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Regulation of connexons composed of human connexin26 (hCx26) by temperature

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Abstract

This report shows that temperature is a latent regulator of the voltage-dependent conductance of hemichannels composed of hCx26. The latter were expressed in *Xenopus* oocytes by injection of a mixture of hCx26 cRNA and antisense of endogenous Cx38 (anti-Cx38). At 24–25 °C, voltage clamp of oocytes at potentials above −40 mV evoked outward currents which were not observed in control oocytes. These currents were reversibly affected by change in temperature. Increasing temperature of the bath solution amplified gradually, whereas decreasing bath temperatures below 20 °C reduced the current. Furthermore analysis revealed that temperature-dependent increase of the conductance of the hemichannels did not correlate with a change of the apparent gating charge, whereas the half-activation voltage $V_{1/2}$ of the hemichannel was affected by a temperature change. It is proposed that this finding correlates with a temperature-dependent transition into an open state above 20 °C. In addition, a temperature-dependent release of Lucifer Yellow from loaded liposomes containing reconstituted purified and hCx26 hemichannels was observed, which indicate that a temperature-dependent regulation of the permeability of hCx26 hemichannels is not related to intracellular mediators. The involvement of temperature to modulate hemichannels as well as of the corresponding gap junction channel composed of hCx26 at physiological condition is discussed.

Keywords: Connexin26; Connexon; Gap junction; Electrophysiology; Voltage; Temperature; Recombinant connexon; Folding

1. Introduction

Human connexin26 (hCx26) is expressed in various tissues by different cell systems like the supporting cells of the organ of Corti and skin cells [1–4]. Mutations of hCx26 have been correlated with different pathologies like the non-syndromic hearing loss and skin disorders such as keratitis-ichthyosis, Vohwinkel syndrome and palmoplantar keratoderma [1,5–8]. Yet, the link between mutations of hCx26 and the pathological phenotype is not well understood, because the regulation and the modulation of the gap junction channels and/or the corresponding hemichannels (connexons) are largely unknown. Using different expression systems like *Xenopus* oocytes or HeLa cells, it was shown that Cx26 of different origin forms voltage-dependent gap junction channels [9]. The macroscopic conductance of the channels (G) was maximal at transjunctional voltage $|V_j| \leq 40$ mV. Increasing $|V_j|$ to 100 mV gradually reduced G to 20% of the maximal G value. Furthermore, it was shown that gap junction channels of Cx26 were modulated by intracellular pH (pH_i). The reduction of pH_i from 7.2 to 6.5 almost inhibited gap junction coupling. Recently, [10] showed that *Xenopus* oocytes injected with a mixture of hCx26 cRNA and an antisense to the oocyte endogenous Cx38 (anti-Cx38) exhibited a current in response to voltage changes. Since this current was not observed in control oocytes which were injected with anti-38, it was concluded that this current was mediated by gap junction hemichannels (connexons) composed of hCx26. Interestingly, it was shown that oocytes expressing the human connexon hCx26 mediated voltage-dependent outward currents [11]. Furthermore it was shown recently that a mutant G45E of hCx26 exhibited increased hemichannel activity with elevated

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Ca^{2+} sensitivity [12]. Hemichannels composed of Cx26 can be now analyzed by their hemichannel function without complications of dual voltage clamp on paired oocytes. Twenty years ago, Santos-Sacchi [13] has shown that a decrease of temperature in bath solution from 35 °C to 15 °C strongly reduced gap junction coupling within the cochlear organ of Corti. Since supporting cells of the inner ear form gap junctions mainly composed of Cx26, it could be postulated that temperature gates the conductance of channels composed of Cx26.

This report shows that hCx26 forms conducting voltage-dependent connexons in the *Xenopus* oocyte membrane which are regulated by the temperature of the bath solution. The effect of temperature on the apparent gating charge and the half-maximal activating voltage were characterized. To study the temperature effect without cellular influences transport experiments with reconstituted and purified hCx26 hemichannel in lipid vesicles were performed.

2. Materials and methods

2.1. Bacterial strains

The *Escherichia coli* strain TOP10 and BL21plys (Invitrogen) was used to host the plasmids pGEMHE and pTrcHisTopo containing the hCx26 gene, respectively [14]. The cells were cultivated in Luria Bertani medium (LB) using ampicillin as selection marker (100 μg ml^{-1}) at 37 °C.

2.2. Oocyte expression and voltage-clamp measurements

Frog oocytes of *Xenopus laevis* were prepared according to [15]. Using the Nanoliter Injector (World Precision Instruments) 23 nl of a solution containing hCx26-cRNA (2 μg μl^{-1}) and anti-Cx38 (0.4 μg/μl) was injected in oocytes. Control oocytes were injected with 23 nl (0.4 μg/μl) of a solution containing only anti-Cx38 (5′-CTG ACT GCT CGT CTG TCC ACA CAG-3′) which was designed according to [16]. For expression, oocytes were conserved for at least 24 h at 16 °C in modified Barth medium composed of (in mM) 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgCl_2$, and 15 HEPES at pH 7.4. Voltage-clamp experiments were performed as described previously [15,17] using a nominal Ca^{2+}-free bath solution composed of (in mM) 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgCl_2$, and 15 HEPES at pH 7.4.

