

# Expression cloning of an epithelial amiloride-sensitive Na<sup>+</sup> channel

## A new channel type with homologies to *Caenorhabditis elegans* degenerins

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A complementary DNA encoding an amiloride-sensitive Na<sup>+</sup> channel has been cloned and characterized from rat colon. The protein encoded by the cDNA has a sequence of 699 amino acids (79 kDa) containing several putative membrane spanning domains and potential phosphorylation sites. It forms a channel that has the electrophysiological and pharmacological properties characteristic of the epithelial Na<sup>+</sup> channel. Homologies (including in transmembrane domains) have been found between a part of the channel sequence and the Mec4 gene product of *Caenorhabditis elegans*, a protein associated with mutation-induced neuronal degeneration.

Sodium channel; Amiloride; Epithelia; Colon; Neuronal degeneration; *Caenorhabditis elegans*

### 1. INTRODUCTION

Amiloride-sensitive Na<sup>+</sup> channels have an important role in sodium and water homeostasis. They are present on the apical membrane of kidney, lung and descending colon cells and in other epithelial tissues (reviewed in refs. 1 and 2). These channels are also present in blood-brain barrier endothelial cells [3] and have a role in sensory perception [2,4]. Amiloride, the typical blocker of this type of channel, is a classical diuretic used in the treatment of hypertension and of a potential use in the treatment of cystic fibrosis [5].

The molecular structure of the amiloride-sensitive Na<sup>+</sup> channel is still unknown. Purifications of epithelial Na<sup>+</sup> channels from different sources have been reported [6,7] and two potential candidates have been cloned [8,9]. One of them from human kidney [8] is a ~100 kDa amiloride binding protein which after transfection into mammalian cells displays a pharmacology similar to that of Na<sup>+</sup> channels but does not express Na<sup>+</sup> channel activity. Another one, which is present in *Xenopus laevis* tissues, has a *M<sub>r</sub>* of 160 kDa but although it is recognized by antibodies thought to bind to the amiloride receptor site, does not display Na<sup>+</sup> channel activity [9].

Rat distal colon is a particularly interesting starting material to isolate a cDNA for the amiloride-sensitive Na<sup>+</sup> channel since very high expression levels of that

channel are induced by aldosterone (or dexamethasone) [2,10,11].

This paper reports the cloning of the rat colon amiloride-sensitive Na<sup>+</sup> channel by expression in *Xenopus* oocytes.

### 2. MATERIALS AND METHODS

#### 2.1. RNA preparation

Wistar rats were infused once a day during 3 days with 3 mg dexamethasone in corn oil and sacrificed the fourth day. Total RNA was extracted from the epithelium of distal colon by the lithium chloride method [12]. To obtain poly(A<sup>+</sup>) RNA, total RNA was loaded on an oligo(dT) cellulose column in Tris-HCl 10 mM at pH 7.5, EDTA 1 mM, NaCl 0.5 M, SDS 0.1%, eluted with water, and precipitated.

#### 2.2. cDNA library synthesis and screening

A size-selected directional cDNA library was synthesized from the rat colon mRNA using a modification of [13].

An oligo(dT)-*Xho*I primer-adaptor was used to prime first strand cDNA synthesis which was performed in the presence of 5-methyl dCTP to protect internal *Xho*I sites. After synthesis of double-stranded cDNA, *Eco*RI adaptors were added and the cDNA was cut with *Xho*I. The resulting cDNA was size fractionated on Sephacryl S1000 and cDNAs greater than 2 kb were ligated into the *Eco*RI/*Xho*I sites of the cloning vector pEXO.

The pEXO vector was derived from pGEM 5-Zf(-) (Promega) by inserting the 5'- and 3'-untranslated regions of *Xenopus* globin [14] which flank an *Eco*RI/*Xho*I polylinker into the *Eco*RV site. A T7 terminator was ligated into the *Sac*I and the *Nde*I sites of the vector and ATG sequences downstream of the T7 promoter were removed by digestion with *Nco*I and *Sph*I followed by blunt end religation. The *E. coli* strain SURE (Stratagene) was transformed with the cDNA library by electroporation.

The 100 000 recombinants obtained were subdivided in 20 subpools of 5,000 and plated on Hybone-N filters. After an overnight growth at 30°C, replicas were made and the template filters were frozen [15]. The replicas were grown again at 37°C, bacteria were scraped off and plasmid was prepared using the alkaline lysis technique.

