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The lung amiloride-sensitive Na^+ channel: Biophysical properties, pharmacology, ontogenesis, and molecular cloning

(human sequence/cystic fibrosis)

NICOLAS VOILLEY*, ERIC LINGUEGLIA*, GUY CHAMPIGNY*, MARIE-GENEVIÈVE MATTÉI†, RAINER WALDMANN*, MICHEL LAZDUNSKI*‡, AND PASCAL BARBRY*

*Institut de Pharmacologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, Université de Nice Sophia Antipolis, 660 route des Lucioles, Sophia Antipolis, F-06560 Valbonne, France; and †Centre de Génétique Médicale, Physiopathologie Chromosomique, Hôpital d'Enfants de la Timone, Boulevard Jean Moulin, 13385 Marseille cedex 05, France

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ABSTRACT **Water balance in the lung is controlled via active Na^+ and Cl^- transport. Electrophysiological measurements on lung epithelial cells demonstrated the presence of a Na^+ channel that is inhibited by amiloride ($K_{0.5}$ = 90 nM) and some of its derivatives such as phenamil ($K_{0.5}$ = 19 nM) and benzamil ($K_{0.5}$ = 14 nM) but not by ethylisopropylamiloride. An amiloride-sensitive Na^+ channel of 4 pS was recorded from outside-out patches excised from the apical membrane. This channel is highly selective for Na^+ ($P_{Na^+}/P_{K^+} \geq 10$). Isolation of a human lung cDNA led to the primary structure of the lung Na^+ channel. The corresponding protein is 669 residues long and has two large hydrophobic domains. An amiloride-sensitive Na^+-selective current apparently identical to the one observed in lung epithelial cells was recorded after expression of the cloned channel in oocytes. The level of the mRNA for the Na^+ channel was highly increased from fetal to newborn and adult stages. This observation indicates that the increased Na^+ reabsorption that occurs at birth as a necessary event to pass to an air-breathing environment is probably associated with control of transcription of this Na^+ channel. The human gene for the lung Na^+ channel was mapped on chromosome 12p13.**

During fetal life, the lungs are filled with liquid, which arises from continuous secretion of epithelial cells (1, 2). At birth, the pulmonary epithelium changes its predominantly active Cl^--secreting properties for predominantly active Na^+-absorbing properties, the result being the clearance of the pulmonary fluid as the lung switches to an air-conducting system (1, 2). In adults, Na^+ transport participates in control of the quantity and composition of the respiratory tract fluid that is necessary for gas exchange and prevention of alveolar collapsing and particle deposition. A number of data suggest that Na^+ absorption by alveolar epithelium mainly occurs through amiloride-sensitive pathways, probably channels, located at the apical membrane of type II pneumocytes (3–6). Surprisingly, available electrophysiological measurements with the patch-clamp technique (7) as well as studies of $^{22}Na^+$ flux into pneumocyte vesicles (8) or binding studies with labeled derivatives of amiloride such as [^{3}H]benzamil (9) or [^{3}H]bromobenzamil (10), which are known to be specific blockers of epithelial Na^+ channels (NaCh) (11), suggest that the amiloride-sensitive NaCh in pneumocytes would be quite different from other NaCh identified in the renal collecting tubule and/or in the renal pars recta (refs. 12 and 13; reviewed in ref. 11).

Because of the importance of the NaCh for pulmonary function, because abnormalities of channel function have been identified in cystic fibrosis (14), and because amiloride is presently assayed as a potential treatment for this major genetic disease (15), it appeared important (*i*) to analyze in detail the biophysical and pharmacological properties of the amiloride-blockable channel in type II pneumocytes, (*ii*) to determine the structure of the human channel by molecular cloning and identify the chromosomal localization of the NaCh gene, and (*iii*) to study its transcription during development.§

MATERIALS AND METHODS

Cell Culture. Primary cultures of fetal alveolar type II cells were carried out according to Orser *et al.* (16). The cells were seeded in minimal essential medium with D-valine, 0.1 mM nonessential amino acids (Sigma), and 10% fetal calf serum (Boehringer Mannheim) and studied 24 hr to 8 days after plating.

