Effect of Amino Terminus of Gap Junction Hemichannel on Its Channel Gating

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Gap junction is an ion channel forming between adjacent cells. It also acts as a membrane channel like sodium or potassium channels in a single cell. The amino acid residues up to the 10th position in the amino (N)-terminus of gap junction hemichannel affect gating polarity as well as current-voltage (I-V) relation. While wild-type Cx32 channel shows negative gating polarity and inwardly rectifying I-V relation, T8D channel in which threonine residue at 8th position is replaced with negatively charged aspartate residue shows reverse gating polarity and linear I-V relation. It is still unclear whether these changes are resulted from the charge effect or the conformational change of the N-terminus. To clarify this issue, we made a mutant channel harboring cysteine residue at the 8th position (T8C) and characterized its biophysical properties using substituted-cysteine accessibility method (SCAM). T8C channel shows negative gating polarity and inwardly rectifying I-V relation as wild-type channel does. This result indicates that the substitution of cysteine residue dose not perturb the original conformation of wild-type channel. To elucidate the charge effect two types of methaenthiosulfonate (MTS) reagents (negatively charged $MTSES^-$ and positively charged $MTSET^+$) were used. When $MTSES^-$ was applied, T8C channel behaved as T8D channel, showing positive gating polarity and linear I-V relation. This result indicates that the addition of a negative charge changes the biophysical properties of T8C channel. However, positively charged $MTSET^+$ maintained the main features of T8C channel as expected. It is likely that the addition of a charge by small MTS reagents does not distort the conformation of the N-terminus. Therefore, the opposite effects of $MTSES^-$ and $MTSET^+$ on T8C channel suggest that the addition of a charge itself rather than the conformational change of the N-terminus changes gating polarity and I-V relation. Furthermore, the accessibility of MTS reagents to amino acid residues at the 8th position supports the idea that the N-terminus of gap junction channel forms or lies in the aqueous pore.

Key words – Gap junction, connexin, hemichannel, gating polarity, charge effect

Gap junction (intercellular channel or cell-to-cell channel) is the membrane structure facilitating the transport of several cellular ions, second messengers, and small metabolites between two apposed cells. The head to head union of two hemichannels (connexons) in series, each of which is composed of six connexin (Cx) subunits, forms a complete gap junction channel. Gap junction channels can be modulated by various stimuli, which include pH, calcium and voltage. Among them, the biophysical properties of gap junction channels modulated by voltage (transjunctional voltage in intercellular channel and membrane potential in hemichannel) are well characterized. Since Cx46 has been cloned and characterized[1,2], studies using hemichannels have been well adapted to characterize the biophysical properties of gap junction channels. The hemichannels share most biophysical properties with their parental (intercellular) channels[3-5]. Experiments using hemichannels over intercellular channels are more useful to obtain single channel recordings, exchange solutions, and observe fast perfusion kinetics.

Single channel studies of both intercellular channels and hemichannels have established that the amino (N)-termini of gap junction subunits provide at least voltage sensor and gate. While Cx26 channel closes at positive potentials, Cx32 channel gates at negative potentials. The reciprocal substitutions or domain swaps between amino acid residues in each N-termini of Cx26 and Cx32 channels change the biophysical properties of their parental channels. For example, the substitution of neutral asparagine (N) residue at the 2nd position of Cx32 with negatively charged glutamate (E) leads parental Cx32 channel to behave as Cx26 channel[6-8]. This Cx32N2E mutant channel now gates at positive potentials as Cx26 channel does. Furthermore, this charge substitution is effective up to the 10th position of amino acid residue in the N-terminus of hemichannel[8,9]. These results have lead us to propose that the first 10 amino acid residues of Cx32 at least lie in the aqueous pore and respond to changes in the voltage field. This voltage sensor and gate model is further supported by a recent re-

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port[10] using one dimensional NMR that the solution structure of an N-terminal Cx26 formed a vestibule at the cytoplasmic entrance of the channel by an open turn around the conserved glycine residue at 12th position.