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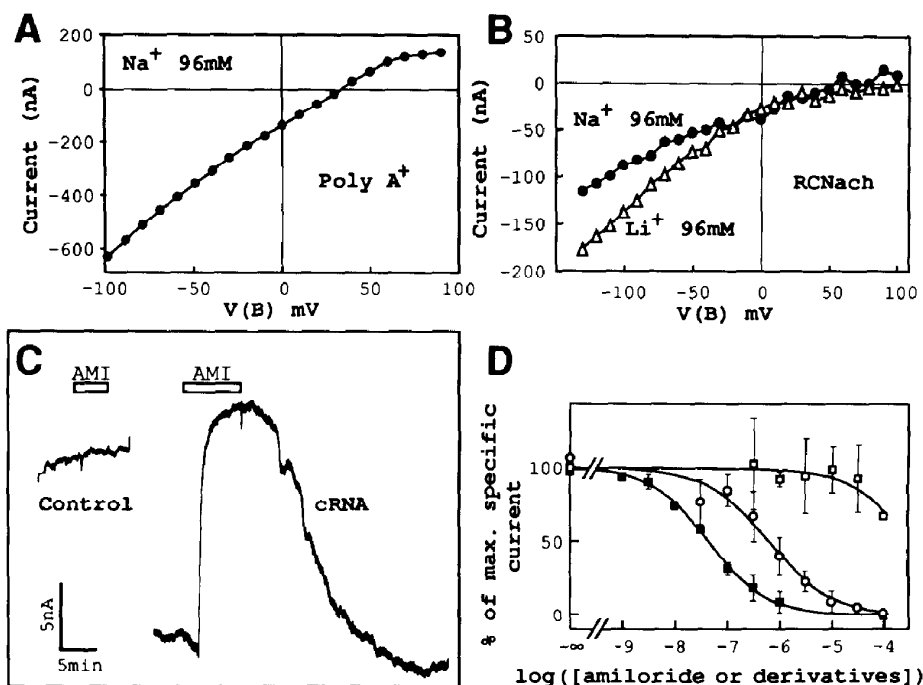


Fig. 1. Electrophysiological and pharmacological characterization of the RCNaCh in *Xenopus* oocytes. A.  $I$ - $V$  relationship of the amiloride-sensitive  $\text{Na}^+$  channel expressed from rat colon poly(A<sup>+</sup>) RNA in a  $\text{Na}^+$  medium (4 days after injection). B.  $I$ - $V$  relationship for RCNaCh cRNA injected oocytes (2 days after injection) in a  $\text{Li}^+$  medium ( $\Delta$ ) and in a  $\text{Na}^+$  medium ( $\bullet$ ). C. Reversible effect of 20  $\mu\text{M}$  amiloride on RCNaCh cRNA expression (24 h after injection). The control corresponds to an oocyte injected with the same cRNA, one hour after injection, i.e. a time too short to produce  $\text{Na}^+$  channel expression. Results similar to those in the control were obtained with water-injected or uninjected oocytes. D. Dose-response curves for amiloride and amiloride derivatives, phenamil ( $\blacksquare$ ) ( $n = 2$ ), amiloride ( $\circ$ ) ( $n = 4$ ) and ethylpropylamiloride ( $\square$ ) ( $n = 3$ ). Voltage was clamped at  $-70$  mV.

In vitro transcription was performed with *NotI*-linearized DNA using a Stratagene kit with T7 RNA polymerase. Capped cRNAs (at 1  $\mu\text{g}/\mu\text{l}$ ) were injected into oocytes (50 nl/egg). After 3 days, the oocytes were tested by  $^{22}\text{Na}^+$  flux measurements with and without amiloride 10  $\mu\text{M}$  as described in [16] and by electrophysiology (measuring the amiloride (20  $\mu\text{M}$ ) sensitive component of the whole egg current).

The frozen filter of the positive fraction was cut into parts and the whole process was repeated until a single positive clone was identified.

### 2.3. Characterization of the clone

The 3081 nucleotide cDNA insert was sequenced in both directions by dideoxy sequencing [17] using the dye terminator kit and automatic sequencing (Applied Biosystems 373A). DNA for sequencing was prepared with the Erase-A-Base system from Promega.

