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A connexin-32 mutation associated with Charcot-Marie-Tooth disease does not affect channel formation in oocytes

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Abstract

Members of the connexin family differ most in their carboxy-termini, both with respect to sequence and length. In order to assess the contribution of this region to channel function, a series of carboxy-terminal deletion mutants were tested in the paired-oocyte expression system. Connexin-32 can be truncated by 64 amino acids without detectable loss of its known channel properties. Removal of additional amino acids results in a progressive loss of function over a stretch of 4 amino acids. In addition to this effect of length the charge of the carboxy-terminus appears to be another determinant of channel function. One of the fully functional deletion mutants, carrying a stop codon after amino acid-219, had been reported to be associated with Charcot-Marie-Tooth disease. The implications of this finding are discussed.

Key words: Connexin-32; Gap junction; Deletion mutants; Channel formation; Charcot-Marie-Tooth disease

1. Introduction

Cells in almost all organized tissues are connected by cell–cell channels which are contained in gap junction plaques. The protein subunits of cell–cell channels, connexins, have been characterized [1–3]. Connexins form a family of related proteins with pronounced tissue specificity and remarkable sequence conservation among different species. It is conceivable that cell–cell channels serve different physiological functions in different tissues. It is known that channels made from different connexins differ in their gating properties [4], their unit conductance [5] and possibly their permeability for different molecules [6].

The various connexins are similar in their extracellular and transmembranous segments but differ in their cytoplasmic portions in particular their carboxy-terminus. It is therefore tempting to look for connexin-specific features in these regions.

In a previous study we had found that connexin-32 can be trimmed by 58 carboxy-terminal amino acids without any apparent change of its known properties [7]. In the present study, we extend the deletion analysis to the remainder of the carboxy-terminus in order to determine the minimum requirement for the formation of functional channels. The questions addressed here have recently received acute interest because of the linkage of connexin-32 mutations with the Charcot-Marie-Tooth disease [8,9]. One of the mutant connexins, found in a family affected by this disorder, has a stop codon after amino acid-219. This particular mutant is part of the present study.

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2. Materials and methods

2.1. Source of connexin mRNA

Connexin-32 mRNA was transcribed in vitro from a cDNA clone [10], that was modified at the 5′-end and subcloned in pGEM-3Z (Promega Biotech). The modification involved the replacement of 31 nucleotides preceding the ATG codon with the sequence GATCC. This removed a silent additional initiation codon and provided a Kozak consensus sequence [11] for efficient translation [7]. Mutagenesis was performed by the Kunkel procedure [12]. All mutants were verified by sequence analysis. Prior to in vitro transcription from the sp6 promoter, the vector DNA was linearized with *Sspl*, located 649 base pairs downstream from the cDNA insertion site. Levels of mRNA were determined by agarose electrophoresis, and concentrations adjusted to yield equal concentrations for injection into oocytes.

2.2. Oocyte expression assay

Oocytes were prepared as described previously [4]. Briefly, they were injected with ~ 50 nl of connexin mRNA (2 μg/μl). 18–24 h later, the vitelline layer was removed with forceps, and the oocytes were then incubated for 20 min with 10 μg/ml soybean agglutinin. Subsequently, the oocytes were washed and paired with the vegetal poles facing each other. Junctional conductance between oocyte pairs was determined 2 h (unless stated otherwise) after pairing with the dual voltage clamp technique [5]. All experiments included uninjected oocyte pairs as negative controls as well as oocytes injected with wildtype connexin-32 as positive controls.

3. Results and discussion

Starting with connexin-32 mutant d225, which has a stop codon after amino acid-225 and still produces channels that with present methods are indistinguishable from wild-type channels [7], we introduced stop codons to give shortened connexins terminating with amino acids-222, -219, -218, -216 and -214. These mutants were tested in the paired-oocyte assay for their ability to form junctional conductance (Table 1).

Somewhat unexpectedly, no sharp decline of conductance was observed but instead a gradual decline in con-

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ductance occurred as the carboxy-terminal tail was shortened. Two additional deletion mutants, with stop codons after amino acids-222 and -219 (d222, d219), were found to be fully functional. Only in one experiment (Experiment 3 in Table 1) did mutant d219 exhibit a somewhat lower conductance than wildtype connexin-32. Mutant d218 yielded approximately 20% of wild-type conductance, and mutant d214 finally gave no conductance.