Electrophysiological Experiments. Resting membrane potential and Na^+ current were recorded at room temperature with the whole-cell patch-clamp technique (17). Outward current refers to the flow of cations from the pipette (cytosolic side) to the bath (external side). The pipette solution contained 100 mM potassium gluconate, 40 mM KCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 4 mM EGTA/KOH (10 nM free Ca^{2+}), 2 mM K_2ATP, and 10 mM Hepes (pH 7.2). The bath solution contained 140 mM NaCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 10 mM Hepes (pH 7.2). The low Na^+ solutions were obtained by substituting NaCl in the bath solution with equal amounts of choline chloride.

Single-channel currents were recorded from outside-out membrane patches (17). In most experiments, solutions were similar to those used in the whole-cell configuration. For experiments in Na^+ symmetrical conditions, K^+ in the pipette solution (see above) was replaced by Na^+. Single-channel data were filtered at 200 Hz. The open probability (P_o) was calculated according to ref. 18.

cDNA Library Construction, Screening, and Sequencing. A cDNA library was synthesized (19) from human adult lung poly(A)$^+$ RNA in Lambda ZAP II phage (Stratagene). Clones (3×10^5) were plated, and five plaques hybridizing with a 614-bp restriction fragment obtained from the rat colon NaCh (20) by digestion with *Eco*RI and *Kpn* I were identified and purified.

For sequencing, DNA was prepared by deletion with the Erase-a-Base system from Promega and sequenced in both directions by dideoxynucleotide sequencing using the dye

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Abbreviations: NaCh, Na^+ channel(s); HLNaCh, human lung NaCh; LE, lung epithelial; EIPA, ethylisopropylamiloride; EPA, ethylpropylamiloride.

‡To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. X76180).

terminator kit and an automatic sequencer (Applied Biosystems model 373A).

Northern Blot Analysis. Northern blotting was carried out by standard techniques (21). The membrane was hybridized with restriction fragments (an *Eco*RI/*Kpn* I 614-bp fragment of the rat colon clone and a *Sau* I/*Hin*cII 814-bp fragment of the human lung clone) labeled with [α-^{32}P]dCTP by random priming.

Human Gene Mapping by *in Situ* Hybridization. *In situ* hybridization was carried out on chromosome preparations obtained from phytohemagglutinin-stimulated human lymphocytes cultured for 72 hr according to ref. 22.

Oocyte Preparation, Microinjection, and Electrophysiological Measurements. These procedures were carried out as described (23). cRNA synthesis of each clone was performed with a Stratagene kit. Capped cRNA (1 μg/μl) was injected into oocytes (50 nl per oocyte). The Na^+ selectivity was tested in the usual buffer without Na^+ (Na^+ replaced by K^+).

RESULTS

Electrophysiological Studies of the NaCh in Rat Pulmonary Cells. Patch-clamp experiments were performed on primary cultures of lung epithelial (LE) cells. Fig. 1*Aa* shows that addition of amiloride to cells in a 140 mM Na^+ medium induced a reversible hyperpolarization. No change in membrane conductance could be observed, suggesting a cell-to-cell electrical coupling. Indeed, in the presence of different blockers of gap junctions (such as 1-heptanol), amiloride produced an increase of cell resistance (data not shown). The mean resting potential was -6.5 ± 13.8 mV (mean $\pm$ SE; $n = 90$) and amiloride (10 μM) induced a mean hyperpolarization of 24.6 ± 13 mV. Removal of Na^+ from the external medium produced the same hyperpolarization as amiloride, and amiloride had no further effect in Na^+-free medium (Fig. 1*Ab*). The amiloride derivative phenamil was more potent than amiloride (in 140 mM Na^+), whereas large concentrations of ethylisopropylamiloride (EIPA) ($\geq$100 μM) were required to see an effect (Fig. 1*Ad*). Apparent $K_{0.5}$ values were 19 and 56 nM for phenamil and amiloride, respectively.