### 2.4. Oocyte preparation, microinjection, electrophysiological measurements

They were carried as described in [18]. For measurement of current-voltage relationships, the clamping voltage was changed in steps from a holding potential of  $-70$  mV and held at the test voltages for 1–2 s.

### 2.5. In vitro translation

In vitro translation was carried out in the presence of [ $^{35}\text{S}$ ]methionine with a reticulocyte lysate (Promega) according to the supplier protocol. For maturation studies, 2.5  $\mu\text{l}$  of canine pancreatic membranes per 25  $\mu\text{l}$  final volume were used.  $\beta$ -Lactamase and the  $\alpha$ -factor were used to control the signal peptide cleavage and glycosylation respectively. In vitro translation products were analyzed by SDS-PAGE and revealed by autoradiography.

## 3. RESULTS AND DISCUSSION

Amiloride-sensitive  $\text{Na}^+$  channels have been previously expressed in *Xenopus* oocytes from poly(A<sup>+</sup>) RNA

from different tissues [16,19]. These expression studies have led to a  $M_r$  weight prediction of 70–80 kDa for the channel protein [19]. Our cloning strategy has used the oocyte expression system and two independent measurements of the  $\text{Na}^+$  channel activity involving electrophysiology and  $^{22}\text{Na}^+$  uptake experiments. Distal rat colon samples expressing amiloride-sensitive short circuit currents higher than 500  $\mu\text{A}/\text{cm}^2$  were selected for poly(A<sup>+</sup>) RNA isolation. A rat cDNA library was then constructed in an expression vector from a high response-evoking sample. A single channel cDNA clone (called RCNaCh for rat colon  $\text{Na}^+$  channel) was isolated by serially subdividing the positive cDNA mixtures.

The electrophysiological properties of the cloned amiloride-sensitive RCNaCh are presented in Fig. 1. This figure shows that  $I$ - $V$  relationships obtained with poly(A<sup>+</sup>) RNA (Fig. 1A) and cRNA (Fig. 1B) are very similar. These responses are also similar to those observed with poly(A<sup>+</sup>) RNA from other tissues [2,19]. The cloned  $\text{Na}^+$  channel, like the channel in intact cells [2], has been found to be permeable to both  $\text{Na}^+$  and  $\text{Li}^+$  (Fig. 1B), with  $I_{\text{Li}}/I_{\text{Na}} = 1.6$  at  $-70$  mV.

An important aspect of epithelial  $\text{Na}^+$  channels is their sensitivity to amiloride. Amiloride derivatives have been developed which can discriminate between the  $\text{Na}^+$  channel and  $\text{Na}^+/\text{H}^+$  or  $\text{Na}^+/\text{Ca}^{2+}$  exchangers [20,21]. Phenamil has a high affinity for  $\text{Na}^+$  channels

