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Analysis of CMTX Mutants Using Connexin Membrane Channels

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Mutations in the human connexin 32 (Cx32) gene are responsible for X-linked Charcot-Marie-Tooth (CMTX) disease. Although over 300 different mutations have been identified the detailed molecular etiology of CMTX disease is poorly understood. Several studies reported that connexin membrane channels share most biophysical properties with their parental gap junction channels. In this study, two connexin mutant membrane channels (one mutant channel called the M34T channel in which the methionine residue at the 34th position of the Cx32 protein is replaced with threonine residue and the other mutant channel called the T86C channel in which the threonine residue at the 86th position is replaced with cysteine residue) associated with CMTX mutations were characterized at the single-channel level instead of using mutant gap junction channels. The biophysical properties of the M34T channel were very similar to those of the gap junction channel formed by M34T mutation. In addition, the mutant membrane channel study revealed the reversal of the gating polarity, the loss of fast gating and the gain of slow gating. The T86C channel also behaves like its parental wild type Cx32 membrane channel. Taken together, these results suggest that a study using connexin membrane channels is useful to characterize CMTX mutants.

Key words : X-linked Charcot-Marie-Tooth disease, gap junction channel, connexin membrane channel

Introduction

X-linked Charcot-Marie-Tooth (CMTX) disease is one type of a heterogeneous group of inherited peripheral neuropathies called Charcot-Marie-Tooth (CMT) disease [7]. The symptoms of CMTX disease manifested by the demyelination of peripheral nerves include loss or progressive weakness of sensation and atrophy of distal muscles. Following a study reporting that CMTX resulted from mutations in the human connexin 32 gene [1], over 300 different mutations have been identified. Most of them are mapped to the coding regions of connexin 32, and some are mapped to the promoter regions of that gene [15]. Although there are few disease models that include pre-matured or lack of connexin 32 protein, abnormal targeting to the cellular membrane, modification in permeability and reduction in open probability of gap junction channels formed by connexin 32 mutant protein [1,9,13] have been postulated, the detailed molecular etiology of CMTX disease is still poorly understood.

Connexin (Cx) is a subunit of the gap junction channel that is a conduit between two apposed cells and thus allows the direct transmission of small molecules, including cellular ions, second messengers, and small metabolites less than 1 kDa [5]. The gap junction channel is formed by two connexons (also called hemichannel or membrane channel), each of which is formed by six connexin subunits. Although the biophysical properties of gap junction channels have been extensively studied, most experiments employing gap junction channels have common technical problems that make it hard to obtain single channel recordings. It is also not feasible to access the intracellular surface of the channel, so it is difficult to perform fast kinetic studies. Two sets of equipment are also required and thus the time and budget needed are doubled. Few connexins such as Cx32, Cx38, Cx46 are known to form functional membrane channels in unapposed cell membranes [3,4,8,12,17]. Studies using connexin membrane channels at the single channel level reported that these channels share most biophysical properties with their parental gap junction channels [8,17,18].

In this study, we chose two CMTX mutants, M34T (the methionine residue at the 34th position of the Cx32 protein is replaced with threonine residue) and T86C (the threonine residue at the 86th position is replaced with cysteine). The connexin membrane channels harboring these mutations were characterized at the single channel level. The

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M34T mutant membrane channel (M34T channel) was used to prove that the biophysical properties of this membrane channel are similar to those of the mutant gap junction channel. A cysteine-substituted CMTX mutant membrane channel (T86C channel) was tested to find out whether the disulfide bonds (cysteine-cysteine) at this position perturb the formation of functional membrane channel.

Materials and Methods

Construction of mutant channels, cRNA synthesis, and oocyte injection

The Cx32 chimera (also called the Cx32*43E1 channel in which the first extracellular loop of Cx32 has been replaced with that of Cx43) was used as a parental template [8]. Threonine (T) and cysteine (C) were introduced at the 34^{th} and the 86^{th} positions of the Cx32 chimeric channel, respectively, by using PCR-based site-directed mutagenesis. Complementary RNA (cRNA) was synthesized from a linearized plasmid template using the 'mMESSAGE mMACHINE T7 kit' (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Approximately 50 nl of 1 ng/nl RNA was co-injected into the *Xenopus* oocyte with 0.3 pmol/nl of an antisense phosphorotioate oligonucleotide complimentary to endogenous *Xenopus* Cx38 to knock down its expression [14]. After the RNA injection, oocytes were kept in a bath solution, containing 88 mM NaCl, 1 mM KCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 0.1% glucose, and 2.5 mM pyruvate, pH 7.6.

