Different Homologous Subunits of the Amiloride-sensitive Na⁺ Channel Are Differently Regulated by Aldosterone*

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Long term regulation of the amiloride-sensitive Na⁺ channel activity by steroid hormones occurs via de novo protein synthesis. The messenger level of RCNaCh1, previously shown by expression cloning to be a component of this channel, was measured in colon from rats fed with a low sodium diet. After 1 week of this diet, the channel activity was increased in an all-or-none fashion, whereas the level of RCNaCh1 remained constant. A cDNA coding for another subunit of the Na⁺ channel was obtained by polymerase chain reaction. The 650-amino acid protein, entitled RCNaCh2, is 58% homologous to RCNaCh1 and displays a similar structure. It had no intrinsic activity when expressed alone in Xenopus oocytes, but its co-expression with RCNaCh1 increased the channel activity 18 ± 5-fold. The increase in messenger level for RCNaCh2 during the time course of the diet is likely to explain the positive regulation of the rat colon Na⁺ channel by steroids. Immunocytochemical localization of the RCNaCh1 subunit revealed an apical labeling in colon from sodium-depleted rats. No labeling was observed in colon from control animals. These results suggest that oligomerization is needed for the proper expression of RCNaCh1 at the cell surface.

Amiloride-sensitive Na⁺ channels mediate the first step of active Na⁺ reabsorption in numerous epithelia, such as distal and collecting tubules of the kidney, distal colon, and salivary and sweat glands (1). The mineralocorticoid hormone aldosterone is the major regulator of Na⁺ reabsorption, acting via an increase of the apical Na⁺ permeability (2). The long term effect of the hormone is blocked by inhibitors of transcription and translation and is thus known to involve de novo RNA and protein synthesis (reviewed in Ref. 3). The rat colon Na⁺ channel has been cloned recently (4, 5). Expression of the cloned protein (699 amino acids) in Xenopus oocytes induces a Na⁺ current, which is inhibited by low concentrations of amiloride and analogs such as phenamil. The present paper reports the molecular cloning of a second channel subunit, with structural homology to the first one, and analyzes the expression of the two subunits during steroid treatment.

EXPERIMENTAL PROCEDURES

Short Circuit Current—Male Wistar rats were fed with a standard and/or a low sodium diet (ICN). The colon was removed and its most distal part mounted in an Ussing chamber. Both sides were perfused with a solution containing 120 mM NaCl, 5.9 mM KCl, 15.5 mM NaHCO₃, 1.2 mM Na₂HPO₄, 1.2 mM MgCl₂, 2.6 mM CaCl₂, 11.6 mM glucose, and 0.5% Triton X-100 (0.2%). After wash of 20 µm amiloride to the luminal side, the increase in messenger level for RCNaCh2 of the hormone is blocked by inhibitors of transcription and protein synthesis. The messenger level of RCNaCh1(5'-GGIARYTGYTWYACIYAAYSA-3') and an antisense primer matching amino acids 419-426 of RCNaCh1 (5'-CCRTYYTYWACRTCRTAAT-3') was detected with an EcoRI-KpnI fragment (614 bp). The 650-amino acid protein, entitled RCNaCh2, is 58% homologous to RCNaCh1 and displays a similar structure. It had no intrinsic activity when expressed alone in Xenopus oocytes, but its co-expression with RCNaCh1 increased the channel activity 18 ± 5-fold. The increase in messenger level for RCNaCh2 during the time course of the diet is likely to explain the positive regulation of the rat colon Na⁺ channel by steroids. Immunocytochemical localization of the RCNaCh1 subunit revealed an apical labeling in colon from sodium-depleted rats. No labeling was observed in colon from control animals. These results suggest that oligomerization is needed for the proper expression of RCNaCh1 at the cell surface.

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**fig. 1.** Nucleotide sequence of RCNaCh2 and deduced amino acid sequence. Transmembrane regions for RCNaCh2 are indicated by straight lines (20), and conserved residues between RCNaCh1 and RCNaCh2 are underlined. Five putative sites of N-glycosylation are observed at Asn21', Asn4', Asn22, Asn48, and Asn289. Putative sites for protein kinase C at Ser145, Ser146, Ser148, Ser149, Ser150, Thr151, and Thr152, for calmodulin kinase II at Ser145, Ser146, Thr147, Thr148, and Thr149, for CR2 protein kinase at Ser141, Ser142, Thr143, Thr144, and Thr145, for MAP kinase at Thr146, Thr147, Thr148, Thr149, and Thr150, and for protein kinase A are found at Ser140 and Thr149, for protein kinase C at Ser145, Ser146, Ser148, Ser149, Thr147, Thr148, and Thr149. For details, see text.