tcagcctgggatcggggacgggtccggacagccccattctgcttcacgcta																														-1	
M	M	L	D	H	T	R	A	P	E	L	N	I	D	L	D	L	H	A	S	N	S	P	K	G	S	M	K	G	N	30	
ATG	ATG	CTG	GAC	CAC	ACC	AGA	GCC	CCT	GAG	CTC	AAC	ATT	GAC	CTA	GAC	CTT	CAC	GCC	TCC	AAC	TCG	CCT	AAG	GGG	TCC	ATG	AAG	GGC	AAC	90	
Q	F	K	E	Q	D	P	C	P	P	Q	P	M	Q	G	L	G	K	G	D	K	R	E	E	Q	G	L	G	G	P	E	60
CAA	TTC	AAG	GAG	CAA	GAC	CCT	TGT	CCT	CCT	CAG	CCC	ATG	CAA	GGA	CTG	GGG	AAG	GGG	GAC	AAA	CGT	GAA	GAG	CAG	GGC	CTG	GGC	CGG	GAA	180	
P	S	A	P	R	Q	P	T	E	E	E	E	A	L	I	E	F	H	R	S	Y	R	E	L	F	Q	F	F	C	N	90	
CCC	TCA	GCA	CCC	CGG	CAG	CCC	ACC	GAG	GAG	GAG	GAG	GCA	CTG	ATT	GAA	TTC	CAC	CGC	TCC	TAC	CGG	GAG	CTC	TTC	CAG	TTC	TTC	TGC	AAC	270	
N	T	T	I	H	G	A	I	R	L	V	C	S	K	H	N	R	M	K	T	A	F	W	A	V	L	W	L	C	T	120	
AAC	ACC	ACC	ATC	CAC	GGG	GCC	ATC	CGC	CTG	GTG	TGC	TCC	AAA	CAC	AAC	CGC	ATG	AAG	ACG	GCC	TTC	TGG	CGC	GTG	CTG	TGG	CTG	TGC	ACC	360	
F	G	M	M	Y	W	Q	T	F	A	L	L	F	E	E	Y	L	S	Y	P	V	S	L	N	I	N	L	N	S	D	K	150
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L	V	F	P	A	V	T	V	C	T	L	N	P	Y	R	A	Y	T	E	I	K	E	E	L	E	E	L	D	R	I	T	180
CTG	GTC	TTC	CCT	GCC	GTC	ACT	GTC	TGC	ACC	CTT	AAT	CCT	TAC	AGA	TAC	ACT	GAA	ATT	AAA	GAG	GAG	CTG	GAA	GAG	CTG	GAC	CTG	ATC	ACG	540	
E	Q	T	L	F	D	L	Y	K	Y	N	S	S	Y	T	R	Q	A	G	A	R	R	R	S	S	R	D	L	L	G	210	
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A	F	P	H	P	L	Q	R	L	R	T	P	P	P	Y	T	S	G	R	T	A	R	S	G	S	S	S	V	R	D	240	
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N	N	P	Q	V	D	R	K	D	W	K	I	G	F	Q	L	C	N	Q	N	K	S	D	C	F	Y	Q	T	Y	S	270	
AAC	AAT	CCC	CAA	GTG	GAC	CGG	AAG	GAC	TGG	AAG	ATC	GGC	TTC	CAA	CTG	TGC	AAC	CAG	AAC	AAA	TCA	GAC	TGT	TTC	TAC	CAG	ACA	TAC	TCC	810	
S	G	V	D	A	V	R	E	W	Y	R	F	H	Y	A	I	N	I	L	S	R	L	S	D	T	S	P	A	L	E	E	300
TCT	GGG	GTG	GAT	GCA	GTG	AGG	GAG	TGG	TAC	CGC	TTC	CAT	TAC	ATC	AAC	ATT	CTG	TCC	AGA	CTG	TCG	GAC	ACC	TCG	CCC	GCT	CTA	GAG	GAA	900	
E	A	L	G	N	F	I	F	T	C	R	F	N	Q	A	P	C	N	Q	A	N	Y	S	K	F	H	H	P	M	Y	330	
GAA	GCC	CTG	GGC	AAC	TTC	ATC	TTC	ACC	TGT	CGC	TTC	AAC	CAG	GCC	TGC	AAC	CAG	CGC	AAT	TAT	TCC	AAG	TTC	CAC	CAC	CCC	ATG	TAC	990		
G	N	C	Y	T	F	N	D	K	N	N	S	N	L	W	M	S	T	M	P	G	V	N	N	G	L	S	L	T	L	360	
GGG	AAC	TGC	TAC	ACT	TTC	AAT	GAC	AAG	AAC	AAC	TCC	AAT	CTC	TGG	ATG	TCC	TCC	ATG	CCT	GGA	GTC	AAC	AAT	GGT	TTG	TCC	CTG	ACA	CTG	1080	
R	T	E	Q	N	D	I	P	L	L	S	T	V	T	G	A	R	V	M	V	H	G	Q	D	E	P	A	F	M	390		
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D	C	T	E	N	G	S	D	V	P	V	K	N	L	Y	P	S	K	Y	T	Q	Q	V	C	I	H	S	C	F	Q	450	
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E	N	M	I	K	K	C	G	C	A	Y	I	F	Y	P	K	P	K	G	V	E	F	C	D	Y	R	K	Q	S	S	480	
GAG	AAC	ATG	ATC	AAG	AAG	TGT	GGC	TGT	GCC	TAC	ATC	TTC	TAC	CCT	AAG	CCC	AAG	GGA	GTG	GAG	TTC	TGT	GAC	TAC	CGA	AAG	CAG	AGC	TCC	1440	
W	G	Y	C	Y	Y	K	L	Q	G	A	F	S	L	D	S	L	G	C	F	S	K	C	R	K	P	C	S	V	I	510	
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N	Y	K	L	S	A	G	Y	S	R	W	P	S	V	K	S	Q	D	W	I	F	E	M	L	S	L	Q	N	N	Y	540	
AAC	TAC	AAA	CTC	TCT	GCC	GGC	TAC	TCA	CGG	TGG	CCA	TCT	GTG	AAG	TCC	CAG	GAT	TGG	ATC	TTC	GAG	ATG	CTG	TCC	TTG	CAG	AAC	AAT	TAC	1620	
T	I	N	N	K	R	N	G	V	A	K	L	N	I	F	F	K	E	L	N	Y	K	T	N	S	E	S	P	S	V	570	
ACT	ATT	AAC	AAC	AAA	AGA	AAC	GGA	GTT	GCA	AAG	CTC	AAC	ATC	TTC	TTC	AAG	GAG	CTG	AAC	TAT	AAA	ACT	AAT	TCG	GAG	TCT	CCT	TCT	GTC	1710	
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g	ga	ac	tc	ag	ct	tc	tt	at	gt	ct	cc	cc	ag	at	gt	ct	cc	cc	cc	ag	at	gt	ct	cc	cc	cc	cc	cc	2900		
ct	ct	gt	gt	gc	gg	ga	tc	gc	at	tt	gt	ct	cc	ct	cc	ct	cc	ct	cc	ct	cc	ct	cc	ct	cc	ct	cc	ct	3020		
cg	cc	cc	cg	cc	cg	cc	cg	cc	cg	cc	cg	cc	cg	cc	cg	cc	cg	cc	cg	cc	cg	cc	cg	cc	cg	cc	cg	cc	3028		