Although LE cells were electrically coupled, stable currents could be recorded (Fig. 1*B*). Addition of amiloride (10 μM) produced a marked, reversible shift in the outward direction of the current recorded at -20 mV (Fig. 1*Ba*). This effect of amiloride was again seen only with Na^+ in the external solution (Fig. 1*Bb*). Amiloride and its derivatives phenamil and benzamil were again effective in reducing the current (Fig. 1*B c* and *d*). The order of potency was benzamil $\approx$ phenamil > amiloride >> EIPA with corresponding apparent $K_{0.5}$ values of 14 nM, 19 nM, 90 nM, and 0.4 mM. Similar results were obtained on currents recorded at -60 mV (data not shown).

Experiments were then performed to study single channels. Amiloride-sensitive NaCh were observed in 17 of 116 outside-out patches. In asymmetrical conditions with K^+ in the pipette and Na^+ in the bath, inward deflections of currents were observed up to 50 mV. At higher potentials, no outward current could be detected up to 100 mV (Fig. 2*A*). These results indicate a high selectivity for Na^+ with a

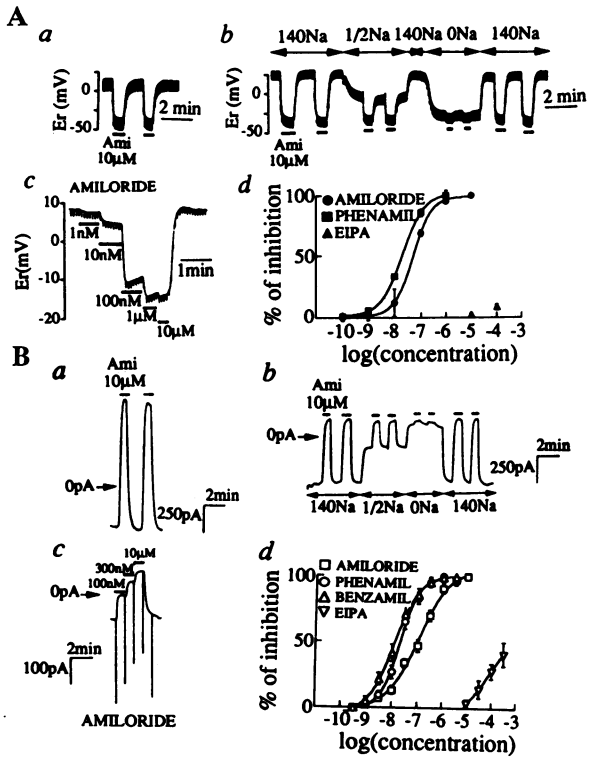

FIG. 1. Electrophysiological analysis of the amiloride-sensitive NaCh in cultures of alveolar type II pneumocytes. (*A*) (*a*) Resting membrane potential (Er) recorded on monolayer in the whole-cell configuration. Membrane resistance was measured by current pulses of -20 pA every 5 s. (*b*) Membrane potential recording at different external Na^+ concentrations with the effects of amiloride. (*c*) Effect of increasing doses of amiloride on the resting potential. (*d*) Dose–response curves for phenamil, amiloride, and ethylisopropylamiloride (EIPA). (*B*) (*a*) Effect of amiloride (10 μM) on the current recorded at -20 mV. (*b*) Dependence of the amiloride-induced decrease of current recorded at -20 mV on the external Na^+ concentration. (*c*) Effect of increasing doses of amiloride on the current recorded at -20 mV; downward deflections correspond to inward current produced by single pulses from -20 to -60 mV and of 400-ms duration. (*d*) Dose–response curves for phenamil, benzamil, amiloride, and EIPA measured on the current recorded at -20 mV. Horizontal bars indicate addition of amiloride (Ami).