The oocytes were clamped at −80 mV and currents were evoked by a voltage change from −100 mV to 60 mV in alternating steps of 20 mV applied for 20 s. Currents obtained after 20 s are named I_{20}. After repolarisation of the oocyte at a holding potential of −80 mV for 5 s a post pulse to −100 mV followed and the cells were clamped after additional 5 s to the holding potential of −80 mV hold at −80 mV for the following 30 s. The evoked currents were measured at the end of the applied voltage pulse, respectively. The I/V, the activation parameter at different temperature were compared. The reversal potential (V_{rev}) of the measured non-leak subtracted I_{20} (V) curve was calculated by a 4-point interpolation polynomial [17]. The resulting conductance ($G(V_{rev})$) was described by a simple Boltzmann distribution according to $G(V)=(A/(1+\exp((V-V_{1/2})zF/RT)+B))$. R, T, and F have their usual meanings, $V_{1/2}$ represents the half-activation voltage at which 50% of the maximal conductance is reached, and z represents the number of apparent equivalent gating charges. The parameter A gives the maximal macroscopic conductance of the hemichannels, B represents the non-voltage-dependent leak conductance. The $G(V_{rev})$ curves were normalized to the maximal value of $G(V_{rev})$, so that the amplitude was given by $A=1$.Values are given as mean±s.e.m., n denotes the number of independent experiments.

The temperature of the bath solution was adjusted during the measurements by a flow chamber, which was mounted under the used cuvette and connected to a temperature-controlled heat supply by a pump. Using a tip sensor placed in the vicinity of the oocyte, the actual temperature (±0.7 °C) of the bath solution was measured at the beginning and the end of each voltage protocol.

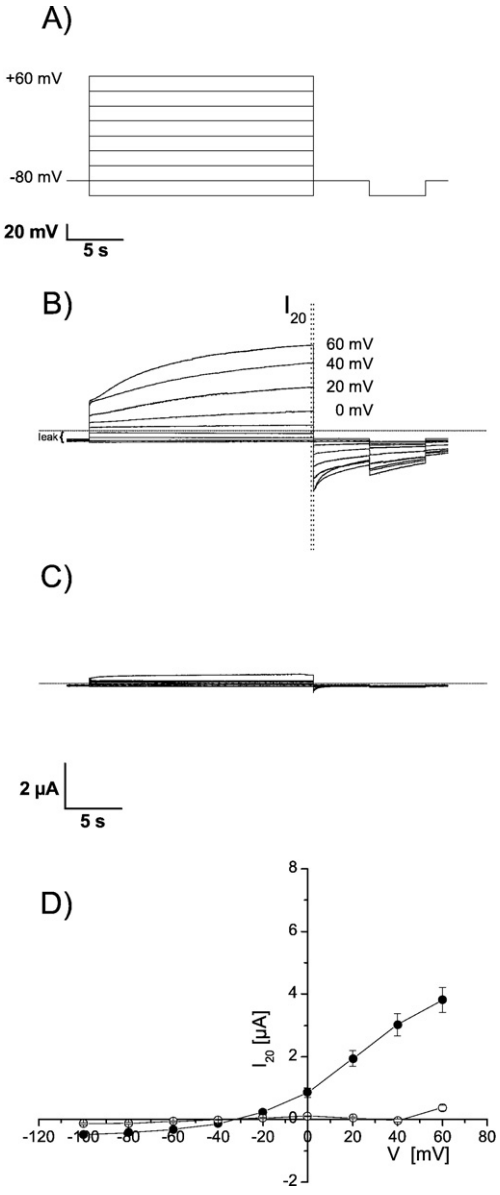

Fig. 1. Analysis of hCx26–ASCx38 activity by voltage clamp. (A) Voltage pulse protocol: starting at a constant holding potential of −80 mV voltages from −100 mV to +60 mV were applied for 20 s in steps of 20 mV. 5 s after returning to holding potential of −80 mV a post pulse to −100 mV for 5 s followed before returning to the holding potential of −80 mV. Between two test pulses, a 30-s resting phase at the holding potential was allowed. (B) Current response to the voltage pulses (pulse protocol above) applied to oocytes expressing hCx26–ASCx38 and (C) control oocytes (ASCx38) at 24.5 °C and in nominally Ca^{2+}-free bath solution (modified Barth medium) (control conditions). n denotes the number of oocytes and m denotes the number of measurements which were introduced in the results. The traces represented here are mean of $n=9$ and $m=24$ for hCx26 expressing oocytes and of $n=5$ and $m=5$ for control oocytes. The dotted line denotes the interval at the end of the 20-s conditioning voltage pulses where the amplitude of the evoked currents were measured (I_{20}). (D) Macroscopic current amplitude (I_{20}) obtained from oocytes expressing hCx26–ASCx38 (●) and control oocytes (ASCx38, ○). The results are given as mean± s.e.m. for $n=9$ and $m=24$ for hCx26 expressing oocytes and for $n=5$ and $m=5$ for control oocytes, n and m have the same meaning as above.

2.3. Functional analysis of purified hCx26

The hCx26 gene was amplified by PCR using the following primers hCx26_forward: 5′-ATG GAT TGG GGC ACG CTG CAG-3′ and hCx26_reverse: 5′-TTA AAC TGG CTT TTT TGA CTT CCC-3′, which were generated from the published sequence of hCx26 (Accession no.: NP_003995). The gene was cloned into the pTrcHis-TOPO vector (Invitrogen). The cloned product phCx26Nt containing the His-tag was transferred into the *E. coli* strain BL21-plys cells which were grown in LB medium over night at 37 °C using an ampicillin concentration of 100 μg ml^{-1}. To induce the protein expression 1 mM IPTG was added to the culture medium and followed by incubation at 37 °C for 6.5 h.