Although several reports provide that the charge substitutions in the N-terminus of channel affect the gating polarity as well as I-V relation, these charge effects can be also resulted from the conformational change (s) of gap junction channel. In this work, we changed one amino acid residue at the 8th position in the N-terminus of Cx32 channel to clarify this issue. It has been known that this position is susceptible to both positive and negative charge substitutions[8,9]. To monitor the charge effect on the biophysical properties of Cx32 channel, we employed substituted-cysteine accessibility method (SCAM)[11]. In this study, methanethiosulfonate (MTS) reagents were used as common reagents and various information regarding the biophysical properties such as gatings and ionic fluxes could be obtained by applying positively charged $MTSET^+$ and negatively charged $MTSES^-$.

Materials and Methods

Construction of mutant channels, cRNA synthesis, and oocytes injection

Cx32*43E1 channel in which the first extracellular loop of Cx32 (E1) has been replaced with that of Cx43 was used as a starting material[7]. Either aspartate (D) or cysteine (C) was introduced at the 8th position of Cx32*43E1 channel by site-directed mutagenesis. Complementary RNA (cRNA) was synthesized from linearized plasmid template using 'mMESSAGE mMACHINE T7 kit' (Ambion) according to the manufacturer's protocol. Approximately 50 nl of 1 ng/nl RNA was co-injected into *Xenopus* oocyte with 0.3 pmol/nl of an antisense phosphorotioate oligonucleotide complimentary to *Xenopus* Cx38. After RNA injection, oocytes were kept in a bath solution, ND96, 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.

MTS reagents

2-trimethylammonioethyl-methane thiosulfonate ($MTSET^+$) and 2-sulfonatoethylmethane thiosulfonate ($MTSES^-$) were purchased from Anatrace (Maumee, OH) and stored by aliquots at -20℃. Prior to each experiment an aliquot was dissolved in ice-cold distilled water or DMSO and stored on ice. Further dilutions were made into ND98 solution (see below) just before application to the desired final concentration (1 mM for $MTSET^+$ and 5 mM for $MTSES^-$, respectively). Activities of MTS reagents were periodically checked using a TNB assay[11]. The bath solution containing MTS reagents was exchanged by gravity perfusion for 40 seconds.

Electrophysiological recordings

Before recording, oocytes were devitellinized 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 same as a bath solution described above, except $CaCl_2$ and $MgCl_2$ were replaced by 2 mM EDTA and 2 mM EGTA (ND98). Single channel data were acquired using pClamp 7.0 software, an Axopatch 200B integrating patch amplifier, and a Digidata 1200A interface (Axon Instruments, Inc.). Data were acquired at 5 kHz and filtered at 1 kHz with a four-pole low pass Bessel filter.

Results and Discussion

T8D*43E1 channel shows positive gating polarity and linear I-V relation

The chimeric Cx32*43E1 channel in which the first extracellular loop of Cx32 (E1) was replaced with that of Cx43 was reported as a conductive membrane channel by Pfahnl et al.[12]. This gap junction hemichannel was further characterized in detail that Cx32*43E1 channel has negative gating polarity and inwardly rectifying I-V relation[7]. It has been proposed that the positive charge associated with the unmodified N-terminal methionine residue (M1) responds to negative potentials. If a negative charge is added to the N-terminus of Cx32, the resultant channel shows opposite gating polarity and modifies I-V relation. For example, Cx32N2E*43E1 channel in which the neutral asparagine (N) residue at the 2nd position of the N-terminus of Cx32 is substituted with negatively charged glutamate (E) shows strong positive gating polarity and linear I-V relation[7]. The charge effects at other positions in the N-terminus of Cx32*43E1 channel on the biophysical properties are also evident[8,9]. Based on the previous reports, we first generated T8D*43E1 channel and compared its biophysical characteristics with those of parental wild-type Cx32*43E1 channel to show its charge effect on gating polarity and I-V relation.