The shortest of the still fully functional deletion mutants, d219, was tested for its gating properties in response to cytoplasmic acidification and transjunctional voltage. Fig. 1 shows that mutant d219 is responsive to cytoplasmic acidification. As previously shown for wild-type connexin-32, mild acidification (30% CO_2) increased the junctional conductance of d219 channels and 100% CO_2 abolished conductance in a reversible manner. Likewise, the voltage dependence of mutant d219 was indistinguishable from that of wild-type connexin-32 (Fig. 1a). In the range of ± 60 mV transjunctional voltage no change in the steady-state transjunctional conductance was observed. However, like wildtype connexin-32, mutant d219 became clearly voltage-dependent upon mild cytoplasmic acidification (Fig. 1c).

There exists a large body of apparently contradictory literature on the voltage sensitivity of connexin-32, the main gap junction protein in liver and pancreas. No voltage dependence has been reported for gap junctions in the liver [13–15] or the pancreas [16], nor for connexin-32 expressed in oocytes [17,18]. On the other hand, voltage gating of connexin-32 has been observed both in transfected cells and oocytes [19,20]. Considering how close the two gating modes are together (see Fig. 1c and [7]), a slight variation in experimental conditions could easily account for these discrepancies.

For the evaluation of the ability of the various mutants to form functional cell–cell channels one has to consider that macroscopic conductance, as measured here, is determined by the number of channels, their unit conductance, and their probability to be in the open state. Each of these determinants could be affected by the deletion. At present, unit conductance and open probability are not accessible to experimental analysis in the oocyte system. Translatability of the various deletion mutants should not be affected by shifting the stop codon. In fact, in vitro translation in the presence of dog pancreas microsomes of mutant d219 was as efficient as that of wildtype connexin-32 producing a truncated protein with the expected mobility shift on PAGE (data not shown). The most likely consequence of the more extensive mutations is misfolding which could affect cytoplasmic transport of the protein, unit conductance of the channel, its open probability, or even docking.

A peculiar feature of the still active deletion mutants of connexin-32 is the presence of positively charged amino acids which are conserved at these positions in most connexins. To test the importance of these positive charges, a series of mutants was generated in which the positively charged amino acids were converted to neutral or negatively charged amino acids. The description of these mutants and the conductances obtained with them are summarized in Fig. 2. It appears that, in addition to length, charge is indeed an important factor in determining activity of these truncated connexins. For example, when in the fully functional deletions mutant d225 all five carboxy-terminal arginine residues were changed to the neutral amino acid asparagine, the conductance was reduced by 75%. This mutant, d225 5R-N, is gated by voltage and cytoplasmic acidification similar to wildtype connexin-32 and mutant d219 (Fig. 1b, and data not shown). Conversion of the same residues to the negatively charged glutamate abolished conductance completely. The same pattern is seen with the shorter connexins. Whether these charges determine the location of the carboxy-terminus in relation to the membrane, or whether they affect junctional conductance in any other way remains to be determined. Charge has been identified as a topogenic determinant for membrane proteins. The positive-inside rule, originally developed by von Heijne [21] and by Hartmann et al. [22], postulates that the orientation of the first transmembrane segment of a protein is determined by a net positive charge of the region of the protein close to the cytoplasmic side of the membrane. Subsequent transmembrane segments of a protein are thought to be determined by the orientation of the first such segment. Boyd and Beckwith [23] radically extended this rule to include other transmembrane segments.

Table 1
Junctional conductances of connexin-32 deletion mutants

Experiment	Control	wt cx32	d222	d219	d218	d216	d214
I	0	17.68 ± 3.29	18.47 ± 4.15	16.38 ± 3.15			
II	0	34.44 ± 2.86			4.59 ± 1.17	0.04 ± 0.02	
III	0	10.66 ± 0.86		4.53 ± 0.56	2.15 ± 0.64		
IV	0.39 ± 0.15	11.34 ± 0.83	11.10 ± 1.29	13.75 ± 3.99		0.92 ± 0.32	
V	0	4.29 ± 2.67					0

All conductances are expressed in μS ± S.E.M. Each value represents the mean of 9 oocyte pairs. Junctional conductance was determined 2 h after pairing with the exception of experiment II, where it was done after 3 h.