Electrophysiological recordings and analysis

Before performing path-clamp experiments, the vitelline membranes of oocytes were removed in a hypertonic solution containing 220 mM Na aspartate, 10 mM KCl, 2 mM $MgCl_2$, and 10 mM HEPES, pH 7.6. In all patch-clamp experiments, the pipette solution was the same as the bath solution described above, except $CaCl_2$ and $MgCl_2$ were replaced with 2 mM EDTA and 2 mM EGTA. Single channel data were acquired using pClamp 7.0 software, an Axopatch 200B integrating patch amplifier, and a Digidata 1200A interface (Molecular Devices, Sunnyvale, CA, USA). Data were acquired at 5 kHz and filtered at 1 kHz with a four-pole low-pass Bessel filter. Microcal Origin (version 6.0, Mocrocal Software, Inc., Northampton, MA, USA) and CorelDRAW (version 9.0, Corel Corporation, Ottawa, Ontario, Canada) were used for data illustration.

Results and Discussion

M34T mutant membrane channel

The single channel activity of the Cx32M34T mutant membrane channel (M34T channel) at a negative potential (-40 mV) is shown in Fig. 1. The mutant channel gates to both open and fully closed states. The fully closed state is defined as the physical conformation of a membrane channel at which there is no conductance. The transition from open to closed states is characterized by a slow event called slow gating [8,17]. Both the Cx32 gap junction channel and the Cx32 membrane channel (the parental membrane channel of the M34T channel) gate to a fully closed state at negative potentials. In addition, those channels gate to different subconductance states, the channel conformation at which there are residual amounts of current flow. This state is also characterized by a fast transition (called fast gating) from the open state. As seen in Fig. 1, the M34T channel shows only slow gating at a negative potential. It is known that all Cx32-derived channels, including the Cx32 gap junction channel and the Cx32 membrane channel, have both fast and slow gating at negative potentials. The loss of fast gating in the M34T channel at negative potentials could be due to the effect of mutation. However, this effect was not observed in the Cx32M34T

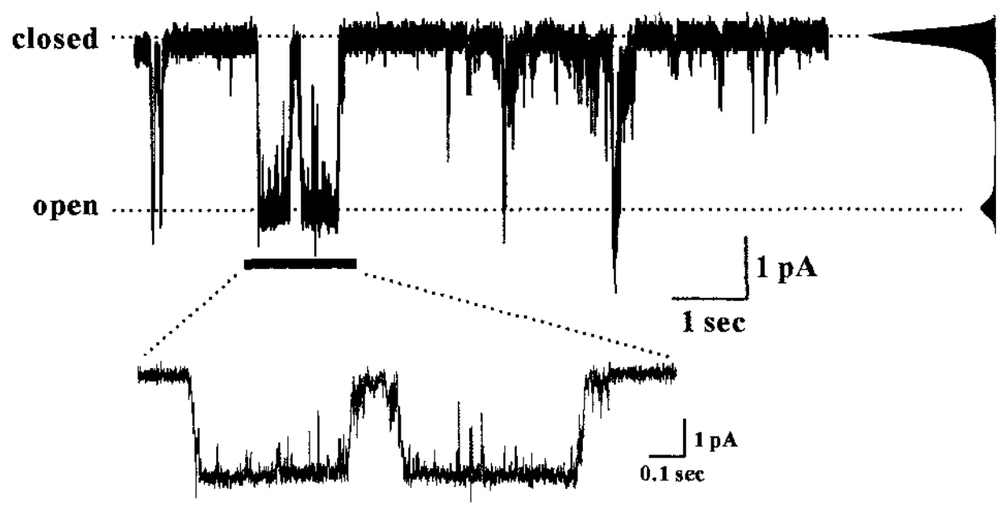

Fig. 1 Voltage dependence of gatings obtained from the Cx32M34T membrane channel. (*top panel*) A representative single channel record at -40 mV holding potential in cell-attached patch mode is shown. An amplitude histogram of this trace is shown on the right side of the record. The open and closed states are indicated by dotted lines, respectively. Most of the time, the channel is in a fully closed state. The open channel probability of this particular channel is ~0.11. Any fast gating event characterized by the transition from open to subconductance states was not observed at this negative potential. (*bottom panel*) A segment of the record (solid bar) is expanded to show the slow gating events of the M34T channel.

