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Single Channel Analysis of *Xenopus* Connexin 38 Hemichannel

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Gap junction channels formed by two adjacent cells allow the passage of small molecules up to ~ 1 kDa between them. Hemichannel (connexon or half of gap junction) also behaves as a membrane channel like sodium or potassium channels in a single cell membrane. Among 26 types of connexin (Cx), Cx32*43E1 (a chimera in which the first extracellular loop of Cx32 has been replaced with that of Cx43), Cx38, Cx46, and Cx50 form functional hemichannels as well as gap junction channels. Although it is known that *Xenopus* oocytes express endogenous connexin 38 (Cx38), its biophysical characteristics at single channel level are poorly understood. In this study, we performed single channel recordings from single *Xenopus* oocytes to acquire the biophysical properties of Cx38 including voltage-dependent gating and permeation (conductance and selectivity). The voltage-dependent fast and slow gatings of Cx38 hemichannel are distinct. Fast gating events occur at positive potentials and their open probabilities are low. In contrast, slow gatings dominate at negative potentials with high open probabilites. Based on bi-ionic experiments, Cx38 hemichannel is anion-selective. It will be interesting to test whether charged amino acid residues in the amino terminus of Cx38 are responsible for voltage gatings and permeation.

Key words : Gap junction, connexin, hemichannel, voltage gating, selectivity

Introduction

Gap junction channel provides a pathway to facilitate the transport of several cellular ions, second messengers, and small metabolites between two apposed cells [7]. 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.

Studies using hemichannels have been well adapted to characterize the biophysical properties of gap junction channels since Cx46 has been cloned and characterized [8,10,15,18]. The advantages of experiments using hemichannels over gap junction channels are 1) relatively easy to get single channel recordings, 2) possible to access the intracelluar surface of channel by changing the bath solution, and 3) feasible to perform fast kinetic studies. Because the hemichannels share most biophysical properties with their parental (gap junction) channels, single channel studies using connexin hemichannels are advantageous to get more detailed information regarding channel functions.

Over 26 members of connexin gene family have been identified [7]. Among them, Cx32*43E1 (a chimera in which the first extracellular loop of Cx32 has been replaced with that of Cx43), Cx38, Cx46, and Cx50 have been reported to form functional hemichannel on a single cell membrane [3,8,12,15]. Studies of the developmental regulation of connexins in *Xenopus* embryo have been suggested that Cx38 is the major connexin in the *Xenopus* oocyte [4,6]. Although *Xenopus* Cx38 hemichannl has some macroscopic properties including outward current induced by depolarization, its blockage by external divalent cations, and temperature sensitivity [3], its detailed hemichannel properties at single channel level are not characterized.

In this experiment, we performed single channel recordings obtained from single *Xenopus* oocytes to acquire the biophysical characteristics of Cx38 including voltage dependence of channel gating, unitary conductance, and ion selectivity.

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Materials and Methods

Construction of Cx38 channel, cRNA synthesis, and oocytes injection

Xenopus Cx38 coding sequence was made from genomic DNA of *Xenopus laevis* using conventional polymerase chain reaction and cloned into pGem-7zf(+) (Promega, Madison, WI, USA) [8]. cRNA was synthesized from linearized plasmid templates using 'mMESSAGE mMACHINE T7 kit' (Ambion, Austin, TX, USA) according to the manu-

facturer's protocol. Approximately 50 ng of RNA was injected into *Xenopus* oocyte with or without 0.3 pmol/nl of an antisense phosphorotioate oligonucleotide complimentary to *Xenopus* Cx38 [13]. 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 pyruvate, pH 7.6.

