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Expression cloning of an epithelial amiloride-sensitive Na^+ channel

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

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A complementary DNA encoding an amiloride-sensitive Na^+ 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^+ 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^+ 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^+ channel is still unknown. Purifications of epithelial Na^+ 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^+ channels but does not express Na^+ channel activity. Another one, which is present in *Xenopus laevis* tissues, has a M_r of 160 kDa but although it is recognized by antibodies thought to bind to the amiloride receptor site, does not display Na^+ channel activity [9].

Rat distal colon is a particularly interesting starting material to isolate a cDNA for the amiloride-sensitive Na^+ 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^+ channel by expression in *Xenopus* oocytes.

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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^+) 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-adapter 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.

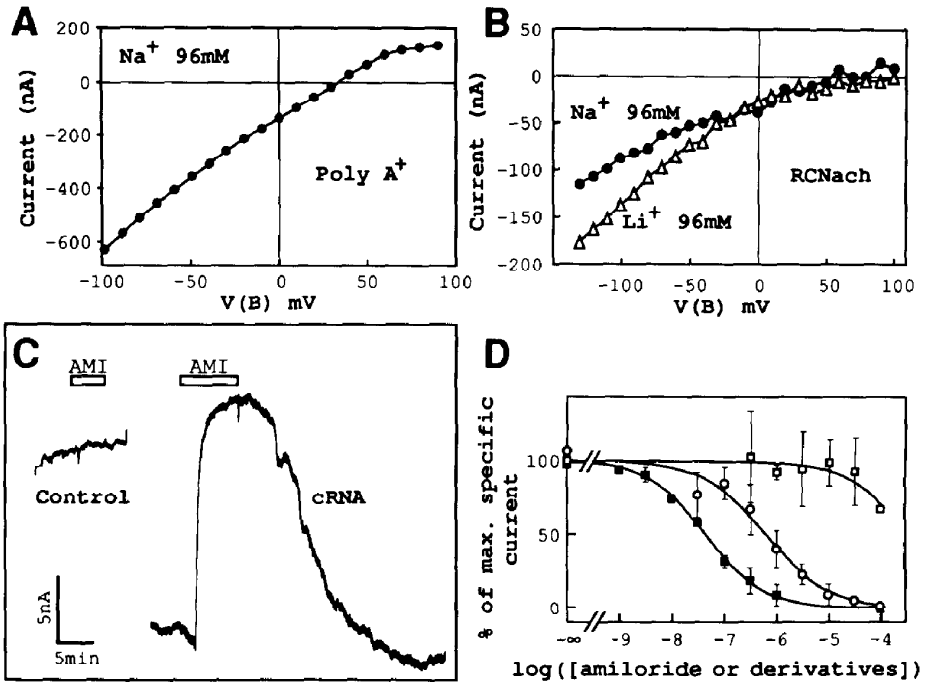

Fig. 1. Electrophysiological and pharmacological characterization of the RCNaCh in *Xenopus* oocytes. A. *I–V* relationship of the amiloride-sensitive Na^+ channel expressed from rat colon poly(A^+) RNA in a Na^+ medium (4 days after injection). B. *I–V* relationship for RCNaCh cRNA injected oocytes (2 days after injection) in a Li^+ medium (△) and in a Na^+ medium (●). C. Reversible effect of 20 μM 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 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 (■) (n = 2), amiloride (○) (n = 4) and ethylpropylamiloride (□) (n = 3). Voltage was clamped at −70 mV.