mutant gap junction channel [9]. It could be explained by the fact that the gating events of the Cx32M34T mutant gap junction channel, whether fast or slow, were too brief to be analyzed carefully. In fact, it has been reported that the Cx32M34T mutant associated with CMTX disease forms a functional gap junction channel with a reduction of open channel probability (~0.02 at -80 mV) [9]. Similar reduced open probability was observed in the M34T channel. The open channel probability of this particular channel was ~0.11 at -40 mV. There are two possible reasons for the increased probability (~0.02 to ~0.11). One reason is the difference of holding potentials (-80 mV versus -40 mV). In general, the gating of both the gap junction channel and the connexin membrane channel are very voltage-dependent, so the channels tend to close at higher potentials. The other reason is the difference in the number of hemichannels (connexin membrane channel) involved in the channel closure. Because the open probabilies of each of the two hemichannels contribute to the closure of a gap junction channel, the open channel probability of a gap junction channel is somewhat smaller than that of a connexin membrane channel. Our previous study showed that the open channel probability of its parental wild type Cx32 membrane channel was ~0.57 to ~0.90 at -80 mV [8]. Therefore, it is certain that the reduced open channel probability of the M34T channel resulted from the mutation at the 34th amino acid position.

The changes in the single channel activity of the M34T channel is more apparent at positive potential. Fig. 2 shows that the M34T channel gates to both subconductance and fully closed states. As mentioned earlier, all Cx32-derived channels including the Cx32 gap junction channel and the Cx32 membrane channel, show fast gating only at negative potentials. At any positive potentials, those channels always reside in an open state. Therefore, the fast gating of the M34T channel at positive potential (+90 mV in Fig. 2) was not expected. This reversal of the gating polarity was observed when the amino acid residues at the amino terminus of Cx32 were replaced by those with negative charges [8,10,11,18]. Although a reason for this result cannot be provided without further experimentation, it is probable that the nonpolar methionine residue embedded in the cell membrane is exposed to the aqueous environment of the channel pore by the replacement of the polar threonine residue, and thus this exposure results in the conformational changes of the channel susceptible to the opposite gating

