J Bioenerg Biomembr (2008) 40:397–405
DOI 10.1007/s10863-008-9151-0

Phosphorylation in the C-terminus of the rat connexin46 (rCx46) and regulation of the conducting activity of the formed connexons

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Received: 1 February 2008 / Accepted: 16 May 2008 / Published online: 31 July 2008

Abstract To analyse the role of PKC-dependent phosphorylation in the C-terminus of rCx46 in regulation of rCx46 connexons, truncated mutants $rCx46_{45.3}$ and $rCx46_{44.2}$ which end before and after PKC-dependent phosphorylation sites respectively were generated. Both $rCx46_{45.3}$ and $rCx46_{44.2}$ formed connexons in *Xenopus* oocytes similar to $Cx46_{wt}$-connexons. They were activated by depolarisation above −40 mV and at voltages above 50 mV, inactivation was spontaneously observed or induced by PKC activator TPA, suggesting that inactivation does not require PKC-dependent phosphorylation in the C-terminus. Three casein-kinase-II-(CKII)-dependent phosphorylation sites were also identified. $rCx46_{37.7}$ and $rCx46_{28.2}$ respectively without two or all of these sites were generated. $rCx46_{37.7}$-connexons were similar to $rCx46_{wt}$-connexons. $rCx46_{28.2}$-connexons comparable to $rCx46_{wt}$-connexons were observed after injection of 50 times more $rCx46_{28.2}$-mRNA (25 ng per oocyte). CKII-blocker inhibited depolarisation-evoked currents in oocytes injected with 0.5 ng per oocyte $rCx46_{37.7}$-mRNA or $rCx46_{wt}$-mRNA. Injection of 25 ng per oocyte $rCx46_{37.7}$-mRNA or $rCx46_{wt}$-mRNA overcame the effect of CKII-inhibitor. We propose that CKII-dependent phosphorylation in the C-terminus accelerates formation of rCx46-connexons.

Keywords Connexin46 · Gap junction · Connexon · PKC · Casein kinase · TPA · Inactivation

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Introduction

Gap junction channels of vertebrates are composed of connexin-monomers (Cx) transcribed from a gene family with 19, 20 and 38 members by rodents, humans, and zebra fish, respectively (Willecke et al. 2002; Eastman et al. 2006). Different studies have shown that all connexin types have the same membrane topology: cytoplasmic N- and C-termini and four transmembrane domains (TM1–4) interconnected by one intracellular and two extracellular loops. Connexins share a large sequence homology in the N-terminus, the TM-domains and the extracellular loops. Variability is mostly found in the intracellular loop and particularly in the C-terminus, which represents in some connexins more than the half of the polypeptide. This region is believed to be involved in voltage, pH and phosphorylation modulation of the gap junction channel as well as in interaction with subsidiary proteins such as ZO, occludin, 14-3-3 proteins and cytoskeletal proteins (Morley et al. 1996; Ek-Vitorin et al. 1996; Calero et al. 1998; Cruciani and Mikalsen 2002; Thomas et al. 2002; Park et al. 2007).

Gap junction channels span the cytoplasmic space between adjacent cells. Thereby they sustain formation of physiological units by allowing exchange of ions and small metabolites between coupled cells. Contradicting old assumption, evidence has accumulated that unopposed gap junction connexons can be opened in different tissues such as the retina (DeVries and Schwarz 1992; Malchow et al. 1993; Kammermans et al. 2001). These findings show that it is necessary to characterise mechanisms regulating the connexons. External Ca^{2+} for instance has been characterised as all round regulator of gap junction connexons. All reports presented so far have shown that gap junction connexons are activated after reduction of the external Ca^{2+} concentration below 0.5 mM (DeVries and Schwarz 1992; Malchow et al. 1993; Kammermans et al.

2001). The ability of human or rodent Cx46 and its homologues such as chicken Cx56 (cCx56) and bovine Cx44 (bCx44) to form connexons was unequivocally demonstrated by experiments, which used *Xenopus* oocytes as heterologous expression system. It was shown that functional connexons were formed after injection of 0.2–1 ng mRNA of these connexins into *Xenopus* oocytes (Paul et al. 1991; Ebihara and Steiner 1993; Gupta et al. 1994; Ebihara et al. 1995). The capability of other connexins such as the lens Cx50, Cx26, Cx32 to form active connexons was also suggested (Zampighi et al. 1999; Castro et al. 1999; Ripps et al. 2004). In contrast to the Cx46 family, however, other connexins form connexons clearly distinguishable from the oocyte background in electrophysiological experiments, if the quantity of injected mRNA is increased to 10–25 ng per oocyte.

For rat Cx46 (rCx46), which was the first connexin shown to form voltage dependent connexons in *Xenopus* oocytes (Paul et al. 1991), it was observed that connexons were inactivated in a voltage dependent manner by activation of PKC (Ngezahayo et al. 1998; Jedamzik et al. 2000). To explain this regulation, a three states model was suggested (Jedamzik et al. 2000). Furthermore it was postulated that this inactivation could be related to direct PKC-dependent phosphorylation of the intracellular C-terminus, which constitutes more than half of the whole molecule (Ngezahayo et al. 1998; Jedamzik et al. 2000).

In this report, analysis of the amino acid sequence of the rCx46 C-terminus revealed the presence of two contiguous putative PKC-dependent phosphorylation sites between amino acids 348 and 410. Furthermore, casein kinase II phosphorylation sites were found along the C-terminus before the PKC-dependent phosphorylation sites. These findings support the hypothesis of a PKC-and CKII-dependent regulation of rCx46-connexons. This hypothesis is also in agreement with the observation that PKC-dependent phosphorylation regulates the conductance of gap junction channels composed of other connexins such as Cx43, Cx50, or Cx37. Furthermore an essential role of casein kinase-dependent phosphorylation for assembly of connexins such as Cx43 or rCx46 was suggested (Lampe and Lau 2004; Zeilinger et al. 2005).

It is shown here that by truncation of the C-terminus induction of the voltage dependent inactivation of rCx46 connexons is not related to PKC-dependent phosphorylation in the C-terminus. Additionally after ablation of all CKII-dependent phosphorylation sites, the quantity of the injected mRNA had to be increased by a factor of 50 from 0.5 to 25 ng per oocyte to form channels clearly recognizable above the background in electrophysiological measurements. The experiments revealed that CKII-dependent phosphorylation regulates formation of active rCx46 connexons. This regulation may take place at the level of either the hexamerisation of the connexin polypeptides into connexons in the ER, or the trafficking of the hexamerised connexons, or insertion of the connexons into the membrane or turnover of the connexins.