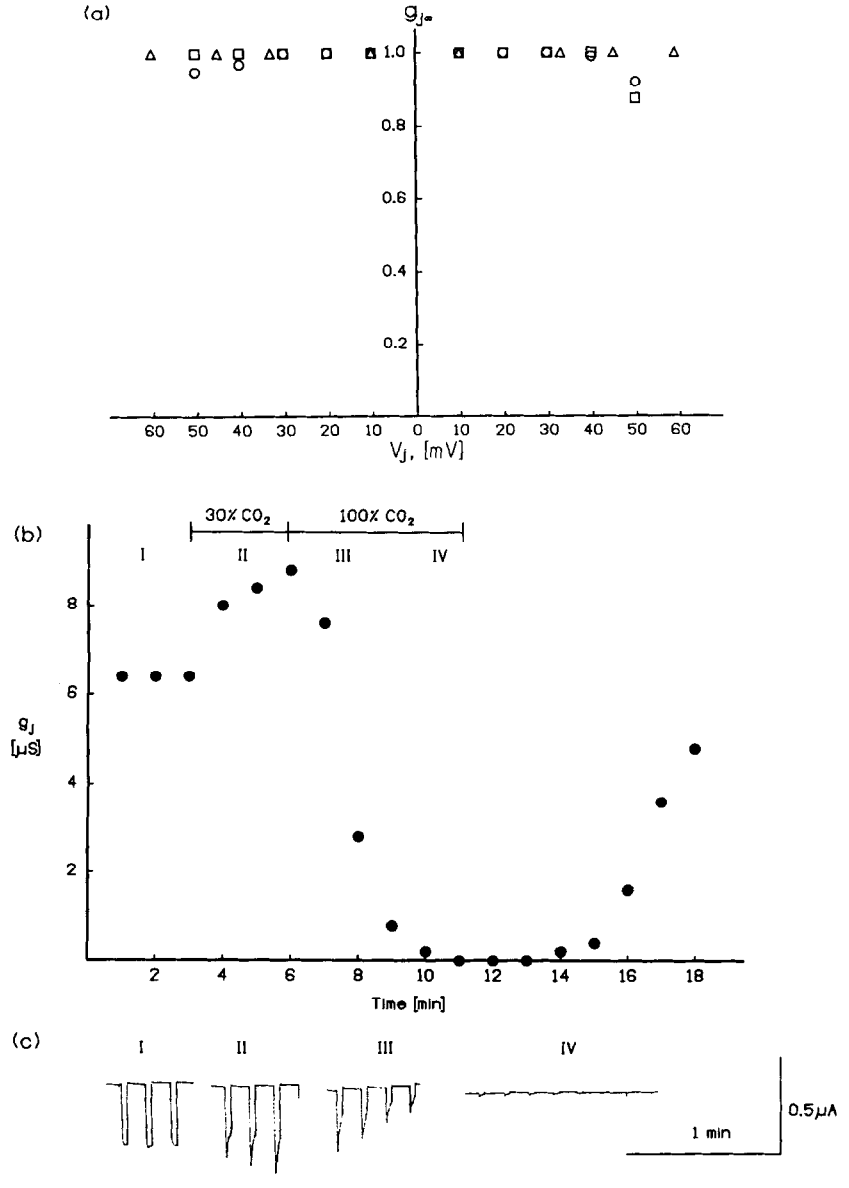

Fig. 1. (a) Effect of transjunctional voltage on cell-cell channels between paired oocytes expressing wild-type connexin-32 (○), mutant d219 (△), or mutant d225 5R-N (□). Junctional conductance, determined at the end of 2 s pulses, is plotted as a function of transjunctional voltage and is expressed as the fraction of current obtained with small transjunctional voltages. Both oocytes were held close to their membrane potential at −50 mV. Transjunctional voltage was established by applying hyperpolarizing or depolarizing voltage steps to one oocyte. (b) Effect of CO_2 on junctional conductance in oocyte pairs expressing mutant d219. Oocytes were continuously perfused with OR2. At the times indicated the OR2 medium was saturated with a 30% CO_2–70% air mixture or with 100% CO_2. Junctional conductance was determined as instantaneous g_j with 50 mV pulses. (c) Samples of junctional currents obtained with 50 mV steps and recorded at the times I–IV indicated in Fig. 1b. Initially the junctional currents do not inactivate with time, but they do in response to incubation with 30% CO_2, indicating the appearance of voltage gating.

4. Conclusion

The present study shows that connexin-32 can be severely truncated without losing channel-forming ability. Only when the stop codon is placed close to the membrane–cytoplasm boundary is channel activity affected. The loss of activity is gradual and occurs over a stretch of five amino acids. In addition to length, positively charged amino acids are an important factor, possibly required for proper folding of this transmembrane protein. For example, partitioning of the carboxy-terminal segment of connexin-32 between cytoplasm and membrane may be affected, although alternate effects have to be considered.