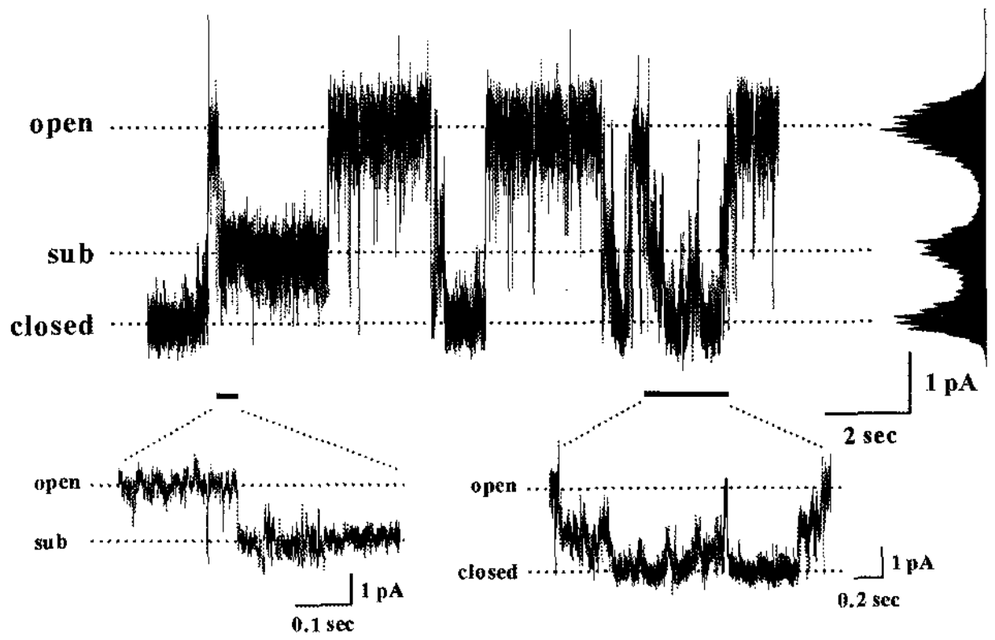

Fig. 2 Voltage dependence of gatings obtained from the Cx32M34T membrane channel. (*top panel*) A representative single channel record at +90 mV holding potential in cell-attached patch mode is shown. An amplitude histogram of this trace is shown on the right side of the record. Three distinct open, subconductance (sub), and closed states are indicated by dotted lines, respectively. (*bottom panels*) Two segments of the record (solid bars) are expanded to show the fast and slow gating events of the M34T channel, respectively. The appearance of the fast and slow gating at positive potential indicates the effect of M34T mutation. The measured conductance of the transition from open to subconductance states is ~22 pS while the transition from open to fully closed states is ~34 pS.

polarity. In addition to the gating polarity reversal, the residence of the M34T channel at a positive potential is different from that of its parental wild type Cx32 membrane channel. At positive potentials, the wild type channel resides at the open state with the open channel probability of ~1.00 [8]. However, the M34T channel gates to both subconductance and fully closed states with a probability of ~0.41. Although fast gating appeared, the slow gating of the M34T channel predominately occurred at the positive potential.

T86C mutant membrane channel

Three point mutations at this position (T86A, T86S and T86N) associated with the disease have been identified [2,16]. To investigate how this position is susceptible to the disease, the threonine residue was replaced with cysteine residue. The cysteine substitution was intended to show the use of the substituted-cysteine accessibility method (SCAM) [6] in future experiment. The SCAM is a useful technique to probe the pore-lining domain of the ion channel although there is a risk that the cysteine-cysteine interaction due to the formation of disulfide bond may perturb

the formation of a functional channel. The single channel activities of the Cx32T86C mutant membrane channel (T86C channel) are shown in Fig. 3. It is likely that the substitution of cyteine residue at the 86th position does not perturb the channel formation. We previously reported that the cysteine substitution at the 8th position (T8C) in the amino terminus of the Cx32 membrane channel induced the flickering event of the T8C mutant membrane channel [18]. However, the flickering event did not occurred in the T86C channel. It appears that all gatings are slow at both positive and negative potentials. There is no fast gating event at negative potential (Fig. 3B). The loss of fast gating of the T86C channel is similar to that of the M34T channel. The open probability of the T86C channel at positive potential (+50 mV) is ~0.53 while that of the channel at negative potential (-50 mV) is ~0.17. These probabilities are very low compared with the open probaility (~1.0) of the parental wild type Cx32 membrane channel at the same holding potentials [8]. The unitary conductance of the T86C channel at -50 mV is ~70 pS (Fig. 3B) while that of the channel at +50 mV is ~36 pS (Fig. 3A). This current rectification of the T86C channel is evident when applying voltage ramps to obtain current-voltage (I-V) relation.