Electrophysiological recordings and analysis

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. Experiments were performed in a RC11 recording chamber (Warner Instruments, Hamden, CT, USA). The bath solution volume was between 500 and 750 ml. In all patch-clamp experiments, the pipette solution contained 100 mM KCl, 2 mM EGTA, 2 mM EDTA, and 10 mM HEPES, pH 7.6 with 1 M KOH. Patches were excised into a bath solution of the same composition and instrumentation offsets were manually corrected. The bath chamber was connected by a 3 M KCl agar bridge to a ground chamber containing the same solution as the pipette solution. When requiring bi-ionic condition, the bath solution was exchanged by gravity perfusion of around 5 ml of solutions containing 10 mM, 20 mM, 50 mM, 200 mM, or 500 mM KCl, 2 mM EGTA, 2 mM EDTA, and 10 mM HEPES, pH 7.6.

Reversal potential (E_{rev}) were determined by fitting a linear or exponential function of voltage to the open state current recorded during voltage ramps. The ratio of cation to anion permeability was determined from reversal potentials using Goldman-Hodgkin-Katz (GHK) equation [5].

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.

Results and Discussion

Endogenous Cx38 and exogenous Cx38

Xenopus oocytes express endogenous Cx38 at later developmental stages [3,4,6]. However, the degree of expression varies depending on oocyte batch. To overcome this inconsistency, we injected recombinant Cx38 cRNA into oocytes and tested whether there is any difference of hemichannel activity between endogenous and exogenous Cx38. We did not observe any significant difference at both macroscopic outward currents and single channel recordings (data not shown). Data shown in this study were obtained from either endogenous Cx38 or exogenous Cx38-injected oocytes.

Voltage dependence of channel gating

It is well established that there are two distinct voltage gatings (fast and slow) of gap junction channels as well as connexin hemichannels [7,11,13-16]. Fast gating refers to instantaneous transition from open state to sub-conductance state (substate) allowing the channel to conduct residual amounts of current. Slow gating is stepwise transition from open to fully closed state without current passing. Cx38 hemichannel showed fast and slow gatings (Fig. 1A). It appears that all voltage gatings were slow at negative potentials,

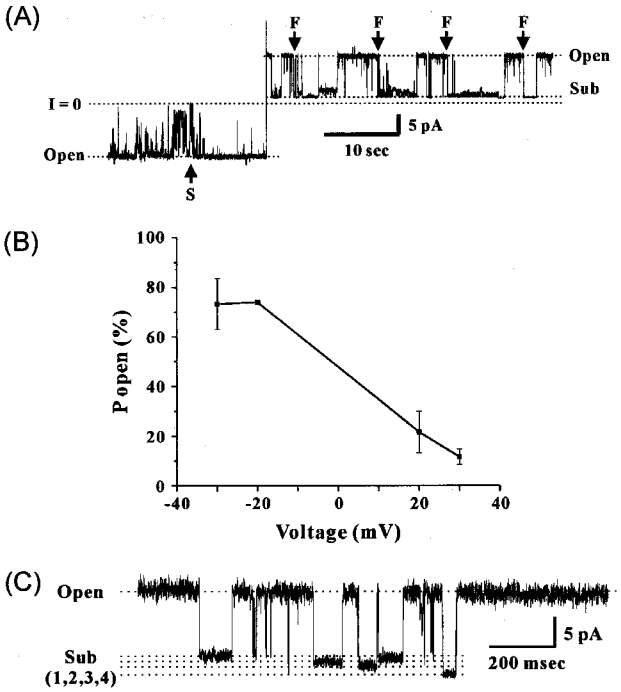

Fig. 1. Voltage dependence of gatings and open probabilities obtained from Cx38 hemichannel. (A) Representative single channel gatings recorded at +/- 30 mV holding potentials in outside-out patch mode are shown. Baseline (I=0), open state (Open), and sub-conductance state (Sub) are indicated by dashed lines. Fast (F) and slow (S) gatings are also indicated by arrows. (B) The measured open probabilities of Cx38 hemichannel are plotted as a function of holding potential. (C) A cell-attached record acquired from single Cx38 hemichannel by the application of voltage at +40 mV is shown to indicate multiple sub-conductance states. Open state (Open) and sub-conductance states (Sub 1, 2, 3, 4) are denoted by dashed lines.