Material and methods

Molecular biology

The *Escherichia coli* strain TOP 10 (Invitrogen) was used to host the plasmid containing the connexin gene. Cells were cultivated with plasmids and grown at 37 °C in Luria Bertani medium (LB) containing ampicillin at a concentration of 100 μg/ml. The $rCx46_{wt}$ and mutant transcripts were generated via PCR, cloned in the pCR2.1-TOPO vector (Invitrogen) and subcloned in the pGEMHE vector for RNA transcription. pGEMHE contains 250 bp of non-coding sequence from *Xenopus* β-globin including a poly-A tract that increases translational efficiency (Krieg and Melton 1984; Liman et al. 1992). mRNA was prepared using a synthesis kit containing SP6 RNA polymerase and CAP analogue purchased from Ambion (Austin, USA). The vector was linearised with *Nhe*I for mRNA transcription. Transcript concentration was estimated spectrophotometrically and analysed on agarose gels.

Expression of $rCx46_{wt}$-and mutants-connexons in *Xenopus* oocytes

Stage V and stage VI *Xenopus* oocytes were harvested from anaesthetized frog and defolliculated by mechanical disruption and a 1.5 h treatment with 120–150 U/ml collagenase Type II (Worthington, Berlin Germany) in OR2 solution consistent of (mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 10 HEPES, pH 7.4 and adjusted with sorbitol to 240 mosmol/l. Isolated oocytes were conserved at 17 °C in ND96 solution containing (mM) 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, pH 7.4 and 240 mosmol/l. The oocytes could be used for 4 days. If not otherwise stated, oocytes were injected with 23 nl of a solution containing connexin mRNA (25 ng/μl) and antisense to the endogenous Cx38 (400 ng/μl). The antisense with the following sequence 5′-GCT GTG AAA CAT GGC AGG ATG-3′ was purchased form Tib Molbiol GmbH (Berlin, Germany). For injection, the Nanoliter Injector (World Precision Instruments, Berlin; Germany) was used. The oocytes were incubated for at least 12 h at 17 °C in ND96 for expression.

Electrophysiological measurements

Recordings of macroscopic currents were performed from single *Xenopus* oocytes 12–36 h after mRNA injection. The

oocytes were transferred into a perfusion chamber containing a bath solution composed of (mM): 100 KCl, 2 $MgCl_2$, 0.2 mM $CaCl_2$, 30 Sorbitol, 10 MES, pH 7.4, 245 mosmol/l at room temperature. The expressed connexons were analysed by two-electrode-voltage clamp (TEV) as described previously (Jedamzik et al. 2000) by use of a voltage-clamp amplifier Turbo TEC-10 CD (npi electronic, Tamm, Germany) connected to a computer via an ITC-16 interface (Instrutech, Port Washington, USA). From a constant holding voltage of −90 mV, test potential pulses were applied from −105 mV to +75 for 20 s in 15 mV steps. The voltage-evoked currents were filtered at 0.125 kHz and sampled at 1 kHz. Pulse protocols, data acquisition, and analysis were performed using Pulse/PulseFit (HEKA, Lambrecht/Pfalz, Germany) and OriginPro 7.5 (Microcal Software, Inc, Northampton, USA). The effect of PKC-dependent phosphorylation of the gap junction connexons was tested by application of the PKC stimulators OAG or TPA as well as the PKC inhibitor calphostin C (Calbiochem-Novabiochem GmbH Schwalbach am Taunus, Germany). The effect of CKII dependent phosphorylation was studied by use of 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) the CKII inhibitor II (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany).

Data analysis

To analyse the voltage dependence of original rCx46-and mutant Cx46-connexons, amplitudes of the steady state current at the end of the voltage pulse were measured and plotted against the corresponding voltage in an I(V) plot. To describe the voltage dependence of the conductance of the connexons, the macroscopic conductance (G) was calculated from the steady state current amplitudes and plotted against the corresponding voltages ($G(V)$ curves). To estimate the activation parameters of the connexons, obtained data points were fitted with a simple Boltzmann distribution as given by the following equation: $G(V)=(A/(1+\exp(-(V-V_{1/2})zF/RT)+B)$. R, T, F have their usual meanings, $V_{1/2}$ represents the half-activation voltage at which 50% of the maximal conductance is reached, z represents the number of apparent equivalent gating charges. The parameter A gives the maximal macroscopic conductance of the connexons, B represents the non voltage-dependent leak conductance. The $G(V)$ curves were normalized to the derived fits by setting $A=1$ and $B=0$.

Results

Previous results have shown that gap junction connexons composed of rCx46 were modulated by PKC-dependent phosphorylation (Ngezahayo et al. 1998; Jedamzik et al. 2000). Sequence analysis allowed identification of two contiguous putative PKC-dependent phosphorylation sites at the end of the C-terminus (Fig. 1). We hypothesised that the observed PKC-dependent inactivation of rCx46-connexons was due to a direct PKC-dependent phosphorylation of the rCx46 C-terminus. In order to verify this hypothesis, we generated $rCx46_{45.3}$ and $rCx46_{44.2}$ by truncation at A409 and P398 respectively. The truncation at A409 to obtain $rCx46_{45.3}$ did not alter the PKC dependent phosphorylation sites. On the contrary, the generation of $rCx46_{44.2}$ by truncation at P398 eliminated both PKC dependent phosphorylation motifs between S401 and R408 (Fig. 1). The generated cDNA mutants as well as the cDNA of wild type were used for in vitro synthesis of corresponding mRNA. Twenty-three nanoliter of a solution containing 0.5 ng of connexin mRNA and 23 ng of the antisense of the endogenous Cx38 (Anti-Cx38) were injected in *Xenopus* oocytes. Activity of expressed connexons was studied by the two-electrode voltage-clamp (TEV) technique 12–36 h after injection. From holding potential of −90 mV, voltage pulses from −105 mV to 75 mV were applied in 15 mV steps for 20 s (Fig. 2a) and the evoked currents (Fig. 2b) were registered.

