence of multiple substates at all holding potentials, which were evident in Fig. 3B. The current-voltage relationship shown in Fig. 3C indicated that the slope conductance of a large channel was $\sim 320 \text{ pS}$. The zero current potential of these channel states was $-17 \text{ mV}$ in the presence of 200 and 75 mM sodium aspartate gradient in the cis and trans chambers, respectively, indicating that they have a preference for cations.

To test the effect of amiloride on these large conductance channels, a stock solution of this drug prepared in water was added to either a cis or a trans channel one at a time, and the behavior of the channels was monitored. It was clear from a number of experiments that the response of these large conductance channels to amiloride could be broadly grouped into...
the following categories. First, the activity of certain large conductance channels could not be altered by the addition of up to 1 mM amiloride to either cis or trans chambers, a behavior similarly observed in the a-ENaC-containing membranes. Second, the addition of 1 µM amiloride brought about complete inhibition of certain large conductance channels, even though many small conductance channels remained active subsequent to the addition of amiloride. Third, the behavior of certain large conductance channels to amiloride could be characterized in the manner described below.

The behavior of the large conductance channels in the aβγ-ENaC membranes at a holding potential of 30 mV is shown in Fig. 4 (traces 1 and 2). At least two major channels with current amplitudes of 3.0 and 4.2 pA are identifiable, although other channels or substates could also be discerned. Within a few seconds after the addition of 1 µM amiloride to the cis chamber, the channel with 3.0 pA current amplitude was closed, as judged by the decrease in the total current amplitude at this holding potential. Interestingly, a channel with current amplitude of 1.5 pA with rapid transitions between open and closed states has appeared. The open and closed times of this new channel were on the order of milliseconds. Inclusion of an additional 1 µM amiloride in the cis chamber resulted in the complete inhibition of this flickery channel activity (not shown). However, the channel with −4.2 pA amplitude was not affected by amiloride.

The flickery block induced by amiloride in the large conductance channels in the aβγ-ENaC containing membranes is dependent on the holding potential, which is shown in Fig. 5. The conductance of the channel in this figure was −409 pS, and representative channel records of this channel at a holding potential of −10 mV were shown in traces 1–3 in Fig. 5. As frequently observed, various substates are also evident in these traces. Traces 4–6 in Fig. 5 show the behavior of this channel in the presence of 2 µM amiloride in the cis chamber. The addition of amiloride significantly changed the gating properties of the channel from slow gating to fast flickery nature. Upon decreasing the holding potential from −10 to −20 mV, the open probability of this flickery channel was greatly reduced (trace 7).

Although not shown, the flickery block induced in these large conductance channels was dependent on the location of amiloride. For example, we have noticed that the addition of amiloride to one side of the bilayer setup, if it did not induce any flickering either at positive or negative holding potentials, did
inhibit these endogenous channels with a variety of channel inhibitors and altered experimental conditions such as changing the salts of sodium without any success.

The most intriguing observation that further complicated the analysis of ENaC activity is the detection of large conductance of >300 pS cation-selective channels in the ENaC-containing membranes. Interestingly, these large conductance channels were not observed in more than 100 planar bilayer experiments using the control membranes. Detection of multiple >300 pS conductance channels every time ENaC-containing membranes fused with bilayer suggested that they must have arisen as a consequence of the ENaC expression in Sf9 cells. Although these large conductance channels in the α-membranes were not sensitive to amiloride up to 500 μM, channels of similar nature detected in the αβγ-ENaC containing membranes were, however, sensitive. As shown in Fig. 5, the flickery block induced by amiloride was more pronounced at a higher holding potential, which was also dependent on the location of amiloride, i.e. cis or trans chamber. Removal of amiloride by perfusion relieved the block. These observations together suggested that amiloride induces reversible flickery block in these large conductance channels. Because of the presence of these large conductance channels in multiple numbers in a given experiment, we were unable clamp the bilayer beyond the reported holding potentials across the bilayer in most of these experiments.