In vitro transcription was performed with *Not*I-linearized DNA using a Stratagene kit with T7 RNA polymerase. Capped cRNAs (at 1 μg/μl) were injected into oocytes (50 nl/egg). After 3 days, the oocytes were tested by $^{22}Na^+$ flux measurements with and without amiloride 10 μM as described in [16] and by electrophysiology (measuring the amiloride (20 μM) 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}S]methionine with a reticulocyte lysate (Promega) according to the supplier protocol. For maturation studies, 2.5 μl of canine pancreatic membranes per 25 μl final volume were used. β-Lactamase and the α-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 Na^+ channels have been previously expressed in *Xenopus* oocytes from poly(A^+) 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 Na^+ channel activity involving electrophysiology and $^{22}Na^+$ uptake experiments. Distal rat colon samples expressing amiloride-sensitive short circuit currents higher than 500 μA/cm^2 were selected for poly(A^+) 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 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^+) RNA (Fig. 1A) and cRNA (Fig. 1B) are very similar. These responses are also similar to those observed with poly(A^+) RNA from other tissues [2,19]. The cloned Na^+ channel, like the channel in intact cells [2], has been found to be permeable to both Na^+ and Li^+ (Fig. 1B), with I_{Li}/I_{Na} = 1.6 at −70 mV.