$rCx46_{wt}$ formed gap junction connexons which were activated when the oocytes were depolarised above −40 mV, whereas hyperpolarising voltages closed the connexons (Fig. 2b). The voltage dependency of connexons is also shown by the steady state current voltage plot ($I(V)$) in which the amplitude of the steady state current (I_{ss}) at the end of the voltage pulse was plotted against the corresponding voltage (Fig. 2c). In some oocytes, a voltage dependent inactivation of the formed $rCx46_{wt}$ connexons could be observed. This inactivation was characterised by a linear increase of the current amplitude with the applied voltage in voltages between −15 mV and 45 mV and a less pronounced increase of the evoked current as the voltage was further increased

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MGDWSFLGRLLENAQEHSTVIGKVWLTVLFIFRILVLGAAAEEVWGDEQS  50
DFTCNTQQPGCENVCYDRAFPISHIRFWALQIIFVSTPTLIYLGHVLHIV 100
RMEEKKKEREEELLRRDNPQHGRGREPMRTGSPRDPPLRDDRGKVRIAGA 150
LLRTYVFNIIFKTLFEVGFIAGQYFLYGFQLQPLYRCDRWPCPNTVDCFI 200
SRPTEKTIFVIFMLAVACASLVLNMLEIYHLGWKKLKQGVTN FNPDASE 250
VRHKPLDPLSEAANSGPPSVSIGLPPYYTHPACPTVQAKATGFPGAPLLP 300
ADFTVVTLNDAQGRGHPVKHCNGHHLTTEQNW SLGAEPQTPASKPSSAA 350
SSPHGRKGLTDSSGSSLEESALVVTPEGEQALATTVEMHSPPLVLLD ER 400
SSKSSSGR RPGDLAI.                                   416
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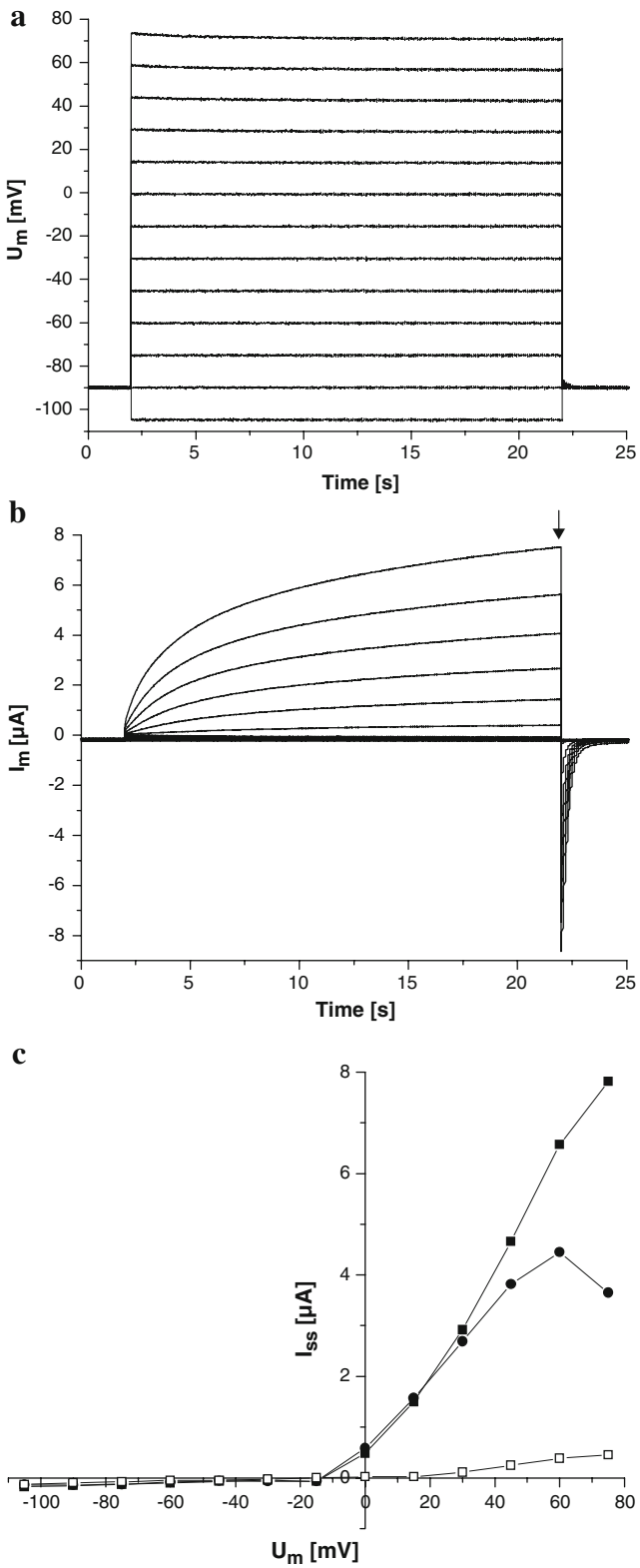
Fig. 1 Amino acid sequence of rCx46. PKC-dependent phosphorylation motifs in a *white box*. CKII-dependent phosphorylation motifs are indicated by a *black box*. S132, a putative PKC-dependent phosphorylation site in the intracellular loop is indicated by *bold type*. The terminal amino acids for the mutants $rCx46_{45.3}$, $rCx46_{44.5}$, $rCx46_{37.7}$, and $rCx46_{28.2}$ are indicated as *white letters* in a *grey box*. The four transmembrane domains are *underlined*



◀ **Fig. 2** **a** Voltage pulses applied to the oocytes under two-electrode-voltage clamp technique. **b** The corresponding currents evoked by the voltage application. The *arrow* indicates the position at which the steady state current (I_{ss}) was measured for the I(V) plot. **c** Example of I(V) plot of (*empty squares*) control oocyte which was injected with the anti-Cx38, (*filled squares*) oocyte which was injected with $Cx46_{wt}$ and in which inactivation was not observed and (*filled circles*) oocyte which was injected with $rCx46_{wt}$ and showed spontaneous voltage dependent inactivation