Recombinant hCx26 was purified from *E. coli* cells using the His-tag as described by Zeilinger et al. [14]. The N-terminal His-tag was removed by enterokinase.

The purified protein was refolded and formed hexamers in the presence of POPC at a protein:lipid ratio of 1:1 (w/w) as described by [14]. Vesicles loaded with Lucifer Yellow (LY) which contained reconstituted hemichannels of hCx26 were formed in a three-step protocol. (i) Lucifer Yellow loaded vesicles are formed spontaneously by addition of a POPC lipid film to a buffer composed of 1.5% LY, 150 mM NaCl and 10 mM Tris–HCl, pH 8. To remove the external dye, the vesicle suspension was centrifuged at 150,000 *g* for 10 min at 4 °C. The supernatant was discarded and in a fresh ice cooled vesicle buffer without LY. After a second centrifugation step the supernatant was discarded and the vesicles

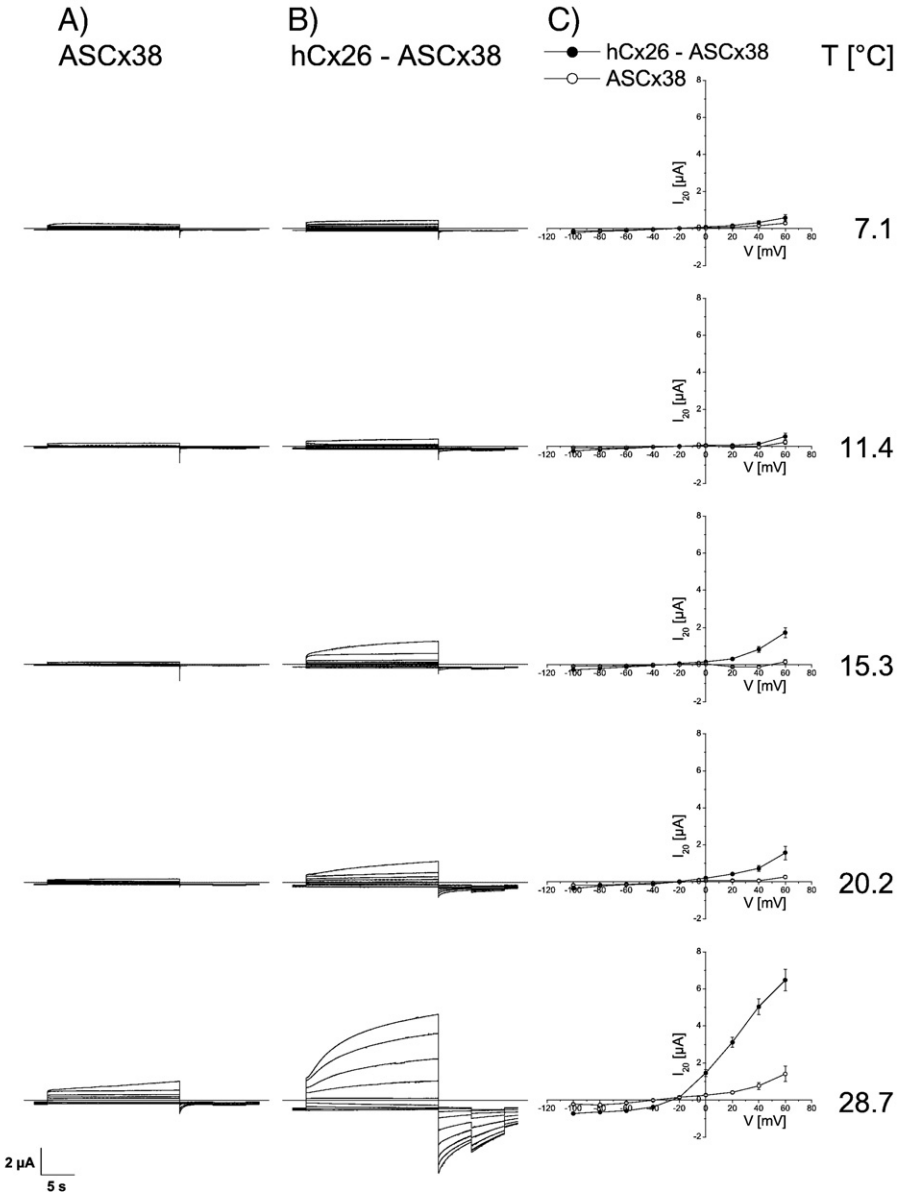

Fig. 2. Current response of the voltage pulses at different temperatures applied during measurements in modified bath medium (Ca^{2+}-free). (A) Control oocytes, ○. (B) Oocyte expressing hCx26, ●. (C) Voltage-dependent amplitude of the macroscopic currents (I_{20}) derived as described in Fig. 1B. The current traces of control oocytes and of hCx26 expressing oocytes are means of a variable number of oocytes and of experiments. The current amplitudes represent mean ± s.e.m. for at least $n=5$ and $m=5$ for each temperature. (ASCx38: 7.1 °C: ($n=9$, $m=12$); 11.4 °C: ($n=6$, $m=8$); 15.3 °C: ($n=5$, $m=9$); 20.2 °C: ($n=5$, $m=9$); 24.5 °C: ($n=5$, $m=5$); 28.7 °C: ($n=5$, $m=7$). hCx26–ASCx38: 7.1 °C: ($n=8$, $m=13$); 11.4 °C: ($n=5$, $m=5$); 15.3 °C: ($n=6$, $m=16$); 20.2 °C: ($n=7$, $m=8$); 24.5 °C: ($n=9$, $m=24$); 28.7 °C: ($n=13$, $m=32$)).

