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

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Abstract To analyse the role of PKC-dependent phosphorylation in the C-terminus of rCx46 in regulation of rCx46 connexons, truncated mutants rCx4645.3 and rCx4644.2 which end before and after PKC-dependent phosphorylation sites respectively were generated. Both rCx4645.3 and rCx4644.2 formed connexons in Xenopus oocytes similar to Cx46wt-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. rCx4637.7 and rCx4628.2 respectively without two or all of these sites were generated. rCx4637.7-connexons were similar to rCx46wt-connexons. rCx4628.2-connexons comparable to rCx46wt-connexons were observed after injection of 50 times more rCx4628.2-mRNA (25 ng per oocyte). CKII-blocker inhibited depolarisation-evoked currents in oocytes injected with 0.5 ng per oocyte rCx4637.7-mRNA or rCx46wt-mRNA. Injection of 25 ng per oocyte rCx4637.7-mRNA or rCx46wt-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

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 inactivation 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 hexam erised 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<sub>wt</sub> 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 polyA 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 *NheI* for mRNA transcription. Transcript concentration was estimated spectrophotometrically and analysed on agarose gels.

**Expression of rCx46<sub>wt</sub>-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<sub>2</sub>, 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<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 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 MgCl₂, 0.2 mM CaCl₂, 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 (Microlab 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 determine the voltage-dependent leak conductance. 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 (Microlab 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). 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 (Microlab 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).

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 rCx46₄₄.₅ and rCx46₄₄.₂ by truncation at A409 and P398 respectively. The truncation at A409 to obtain rCx46₄₄.₅ did not alter the PKC dependent phosphorylation sites. On the contrary, the generation of rCx46₄₄.₂ 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 anti-sense 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.

cx46₄₄.₅ 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 (Iₛₛ) 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 cx46₄₄.₅ 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.
resulting in a kink in the \( I(V) \) curve (Fig. 2c). Since this inactivation has been correlated with activation of PKC (Ngezhay et al. 1998; Jedamzik et al. 2000), application of PKC activator TPA could induce the voltage dependent inactivation.
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).

rCx4645.3 as well as rCx4644.2 formed connexons which behaved similar to the rCx46wt 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 rCx46wt 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 \(-10.0\pm16.7\) mV, \(-2.9\pm7.4\) and \(1.1\pm10.4\) mV as well as apparent gating charge \(z\) of \(2.03\pm0.57, 1.88\pm0.27\) and \(1.89\pm0.06\) were estimated for rCx46wt, rCx4645.3 and rCx4644.2 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, rCx4637.7 and rCx4628.2, which were truncated behind A333 and H243 respectively were generated. rCx4637.7 contains one CKII

<table>
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<th>Connexin</th>
<th>(V_{1/2} [\text{mV}])</th>
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<td>Cx46wt</td>
<td>(-10.0\pm16.7)</td>
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<tr>
<td>Cx4645.3</td>
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<tr>
<td>Cx4628.2</td>
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The data are average±SEM for at least \(n=4\) for each connexin

Fig. 4 I(V) plots obtained from oocytes expressing rCx46wt (filled squares), rCx4637.7 (filled inverted triangles), rCx4628.2 (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, rCx46wt as well as rCx4637.7 formed connexons, which could clearly be observed above the background, whereas rCx4628.2 formed connexons, which could not be distinguished from the background. It is noteworthy that rCx4637.7 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 rCx4628.2. 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 Cx4628.2 connexons. I(V) plot obtained under control conditions (empty diamonds) and 30 min after application of 50 nM TPA (empty upright triangles)
dependent phosphorylation site whereas rCx4628.2 contains no CKII dependent phosphorylation motif anymore (Fig. 1). Moreover rCx4628.2 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). rCx4637.7 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 rCx4628.2, 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 rCx4628.2 has about the same length as hCx26, the amount of injected rCx4628.2-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 rCx4628.2 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 rCx4637.7 or Cx46 wt. Oocytes were injected with 0.5 ng mRNA of rCx4637.7 or Cx46 wt, and incubated in presence of 10 µM of the CKII inhibitor 2-dimethylamino-4,5,6,7-tetramethylimidazole (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 rCx4637.7 (Fig. 7) or Cx46 wt (result not shown). By increasing the quantity of the injected mRNA to 25 ng per oocyte, currents with amplitude comparable to the control

![Fig. 6](Image) (V) plot obtained from oocytes expressing rCx46 wt (filled squares), rCx4637.7 (filled circles), rCx4628.2 (filled triangles), and rCx4644.2 (filled diamonds). For rCx4628.2, 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.

![Fig. 7](Image) a Representative currents evoked by depolarisation of oocytes to 60 mV, which were injected with the rCx4637.7. 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 rCx4628.2 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 rCx4645.3, rCx4644.2, rCx4637.7 and even rCx4628.2 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 rCx4645,3 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 Cx56 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; Zampighi et al. 1999; Chappell et al. 2003; Ripps et al. 2004). Since rCx4628.2 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 rCx4628.2-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
Effect observed by Cx4628.2 are not related to the reduction of oocytes. These pharmacological results suggest that the injected with 25 ng mRNA of Cx4628.2 support this hypothesis. In the experiments in which TEV was applied on oocytes connexons recognisable above the background. The result of the experiments in which TEV was applied on oocytes injected with 25 ng mRNA of Cx4628.2 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 (rCx4628.2) 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 Cx4628.2 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 rCx4637.7 or rCx46wt 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 in 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 rCx4637.7 or Cx46wt mRNA showed huge currents which deteriorated the oocytes. Comparable pharmacological results were observed for rCx46wt. Furthermore DMAT did not affect the formation of active Cx4628.2 connexons after injection of 25 ng mRNA into the oocytes. These pharmacological results suggest that the effect observed by Cx4628.2 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 Cx4628.2, 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 Cx4617.7 (Fig. 7) or Cx4637.7 by inhibition of the CKII-dependent phosphorylation (Fig. 7), it is tempting to speculate that Cx4628.2 or non phosphorylated Cx4637.7 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.

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