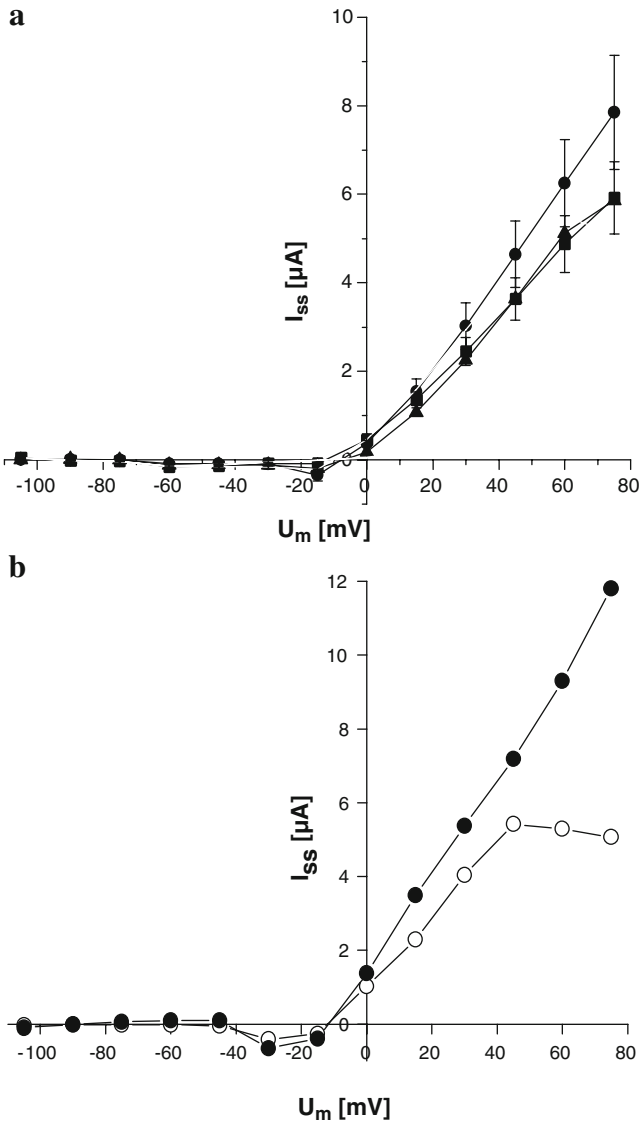


Fig. 3 **a** I(V) plots obtained from oocyte expressing (*filled squares*) $rCx46_{wt}$, (*filled upright triangles*) $rCx46_{45.3}$ and (*filled circles*) $rCx46_{44.2}$. The results are average±SEM for at least five experiments for each type of connexin. For $rCx46_{45.3}$ and $rCx46_{44.2}$, voltage dependent inactivation was spontaneously observed or induced by the PKC activator TPA. **b** A representative example of I(V) plot obtained from oocyte expressing $rCx46_{44.2}$ which did not show voltage dependent inactivation (*filled circles*). Application of TPA induced voltage dependent inactivation (*empty circles*)

resulting in a kink in the *I*(*V*) curve (Fig. 2c). Since this inactivation has been correlated with activation of PKC (Ngezahayo et al. 1998; Jedamzik et al. 2000), application of PKC activator TPA could induce the voltage dependent

Table 1 The activation parameter as estimated by Boltzmann fitting of the G(V) plot

Connexin	V1/2 [mV]	*z*
$Cx46_{wt}$	−10.0±16.7	2.03±0.57
$Cx46_{45.3}$	−2.9±7.4	1.88±0.27
$Cx46_{44.2}$	1.1±10.3	1.89±0.07
$Cx46_{37.7}$	−6.9±12.0	2.25±0.66
$Cx46_{28.2}$	−7.1±7.9	2.25±0.43

The data are average±SEM for at least $n=4$ for each connexin

inactivation in oocytes that primarily did not show this inactivation. In parallel, application of the PKC inhibitor Calphostin C could suppress the voltage dependent inactivation in oocyte where this voltage dependent inactivation was spontaneously observed (result not shown).

$rCx46_{45.3}$ as well as $rCx46_{44.2}$ formed connexons which behaved similar to the $rCx46_{wt}$ connexons. They showed a voltage dependence (Fig. 3a) and a PKC related induction of voltage dependent inactivation (Fig. 3b). When the voltage dependent inactivation was observed, it could be suppressed by the PKC inhibitor Calphostin C (result not shown). In oocytes where the voltage dependent inactivation was not primarily observed, it could be induced by application of PKC activator TPA (Fig. 3b). The similarity of the connexons formed by the $rCx46_{wt}$ and the mutants is also reflected in the activation parameters estimated from the Boltzmann fit of the voltage conductance $G(V)$ plot (Fig. 6, Table 1). Half activation voltage ($V_{1/2}$) of −10.0± 16.7 mV, −2.9±7.4 and 1.1±10.4 mV as well as apparent gating charge z of 2.03±0.57, 1.88±0.27 and 1.89±0.06 were estimated for $rCx46_{wt}$, $rCx46_{45.3}$ and $rCx46_{44.2}$ respectively (Table 1).

Other kinases such as casein kinase are known to phosphorylate connexins (reviews: Lampe and Lau 2000, 2004). In the C-terminus of rCx46, three putative CKII-dependent phosphorylation site could be found at positions 384, 365 and 307 (Fig. 1). Two mutants, $rCx46_{37.7}$ and $rCx46_{28.2}$, which were truncated behind A333 and H243 respectively were generated. $rCx46_{37.7}$ contains one CKII

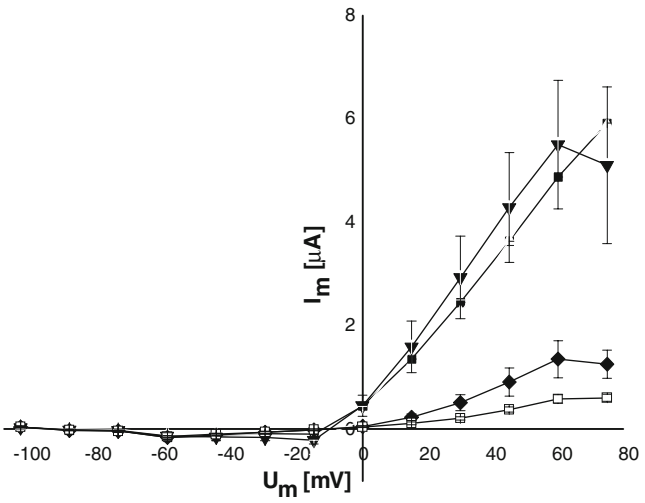


Fig. 4 I(V) plots obtained from oocytes expressing $rCx46_{wt}$ (*filled squares*), $rCx46_{37.7}$ (*filled inverted triangles*), $rCx46_{28.2}$ (*filled diamonds*) and control oocytes which were injected with anti-Cx38. The cells were always injected with 0.5 ng connexin-mRNA/oocytes. With this quantity, $rCx46_{wt}$ as well as $rCx46_{37.7}$ formed connexons, which could clearly be observed above the background, whereas $rCx46_{28.2}$ formed connexons, which could not be distinguished from the background. It is noteworthy that $rCx46_{37.7}$ showed spontaneous inactivation at large depolarising voltages. The results are averages± SEM for at least five experiments for each type of connexin