were resuspended in fresh LY-free vesicle solution. (ii) Solubilized hCx26-hexamers were added at 100 μg/ml in a solution containing 30 mM octyl-glucosid, 150 mM NaCl and 10 mM Tris–HCl, pH 8 to LY loaded vesicles at 10 mg lipid/ml solution and incubated on ice for 30 min. (iii) To remove the detergent, the vesicle suspension was dialysed against a solution (about 250-fold of the vesicle volume) composed of 150 mM NaCl and 10 mM Tris–HCl, pH 8 at 4 °C. After 24 h this dialysis solution was replaced by a fresh solution and applied for additional 24 h. Afterwards the vesicle suspension was kept on ice and could be used for the next 4 h. For analysis of LY-release, 100 μl of vesicle suspension was filled in PCR tubes and incubated for 5 min at the desired temperature. The release was stopped by addition of 200 μl ice cooled LY-free vesicle buffer and the vesicle suspension was centrifuged at 150,000 for 10 min at 4 °C. The supernatant was excited at 480 nm in a microtiter plate and the resulting emission at 530 nm was analyzed. In parallel a suspension of LY loaded vesicles without hCx26 was exposed to the corresponding temperature and used as control.

3. Results

The conducting activity of hCx26 gap junction hemichannels was analyzed by two electrode voltage-clamp experiments on oocytes, which were injected with a mixture of hCx26 cRNA and anti Cx38 24–60 h before. Oocytes injected with only anti-Cx38 were used as control. The cells were clamped at −80 mV. Voltage pulses from −100 mV to 60 mV in 20 mV steps were applied for 20 s (Fig. 1A) and the evoked currents were recorded (Fig. 1B, C). To evaluate the voltage–current relationship, the amplitude of evoked currents (I_{20}) was measured after 20 s of the conditioning pulse and is given as voltage–current (V (I)) plot (Fig. 1D). At 24.5 °C and external nominal Ca^{2+}-free solution (control conditions), application of the voltage protocol on oocytes which were injected with the hCx26/anti-Cx38 mixture 24–60 h prior to start of the experiment, evoked significant outward currents at positive potentials. Such currents were not observed in control oocytes, which were injected with anti-Cx38 only (Fig. 1B, C, D). Currents mediated by hCx26 were activated if the depolarizing voltage pulse exceeded

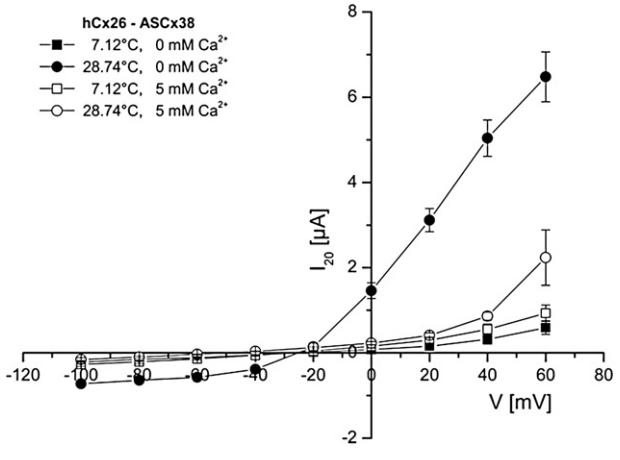

Fig. 3. Current voltage relation ($I_{20}(V)$) derived from oocytes in the presence of 5 mM Ca^{2+} (○, □) and in the absence of Ca^{2+} (0 mM Ca^{2+}: ●, ■) at two different temperatures, (7.1 °C: ■ and □, 28.7 °C: ● and ○) expressing hCx26. The results are given as mean±s.e.m. $n=8$ and $m=13$ for (■ : 0 mM Ca^{2+} at 7.1 °C); $n=13/m=32$ for (●:0 mM Ca^{2+} at 28.7 °C); $n=5/m=8$ for (□: 5 mM Ca^{2+} at 7.1 °C) and for $n=3/m=4$ (○: 5 mM Ca^{2+} at 28.7 °C), n and m have the same meaning as in Fig. 1.

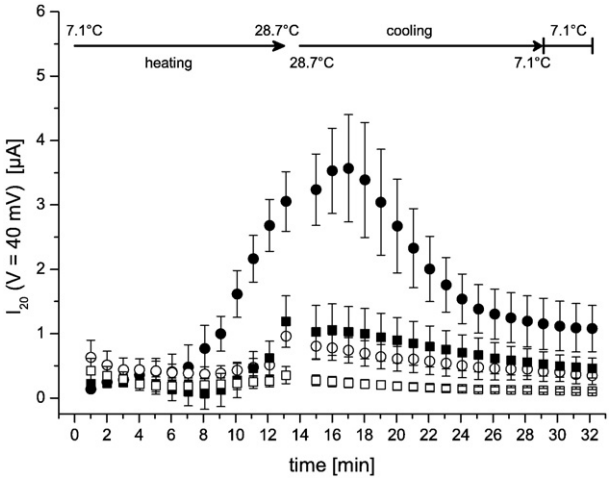

Fig. 4. Amplitude (I_{20}) of the macroscopic current in response to a voltage of 40 mV during warming and cooling periods and in the presence or absence of Ca^{2+} in the bath solution. The results are given as mean±s.e.m. for at least $n=5$ experiments for oocytes at 0 (●)and 5 mM Ca^{2+} (○) expressing hCx26 and control oocytes at 0 (■) and 5 mM Ca^{2+} (□). Suggesting a rate around 1.87–2 °C/min, the initiation temperature is around 18–19 °C for warming.