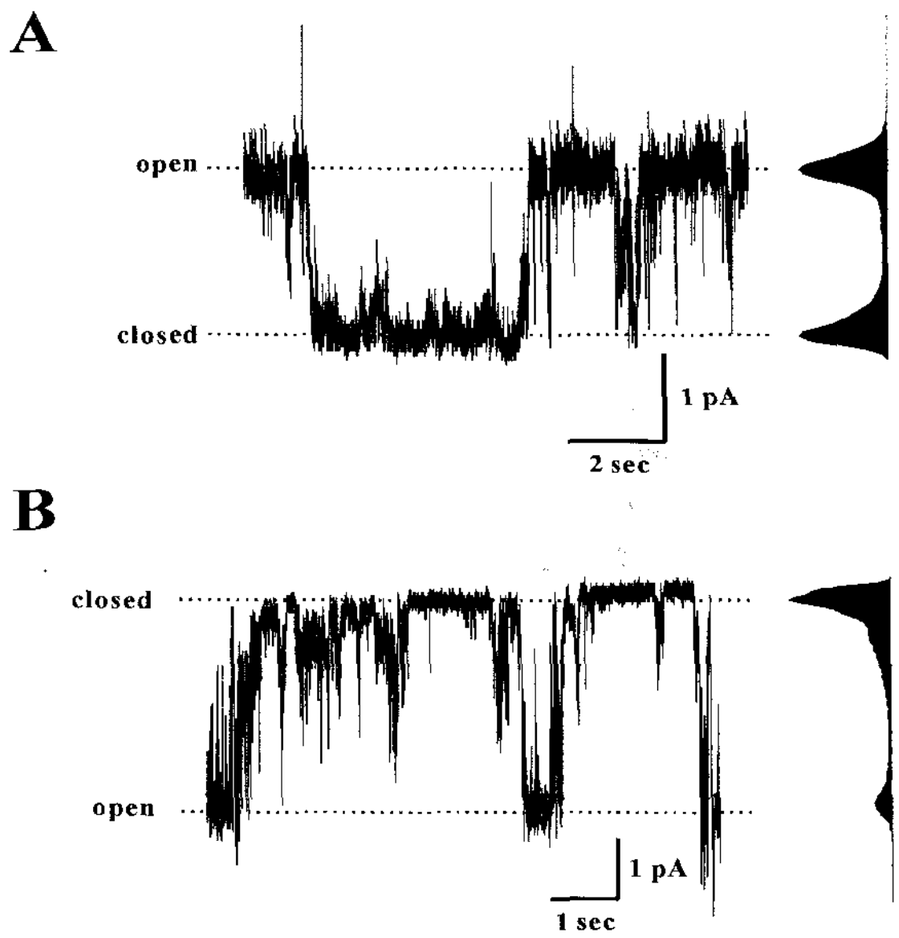

Fig. 3. Representative single channel recordings obtained from the Cx32T86C membrane channel. (A) A cell-attached record obtained by the application of +50 mV holding potential is shown. An amplitude histogram of this trace is shown on the right side of the record. The open and closed states are indicated by dotted lines. The open channel probability and the unitary conductance of this particular channel are ~0.53 and ~36 pS, respectively. (B) A cell-attached record at -50 mV holding potential is shown. An amplitude histogram of this trace is shown on the right side of the record. No fast gating at negative potential was observed. The open channel probability and the unitary conductance of this T86C channel are ~0.17 and ~70 pS, respectively.

The I-V relation the of T86C channel is presented in Fig. 4. The ionic current of the T86C channel is inwardly rectified. The amounts of current passing through the open T86C channel at negative potentials are twice as high as those at positive potentials. This two-fold current rectification is also observed in the wild type Cx32 membrane channel. Although it is not feasible to obtain the unitary conductance of the T86C channel to precisely compare it with that of the wild type Cx32 membrane channel, the similar degree of rectification in both channels indicates that T86C mutation does not interfere with the overall channel formation.

In conclusion, we chose two CMTX mutants to test whether the approach of using connexin membrane channels harboring these mutations was useful for characterizing the mutants instead of using mutant gap junction channels. The biophysical properties of the M34T mutant channel were very similar to those of the gap junction channel formed by M34T mutation. These include the reduced open channel probability and the slow gating at negative potential. In addition, new facts about the M34T channel were revealed: the loss of fast gating at negative