Fig. 2. cDNA and deduced amino acid sequence of RCNaCh.

and a poor affinity for  $\text{Na}^+/\text{H}^+$  and  $\text{Na}^+/\text{Ca}^{2+}$  exchangers. It is considered to be the most specific blocker of the  $\text{Na}^+$  channel [21]. On the other hand ethylpropylamiloride is known to be a potent blocker of the  $\text{Na}^+/\text{H}^+$  exchanger and a very weak effector of the  $\text{Na}^+$  channel [21]. Amiloride reversibly blocks  $\text{Na}^+$  channel activity from oocytes injected with the cRNA (Fig. 1C). Dose-response curves for the inhibition of the  $\text{Na}^+$  channel activity of the cloned protein by amiloride and derivatives are presented in Fig. 1D.  $\text{ED}_{50}$ 's are 44 nM, 620 nM, and  $>300 \mu\text{M}$  for phenamil, amiloride and ethylpropylamiloride, respectively. These values are very

similar to those previously described for the native channel in colon of dexamethasone-treated rats [22].

The nucleotide sequence of the cloned cDNA (3081 bp) is presented in Fig. 2. It contains one open reading frame of 2097 base pairs (bp). The predicted polypeptide consists of 699 amino acids. The calculated molecular weight of 79 kDa is in good agreement with results from in vitro translation experiments (Fig. 3, lanes a and b), which produced in the absence of microsomal membranes a polypeptide with an apparent molecular weight of 75 kDa in SDS-PAGE. The apparent  $M_r$  increased to 92 kDa (Fig. 3, lane c) when translation was