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

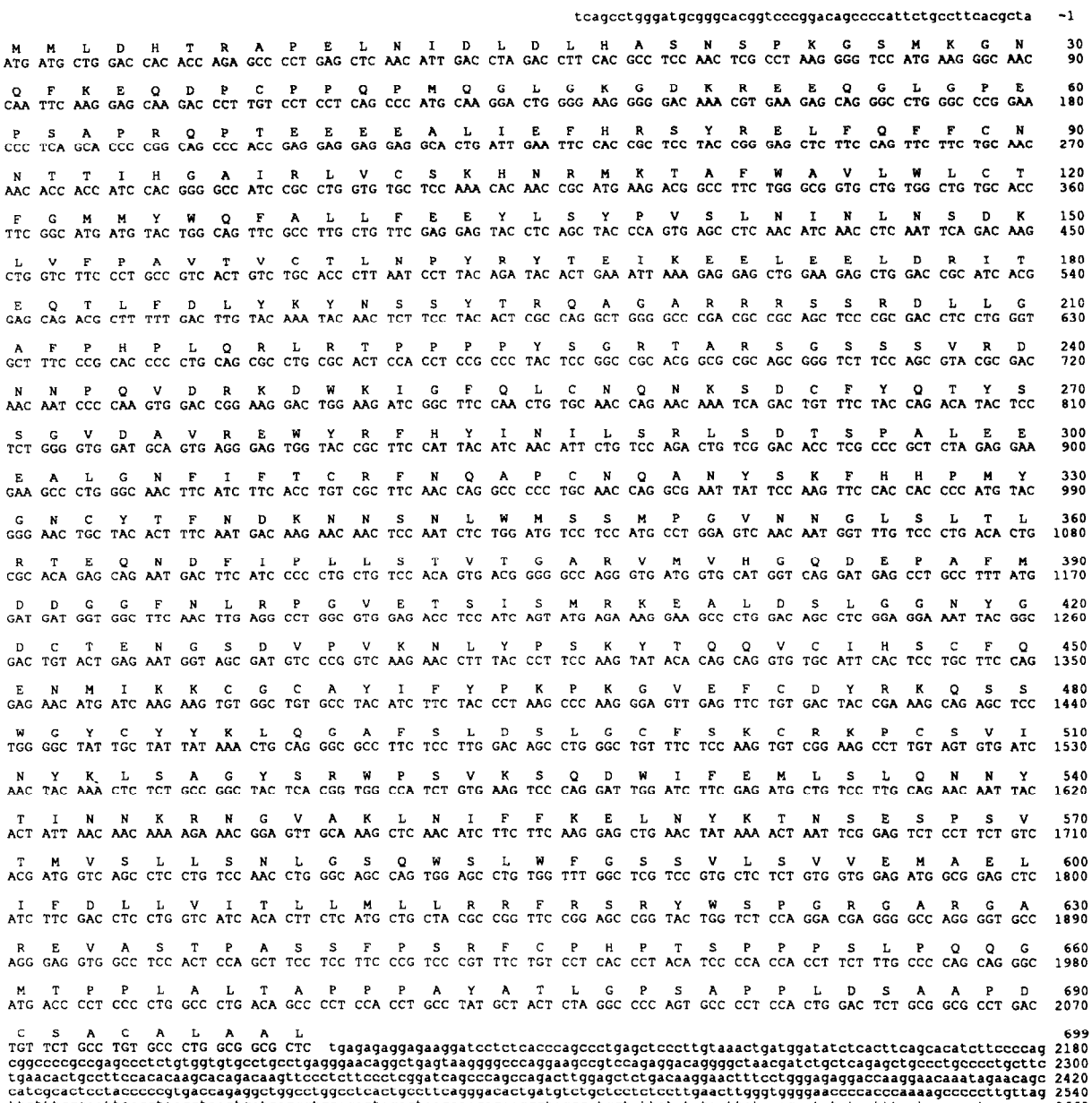

Fig. 2. cDNA and deduced amino acid sequence of RCNaCh.

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

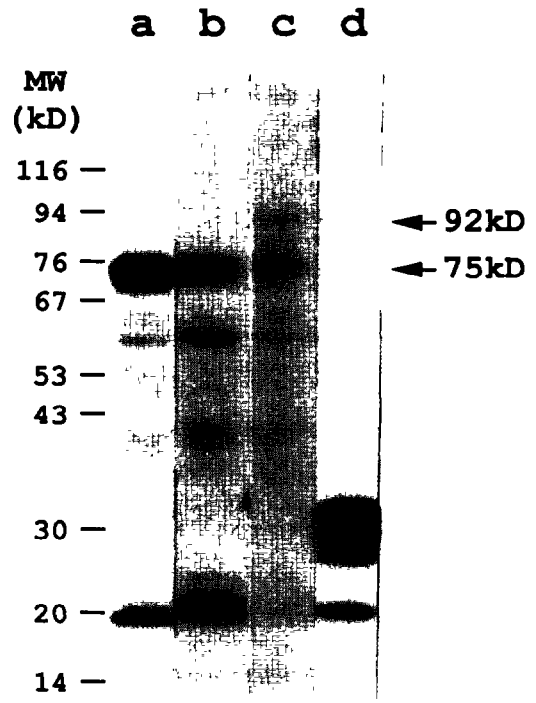

Fig. 3. Electrophoresis pattern of in vitro translation products of the Na^+ channel cRNA. M_r (kDa) are shown on left. Lane a, cRNA with reticulocyte lysate; lane b, same conditions with 0.5% of Triton X-100; lane c, cRNA with reticulocyte lysate and canine pancreatic microsomes; lane d, control cRNAs for α-factor and β-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 Na^+ channel protein shows no significant sequence homology with other previously cloned channels (including the voltage-sensitive 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 Ca^{2+} channel both capable to introduce deleterious concentrations of 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 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-