Single channel records obtained from T8D*43E1 are shown on Fig. 1. The clear gatings of T8D*43E1 channels appear at positive potential (+50 mV) although brief flickering events appear at negative potential (-50 mV) (Fig. 1A). These flicker-

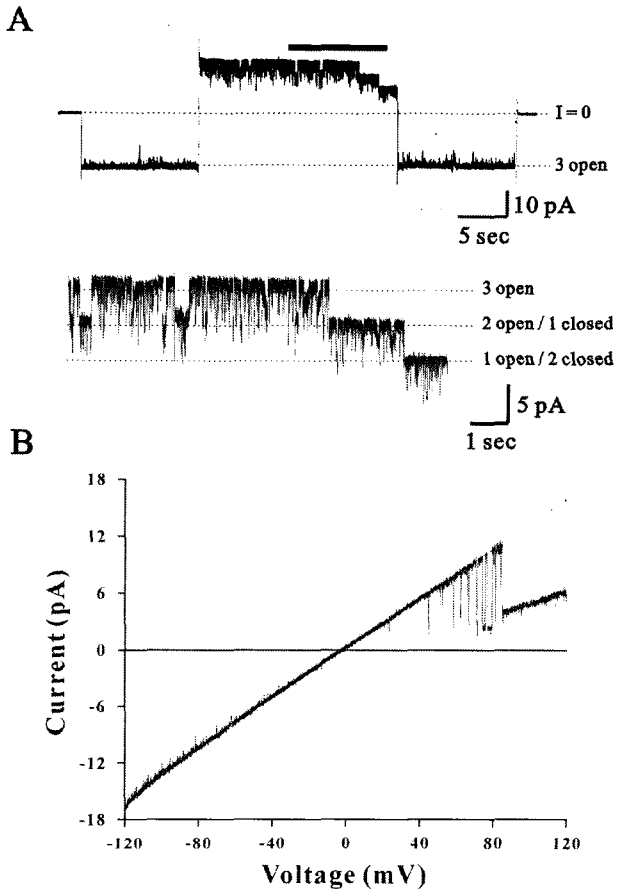

Fig. 1. Representative recordings obtained from T8D*43E1 channel. (A) (*Top panel*) A cell-attached record acquired from T8D*43E1 channel by the application of voltage steps (+/-50 mV) is shown. (*Bottom panel*) Ten seconds of record from top panel (solid bar) is expanded to show the positive gating events of T8D*43E1 channel. This particular patch contains three active channels. (B) Current-voltage relation obtained by the application of voltage ramp (from -120 to +120 mV) shows positive gatings and linear current flux through open channel. The linear line is the fit to the current trace.

ing events at negative potentials were previously reported by Purnick et al.[9]. We showed that the negative gating events of T8D*43E1 channel appear at higher negative potentials (beyond -100 mV) and proposed that the gating of T8D*43E1 channel is bipolar. The bipolarity of T8D*43E1 channel could be explained by postulating the existence of at lest two open channel states. In one open channel state, the T8D residue would lie within the electric field and thus would gate at positive potentials. In the second open state, T8D would lie outside the electric field and instead positive charge associated with the unmodified N-terminal methionine residue would respond to negative potentials. This bipolarity of T8D*43E1 channel needs to be carefully assessed and analyzed in detail. Because the effect of T8D residue on positive gating was evident, we did not consider the negative gating at higher potentials in this report.

The conductance of fast transitions between open and closed states at +50 mV (Fig. 1A) was about 96~99 pS. The positive gating of T8D*43E1 channel was more evident when voltage ramp (from -100 to +100 mV) protocol was applied (Fig. 1B). The I-V curve was linear and the slope conductance was about 130 pS. We previously reported that wild-type Cx32*43E1 channel shows negative gating and inwardly rectifying I-V relation[7]. The linearity of current flows and positive gating in T8D*43E1 channel compared with those in parental wild-type channel reflect the charge effect of aspartate (D) residue in the N-terminus. It may be also possible that these changes are resulted from the conformational change(s) induced by aspartate residue. However, our recent report that channels carrying a positive charges at the 8th position (T8K or T8R) maintain the negative gating polarity of the parental channel provides another evidence for the charge effect[8].

Although it is unlikely that both negative and positive charges at the 8th position of N-terminus exert similar conformational changes, it is hard to compare conformational changes after harboring the different types of amino acid residues (aspartate versus lysine or arginine in this case). To clarify this issue, we generated a mutant channel carrying a common cysteine residue at the 8th position in the N-terminus of Cx32 and assessed the charge effect at this position by introducing either positive or negative charges.

T8C*43E1 channel shows negative gating polarity and inwardly rectifying I-V relation.