while fast gatings were apparent at positive potentials. This result was similar to that obtained from Cx46 hemichannel [15] and different from that of Cx32*43E1 hemichannel [8,10,12]. While Cx46 shows fast gating at positive potentials and slow gatings at negative, Cx32*43E1 has both fast and slow at negative potentials. Fast gating (also called V_j gating) and slow gating (called loop gating) at single channel level using a single oocyte membrane were originally postulated by Trexler *et al.* [15] to describe voltage gating and slow transition of Cx46 hemichannel at positive and negative potentials, respectively. They also suggested that amino terminus of Cx46 is responsible for fast V_j gating while extracellular loops for slow gating. Although charged amino acid residues on amino termini of Cx46 and Cx32* 43E1, particularly amino acid at second position, has strong effect on fast gatings, site or domain responsible for slow gatings is not specified yet. According to sequence, there are five charged amino acid residues on amino terminus of Cx38. They include glutamate at the 5th position, lysine at the 8th, aspartate at the 12th and the 13th, and glutamate at the 16th. It is interesting to know whether loss of any charge at one of these positions affects fast gating of Cx38 hemichannel at positive potentials.

To examine whether the gating events are voltage dependent, we measured open probabilities (P_{open}) of Cx38 hemichannels at different potentials (Fig. 1B). Open probabilities were calculated from the ratio of the areas under the peaks of all point histograms. Cx38 hemichannels dominantly reside in open state at negative potentials (P_{open} = 74% at -30 mV). However, channels stay in sub-conductance state at positive potentials (P_{open} = 12% at 30 mV). The relation between gatings and open probabilities of Cx38 hemichannels (slow gatings with high P_{open} at negative potentials and fast gatings with low P_{open} at positive potentials) could be explained by the fact that fast gating requires only one out of six connexin subunits to initiate channel closing [8,10] while slow or loop gating needs total twelve extracellular loops. In addition, the amino terminus of connexin subunit responsible for fast gating is relatively short (around 22 amino acids) and has one open end thus more flexible to voltage. However, extracellular loops for slow gating are long (around 35 amino acids for the first extracellular loop and 18 amino acids for the second loop) and total twelve loops (two of each subunit) are believed to contribute to channel closing. Thus, it is assumed that short and flexible amnino termini of Cx38 hemichannel are more susceptible for voltage sensing to initiate fast gating than twelve long loops to induce slow gating.

Fast gatings of Cx38 hemichannel induced multiple substates at positive potentials. Fig. 1C showed at least four distinctive sub-conductance states with different residual currents. Multiple substates observed from Cx38 hemichannel are similar to those from Cx26, Cx32, Cx43, and Cx46 channels by several studies [2,8,11,15]. It is not known whether each substate represents each connexin subunit at fast gating events. The view that only one subunit is enough to initiate fast gating is currently favorable to explain multiple substates of fast gating.

Unitary conductance and ion selectivity

Gap junction channels are not passive conduits. They provide active pathways by limiting molecules to be passed although they have larger diameters of channel pores than those of other cellular membrane channels such as potassium and sodium channels [7]. Although size and charge of a molecule affect conductance and selectivity of gap junction channels, fixed charges on their subunits are major determinants for the measured permeability of gap junctions [1,8,9-11,18].

To measure the unitary conductance of Cx38 hemichannel, we performed single channel recordings by applying either voltage steps or ramps at different salt conditions. Unitary conductances at different salt concentrations are plotted as a function of voltage in Fig. 2. At

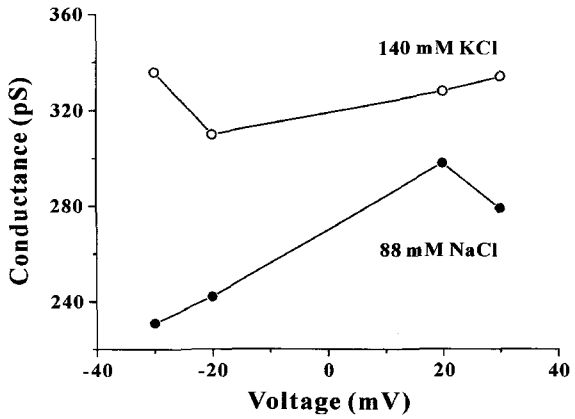

Fig. 2. Voltage dependence of unitary conductances measured from Cx38 hemichannel. The measured unitary conductances at different salt conditions are plotted as a function of voltage. Unitary conductances at low salt condition (88 mM NaCl as a major salt) are indicated by closed circles, while conductances at high salt (140 mM KCl) are denoted by open circles.