−40 mV (Fig. 2) and were blocked by hyperpolarizing voltages (Fig. 1B). This result confirms the capability of hCx26 to form active hemichannels as described by [10]. In line with the observation of Ripps et al. [10], we observed that addition of Ca^{2+} to the bath in the millimolar range suppressed the conductance of hCx26 hemichannels (Fig. 3).

Previously, it has been shown that gap junction coupling in the organ of Corti was modulated by temperature. Reduction of temperature from 35 °C to 17 °C correlated with a decrease of the electrical coupling of 75% [13]. Since Cx26 accounts for the major population of connexins in the formation of those gap junctions, we analyzed whether temperature regulates the conductance of hCx26 hemichannels. As presented in Fig. 4, increase in temperature of the bath solution from 7.1 °C to 28.7 °C correlates with a significant increase of the current evoked by the applied voltage pulses. Reduction of the temperature in the bath gradually decreased the voltage-dependent current (Fig. 4). For a temperature range of 7.1 °C to 28.7 °C, the effect of the temperature on the current was reversible (Fig. 4). It is interesting to note that the Ca^{2+} dependent blockade of the hemichannel was not affected by changes of temperature.

To describe the regulation by temperature for the voltage-dependent hCx26 hemichannels, the macroscopic conductance at the end of the voltage pulses was determined at different temperatures (Fig. 5A). To compare the relative conductance the parameters of the temperature related regulation of the hCx26 hemichannels, the half-activation voltage ($V_{1/2}$) and the apparent gating charge z are determined by using Boltzmann distribution. Fig. 5B shows that an increase of temperature above 20 °C temperature affected these parameters. At 28.7 °C and 24.5 °C $V_{1/2}$ changed from 7.82±1.25 mV to 20.81±3.1 mV and the apparent gating charges z from 1.6±0.1 to 1.3±0.18. The deactivation of voltage-dependent outward currents at two temperatures (24.5 °C and 28.7 °C) revealed that temperature

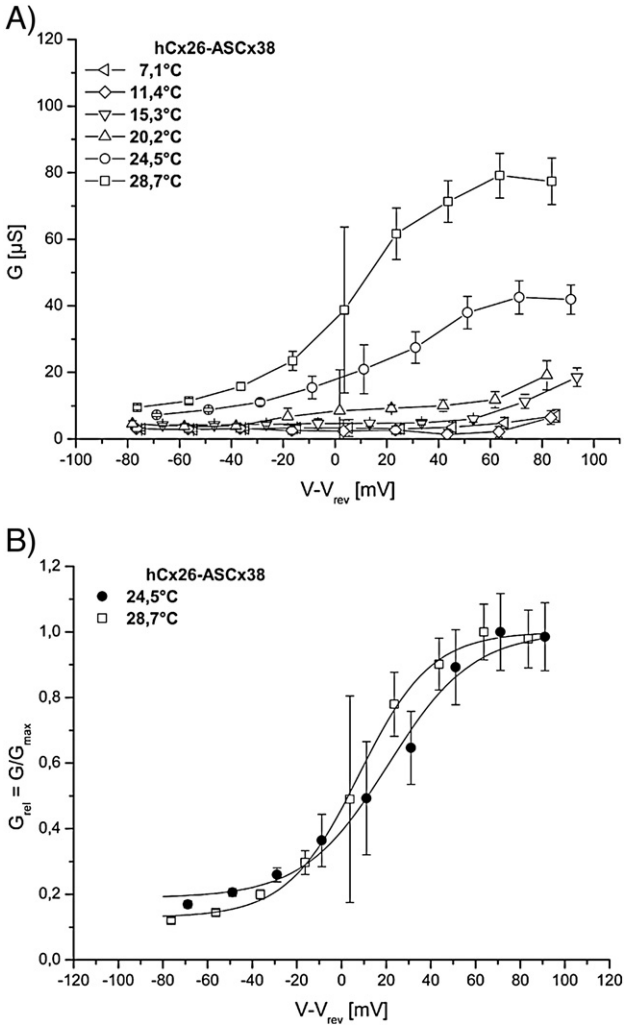

Fig. 5. A) Voltage dependence of macroscopic conductance (G) of the hCx26 hemichannels at different temperatures. For better comparison V_{rev}'s are subtracted at (7.1 °C) −25.41 mV; (11.4 °C) −23.30 mV; (15.3 °C) −33.41 mV; (20.2 °C) −21.80 mV; (24.5 °C) −31.12 mV; and (28.7 °C) −23.67 mV. B). The relative conductance $G_{rel}=G/G_{max}$ is given as function of voltage at 24.5 °C (●) and at 28.7 °C (□). The results are given as mean±s.e.m. as indicated in the figure. The solid lines show fits of the data by a simple Boltzmann function (see Materials and methods) with 24.5 °C: $V_{1/2}$ (20.81±3.08) mV, z 1.35±0.18, B 0.18916±0.02994 and 28.7 °C: $V_{1/2}$ (7.82±1.26) mV, z 1.61±0.11, B 0.1296±0.0433 for the fit. (hCx26–ASCx38: 7.1 °C: (n=8, m=13); 11,4 °C: (n=5, m=5); 15.3 °C: (n=6, m=16); 20.2 °C: (n=7, m=8); 24.5 °C: (n=9, m=24); 28.7 °C: (n=13, m=32)).

significantly affects the amplitude of the deactivating currents, but not the time rates.