T8C*43E1 channel in which threonine residue at the 8th position in the N-terminus of Cx32 is replaced with a cysteine residue formed a conductive membrane channel. This indicates that any perturbation by forming disulfide bond(s) between cysteine residues located in six subunits was not occurred. As we expected, the substitution of neutral residue at the 8th position in the N-terminus did not change the main features of wild-type channel. The biophysical properties of T8C*43E1 channel are similar to those of the parental wild-type channel, showing negative gating polarity and inwardly rectifying I-V relation (Fig. 2). However, the flickering events at both positive and negative potentials were more frequent than those of wild-type channel (Fig. 2A). Although not qualitatively analyzed, this increased frequency of the flickering events might be resulted from the spontaneous binding

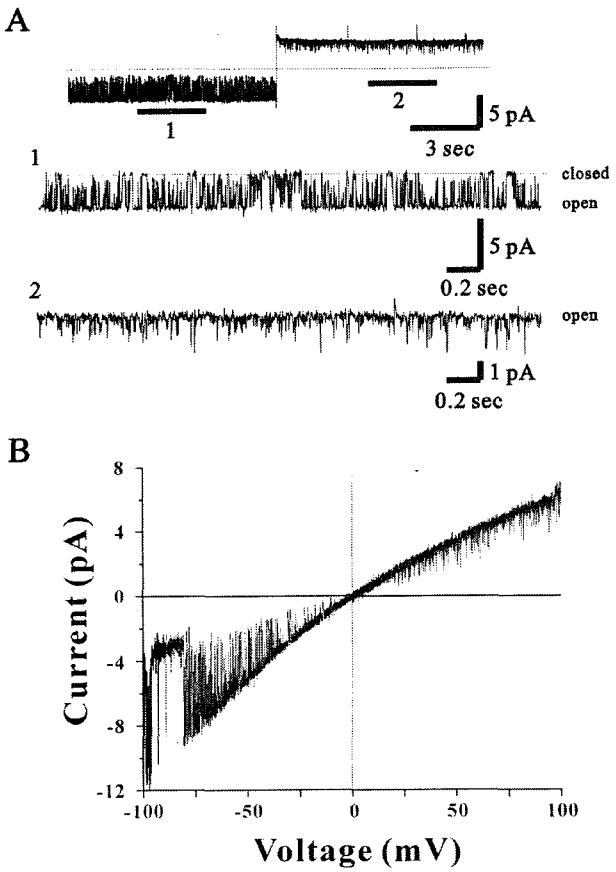

Fig. 2. Representative recordings obtained from T8C*43E1 channel. (A) (*Top panel*) A cell-attached record acquired by the application of voltage step (from -50 to 50 mV) is shown. (*Bottom panels*) Two portions of record (1 and 2 on top panel) are expended to compare the negative gating events (1) with the positive gating events (2). (B) Current-voltage relation obtained from T8C*43E1 channel by the application of voltage ramp (from -100 to +100 mV) shows negative gating events and inwardly rectifying current flux.

between cysteine residues in subunits. The conductance (66 pS) of fast transition measured from T8C*43E1 channel was somewhat smaller than that (about 96~99 pS) from the parental wild-type channel. The fact that T8C*43E1 channel maintains the main features of wild-type channel guarantees the following cysteine-modifying experiments to succeed.

A negative charge confers T8D effect on T8C*43E1 channel

Total six cysteine residues (one from each connexin subunit) in the N-terminus of T8C*43E1 are able to respond to MTS reagents which react with a sulfhydryl group of cysteine residue to form a disulfide bond. MTS reagents used in this work are small (MW 242.21 Da for $MTSES^-$ and 278.23 Da for $MTSET^+$, respectively) and charged. When negatively charged $MTSES^-$ reagent (5 mM) was applied, T8C*43E1 channel acquired the biophysical properties similar to those of T8D*43E1 channel. The reacted channel shows positive gating and linear I-V relation (Fig. 3A) while un-reacted channel shows negative gating and inwardly rectifying I-V relation (Fig. 2B). For