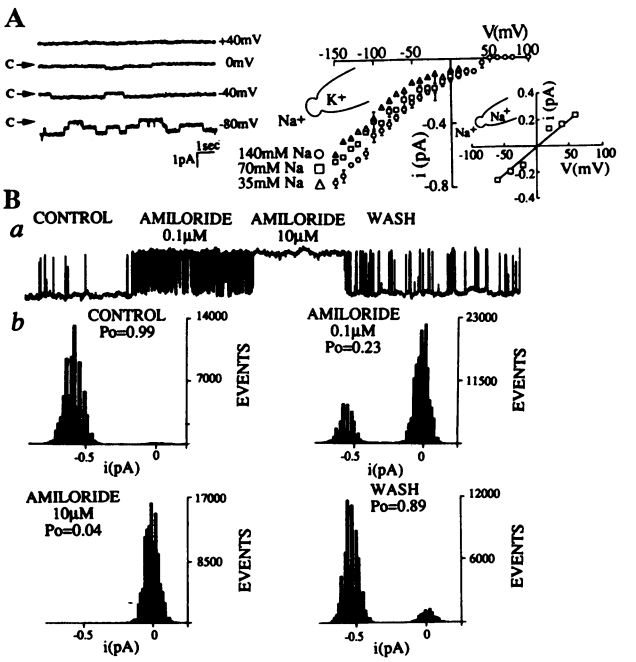

FIG. 2. Single-channel properties of the amiloride-sensitive NaCh. (*A*) (*Left*) Single-channel current traces recorded at different pipette potentials from an outside-out patch. (*Right*) Mean *I–V* relationships measured with different external Na^+ concentrations and K^+-containing solution in the pipette. (*Inset*) *I–V* relationship measured in Na^+ symmetrical condition from two different outside-out patches. (*B*) (*a*) Long-lasting outside-out patch recording at -100 mV, showing the blocking effect of amiloride. (*b*) Corresponding amplitude histograms from which P_o values have been calculated.

P_{Na^+}/P_{K^+} ratio > 10. In Na^+ symmetrical conditions the *I–V* relationship crossed the voltage axis near 0 mV. The unitary conductance was 4.4 pS from −100 mV to 0 mV. The activity of this channel was also characterized by very slow kinetics with opening and closing times in the range of seconds. This channel was highly sensitive to amiloride (Fig. 2*B*). Amiloride (100 nM) caused a 77% decrease in P_o (n = 5).

Cloning and Amino Acid Sequence of Human Lung NaCh (HLNaCh). A human lung cDNA library was prepared and screened with a fragment of the rat colon NaCh cDNA (20). Five positive clones were identified with sizes ranging from 2.7 to 3.8 kb. They were transcribed *in vitro* and injected into *Xenopus* oocytes. One expressing clone was sequenced (3151 bp). It contained a 2007-bp open reading frame encoding a 669-amino acid protein with a predicted size of 76 kDa (Fig. 3). The Kyte and Doolittle hydrophobicity analysis (24) indicates the presence of two large hydrophobic domains (from amino acid 85 to 135 and 542 to 585). There are seven motifs of N-glycosylation and a number of consensus sites for phosphorylation including one site for protein kinase A, five sites for protein kinase C, and nine sites for casein kinase II.

Northern Blot Analysis and Ontogenesis of NaCh. The mRNA for NaCh was specifically detected in human as well as in rat Na^+-reabsorbing epithelial tissues (Fig. 4). In humans, a 3.8-kb messenger was present in low abundance in pancreas but was expressed at a higher level in thyroid and