To test whether the effect of the temperature is related to binding of connexins to regulatory proteins, the effect of temperature was studied on purified hexameric recombinant hCx26 reconstituted in vesicles by measurement of Lucifer Yellow (LY)-release (Fig. 6A, lanes 1, 2). Fig. 6B shows that a temperature increase from 15 °C to 35 °C stimulated LY-release. The relationship between temperature and LY-release (T(LY-release)) followed a sigmoid curve with a half-maximal release at 23 °C–25 °C (Fig. 6B). This result indicates that intracellular modulators seem not to be involved in the regulatory effect of temperature on the conductance of hCx26 hemichannels.

4. Discussion

In this report, activation of hCx26 hemichannels expressed in *Xenopus* oocytes is shown. The hemichannels were activated by depolarizing voltages at reduced Ca^{2+}-concentration (see also Fig. 1). It can be questioned whether the activation of hemichannels revealed in expression systems has any relevance for their physiological functions. Active hemichannels composed of Cx26 in retinal horizontal cells of carp have been postulated [18]. Reduction of concentration of extracellular Ca^{2+} was shown to stimulate the activation of hemichannels in different cell types [19,20]. With regard to the physiological function of gap junction hemichannels, recent experiments have shown that the opening of hemichannels by reduction of extracellular Ca^{2+}-concentration may be involved in cellular secretion of metabolites e.g. ATP or NAD, which have neurotransmitter/hormonal activity [21]. Moreover, our results presented in this report about active hCx26 hemichannels are in line with the observation of Ripps et al. [10] that hCx26 formed active gap junction hemichannels in *Xenopus* oocyte. This report shows that hCx26 forms active voltage-dependent hemichannels as previously reported. The voltage dependence is in agreement with that observed in experiments using paired oocytes or HeLa cells expressing hCx26 gap junctions [22,23]. In these experiments, it could be observed that the macroscopic conductance of gap junctions (G_j) was maximal at transjunctional voltages of $|V_j| \leq 40$ mV. An increase in $|V_j|$ correlates with reduction of G_j to less than 20% of the maximal value. In line with this result, it is shown that hCx26 hemichannels are activated at voltages more positive than −40 mV (Fig. 1). It was postulated that the voltage dependency of gap junction conductance, based on hCx26 is related to the N-terminus of hCx26 [9]. Since the N-terminus is localized in the intracellular space, it is therefore not involved in connexon–connexon docking.

4.1. Regulation of the conductance of hCx26 hemichannels by temperature

Our data indicate that heating from 7.1 °C to 28.7 °C causes an increase in the conductance mediated by hCx26 hemichannels. As shown in Fig. 4 heating as well as cooling dependent effects on membrane conductance was reversible. Furthermore this temperature-dependent reduction of gap junction conductance correlates with the reduction of the molecular permeability of reconstituted hCx26 channels, since the LY-release could be suppressed by a reduction of the temperature (Fig. 6). It is interesting to note that the temperature-dependent gating effect on hCx26 conductance and on LY-release occurs in a similar temperature range (20–25 °C). Since the LY-release experiments were performed with hCx26 channels reconstituted in lipid vesicles, it can be assumed that the temperature effect is not related to an intracellular mediator.

The voltage-dependent conductance of the hemichannels at 28.7 °C, 24.5 °C, 20.2 °C and 15.3 °C was calculated and saturating conductances at 28.7 °C and 24.5 °C were fitted by Boltzmann distribution. At 24.5 °C, a $V_{1/2}$ of about 20.8 mV is estimated, whereas at 28.7 °C a $V_{1/2}$ of about 7.8 mV is found.

This result may indicate, that beside the conductance of hCx26 hemichannels the temperature also affects voltage sensitivity of the hemichannels. The observed effect of temperature on conductance and voltage sensitivity shows a non-linear behaviour with a transition temperature above 20 °C. Such a behaviour cannot be related to different classes of channels with different conductances and temperature sensitivities as observed for embryonic cardiac gap junctions [24]. With respect to the apparent gating charge, it is observed that temperature does not significantly affect this parameter (Fig. 5). Moreover, a relatively small apparent gating charge was observed, which corresponds to activation in a large voltage range as seen in Fig. 5.

Examination of the temperature effect on the deactivation reveals that temperature influences the amplitude, but not the time rates. Single channel measurements on Cx30 which is very similar to Cx26 identified high and low conducting states [25,26]. Interestingly it was found that the Cx30 channels are controlled by two types of gates, a fast one responsible for $|V_j|$ gating involving transitions between open states, and a slow one correlated with a putative chemical gating involving transitions between the closed state and an open state [26]. Therefore it is possible that the temperature influences the transition between these two states which results in an increase in the single channel conductance by shifting towards a main open state. However, a two state model with closed to open transition may describe the open gap junction hemichannel, but it is unknown whether a two state model can also include the activation by temperature. The recently published structure of hCx26 at a resolution of 5 Å on 2D crystals identified a pore diameter of around 17 Å and huge plug in the pore [27]. Therefore the open channel can mediate transport of large molecules like LY (Fig. 6). If the increase in single channel conductance correlates with an increase in pore diameter, additional ions as well as small molecules like nucleotides and peptides may pass and this is in agreement with the temperature-dependent LY-release (Fig. 6). Conductance and LY-release mediated by hCx26 was initiated within a similar temperature range. But there exists a significant difference: conducting ions have diameter of few angstroms, but permeation of LY requires an open pore diameter of about 13 Å. Those open pores may enable the transport of large molecules like ATP and MHC I peptides [28].