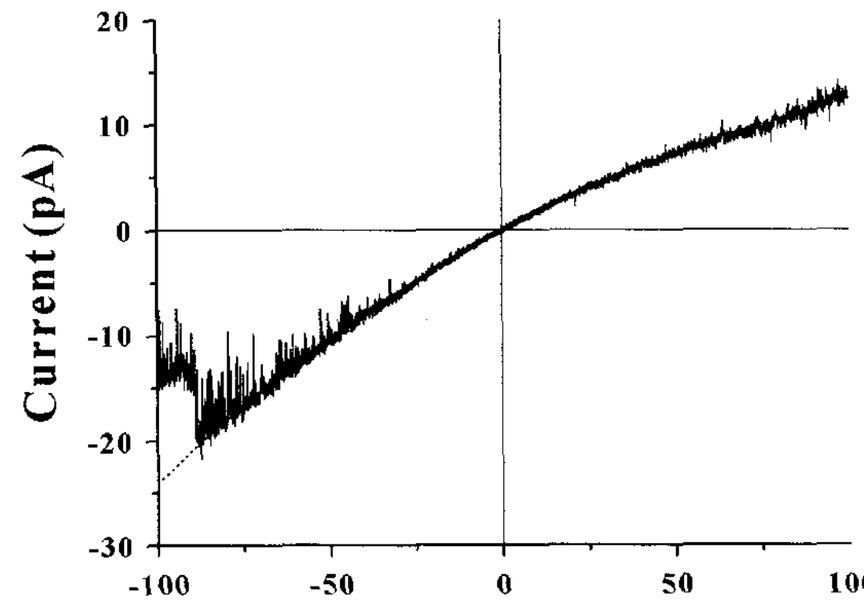

Fig. 4. Current-Voltage relation of the Cx32T86C membrane channel. The current trace was obtained by the application of voltage ramp (from -100 to +100 mV). There are two T86C channels in this particular cell-attached patch. The current flux through two open channels is inwardly rectified. The dotted line is the exponential curve fit to the current trace.

potential and the gain of fast gating (the reversal of the gating polarity) and slow gating at positive potentials. These findings could be obtained by employing the mutant membrane channel rather than using the gap junction channel. Therefore, the findings of this study strongly suggest that researchers performing membrane channel experiments can overcome the technical barriers imposed by using the gap junction channels. The reduced open probability and the polarity reversal of the M34T channel can be explained by the conformational change of the wild type channel caused by the mutation. In contrast to the M34T channel, the conformational change in the T86C channel is not the main effect caused by the mutation because the shape and the degree of current rectification of the T86C channel are not changed. Both M34T and T86C mutations are located in the 1st and the 2nd transmembrane domain, respectively. It has been postulated that both the 1st and the 2nd transmembrane domains contribute to the permeation pathway of the Cx32 channel [9]. Therefore, it will be useful to test whether these positions are accessible by using SCAM. Taken together, the results of this study suggest that the changes in biophysical properties of the CMTX mutants, including reduced open channel probability, reversed gating polarity, and modification of slow and fast gating, resulted from the mutations and that a new experimental paradigm using the connexin membrane channel instead of the gap junction channel is very useful to characterize the CMTX mutants.