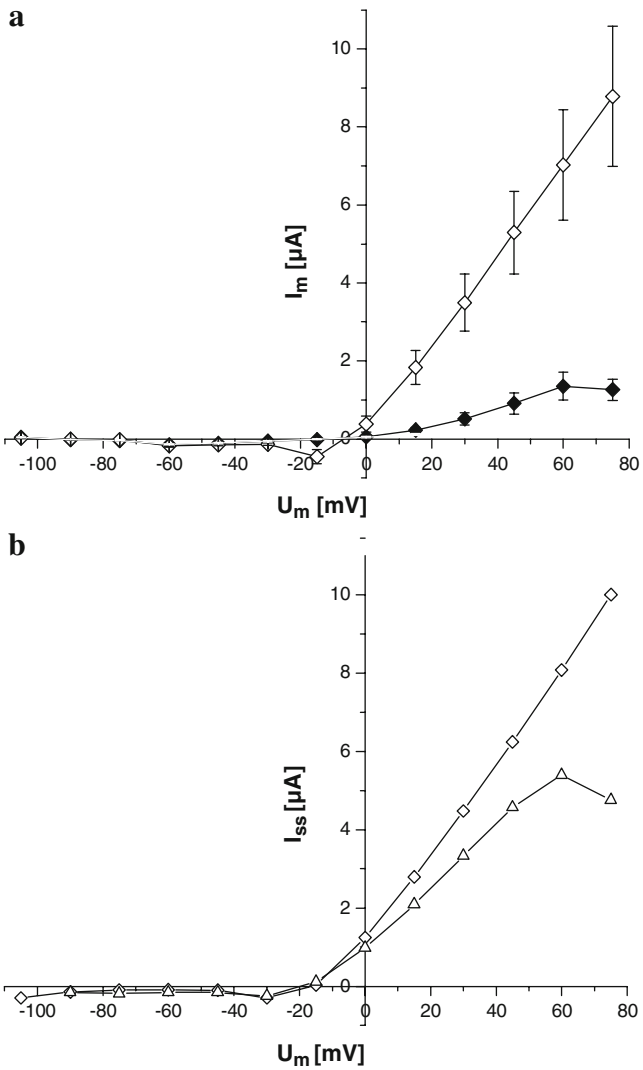


Fig. 5 I(V) plot obtained from oocytes which were injected with $rCx46_{28.2}$. **a** Oocytes were injected with 0.5 ng/oocyte (*filled diamonds*) and 25 ng/oocyte (*empty diamonds*) The results are averages±SEM for at least five experiments for each type of connexin. **b** A demonstrative example of TPA stimulated induction of voltage dependent inactivation of $Cx46_{28.2}$ connexons. I(V) plot obtained under control conditions (*empty diamonds*) and 30 min after application of 50 nM TPA (*empty upright triangles*)

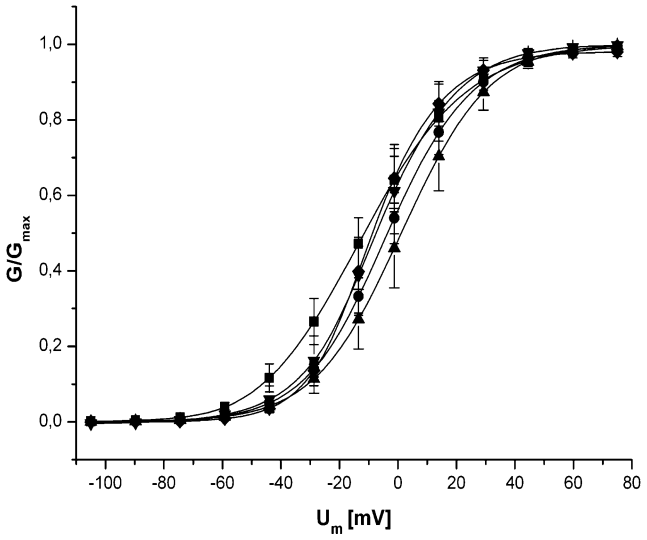


Fig. 6 G(V) plot obtained from oocytes expressing $rCx46_{wt}$ (*filled squares*), $rCx46_{45.3}$ (*filled circles*), $rCx46_{44.2}$ (*filled upright triangles*), $rCx46_{37.7}$ (*filled inverted triangles*), and $rCx46_{28.2}$ (*filled diamonds*). For $rCx46_{28.2}$, oocytes injected with 25 ng were used to estimate the conductance. The points represent averages±SEM for at least four different experiments for each connexin

dependent phosphorylation site whereas $rCx46_{28.2}$ contains no CKII dependent phosphorylation motif anymore (Fig. 1). Moreover $rCx46_{28.2}$ has approximately the same amino acid sequence length as the human or rodent Cx26, which is known to be not phosphorylated (Traub et al. 1989). $rCx46_{37.7}$ formed connexons comparable to those formed by $rCx46_{wt}$ in terms of voltage dependency (Fig. 4 and 6). Moreover, like $rCx46_{wt}$, spontaneous voltage dependent inactivation could still be observed in some oocytes (Fig. 4). Again in oocytes which showed spontaneous inactivation, it could be suppressed by Calphostin C and it could be induced by TPA in oocytes which did not show the spontaneous inactivation. For $rCx46_{28.2}$, the voltage dependent gap junction current could not be detected by TEV applied on oocytes which were injected with the same quantity of mRNA (0.5 ng per oocyte) as done for expression of $rCx46_{wt}$ and the other generated rCx46 mutants (Figs. 4 and 5a). Recent publications have shown that injection of 25 ng of hCx26 mRNA in *Xenopus* oocytes produced gap junction connexons which could be detected by TEV (Ripps et al. 2004; Gonzalez et al. 2006). Since $rCx46_{28.2}$ has about the same length as hCx26, the amount of injected $rCx46_{28.2}$-RNA was increased by about 50 times to 25 ng per oocyte. Those cells showed voltage dependent currents with amplitudes comparable to those observed in oocytes injected with 0.5 ng mRNA of $rCx46_{wt}$ (Fig. 5a). These $rCx46_{28,2}$ connexons were sensitive to the stimulator of PKC (Fig. 5b) and showed similar activation parameter as $rCx46_{wt}$ and the other generated rCx46 mutants (Fig. 6 and Table 1). Since the ablation of all CKII-dependent phosphorylation positions seemed to interfere with the formation of the connexons, it was tested whether pharmacological blockade of CKII would have an influence on the formation of connexons by $rCx46_{37.7}$ or $Cx46_{wt}$. Oocytes were injected with 0.5 ng mRNA of $rCx46_{37.7}$ or $Cx46_{wt}$ and incubated in presence of 10 µM of the CKII inhibitor 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) during expression period (12–18 h). At this concentration, DMAT is specific for CKII (Pagano et al. 2004). The expressed connexons were measured with TEV. It was observed that the presence of DMAT during connexon expression strongly reduced the current amplitude evoked by a depolarisation to 60 mV of the oocyte expressing $rCx46_{37.7}$ (Fig. 7) or $Cx46_{wt}$ (result not shown). By increasing the quantity of the injected mRNA to 25 ng per oocytes, currents with amplitude comparable to the control