4.2. Regulation of the hCx26 hemichannel by Ca^{2+}

In agreement with the observation of [10], we show (Fig. 3) that hCx26 hemichannels are blocked by increased external free Ca^{2+} concentrations indicating the role of Ca^{2+} as regulator of the hemichannel activity *in vivo*. In oocytes the necessary Ca^{2+} concentration is not specific for hCx26 hemichannels, since comparable concentrations were also observed for rCx46 hemichannels [15,17,29]. Interestingly these high concentrations were observed in cell systems where hemichannels could be detected [18–20]. For hemichannels composed of rCx46, it was proposed that external Ca^{2+} inhibits the hemichannel by an interaction with negative charges in the channel pore, which induces a conformational change to the closed state [17,29]. It was speculated, that the Ca^{2+} binding to the pore is weak so that,

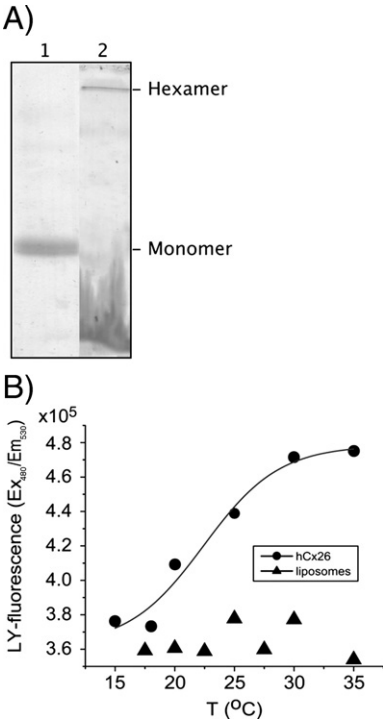

Fig. 6. SDS-PAGE analysis of purified hCx26. Lane 1 shows purified hCx26Nt after elution from affinity column without additional lipid (POPC) at ~28.6 kDa. Lane 2 shows oligomer of hCx26Nt at ~179 kDa in the presence of POPC (10 mg ml^{-1}). B) Temperature-dependent Lucifer Yellow release from vesicles reconstituted with purified hexameric hCx26Nt (●) and control vesicles without hCx26 hemichannels (▲). The solid lines show fits of the data by a simple Boltzmann function.

high Ca^{2+} concentrations are necessary. As a high homology in the amino acid sequence of the transmembrane domains and the first external loop is observed in different connexins, it appears likely that the proposed Ca^{2+} binding site in the external pore of Cx46 hemichannels is also present in hemichannels composed of other connexins like hCx26. The observation that the Ca^{2+} induced inhibition of hCx26 hemichannel mediated conductance was not affected by temperature shows that the Ca^{2+} binding on the hemichannels is not affected by temperature above 20 °C. It is possible that the interaction between channels and Ca^{2+} is affected by low temperatures, but since the channels are closed at the lower temperature it is not possible to detect this effect.

4.3. Physiological significance of temperature dependence of hCx26 mediated conductance

The skin covers the body, appearing to have the largest surface area of all organs and forms an anatomical barrier between the internal and external environment in bodily protection. Beside the important function of protection, temperature sensors for cold and heat are also localized in the skin such as the family of transient receptor potential (TRP) ion channels. They are known to mediate a wide range of sensory modalities, including thermo-sensation and taste [30]. In an extended context additional components required for temperature sensation and temperature and pain are linked to different channel

types e.g. sodium channel function or perhaps also connexins [30,31]. Interestingly, hCx26 is expressed in skin. Therefore the observed temperature-dependent gating of Cx26 hemichannels could indicate an involvement of the corresponding Cx26 cell-to-cell channels in thermo regulation, and an important role in transport of small molecules through hemichannels [13]. Our observation provides a concept for the analysis of different skin pathologies related to mutations of the hCx26 gene, which lead to expression of an incorrectly regulated hCx26 mutant. Analysis of the activity of the mutants and their regulation by temperature is needed to clarify this issue.

5. Conclusion

The results presented in this report show that hCx26 expressed in *Xenopus* oocytes forms conducting hemichannels which, as already reported [10], exhibit a voltage-dependent activation. In addition, we observed a specific regulation by temperature. It can be speculated that the corresponding gap junction channels preferentially composed of hCx26 are also gated by temperature and thereby could be involved in thermoregulation especially of the skin.