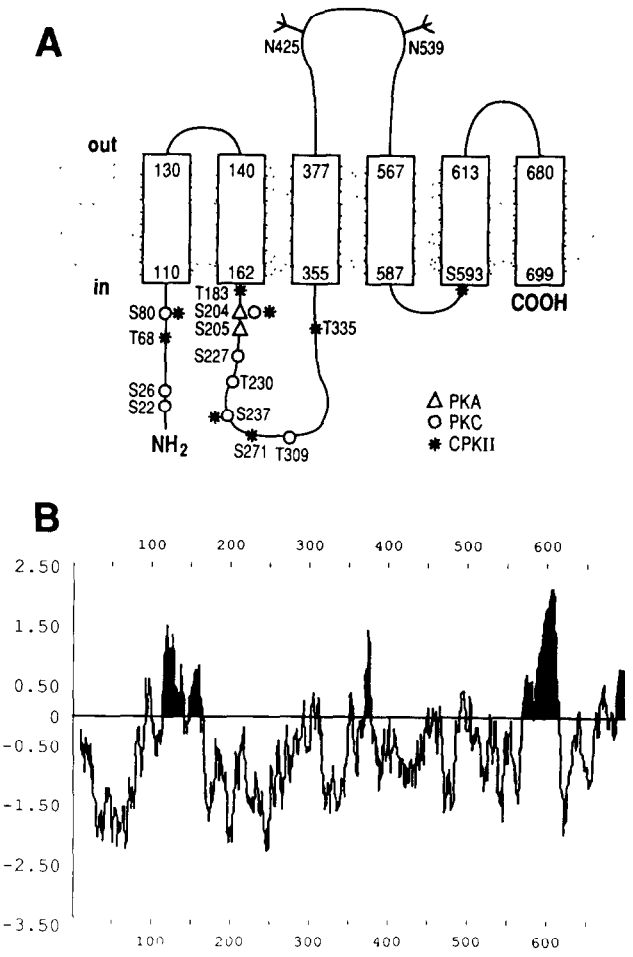

Fig. 4. (A) A putative structural model of the amiloride-sensitive Na^+ channel. (B) Hydrophobicity plot according to [25] using a window of 15 amino acids. Hydrophobic segments are presented in black.

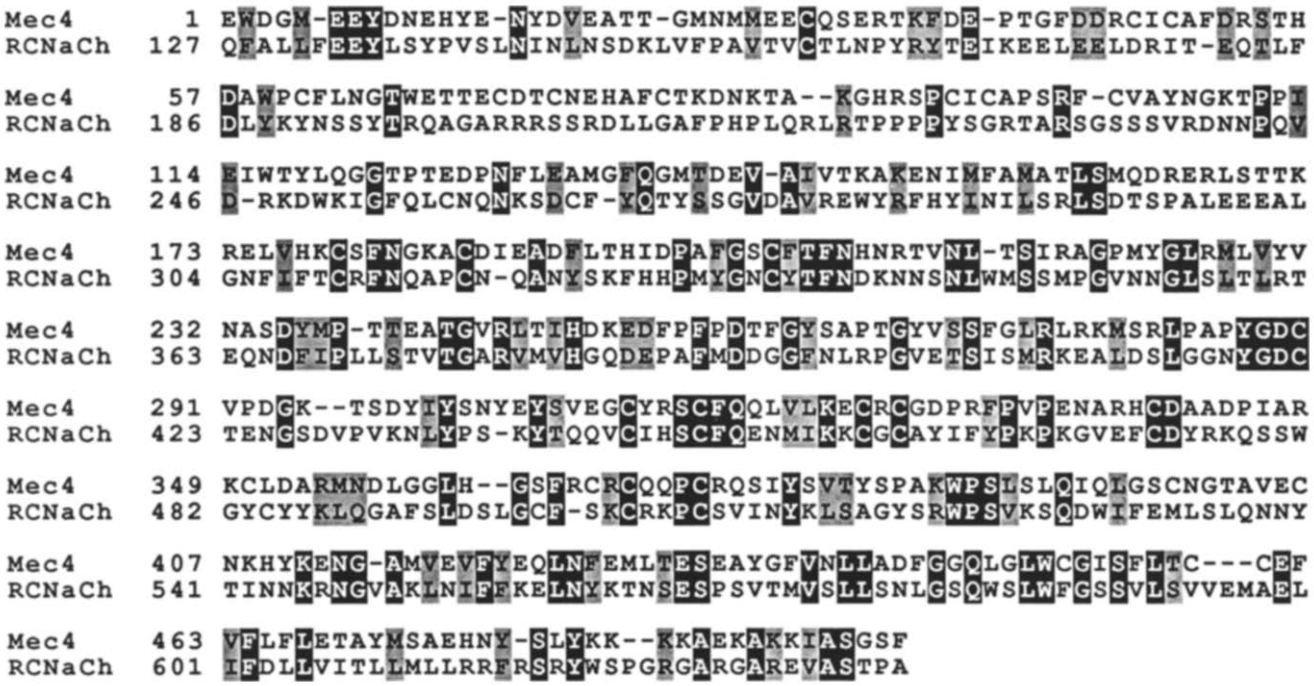

Fig. 5. Protein sequence homology between RCNaCh and the known sequence of Mec4 degenerin. Identical amino acids are labelled in black, and amino acids with similar side chains are in grey.

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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