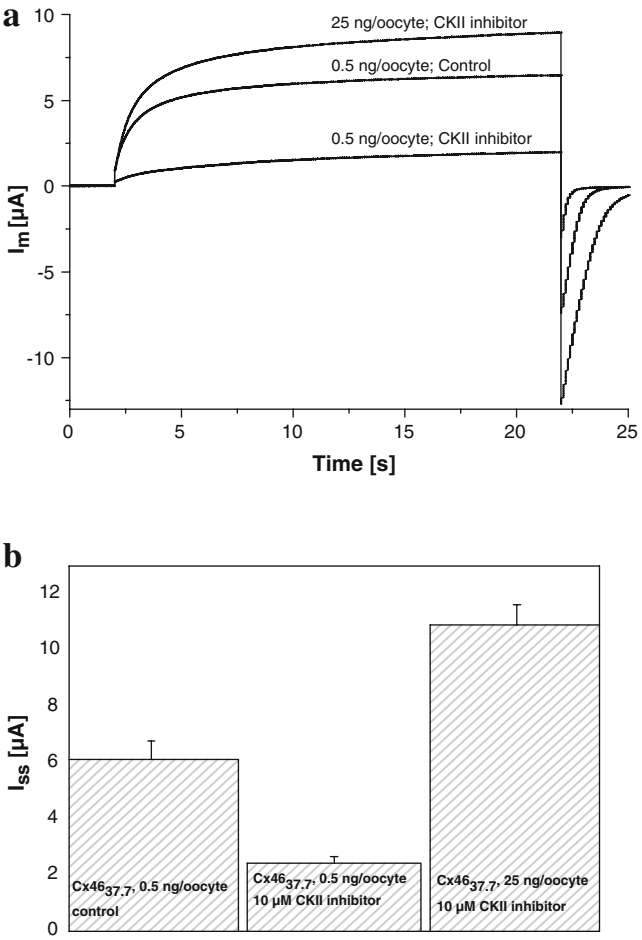


Fig. 7 **a** Representative currents evoked by depolarisation of oocytes to 60 mV, which were injected with the $rCx46_{37.7}$. The quantity of mRNA that was injected and the incubation conditions during the expression time are stated. **b** The amplitude of currents as measured in **a**. The *columns* represent average±SEM for at least six independent experiments for each treatment. The amplitudes are significantly different ($p<0.01$: student *t*-test). Comparable results were obtained for $Cx46_{wt}$

experiments could be evoked even if the oocytes were incubated with DMAT during the expression phase (Fig. 7). It is noteworthy that DMAT did not affect the formation of $rCx46_{28.2}$ connexons after injection of 25 ng mRNA/oocyte (result not shown).

Discussion

Formation of active connexons by truncated rCx46

In this report, it is shown that $rCx46_{45.3}$, $rCx46_{44.2}$, $rCx46_{37.7}$ and even $rCx46_{28,2}$ formed active connexons in *Xenopus* oocytes. The results represent an experimental demonstration for the assumption that the bulk of the C-terminus is not essential in assembly, trafficking to the plasma membrane and formation of gap junctions (Saez et al. 2003; Evans et al. 2006). The results however contradict recently published observation by Zeilinger et al. (2005) that ablation of the last seven amino acids in the C-terminus to produce $rCx46_{45.3}$ strongly reduced the capability of the rCx46 to form active gap junction connexons. The electrophysiological results from truncated connexin by Zeilinger et al. (2005) were obtained after injection into oocytes of mRNA synthesized from PCR product which did not contain the non coding sequence for *Xenopus* β-globin and the poly-A tract (Zeilinger personal communication). Both the non coding sequence for *Xenopus* β-globin and the poly-A tract are known to increase the efficiency of translation (Krieg and Melton 1984; Liman et al. 1992). Therefore the results by Zeilinger et al. (2005) are not comparable to the results obtained from the $rCx46_{wt}$ or to the results of the present report which were obtained after injection of mRNAs containing the non coding sequence for *Xenopus* β-globin and the poly-A tract.

PKC-dependent phosphorylation of the C-terminus is not involved in induction of voltage dependent inactivation of rCx46-connexons

The present report shows that suppression of the PKC-dependent phosphorylation positions in the C-terminus of the rCx46 does not alter the inactivation related to PKC stimulation (Fig. 3). Even ablation of almost the whole C-terminus was not able to suppress the PKC related induction of voltage dependent inactivation (Figs. 4b and 5b). These results confirm our previous result that stimulation of PKC is involved in induction of voltage dependent inactivation of rCx46 connexons (Ngezahayo et al. 1998; Jedamzik et al. 2000). The results contradict however our previous hypothesis that the PKC related voltage dependent inactivation was a result of a direct PKC-dependent phosphorylation of the rCx46 C-terminus (Ngezahayo et al. 1998; Jedamzik et al. 2000). How the PKC-dependent induction of voltage dependent inactivation of rCx46 connexons is achieved can only be a matter of speculation. S132 in the intracellular loop (Fig. 1) is a PKC-dependent phosphorylation position. It was observed that TPA stimulated phosphorylation of a comparable serine residue at position 118 in the intracellular loop of cCx56 the chicken homologous of rCx46 (Berthoud et al. 1997). The observed PKC-dependent induction of voltage dependent inactivation of wild type as well as the truncated rCx46-connexons could therefore be related to phosphorylation at S132. Comparison studies of these connexins combined with point mutation analysis are needed for more clarification. Additionally, it is known that connexins interact with accessory proteins such as calmodulin, ZO, protein 14-3-3, occludin and integrin, which form dynamic scaffold complexes that function as platform for assembly of signalling (Cruciani and Mikalsen 2002; Thomas et al. 2002; Park et al. 2007). It is tempting to speculate that this dynamic association of proteins and rCx46 could be regulated by PKC-dependent phosphorylation and thereby could participate in induction of the voltage dependent inactivation of the connexons. Combination of biochemical, molecular biological, pharmacological and physiological studies far beyond the present report are needed to clarify the mechanisms on the basis of the PKC related regulation of the rCx46-connexons.