low concentration (88 mM NaCl as a major salt), the values of unitary conductance are ranging from ~ 230 pS to ~ 300 pS. It appears that conductances at positive potentials are larger than those at negative potentials. Similar observations have been reported that a negatively charged amino acid residue at amino terminus of Cx32*43E1 hemichannel significantly increases unitary conductance of its parental channel [8,10]. This indicates that a charged residue at amino terminus increases local concentration of ions and thus these localized ions affects the channel conductance. Among five charged residues of Cx38 amino terminus, glutamate at the 5th position and lysine at the 8th position are best candidates. Mutant analysis at those positions is required further to examine the determinant of channel conductance. At high concentration (140 mM KCl), it seems that unitary conductances at different voltages were saturated. This can be explained by two ways. The upper limit of conductance in Cx38 channel due to pore diameter and channel length causes this saturation if we assume that there is no difference in permeability between sodium and potassium ions. If there is any difference in permeability, it suggests that potassium ion is more permeable to Cx38 channel than sodium ion.

To examine the ion selectivity of Cx38 hemichannel, we measured the reversal potentials at bi-ionic condition in inside-out patch mode. The absolute values of the reversal potentials are plotted as a function of the bath salt concentration in Fig 3A. The measured reversal potential, E_{rev} = 26.5 mV (at 500 mM salt) corresponds to a P_K/P_{Cl} ~ 0.1, indicating Cx38 hemichannel is anion-selective. The anion selectivity decreases as the salt concentration of the external bath solution is decreased (E_{rev} = -14.47 mv and P_K/P_{Cl} ~ 0.5 at 10 mM salt). Several studies have been reported that Cx32 channel is anion selective while Cx37 and Cx46 are cation selective [9,15,17]. Current traces and their exponential fits are plotted as a function of voltage in Fig 3B and 3C, respectively. Although we have not ruled out the possible involvement of extracellular loops for anion selectivity, it is reasonable to perceive that lysine residue at the 8th position in amino terminus of Cx38 is one of candidate(s) for selectivity determinant.

In conclusion, Cx38 expressed in *Xenopus* oocyte forms functional hemichannels on a single oocyte membrane. Cx38 hemichannel has two distinct voltage gatings, fast and slow. At positive potentials, Cx38 hemichannel showed fast gating events with low open probabilities. In