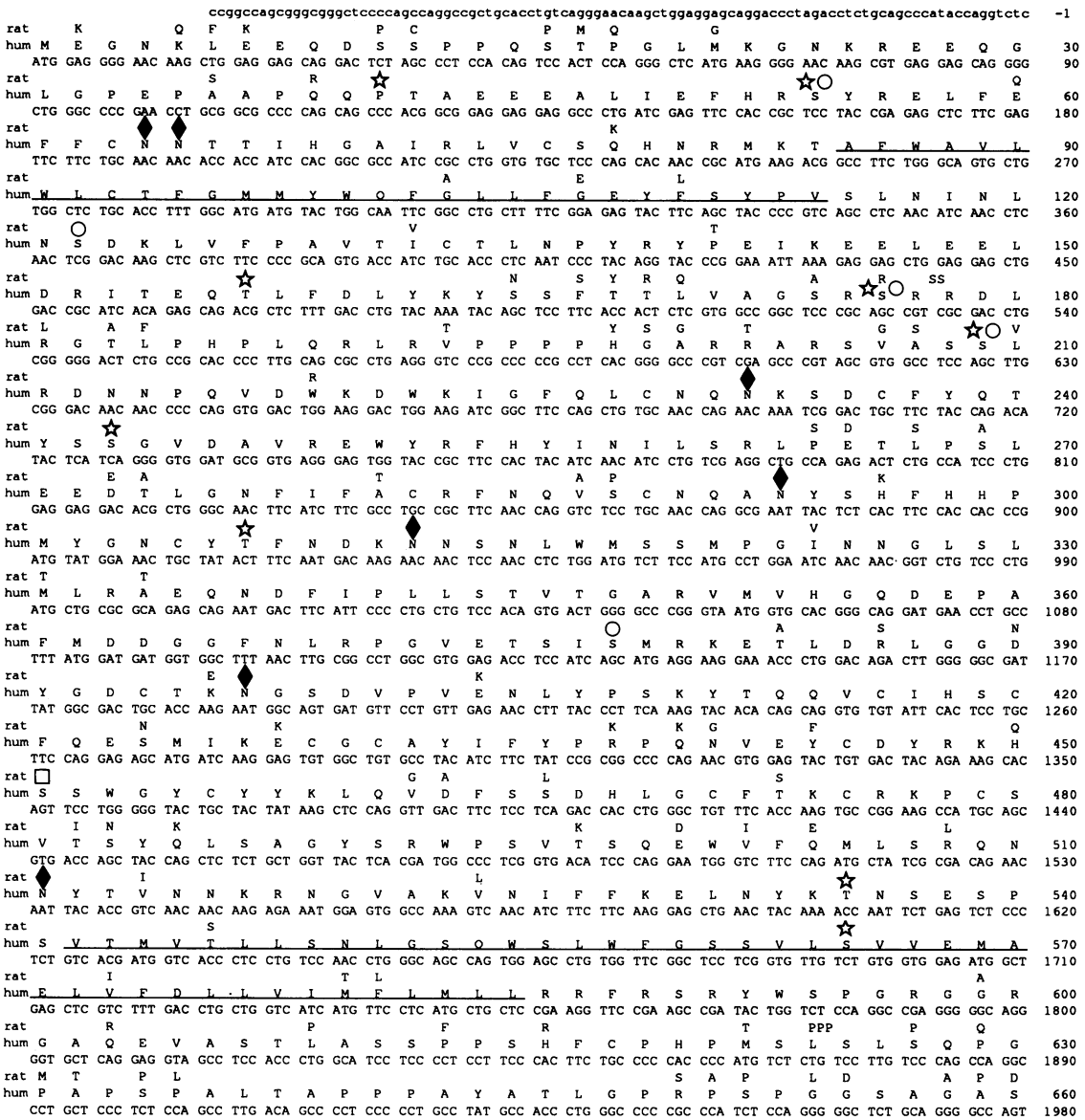

FIG. 3. Nucleotide sequence of HLNaCh cDNA and the corresponding protein and alignment with the rat colon NaCh. In the rat sequence, only divergent residues are shown. ◆, Putative conserved N-glycosylation sites; □, phosphorylation sites by kinase A; ○, phosphorylation sites by kinase C; ☆, phosphorylation sites by casein kinase II.

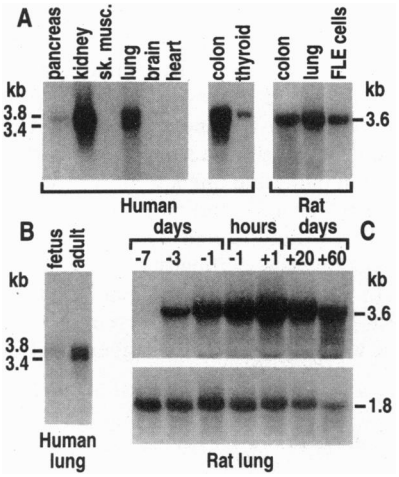

FIG. 4. Distribution of NaCh analyzed by Northern blot experiments. (*A*) Human tissues [2 μg of poly(A)$^+$ except for thyroid (4 μg)] and rat tissues (6 μg of total RNA). FLE, fetal LE cells. (*B*) Human lung [1.2 μg of poly(A)$^+$], fetus (20–25 weeks), and adult. (*C*) Rat lung (6 μg of total RNA). (*Upper*) Hybridization with rat colon NaCh probe. (*Lower*) Control hybridization with a β-actin probe. Days before birth (−) and after birth (+).

even more in human kidney. This same mRNA was found in lung and colon. In these two tissues, another 3.4-kb transcript was also observed (Fig. 4*A*).