Acknowledgments

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References

[1] H.B. Zhao, T. Kikuchi, A. Ngezahayo, T.W. White, Gap junctions and cochlear homeostasis, J. Membr. Biol. 209 (2006) 177–186.
[2] T. Kikuchi, R.S. Kimura, D.L. Paul, T. Takasaka, J.C. Adams, Gap junction systems in the mammalian cochlea, Brain Res. Brain Res. Rev. 32 (2000) 163–166.
[3] A. Forge, D. Becker, S. Casalotti, J. Edwards, N. Marziano, G. Nevill, Gap junctions in the inner ear: comparison of distribution patterns in different vertebrates and assessment of connexin composition in mammals, J. Comp. Neurol. 467 (2003) 207–231.
[4] A. Forge, N.K. Marziano, S.O. Casalotti, D.L. Becker, D. Jagger, The inner ear contains heteromeric channels composed of cx26 and cx30 and deafness-related mutations in cx26 have a dominant negative effect on cx30, Cell Commun. Adhes. 10 (2003) 341–346.
[5] D.A. Gerido, T.W. White, Connexin disorders of the ear, skin, and lens, Biochim. Biophys. Acta 1662 (2004) 159–170.
[6] M.A. van Steensel, Gap junction diseases of the skin, Am. J. Med. Genet. C Semin. Med. Genet. 131C (2004) 12–19.
[7] D.P. Kelsell, W.L. Di, M.J. Houseman, Connexin mutations in skin disease and hearing loss, Am. J. Hum. Genet. 68 (2001) 559–568.
[8] R. Rabionet, N. Lopez-Bigas, M.L. Arbones, X. Estivill, Connexin mutations in hearing loss, dermatological and neurological disorders, Trends Mol. Med. 8 (2002) 205–212.
[9] S. Oh, J.B. Rubin, M.V. Bennett, V.K. Verselis, T.A. Bargiello, Molecular determinants of electrical rectification of single channel conductance in gap junctions formed by connexins 26 and 32, J. Gen. Physiol. 114 (1999) 339–364.
[10] H. Ripps, H. Qian, J. Zakevicius, Properties of connexin26 hemichannels expressed in *Xenopus* oocytes, Cell. Mol. Neurobiol. 24 (2004) 647–665.
[11] D. Gonzalez, J.M. Gomez-Hernandez, L.C. Barrio, Species specificity of mammalian connexin-26 to form open voltage-gated hemichannels, FASEB J. 20 (2006) 2329–2338.
[12] D.A. Gerido, A.M. Derosa, G. Richard, T.W. White, Aberrant hemichannel properties of Cx26 mutations causing skin disease and deafness, Am. J. Physiol., Cell Physiol. 293 (1) (2007) C337–C345.
[13] J. Santos-Sacchi, The temperature dependence of electrical coupling in the organ of Corti, Hear. Res. 21 (1986) 205–211.
[14] C. Zeilinger, M. Steffens, H.A. Kolb, Length of C-terminus of rCx46 influences oligomerization and hemichannel properties, Biochim. Biophys. Acta 1720 (2005) 35–43.
[15] A. Ngezahayo, C. Zeilinger, I.I. Todt, I.I. Marten, H. Kolb, Inactivation of expressed and conducting rCx46 hemichannels by phosphorylation, Pflugers Arch. 436 (1998) 627–629.
[16] R. Bruzzone, J.A. Haefliger, R.L. Gimlich, D.L. Paul, Connexin40, a component of gap junctions in vascular endothelium, is restricted in its ability to interact with other connexins, Mol. Biol. Cell 4 (1993) 7–20.
[17] B. Jedamzik, I. Marten, A. Ngezahayo, A. Ernst, H.A. Kolb, Regulation of lens rCx46-formed hemichannels by activation of protein kinase C, external Ca(2+) andprotons, J. Membr. Biol. 173 (2000) 39–46.
[18] M. Kamermans, I. Fahrenfort, K. Schultz, U. Janssen-Bienhold, T. Sjoerdsma, R. Weiler, Hemichannel-mediated inhibition in the outer retina, Science 292 (2001) 1178–1180.
[19] S.H. DeVries, E.A. Schwartz, Hemi-gap-junction channels in solitary horizontal cells of the catfish retina, J. Physiol. 445 (1992) 201–230.
[20] R.P. Malchow, H. Qian, H. Ripps, A novel action of quinine and quinidine on the membrane conductance of neurons from the vertebrate retina, J. Gen. Physiol. 104 (1994) 1039–1055.
[21] L. Ebihara, New roles for connexons, News Physiol. Sci. 18 (2003) 100–103.
[22] K.C. Young, C. Peracchia, Opposite Cx32 and Cx26 voltage-gating response to CO2 reflects opposite voltage-gating polarity, J. Membr. Biol. 202 (2004) 161–170.
[23] J.B. Rubin, V.K. Verselis, M.V. Bennett, T.A. Bargiello, A domain substitution procedure and its use to analyze voltage dependence of homotypic gap junctions formed byconnexins 26 and 32, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 3820–3824.
[24] Y.H. Chen, R.L. DeHaan, Temperature dependence of embryonic cardiac gap junction conductance and channel kinetics, J. Membr. Biol. 136 (1993) 125–134.
[25] V. Valiunas, H. Niessen, K. Willecke, R. Weingart, Electrophysiological properties of gap junction channels in hepatocytes isolated from connexin32-deficient and wild-type mice, Pflugers Arch. 437 (1999) 846–856.
[26] V. Valiunas, R. Weingart, Electrical properties of gap junction hemichannels identified in transfected HeLa cells, Pflugers Arch. 440 (2000) 366–379.
[27] A. Oshima, K. Tani, Y. Hiroaki, Y. Fujiyoshi, G.E. Sosinsky, Three-dimensional structure of a human connexin26 gap junction channel reveals a plug in the vestibule, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 10034–10039.
[28] J. Neijssen, C. Herberts, J.W. Drijfhout, E. Reits, L. Janssen, J. Neefjes, Cross-presentation by intercellular peptide transfer through gap junctions, Nature 434 (2005) 83–88.
[29] L. Ebihara, E. Steiner, Properties of a nonjunctional current expressed from a ratconnexin46 cDNA in *Xenopus* oocytes, J. Gen. Physiol. 102 (1993) 59–74.
[30] K. Venkatachalam, C. Montell, TRP Channels, Ann. Rev. Biochem. 76 (2007) 387–417.
[31] K. Zimmermann, A. Leffler, A. Babes, C.M. Cendan, R.W. Carr, J. Kobayashi, C. Nau, J.N. Wood, P.W. Reeh, Sensory neuron sodium channel Nav1.8 is essential for pain at low temperatures, Nature 447 (2007) 855–858.