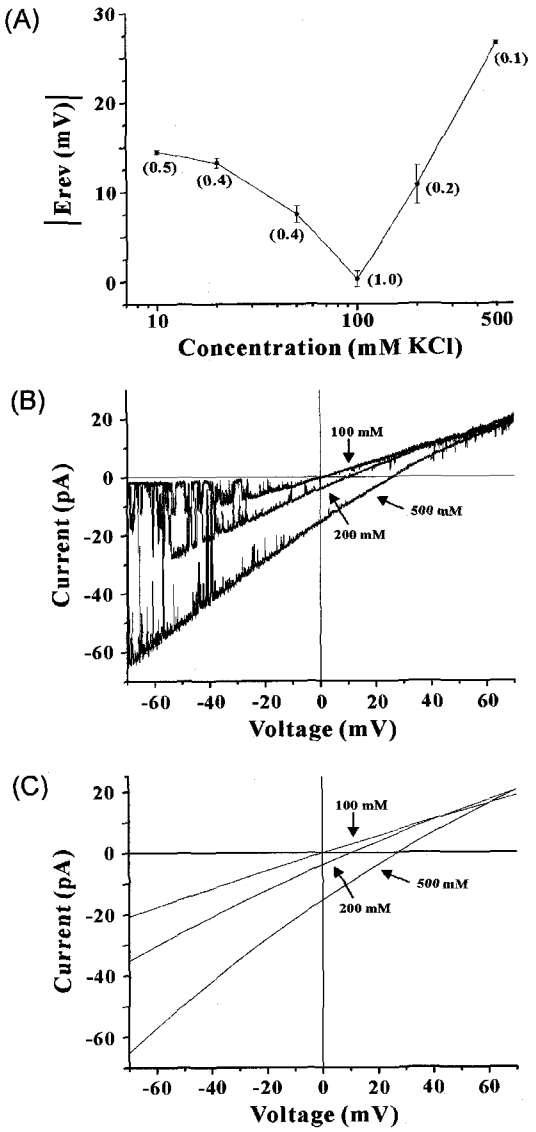

Fig. 3. Ion selectivity of Cx38 hemichannel. (A) The absolute values of measured reversal potentials are plotted as a function of bath concentration (in mM KCl). The calculated P_K/P_{Cl} ratios by applying the measured reversal potentials to GHK equation are denoted in parentheses. (B) Representative current traces obtained from a single Cx38 hemichannel by the application of voltage ramps (+/-70 mV, ~ 1.8 sec.) are shown. The concentration of the KCl (mM) in the bath solution is indicated by arrow. (C) The exponential fits for the current traces in (B) are shown. The concentration of the KCl (mM) in the bath solution is indicated by arrow.

contrast, slow gatings with high open probabilites dominated at negative potentials. The ion selectivity of Cx38 hemichannel was anion-selective as Cx32. There are five charged amino acid residues in the amino terminus of Cx38. It will be very interesting to examine whether these

residues are responsible for voltage gatings and selectivity by mutant studies using charge substitutions in these positions.

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초록 : 제노푸스 Cx38 세포막채널의 단일채널분석

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간극결합(intercellular channel)은 인접하는 두 세포사이에 형성된 이온채널이며 이를 통해서 각종 이온, 이차 신호전달물질, 그리고 1 kDa 미만의 대사물질들이 통과한다. 아울러, sodium 혹은 potassium 이온채널처럼 반쪽의 간극결합(connexon 혹은 hemichannel)도 세포막채널로서 작용을 한다. 현재까지 간극결합을 구성하는 connexin (Cx) 단위체는 26종류 이상이 확인되었다. 이 가운데, Cx32, Cx38, Cx46 그리고 Cx50 만이 간극결합채널뿐만 아니라 세포막채널로서도 기능을 수행한다. *Xenopus* oocytes에서 connexin 38 (Cx38)이 발현하는 것으로 알려져 있지만 Cx38의 생물리학적 특성이 단일채널수준에서 연구가 진행된 경우는 없다. 이번 연구에서는 Cx38 채널의 생물리학적 특성, 즉 전압-의존적 개폐와 투과성(전기전도도와 이온선택성)을 알아보고자 단일채널기록을 수행하였다. Cx38 hemichannel은 전압-의존적인 빠른 개폐와 느린 개폐의 특성을 보였다. 양성전압 환경에서는 Cx38 채널이 낮은 열릴 확률(open probability)로 빠른 개폐가 유도된 반면, 음성전압에서는 느린 개폐가 높은 열릴 확률로 유도되었다. bi-ionic 실험을 통하여, Cx38 채널은 양이온보다 음이온을 더 선택적으로 통과시킨다는 점을 알게 되었다. Cx38의 아미노산서열을 살펴보면, 아미노말단부위에 전하를 띠는 5개의 아미노산 잔기가 존재한다. 앞으로 이들 잔기를 치환시킨 돌연변이 Cx38 채널을 이용하여 과연 이들 아미노산 부위가 전압-의존적 개폐와 투과성에 관여하는 지 여부를 조사하는 연구는 매우 흥미로운 결과를 도출할 것으로 기대한다.