Casein kinase dependent phosphorylation in C-terminus of rCx46 tunes rCx46 connexon formation

Ablation of the whole C-terminus reduced the voltage dependent currents (Fig. 5a). Many findings in the literature show that different connexins form gap junction connexons in expression systems or in the natural cellular environment. The human and rodent lens fibre Cx46 and its homologues bovine Cx44 and chicken Cx56 differ from the other connexins because they form active connexon after injection of a minimal quantity of mRNA. After injection of 0.2–1 ng per oocyte, Cx46 and its homologues form connexons, with currents clearly distinguishable from the oocyte background (Paul et al. 1991; Ebihara and Steiner 1993; Gupta et al. 1994; Ebihara et al. 1995; Fig. 2). In comparison other connexins such as human Cx26, Cx32, and Cx50 or the zebra fish Cx35, only formed connexons recognisable above the oocyte background after injection of 10–50 ng mRNA per oocyte (Castro et al. 1999; Zampighi et al. 1999; Chappell et al. 2003; Ripps et al. 2004). Since $rCx46_{28.2}$ which was reduced to the length of Cx26 did not form connexons distinguishable above the oocyte background after injection of 0.5 ng per oocyte $rCx46_{28.2}$-mRNA (Fig. 4a), we hypothesised that an increase of the amount of injected mRNA to the quantity observed for Cx26 (Ripps et al. 2004; Steffens et al. 2008) would yield

connexons recognisable above the background. The result of the experiments in which TEV was applied on oocytes injected with 25 ng mRNA of $Cx46_{28.2}$ support this hypothesis (Fig. 5a). Three hypothesis can be postulated to explain this observation. (a) Formation of connexons in the membrane by polypeptides translated from the short mRNA ($rCx46_{28.2}$) needs a large amount of connexin polypeptides for either hexamerisation, trafficking, or insertion of the connexons in the membrane. (b) The connexons are formed in the membrane but the open probability of the single channels is reduced. (c) The connexons are formed with a reduced single channel conductance. If the open probability or the conductance of single channels were the problem, it would be reasonable to assume, that activation parameters estimated from the Boltzmann fitting of the voltage dependent conductance would be affected. Figure 6 and Table 1 show however that neither the half activation voltage $V_{1/2}$ nor the apparent gating charge z of the rCx46 connexons or those formed by the generated mutants significantly differed, suggesting that the open probability or the conductance of the single channels are not affected. As for the formation of active connexons, three CKII kinase-dependent phosphorylation sites in the C-terminus were identified (Fig. 1). The ablation of the C-terminus to generate $Cx46_{28.2}$ with a comparable amino acid sequence length as Cx26 correlated with the ablation of all CKII-dependent phosphorylation sites. It is therefore tempting to postulate that CKII-dependent phosphorylation is involved in either the hexamerisation of the rCx46 connexins, or the trafficking of the hexamerised connexons or the insertion of the connexons in the membrane. To approach this hypothesis, *Xenopus* oocytes injected with 0.5 ng mRNA for $rCx46_{37.7}$ or $rCx46_{wt}$ were incubated during the expression period and electrophysiological experiments in presence of 10 μM CKII inhibitor DMAT. The inhibition of CKII strongly reduced the current evoked by depolarisation to 60 mV (Fig. 7). Interestingly an increase of the amount of injected mRNA to 25 ng per oocyte restored the amplitude of depolarisation-evoked currents (Fig. 7). In absence of DMAT, oocytes injected with 25 ng $rCx46_{37.7}$ or $Cx46_{wt}$ mRNA showed huge currents which deteriorated the oocytes. Comparable pharmacological results were observed for $rCx46_{wt}$. Furthermore DMAT did not affect the formation of active $Cx46_{28.2}$ connexons after injection of 25 ng mRNA into the oocytes. These pharmacological results suggest that the effect observed by $Cx46_{28.2}$ are not related to the reduction of the length of the connexins but to activity of CKII. This result also suggests that a total ablation of CKII dependent phosphorylation interferes with the formation of active rCx46 connexons. This hypothesis is in agreement with the suggestion by Zeilinger et al. (2005) who showed that casein kinase was important for hexamerisation of rCx46 using a biochemical assay. Additionally, it was shown that while connexons of other connexins such as Cx43, Cx32 are transported along the endoplasmic reticulum and Golgi apparatus to reach the plasma membrane, Cx26-connexons travelled directly from the ER into the membrane without the passage in Golgi apparatus (George et al. 1999). Since $Cx46_{28.2}$, which is similar to Cx26, formed connexons under conditions comparable to those of Cx26 (Fig. 5a; Ripps et al. 2004; Gonzalez et al. 2006; Steffens et al. 2008) and comparable results could be obtained for $Cx46_{37.7}$ (Fig. 7) or $Cx46_{wt}$ by inhibition of the CKII-dependent phosphorylation (Fig. 7), it is tempting to speculate that $Cx46_{28.2}$ or non phosphorylated $Cx46_{37.7}$ may form connexons which take the same trafficking pathway as Cx26-connexons. From the results presented in this report and in agreement with the assertion that specific amino acids in the C-terminus control the oligomerisation of connexins and the intracellular trafficking of connexons (Saez et al. 2003; Evans et al. 2006), it can also be postulated that the CKII-dependent phosphorylation facilitates and thereby accelerates either the hexamerisation of the rCx46 connexins, or a correct trafficking of the hexamerised connexons or the insertion of the formed connexons in the membrane. The identification of CKII-dependent phosphorylation motifs in the published sequences of bCx44 (Gupta et al. 1994) and cCx56 (Rupp et al. 1993) the homologues of rCx46 known to form active connexons in oocytes after injection of 0.2–1 ng per oocyte support this hypothesis. Additionally, it is shown that the CKII-dependent phosphorylation at position T307 suffices to accelerate the formation of active rCx46 connexons (Fig. 7). Whether the CK-II dependent phosphorylation of this single site is essential or whether there is a redundancy in CKII-dependent phosphorylation sites is not known and should be examined in the near future.

Acknowledgments The authors thank Dr. David Paul; Harvard Medical School for providing the rCx46 cDNA, Iva Maxwell for proofreading the manuscript and Ingrid Buchwald for excellent technical support.

The work was partly supported by the project NANOTOME; Biophotonik III.

Dr. Zeilinger was supported by the Helmholtzgemeinschaft Virtual Institute for Biological Structure Research (VIBS); VH-VI-013.