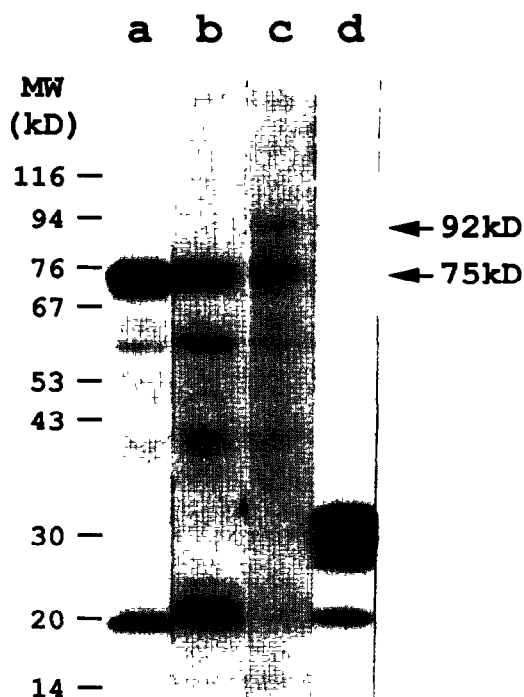


Fig. 3. Electrophoresis pattern of in vitro translation products of the  $\text{Na}^+$  channel cRNA.  $M_r$  (kDa) are shown on left. Lane a, cRNA with reticulocyte lysate; lane b, same conditions with 0.5% of Triton X-100; lane c, cRNA with reticulocyte lysate and canine pancreatic microsomes; lane d, control cRNAs for  $\alpha$ -factor and  $\beta$ -lactamase with reticulocyte lysate and pancreatic microsomes.

carried out in the presence of microsomal membranes, suggesting a maturation process probably associated with glycosylation.

A hydrophobicity analysis of RCNaCh showed the presence of four main hydrophobic domains consisting of more than 20 amino acids residues (Fig. 4B). Two of these domains from 110 to 162 and from 567 to 613 are sufficiently long to be split into 2 transmembrane segments. Therefore we have tentatively built a model (Fig. 4A) which presents 6 transmembrane regions. Since there is no signal peptide, the N-terminal end of the channel was assumed to be cytoplasmic. The main extracellular loop from 378 to 566 contains 2 *N*-glycosylation sites in positions 425 and 539 (the other potential *N*-glycosylation sites are not indicated in this model because they are situated on intracellular segments).

Two potential consensus phosphorylation sites for kinase A have been identified at Ser-204 and Ser-205 as well as 8 consensus sites for phosphorylation by kinase C and 8 sites for casein kinase II (Fig. 4A). Therefore multiple types of regulations of this channel protein are theoretically possible and might account for the well known hormonal regulation of the channel [1,2].

The  $\text{Na}^+$  channel protein shows no significant sequence homology with other previously cloned channels (including the voltage-sensitive  $\text{Na}^+$  channel). A particularly interesting sequence homology has been found

between RCNaCh and the Mec4 gene product from *C. elegans* which has been called a degenerin [23], a transmembrane protein whose mutation provokes neuronal degeneration. Sequence homologies between Mec4 (a 497 amino acids segment) and the 127 to 638 region of RCNaCh are presented in Fig. 5. This sequence homology suggests that, as previously discussed [23], Mec4 degenerin might be an ionic channel which could be speculated to be a non selective channel or a new type of  $\text{Ca}^{2+}$  channel both capable to introduce deleterious concentrations of  $\text{Ca}^{2+}$  into cells. It is particularly interesting to note that on one hand Mec4 is associated with touch cells that are required for mechanosensation [23] while, on the other hand, mechanosensitive channels are blocked by high concentrations of amiloride [24].

In conclusion amiloride-sensitive  $\text{Na}^+$  channel activity is generated by a single protein of 79 kDa which seems to have most or all of the biophysical and pharmacological properties of the native channel in the epithelial tissue. However, one cannot eliminate that other types of subunits associate to this channel protein for regulation of its activity, as observed for most voltage-