A large increase of expression of NaCh was found from fetal to adult stage in human lung (Fig. 4*B*). During development of rat lung (Fig. 4*C*), NaCh mRNA appeared a few days before birth, with a large increase in expression around the time of birth. The level of expression remained high in the adult stage. NaCh mRNA was also detected in a primary culture of LE cells used for electrophysiology (Fig. 4*A*).

Human Chromosome Localization. In the 100 metaphase cells examined after *in situ* hybridization, there were 184 silver grains associated with chromosomes, and 63 of these (34.2%) were located on chromosome 12. The grain distribution on this chromosome was not random; 50/63 (79.4%) mapped to the p13 band of the short arm of chromosome 12 (Fig. 5).

Functional Expression of the HLNaCh in *Xenopus* Oocytes. Oocytes injected with cRNA expressed an amiloride-sensitive current in 98 mM Na^+ medium (Fig. 6 *Inset*, trace A). When external Na^+ was replaced by K^+ (trace B), no amiloride-sensitive current was observed. Fig. 6 gave $K_{0.5}$ values of 50 and 80 nM for inhibition by phenamil and amiloride, respectively. Ethylpropylamiloride (EPA) at 10 μM had no effect on NaCh activity.

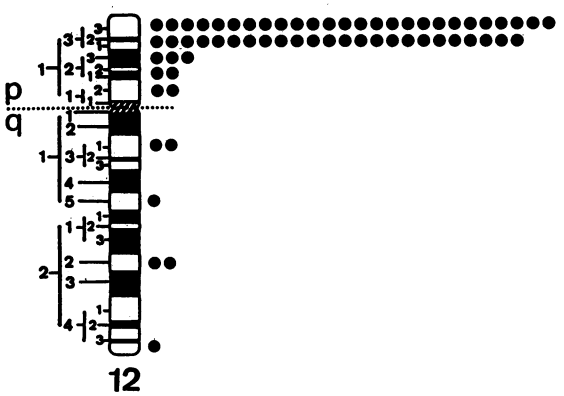

FIG. 5. Idiogram of human G-banded chromosome 12 illustrating distribution of labeled sites for the HLNaCh probe.

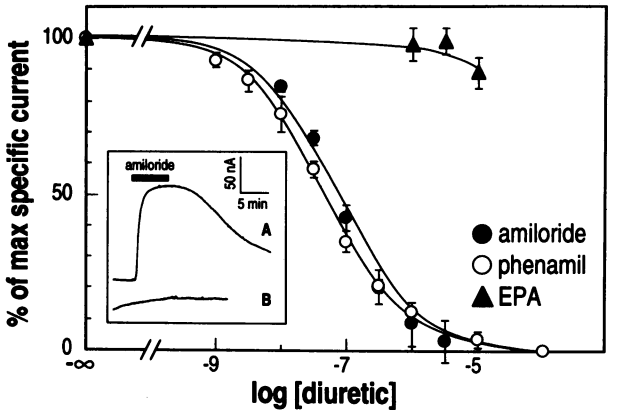

FIG. 6. Electrophysiological and pharmacological characterization of HLNaCh in *Xenopus* oocytes. Dose–response curves for amiloride and its derivatives phenamil and EPA (n = 4 for each compound). (*Inset*) Effect of 20 μM amiloride on HLNaCh cRNA expression. Trace A, reversible effect of amiloride in 98 mM Na^+; trace B, selectivity for Na^+ measured in a Na^+-free buffer (replacement of Na^+ by K^+).

DISCUSSION

The existence of amiloride-sensitive conductive pathways in alveolar epithelial cells has been demonstrated by macroscopic measurements (3–6). Patch-clamp studies have identified an amiloride-sensitive Ca^{2+}-activated nonselective channel (P_{Na^+}/P_{K^+} = 0.9) in fetal alveolar epithelial cells (16). However, because of the high intracellular Ca^{2+} concentration required to activate this channel (25), its role in Na^+ absorption seems difficult to explain.