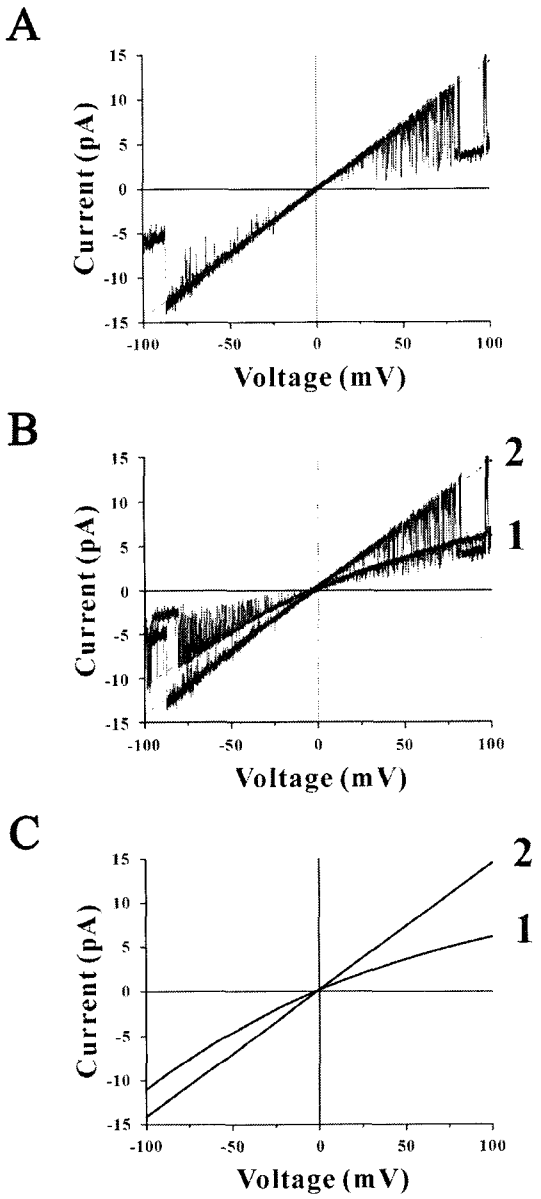

Fig. 3. Effect of $MTSES^-$ on T8C*43E1 channel. (A) Current-voltage relation obtained from T8C*43E1 channel after the application of $MTSES^-$ is shown. The voltage ramp was applied from -100 to +100 mV for 8 seconds. (B) Two current-voltage curves before (1) and after (2) the application of $MTSES^-$ are superimposed for comparison. (C) The exponential (1) and linear (2) fits to the data from Fig. 3B were superimposed to compare un-reacted (1) and reacted (2) T8C*43E1 channels.

comparison, two I-V curves of Fig. 2B and 3A are superimposed (Fig. 3B). These changes induced by the negative charge of $MTSES^-$ are very similar to the changes by the substitution of negatively charged residue found in T8D*43E1 channel (Fig. 1B). The linear and exponential fits to the data from each single ramps of reacted and un-reacted T8C*43E1 channels with $MTSES^-$ were superimposed on Fig. 3C. It is likely that the I-V curve might be dominated by the appearance of a negative charge in the N-terminus of T8C*43E1 over the unmodified N-terminal methionine residue (M1). The increased conductance of reacted T8C*43E1 channel can also be explained if the negative charge obtained from $MTSES^-$ increases the local concentration of positive ions by electrostatic attraction. Furthermore, the modification of cysteine residue at the 8^{th} position in the N-terminus by $MTSES^-$ indicates that this site is accessible and thus supports that the N-terminus of connexin forms or lies in the channel pore.

A positive charge maintains the biophysical properties of T8C*43E1 channel

The charge effect induced by positively charged $MTSET^+$ on the biophysical properties of T8C*43E1 channel was also observed. As expected, a positive charge introduced by $MTSET^+$ to the N-terminus maintained the negative gating and inwardly rectifying I-V relation of T8C*43E1 channel (Fig. 4A). The superimposed exponential fits to the data obtained from each single ramps of reacted and un-reacted T8C*43E1 channels show that the extent of current rectification from $MTSET^+$-reacted channel is more enhanced than that from un-reacted channel (Fig. 4B). The summation of two positive charges in the N-terminus (one from $MTSET^+$ reagent and the other from unmodified N-terminal methionine residue) might be responsible for the increased local concentration of negative ions and thus the enhanced current flows. However, this kind of charge summation in the N-terminus is not always occurred. According to our recent report[8], the mutant channels in which uncharged residues at the 2^{nd}, 5^{th}, and 8^{th} positions in the N-terminus of wild-type Cx32*43E1 channel are substituted with positively charged residues (arginine or lysine) have smaller slope conductances (from 70 pS to 80 pS) than that of the parental wild-type channel (115 pS). The changes in the slope conductances of these mutant channels can be explained either by invoking changes in the free energy of the open and/or closed states or by changes in the force acting on the voltage sensor due to local distortions in the voltage profile. As $MTSES^-$ experiment,