References

Berthoud VM, Beyer EC, Kurata WE, Lau AF, Lampe PD (1997) The gap-junction protein connexin 56 is phosphorylated in the intracellular loop and the carboxy-terminal region. Eur J Biochem 244:89–97

Calero G, Kanemitsu M, Taffet SM, Lau AF, Delmar M (1998) A 17mer peptide interferes with acidification-induced uncoupling of connexin43. Circ Res 82:929–935

Castro C, Gomez-Hernandez JM, Silander K, Barrio LC (1999) Altered formation of connexons and gap junction channels caused by c-terminal connexin-32 mutations. J Neurosci 19:3752–3760

Chappell RL, Zakevicius J, Ripps H (2003) Zinc modulation of connexon currents in *Xenopus* oocytes. Biol Bull 205:209–211

Cruciani V, Mikalsen SO (2002) Connexins, gap junctional intercellular communication and kinases. Biol Cell 94:433–443

DeVries SH, Schwarz BA (1992) Hemi-gap-junction channels in solitary horizontal cells of the catfish retina. J Physiol 445: 201–230

Eastman SD, Chen THP, Falk MM, Mendelson TC, Iovine MK (2006) Phylogenetic analysis of three complete gap junction gene families reveals lineage-specific duplications and highly supported gene classes. Genomics 87:265–274

Ebihara L, Steiner E (1993) Properties of a nonjunctional current expressed from a rat connexin46 cDNA in *Xenopus* oocytes. J Gen Physiol 102:59–74

Ebihara L, Berthoud VM, Beyer EC (1995) Distinct behavior of connexin56 and connexin46 gap junctional channels can be predicted from the behavior of their hemi-gap-junctional channels. Biophys J 68:1796–1803

Ek-Vitorin J, Calero G, Morley GE, Coombs W, Taffet SM, Delmar M (1996) pH regulation of connexin43: molecular analysis of the gating particle. Biophys J 71:1273–1284

Evans WH, De Vuyste E, Leybaert L (2006) The gap junction cellular internet: connexin connexons enter the signalling limelight. Biochem J 397:1–14

George CH, Kendall JM, Evans WH (1999) Intracellular trafficking pathways in the assembly of connexins into gap junctions. J Biol Chem 274:8678–8685

Gonzalez D, Gomez-Hernandez JM, Barrio LC (2006) Species specificity of mammalian connexin-26 to form open voltage-gated connexons. FASEB J 20:2329–2338

Gupta VK, Berthoud VM, Atal N, Jarillo JA, Barrio LC, Beyer EC (1994) Bovine connexin44, a lens gap junction protein: molecular cloning, immunologic characterization, and fundamental expression. Invest Ophthalmol Vis Sci 35:3747–3758

Jedamzik B, Marten I, Ngezahayo A, Ernst A, Kolb HA (2000) Regulation of lens rCx46-formed connexons by activation of protein kinase C, external Ca^{2+} and protons. J Membr Biol 173:39–46

Kamermans M, Fahrenfort I, Schultz K, Janssen-Bienhold U, Sjoerdsma T, Weiler R (2001) connexon-mediated inhibition in the outer retina. Science 292:1178–1180

Krieg PA, Melton DA (1984) Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. Nucleic Acids Res 12:7057–7070

Lampe PD, Lau AF (2000) Regulation of gap junctions by phosphorylation of connexins. Arch Biochem Biophys 384:205–215

Lampe PD, Lau AF (2004) The effects of connexin phosphorylation on gap junctional communication. Int J Biochem Cell Biol 36: 1171–1186

Liman ER, Tytgat J, Hess P (1992) Subunit stoichiometry of a mammalian K^+ channel determined by construction of multimeric cDNAs. Neuron 98:861–871

Malchow RP, Qian H, Ripps H (1993) Evidence for hemi-gap junctional channels in isolated horizontal cells of the skate retina. J Neurosci Res 35:237–245

Morley GE, Taffet SM, Delmar M (1996) Intramolecular interactions mediate pH regulation of connexin43 channels. Biophys J 70:1294–1302

Ngezahayo A, Zeilinger C, Todt I, Marten I, Kolb HA (1998) Inactivation of expressed and conducting rCx46 connexons by phosphorylation. Pflügers Arch 436:627–629

Pagano MA, Meggio, F, Ruzzene M, Andrzejewska M, Kazimierczuk Z, Pinna LA (2004) 2-Dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole: a novel powerful and selective inhibitor of protein kinase CK2. Biochem Biophys Res Commun 321:1040–1044

Park DJ, Freitas TA, Wallick CJ, Guyette CV, Warner-Cramer BJ (2007) Molecular dynamics and in vitro analysis of connexin43: a new 14-3-3 mode-1 interacting protein. Protein Sci 15:2344–2355

Paul DL, Ebihara L, Takemoto LJ, Swenson KI, Goodenough DA (1991) Connexin46, a novel lens gap junction protein, induces voltage-gated currents in nonjunctional plasma membrane of *Xenopus* oocytes. J Cell Biol 115:1077–1089

Ripps H, Qian H, Zakevicius J (2004) Properties of connexin26 connexons expressed in *Xenopus* oocytes. Cell Mol Neurobiol 24:647–667

Rup DM, Veenstra RD, Wang HZ, Brink PR, Beyer EC (1993) Chick connexin-56, a novel lens gap junction protein. Molecular cloning and functional expression. J Biol Chem 268:706–712

Saez JC, Berthoud VM, Branes MC, Martinez AD, Beyer EC (2003) Plasma membrane channels formed by connexins: their regulation and functions. Physiol Rev 83:1359–1400

Steffens M, Göpel F, Ngezahayo A, Zeilinger C, Ernst A, Kolb HA (2008) Regulation of connexons composed of human connexin26 (hCx26) by temperature. Biochim Biophys Acta 1778:1206–1212

Thomas MA, Huang S, Cokoja A, Riccio O, Staub O, Suter S, Chanson M (2002) Interaction of connexins with protein partners in the control of channel turnover and gating. Biol Cell 94:445–456

Traub O, Loo J, Dermietzel R, Brümmer F, Hülser D, Willecke K (1989) Comparative characterization of the 21-kD and the 26-kD gap junction proteins in murine liver and cultured hepatocytes. J Cell Biol 108:1039–1051

Willecke K, Eiberger J, Degen J, Eckardt D, Romualdi A, Guldenagel M, Deutsch U, Sohl G (2002) Structural and functional diversity of connexin genes in the mouse and human genome. Biol Chem 383:725–737

Zampighi GA, Loo DDF, Kreman M, Eskandari F, Wright EM (1999) Functional and morphological correlates of connexin50 expressed in *Xenopus laevis* Oocytes. J Gen Physiol 113:507–523

Zeilinger C, Steffens M, Kolb HA (2005) Length of C-terminus of rCx46 influences oligomerization and connexon properties. Biochim Biophys Acta 1720:35–43