RESULTS AND DISCUSSION

The previously cloned Na+ channel subunit is homologous with three proteins identified in the nematode Caenorhabditis elegans, called Mec-4, Mec-10 and Deg-1 (4, 5, 8), which are all expressed in mechanosensory neurons and are involved in mechanosensitive function (9). Their expression in the same cell type has suggested that they might be associated within a multimeric structure to form an ion channel (8), as observed for ligand-gated ionic channels (10-13). Multimeric associations of homologous subunits have also been reported for other channel types (14, 15).

Sequence alignments between the cloned rat colon Na+ channel (5) and human lung Na+ channel (16), on the one hand, and Mec-4, Mec-10, and Deg-1 (4, 5, 8), on the other hand, have revealed several regions of significant identity. Two of them, corresponding to amino acids 331-338 (GNCYTFND) and amino acids 419-426 (YGDCTENG), were used to design degenerate oligonucleotides. PCR amplification of rat colon cDNA gave one band of 280 bp. Analysis by restriction enzyme digestion revealed that this PCR product actually contained two distinct products of identical size. The first product corresponded to the previously cloned subunit, and the second displayed 57% homology with the first. The screening of a rat colon cDNA library led to the isolation of a cDNA containing a 1950-nucleotide open reading frame. It corresponds to a protein of 650 amino acids, with a predicted molecular mass of 74 kDa (Fig. 1). The overall homology between the two rat colon proteins is 58%, and there is a structural conservation of both the two hydrophobic domains and the cysteine-rich region (Fig. 1). The terminology that will be subsequently used is as follows. The first clone will now be called RCNaCh1 and the new one RCNaCh2.

Fig. 2 shows the results obtained by co-expression of the two subunits in Xenopus oocytes. Injection of the RCNaCh2 alone did not increase ²²Na+ uptake (5 ng of RNA injected/oocyte), while a small but significant amiloride-sensitive uptake was measured after expression of RCNaCh1 alone, as previously described (5). A large potentiation of the amiloride-sensitive ²²Na+ uptake was observed when the two subunits were co-expressed (Fig. 2a). The stimulation factor was 18 _±_ 5 (mean _±_ S.D.; _n_ = 4). Similar results were observed by measuring the amiloride-sensitive Na+ current under voltage-clamped conditions (Fig. 2b). The current observed after expression of RCNaCh1 alone was 24 _±_ 6 nA, while the current observed after co-expression of the two subunits was 295 _±_ 38 nA. This intensity roughly corresponds to the signal obtained by injection of total messenger RNAs from a steroid-treated colon. Clearly, in
oocytes, RCNaCh2 potentiates the channel activity of RCNaCh1.

Fig. 3a shows the time dependence of expression of the two rat colon subunits during a low sodium diet, which raises plasma aldosterone concentration and thus increases Na⁺ channel activity (17). After a switch to a low sodium diet, there was a time-dependent increase of the amiloride-sensitive short circuit current, which reached a steady state after 2 weeks (Fig. 3a). A parallel rise in aldosterone levels was observed (not shown). Level of messenger RNA for RCNaCh1 was already high in the control and remained nearly constant (Fig. 3b). In 5 out of 11 independent experiments, an increase in RCNaCh1 RNA level was observed after steroid treatment. Because variations in RCNaCh1 RNA level existed between colons with similar short circuit currents, it is likely that other factors might also regulate its expression. The mRNA for RCNaCh2 was hardly detectable in control animals, and its level steadily increased as the amiloride-sensitive short circuit current became higher (Fig. 3b). The positive regulation of the rat colon Na⁺ channel activity by aldosterone can therefore be explained by the large increase in transcription of RCNaCh2.