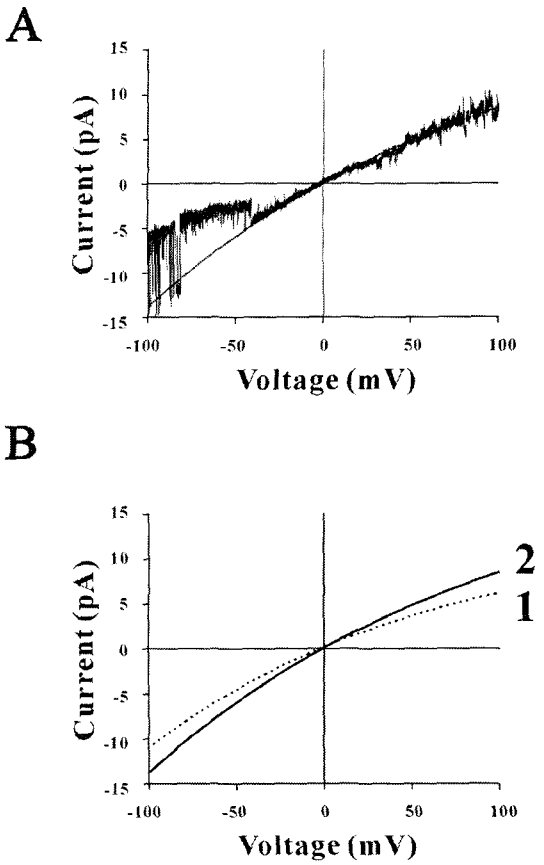

Fig. 4. Effect of $MTSET^+$ on T8C*43E1 channel. (A) Current-voltage relation obtained from T8C*43E1 channel after the application of $MTSET^+$ is shown. The voltage ramp was applied from -100 to +100 mV for 16 seconds. The solid line is exponential fit to the current trace of open channel. (B) The exponential fits to the data from Fig. 4A and 3C were superimposed to compare un-reacted (1) and reacted (2) T8C*43E1 channels.

the modification of cysteine residue at the 8^{th} position in the N-terminus of Cx32 channel by $MTSET^+$ also supports our model in which the N-terminus of connexin forms or lies in the aqueous pore[7-9].

The T8C*43E1 channel behaves as the parental Cx32*43E1 channel, showing negative gating polarity and inwardly rectifying I-V relation. It is likely that the substitution of cysteine residue at the 8^{th} position dose not perturb the original conformation of wild-type Cx32*43E1 channel. It is also likely that the addition of a charge by small MTS reagents does not distort the conformation of the N-terminus. Therefore, the opposite effects of $MTSES^-$ and $MTSET^+$ on T8C*43E1 channels (Fig. 5) suggest that the addition of a charge itself rather than the conformational change of the N-terminus modifies gating polarity and I-V curve. Our results are consistent with the observations found in Cx46 hemichannels by Kronengold et

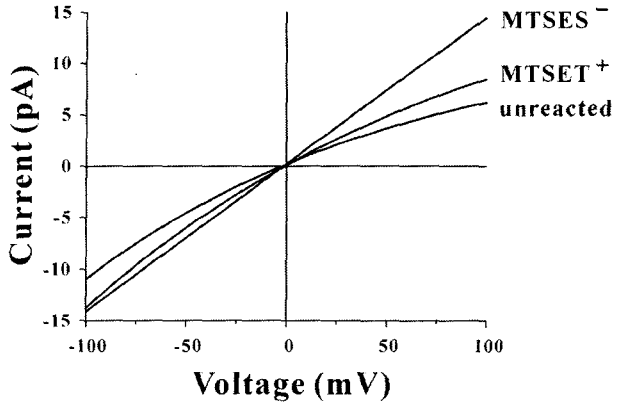

Fig. 5. Effects of MTS reagents on the current-voltage relation of T8C*43E1 channel. The exponential and linear fits to the data obtained from current-voltage curves of T8C*43E1 channel before and after MTS reagents treatments were superimposed.