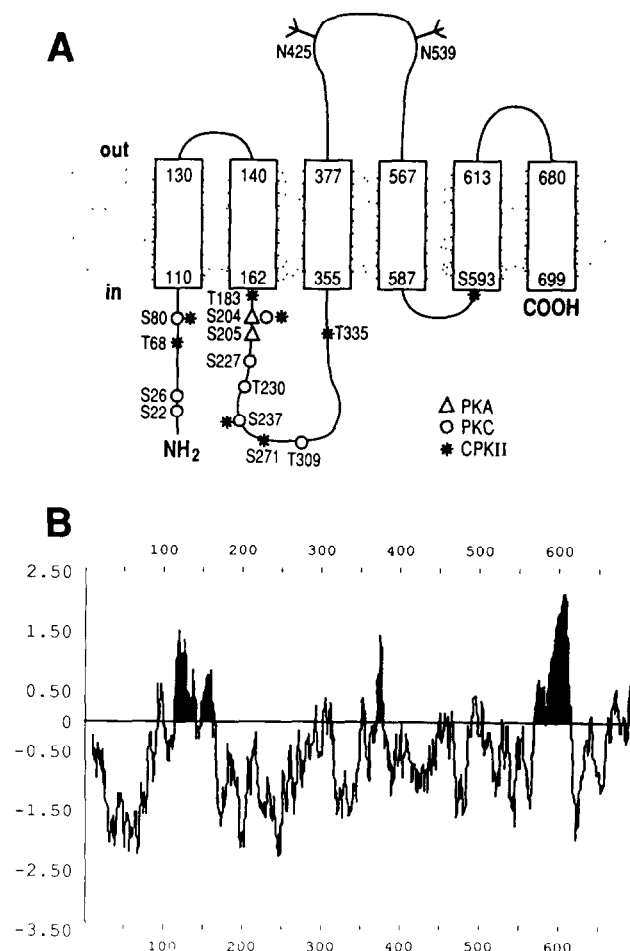


Fig. 4. (A) A putative structural model of the amiloride-sensitive  $\text{Na}^+$  channel. (B) Hydrophobicity plot according to [25] using a window of 15 amino acids. Hydrophobic segments are presented in black.

Mec4	1	EWDGM-EEYDNEHYE-NYDVEATT-GMNMEECQSERTKFDE-PTGFDDRCICAFDRSTH
RCNaCh	127	QEALFEEYLSYPVSLNINLNSDKLVFPVAVTVCTLNPHYRYTEIKEELEELDRIT-EQTLF
Mec4	57	DAWPCFLNGTWETTECDTCNEHAFCTKDNKTA--KGHRSPICCAPSRF-CVAYNGKTPPI
RCNaCh	186	DLYKYNSSYTRQAGARRRSSRDLLGAFPHPLQRLRTPPPPYSGRTARSGSSSVRDNNPQV
Mec4	114	EIWTYLQGGTPTEDPNFLEAMGFQGMTDEV-AIVTKAKENIMFAMATLSMODRERLSTTK
RCNaCh	246	D-RKDWKIGFQLCNQNKSDCF-YQTYSSGVDVAVREWYRFHYINILSRISDTSPALEEEAL
Mec4	173	RELVHKCSFNGKACDIEADFLTHIDPAFGSCFTFNHNRTVNL-TSIRAGPMYGLRMLVYV
RCNaCh	304	GNFIFTCRFNQAPCN-QANYSKFHHPMYGNCYTFNDKNNSNLWMSSMPGVNNGLSITLRT
Mec4	232	NASDYMP-TTEATGVRLTIHDKEDFFFPDFTFGYSAPTGYVSSFGLRLRKMSRLPAPYGDC
RCNaCh	363	EQNDFEIPLLSTVTGARVMVHGQDEPAEMDDGGFNLRPGVETSIISMRKEALDSLGGNYGDC
Mec4	291	VPDGGK--TSDYIYSNYEYSVEGCRYSCFOQLVLKECRCGDPRFPVPENARHCDAADPIAR
RCNaCh	423	TENGSDVPVKNLYPS-KYTQQVCIHSCFOENMIKKCGCAYIFYPKPKGVEFCDYRKQSSW
Mec4	349	KCLDARMNDLGGLH--GSFRCRCQOPCRQSIYSVTYSPAKWPSLSLQIQLGSCNGTAVEC
RCNaCh	482	GYCYYKLGAFSLDSLGCFSKCRKPCSVINYKLSAGYSRWPSVKSQDWIFEMLSLQNNY
Mec4	407	NKHYKENG-AMVEVFYEQLNEMLTESEAYGFVNLLADFGGQLGLWCGISFLTTC--CEF
RCNaCh	541	TINNKRNQVAKLNIFEKELNKKTNSESFSVTMVSLLSNLGSSQWSLWFGSSVLSVVEMAEL
Mec4	463	VFLFLETAYMSAEHNY-SLYKK--KKAEEKAKKIASGSF
RCNaCh	601	IFDLLVITLLMLLRFRSRYWSPGRGARGAREVASTPA

Fig. 5. Protein sequence homology between RCNaCh and the known sequence of Mec4 degenerin. Identical amino acids are labelled in black, and amino acids with similar side chains are in grey.

sensitive channels. The structure described here is that of a new channel type which seems to include degenerins and might also include many other ionic channels that have been identified electrophysiologically but not yet cloned.

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