An immunohistochemical localization of the RCNaCh1 protein was performed with polyclonal antibodies, in order to assess the effect of diet on the protein level (Fig. 4). These antibodies raised against peptides of RCNaCh2 have been characterized previously (7). Normal and sodium-depleted animals displayed a striking difference in protein expression. No signal was detected in colon from control rats (Fig. 4a), whereas an apical labeling was detected with sodium-depleted animals expressing high levels of Na⁺ channel activity (Fig. 4c and e). Similar results were obtained with two other polyclonal sera raised against two distinct epitopes of RCNaCh1 (not shown) and with anti-peptide antibodies purified by affinity chromatography on a peptide column (Fig. 4e). Moreover, the labeling was inhibited by a previous incubation with an excess of immunogenic peptide (Fig. 4f) and was absent after incubation with the preimmune serum (Fig. 4, b and d). Only the borders were labeled, and no labeling was observed in the crypts (Fig. 4, c and e). The increase in RCNaCh1 transcription hardly explained the differences between control and sodium-depleted colon. It is therefore likely that the expression of RCNaCh1 on the cell surface is dependent of the co-expression of other subunit(s), as previously demonstrated for the nicotinic receptor (18); in that case, β, γ, and δ subunits stabilize the α subunit, as measured by the binding of α-bungarotoxin (19). A similar model might be built for the Na⁺ channel, which would explain its regulation by aldosterone. In control animals, the RCNaCh1 subunit would be synthesized but would be rapidly degraded in the absence of RCNaCh2. When the animals are exposed to higher aldosterone concentrations, for instance after sodium depletion, at least one other component, RCNaCh2, would be expressed, and this expression would permit the processing of RCNaCh1 to the apical membrane. These two distinct regulatory mechanisms (transcription and stabilization of the subunits) would permit large variations of Na⁺ reabsorption to occur for Na⁺ homeostasis. The present article shows that, in colon, RCNaCh2 is one limiting factor for Na⁺ channel expression, whereas RCNaCh1 messenger seems to be constitutively expressed. Oligomerization of the different subunits appears to be an early event during the sorting of the channel complex, since RCNaCh1 is
not detected on the apical membrane when RCNaCh2 is not expressed.

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Note Added in Proof—Canessa et al. (21) have very recently cloned two new subunits for the Na⁺ channel called β and γ. Their γ subunit corresponds to our RCNaCh2 subunit. We have now observed that the expression of the β subunit is also induced by aldosterone.

REFERENCES

1. Garty, H., and Benos, D. J. (1988) Physiol. Rev. 68, 309-372
2. Crabbé, J. (1961) J. Clin. Invest. 40, 2103-2110
3. Rosier, B. C., and Palmer, L. G. (1992) The Kidney: Physiology and Pathophysiology (Seldin, D. W., and Giebisch, G., eds) 2nd Ed., pp. 1373-1409, Raven Press, Ltd., New York
4. Canessa, C. M., Horisberger, J. D., and Rosier, B. C. (1993) Nature 361, 477-480
5. Linguaglia, E., Voilley, N., Waldmann, R., Lazdunski, M., and Barbry, P. (1993) FEBS Lett. 318, 95-99
6. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467
7. Renard, S., Linguaglia, E., Voilley, N., Lazdunski, M., and Barbry, P. (1994) J. Biol. Chem. 269, 12981-12986
8. Chalfie, M. (1993) J. Neurobiol. 24, 1433-1441
9. Driscoll, M., and Chalfie, M. (1991) Nature 349, 588-593
10. Bets, H. (1990) Biochemistry 29, 3591-3599
11. Galry, J.-L., Revah, F., Besias, A., and Changeux, J.-P. (1991) Annu. Rev. Pharmacol. 31, 57-72
12. Karlin, A. (1993) Curr. Opin. Neurobiol. 3, 299-309
13. Wisden, W., and Seeburg, P. H. (1993) Curr. Opin. Neurobiol. 3, 291-298
14. Jan, Y. J., and Jan, Y. N. (1992) Cell 69, 715-718
15. Pongs, O. (1992) Physiol. Rev. 72, 969-986
16. Voilley, N., Linguaglia, E., Champigny, G., Mattéi, M.-G., Waldmann, R., Lazdunski, M., and Barbry, P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 247-251
17. Will, P. C., Lefebvre, J. L., and Hopfer, U. (1980) Am. J. Physiol. 238, F261-F266
18. Blount, P., and Merlie, J. P. (1988) J. Biol. Chem. 263, 1072-1080
19. Green, W. N., and Claudio, T. (1993) Cell 74, 57-69
20. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105-132
21. Canessa, C. M., Schild, L., Buell, G., Thorens, B., Gautachl, I., Horisberger, J. D., Rosier, B. C. (1994) Nature 367, 463-467