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References

1. Bergoffen, J., S. S. Scherer, S. Wang, M. O. Scott, L. J. Bone, D. L. Paul, K. Chen, M. W. Lensch, P. F. Chance and K. H. Fischbeck. 1993. Connexin mutations in X-linked Charcot-Marie-Tooth disease. *Science* **262**, 2039-2042.
2. Bone, L. J., S. M. Deschênes, R. J. Balice-Gordon, K. H. Fischbeck and S. S. Scherer. 1997. Connexin32 and X-linked Charcot-Marie-Tooth disease. *Neurobiol. Dis.* **4**, 221-230.
3. Cheon, M. and S. Oh. 2007. Single channel analysis of Xenopus connexin 38 hemichannel. *J. Life Sci.* **17**, 1517-1522.
4. Ebihara, L. 1996. *Xenopus* connexin38 forms hemi-gap-junctional channels in the nonjunctional plasma membrane of *Xenopus* oocytes. *Biophys. J.* **71**, 742-748.
5. Harris, A. L. 2001. Emerging issues of connexin channels: Biophysics fills the gap. *Q. Rev. Biophys.* **34**, 325-472.
6. Karlin, A. and M. H. Akabas. 1998. Substituted-cysteine accessibility method. *Methods Enzymol.* **293**, 123-145.
7. Keller, M. P. and P. F. Chance. 1999. Inherited peripheral neuropathy. *Semin. Neurol.* **19**, 353-362.
8. Oh, S., C. K. Abrams, V. K. Verselis and T. A. Bargiello. 2000. Stoichiometry of transjunctional voltage-gating polarity reversal by a negative charge substitution in the amino terminus of a connexin32 chimera. *J. Gen. Physiol.* **116**, 13-31.
9. Oh, S., Y. Ri, M. V. Bennett, E. B. Trexler, V. K. Verselis and T. A. Bargiello. 1997. Changes in permeability caused by connexin 32 mutations underlie X-linked Charcot-Marie-Tooth disease. *Neuron* **19**, 927-938.
10. Oh, S., S. Rivkin, Q. Tang, V. K. Verselis and T. A. Bargiello. 2004. Determinants of gating polarity of a connexin 32 hemichannel. *Biophys. J.* **87**, 912-928.
11. Oh, S., J. B. Rubin, M. V. L. Bennett, V. K. Verselis and T. A. Bargiello. 1999. Molecular determinants of electrical rectification of single channel conductance in gap junctions formed by connexin 26 and 32. *J. Gen. Physiol.* **114**, 339-364.
12. Pfahnl, A., X. W. Zhou, R. Werner and G. Dahl. 1997. A chimeric connexin forming gap junction hemichannels. *Pflugers Arch.* **433**, 773-779.
13. Ressot, C., P. Latour, F. Blanquet-Grossard, F. Sturtz, S. Duthel, J. Battin, E. Corbillon, E. Ollagnon, F. Serville, A. Vandenberghe, A. Dautigny and D. Pham-Dinh. 1996. X-linked dominant Charcot-Marie-Tooth neuropathy (CMTX): new mutations in the connexin32 gene. *Hum. Genet.* **98**, 172-175.
14. Rubin, J. B., V. K. Verselis, M. V. Benett and T. A. Bargiello. 1992. A domain substitution procedure and its use to analyze voltage dependence of homotypic gap junctions formed by connexins 26 and 32. *Proc. Natl. Acad. Sci. USA* **89**, 3820-3824.
15. Shy, M. E. 2004. Charcot-Marie-Tooth disease: an update. *Curr. Opin. Neurol.* **17**, 579-585.
16. Sorour, E. and M. Upadhyaya. 1998. Mutation analysis in Charcot- Marie-Tooth disease type 1 (CMT1). *Hum. Mutat.* **Suppl. 1**, S242-S247.
17. Trexler, E. B., M. V. Bennett, T. A. Bargiello and V. K. Verselis. 1996. Voltage gating and permeation in a gap junction hemichannel. *Proc. Natl. Acad. Sci. USA* **93**, 5836-5841.
18. Yim, J., M. Cheon, J. Jung and S. Oh. 2006. Effect of amino terminus of gap junction hemichannel on its channel gating. *J. Life Sci.* **16**, 37-43.

초록 : 커넥신 세포막채널을 이용한 씨엠티엑스 돌연변이체의 분석

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커넥신(connexin) 32 유전자의 돌연변이가 씨엠티엑스(CMTX, X-linked Charcot-Marie-Tooth) 질환과 관련이 있다. 현재까지 300여개 이상의 돌연변이가 보고가 되었으나 이 질환에 대한 상세한 분자병리학적 원인을 거의 알려져 있지 않고 있다. 여러 연구를 통해서 커넥신 세포막채널이 간극결합채널이 갖고 있는 대부분의 생물리학적 특성을 갖고 있는 것으로 판명되었다. 이번 연구에서는 씨엠티엑스 질환과 관련된 두 개의 돌연변이체를 선정하여 간극결합채널 대신 돌연변이체로 구성된 커넥신 세포막채널을 이용하여 단일채널수준에서 이들 돌연변이체의 특성을 조사하였다. M34T 돌연변이 세포막채널의 생물리학적 특성은 이들로 구성된 돌연변이 간극결합채널의 특성과 거의 유사하였다. 더욱이, 돌연변이 세포막채널을 이용한 연구를 통해서 간극결합채널을 이용한 연구에서는 밝혀지지 않았던 개폐극성의 역전, 빠른 개폐의 소실과 느린 개폐의 생성과 같은 새로운 사실을 알게 되었다. T86C 돌연변이 세포막채널 또한 이의 모체가 되는 커넥신 32 세포막채널과 유사한 특성을 갖고 있음을 알게 되었다. 이상의 결과를 통해서 커넥신 세포막을 이용한 연구가 씨엠티엑스 질환의 돌연변이체를 연구하는데 매우 유용할 것으로 생각된다.