The electrophysiological data presented in this paper show that the NaCh in LE cells has a conductance of 4 pS, a high selectivity for Na^+ versus K^+, slow kinetics, a relatively high affinity for its blocker amiloride ($K_{0.5}$ = 90 nM) and for amiloride derivatives such as phenamil and benzamil, and a quasi-insensitivity to the Na^+/H^+ exchange blocker EIPA (26). These properties are very similar, if not identical, to those described for the amiloride-sensitive NaCh in the renal collecting tubule (12, 27).

The cloned human lung NaCh is a 76-kDa protein with a structure similar to that established for the rat colon NaCh (20, 28), with which it has 81% identity if Met-27 of the rat colon structure (20) is aligned with the first methionine (Met-1) of the human sequence.

Labeled [^{3}H]benzamil (9) and [^{3}H]bromobenzamil (10) have been used in an attempt to biochemically characterize the amiloride-sensitive NaCh in pneumocytes. The corresponding binding sites have properties similar to those of two recently cloned amiloride binding proteins (ABPs) (29). Transfected cells expressing long or short ABPs expressed high levels of binding for amiloride and amiloride derivatives but did not express NaCh activity (29). There is no structural homology between the HLNaCh cloned in this work and the ABPs.

Both the ionic selectivity and the pharmacology of HLNaCh expressed in *Xenopus* oocytes are very similar to those found by patch-clamp techniques on cultured pneumocytes. The cloned channel is Na^+ selective versus K^+ and its $K_{0.5}$ values for amiloride (80 nM) and for phenamil (50 nM) are very similar to those observed in intact pneumocytes. The channel is essentially insensitive (up to 10 μM) to EPA, an amiloride derivative that blocks Na^+/H^+ exchange.

For all these reasons, there is little doubt that the cloned protein is an essential element of the NaCh structure. However, the relatively low intensity of the expressed amiloride-sensitive Na^+ current observed after injection of HLNaCh

cRNA into oocytes (50–100 nA) might suggest that other subunits are required for generating a higher level of activity, as previously observed for other ionic channels (30). These other subunits might participate in Na^+ permeation or, alternatively, might be components necessary for a proper maturation of the cloned protein.

Two distinct forms of transcripts were detected in human tissues at 3.4 and 3.8 kb. Expression in lung, kidney, and colon has very similar characteristics. The case of the thyroid, which expresses only the 3.8-kb messenger, might be due to the fact that, in this tissue, the amiloride-sensitive NaCh is different. It has a low Na^+ versus K^+ selectivity (31).

The expression of HLNaCh is highly regulated during development. The channel mRNA is not expressed at the fetal stage (20–25 weeks) but is, as expected, fully expressed in adults. This observation is to be linked to the fact that fetal lung behaves as a Cl^--secreting epithelium and adult lung behaves as a Na^+-reabsorbing one. A more detailed analysis of NaCh ontogenesis in the rat confirmed that expression of mRNA increased considerably just before birth. Because steroids are well-known regulators of amiloride-sensitive NaCh function in kidney (11, 32) and colon and because corticosteroids play a role in terminal lung differentiation (reviewed in ref. 33), steroids appear as excellent potential candidates for transcriptional regulators during lung development.

One particularly important aspect of the lung NaCh is its involvement in cystic fibrosis. The disease is now well known to be associated with mutations of CFTR, a cAMP-dependent Cl^- channel (34). However, while the CFTR Cl^--channel activity is drastically reduced for the most severe mutations such as Phe-508 deletion (18), NaCh activity, in the meantime, is significantly increased (14). The combination of the two abnormalities results in a severe modification of the water balance that is the central feature of the disease. A consequence of the increased functional expression of NaCh in cystic fibrosis is that aerosol administration of amiloride has been proposed as a treatment of the disease (15). It is probably important to know in that respect that the HLNaCh has the same pharmacology as kidney and colon NaCh with a particularly high affinity for amiloride.

Numerous human diseases are associated with channel function (reviewed in ref. 35). It would not be surprising that mutations of NaCh are also associated with diseases in relation to transepithelial Na^+ movements. This work provides probes to analyze the possible implications of this channel type in genetic diseases and indicates that the HLNaCh gene is localized on chromosome 12.

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