The molecular basis for the presence of large conductance channels that are amiloride-insensitive in the α-ENaC-containing membranes and amiloride-sensitive in the αβγ-ENaC membranes is not clear at present. However, the following arguments may provide a plausible explanation for these observations. It is likely that these large conductance channels are cryptic channels endogenously present in the Sf9 cells. Because the β-ENaC, γ-ENaC, P-glycoprotein (MDR1 gene product), and breast cancer resistance protein, all of which are bona fide integral membrane proteins, did not elicit the activity of these large conductance channels, it is possible that the α-subunit activates these endogenous channels. In association with the β and γ subunits, these α-subunit-activated endogenous channels, as in the αβγ-containing membranes, acquired amiloride sensitivity.

On the other hand, Miller and co-workers (47) have predicted that the presence of high density channel protein in membrane vesicles would result in channel appearance in multi-channel packages. Because ENaC is produced relatively in large amounts and multiple large conductance channels were consistently observed in the ENaC-containing membranes, it is reasonable to suggest that the large conductance channel activity is due to ENaC. The unusually large conductance could be the result of aggregation of the expressed protein. However, it remained unclear at present whether the large conductance channel activity is due to normally folded or aggregated ENaC.

Interestingly, structurally similar ion channels including the inwardly rectifying K+ channel (Kir), mechanosensitive small channel of E. coli (MscL), and the ATP-gated cation channel (P2X receptor), all of which contain two-transmembrane segments (54), are known to exhibit channel activity of large conductance when compared with ENaC. In particular, the MscL, a homo-hexamer, exhibits a channel activity of ~3500 pS (55). Thus, it is conceivable that ENaC with a subunit composition of four to nine subunits (16–18), which is not very different from the structure of MscL, could also exhibit higher conductance channel activity. Because proteins including CFTR and actin are known to interact with ENaC (29, 34), it is also possible that ENaC could exhibit high conductance in the absence such interactions. Characterization of the purified ENaC will be necessary to resolve the biophysical properties of this interesting channel.

**FIG. 5. Effect of holding potential on the behavior of αβγ-ENaC channels in the presence of amiloride.** The conductance of the channel shown here is ~409 pS. Traces 1–3 are the continuous channel records at a holding potential of ~10 mV. Traces 4–6 are the continuous channel records upon addition of 2 μM amiloride in the cis chamber at a holding potential of ~10 mV. The holding potential was changed to ~20 mV (indicated with an arrow in trace 6), and we continued recording (trace 7). The maximal open state (O) of the channel activity is marked with a dotted line.

Indeed, induce flickering once amiloride was added to the other chamber. These data suggested that these channels contain an amiloride-binding site that is asymmetrically located. In addition, perfusion of chambers with buffers without amiloride eliminated the flickering and restored normal appearance of these channels, which suggested that amiloride binding is reversible.

**DISCUSSION**

To study the structure of ENaC and its interactions with a variety of regulatory proteins, we have adapted the Sf9 insect cell-BV expression system, which is widely used to express receptors, channels, enzymes, and other proteins in a number of laboratories (39–46). Unlike the Shaker K+ channel, for instance, similarly expressed in the Sf9 cells (47), major amounts of the ENaC subunits were fully N-glycosylated and migrated with molecular masses similar to these subunits expressed in Xenopus oocytes, Madin-Darby canine kidney cells, and in vitro translation in the presence of canine pancreatic microsomal membranes (15, 19, 48). This suggested that the Sf9 insect cells carry out post-translational modifications of ENaC subunits similar to the above expression systems. Because all of the three ENaC subunits are co-expressed in each Sf9 insect cell via infection with the αβγ-BV (35), it is reasonable to assume that at least some portion of the ENaC subunits could be in the form of an ENaC complex.

The membranes prepared from control Sf9 cells contained several cation-selective channels with conductances ranging from ~7.5 to 145 pS. The ~7.5 pS channels in the control membranes have particularly posed obvious difficulties in determining whether or not the expressed ENaC is functional, because the gating properties of this channel were similar to that of the ENaC. As pointed out by Gabriel et al. (49) with regards to expression of CFTR, it is highly desirable to express ENaC in a heterologous expression system with no endogenous channels that exhibit characteristics resembling that of the ENaC. Because it is unlikely that an ideal experimental system exists for ENaC, we have attempted, as an alternative, to inhibit these endogenous channels with a variety of channel blockers and altered experimental conditions such as changing the salts of sodium without any success.
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