al.[13]. They showed that a few residues in the first extracellular loop and first transmembrane domains of Cx46 hemichannels are accessible to modifications by MTS reagents and that MTS reagents differing in charges have opposite effects on Cx46 hemichannels. Although changes in gating polarity and I-V curve can be explained by simple charge model, changes in conductance can not be explained by charge effect. Accordingly, it needs to be analyzed further. One approach is to utilize the one dimensional Poisson-Nernst-Plank (PNP) model[14] to determine the voltage profile of simple cylindrical channels with fixed permanent charge(s) within the channel pore. This PNP model applied to Cx32*43E1 channel was used to demonstrate how local variations in the electric field may influence the gating polarity and voltage sensitivity[7,8].

This report is a part of our continuing efforts to obtain the detailed information regarding the biophysical properties of gap junction channels and to map the voltage sensor and gate of Cx32 channel as a model. We have shown that the addition of negatively charged amino acid residues at several positions within the first 10 amino acid residues reverses the gating polarity of their parental Cx32 channel and proposed that voltage sensor and gate are located within a segment of the N-terminus. Several evidences support that the N-terminus of connexin channel contributes to the formation of the channel pore. In this report, we provide that the charge itself rather than the conformational change modifies the biophysical properties of Cx32 channel and suggest that the N-terminus of Cx32 channel contains the voltage sensor and gate.

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초록 : 간극결합채널의 아미노말단이 채널개폐에 미치는 영향

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간극결합은 이웃하는 두 세포 사이에 형성된 이온채널이며 또한 단일세포막에서도 작용한다. 간극결합채널을 형성하는 아미노 말단의 10번째 아미노산 잔기 부위까지가 개폐극성(gating polarity)과 전류-전압관계에 영향을 미친다. 정상적인 Cx32 채널은 음성의 개폐극성과 내향적인 정류현상을 보이는 반면, 음성전하를 띠는 aspartate로 치환된 T8D 채널은 반대의 개폐극성과 직선의 정류현상을 보인다. 이러한 개폐극성과 정류현상의 변화가 전하 자체에 의한 것인지 아니면 아미노 말단의 구조적인 변화에 의한 것인지는 아직 불명확하다. 이러한 문제점을 규명하기 위하여 아미노 말단의 8번째 아미노산 잔기를 cysteine기로 치환시킨 T8C 채널을 만들어 substituted-cysteine accessibility method (SCAM) 방법으로 이 채널의 생물리학적 특성을 조사하고자 하였다. T8C 채널은 정상적인 Cx32 채널처럼 음성의 개폐극성과 내향적인 정류현상을 보였으며, cysteine기로 치환이 정상적인 Cx32 채널의 원래 구조를 변화시키지 않았다는 것을 의미한다. 본 연구에서는 이런 전하효과를 규명하기 위하여 음성전하를 갖는 MTSES-와 양성전하를 갖는 MTSET+를 사용하였다. MTSES-를 처리하면 T8C 채널은 T8D 채널의 특성처럼 양성의 개폐극성과 직선의 정류현상을 보였다. 그러나 양성전하를 갖는 MTSET+를 처리한 경우에는 T8C 채널은 본래의 특성을 그대로 유지하였다. 작은 분자의 MTS에 의해서 부여된 전하가 아미노 말단의 구조적인 변화를 초래하지는 않을 것으로 생각된다. 따라서 반대의 전하를 띠는 MTSES-와 MTSET+가 서로 상반대는 영향을 미치는 것으로 보아 본 연구에서 관찰된 개폐극성과 전류-전압의 변화는 아미노말단의 구조적인 변화라기보다는 MTS에 의해서 부여된 전하 자체에 기인한다고 할 수 있다. 또한 MTS가 아미노말단의 8번째 부위에 접근하여 반응을 일으킬 수 있다는 결과는 간극결합채널의 아미노말단이 채널의 통로(pore)를 형성한다는 가설을 뒷받침한다.