One of the deletion mutations, d219, that was found to be fully functional in this study, has recently been found in a family affected by the Charcot-Marie-Tooth disease [9]. Charcot-Marie-Tooth disease comes in three forms with different chromosomal locations. One of the forms is X-linked and maps near the chromosomal loca-

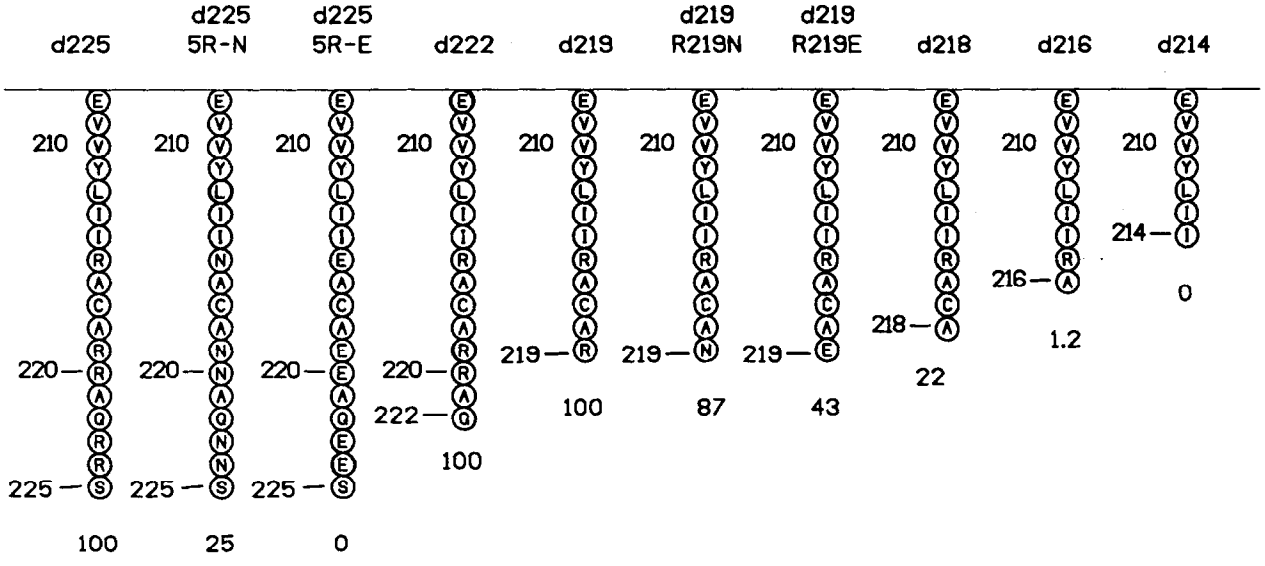

Fig. 2. Summary of all mutants. The relative junctional conductances are indicated below each mutant (expressed as % of wild-type connexin-32).

tion of the connexin-32 gene. Furthermore, families of this group have been reported to carry connexin-32 mutations [8,9]. Several of these mutations are in regions of the protein where previous studies have shown that such mutation abolishes function, while others are in regions where one would expect functional consequences [24,25]. It is, therefore, surprising that the d219 mutation is without functional consequence as detectable by the oocyte assay. This applies not only to the macroscopic conductance obtained with this mutant in oocyte pairs but also to the gating properties. Within the resolution of the assays used, this mutant is gated by cytoplasmic acidification and by voltage in a way indistinguishable from wild-type connexin-32.

This result could mean that there is an important function associated with the carboxy-terminus of connexin-32 that is neither related to basic channel formation nor to known gating mechanisms. This function may not become apparent in the oocyte assay but may be required in Schwann cells, the cells reported to be affected by the disease. On the other hand, the apparent discrepancy between the presumably nonfunctional connexin-32 mutant in humans and the full functioning of the same mutant in oocytes could be explained by a difference in folding. For example, the protein in the oocyte is assayed at a temperature that is much lower than the one prevailing in a human cell. In this context, the d119 mutation could be regarded as a temperature sensitive mutation, with 20°C being the permissive temperature. A difference in folding could also be the result of a difference in chaperone proteins or any other factor affecting the function of the protein. Alternatively, the d219 mutation found in a family with Charcot-Marie-Tooth disease may not be the cause of the disease. For example, the disease-causing defect could be in a different location of the gene, e.g. the promoter, and the stop codon after position 219 could be coincidental. These possibilities need to be further investigated.

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