Pflügers Arch – Eur J Physiol (1998) 436:627–629

SHORT COMMUNICATION

A. Ngezahayo · C. Zeilinger · I. Todt · I. Marten
H.-A. Kolb

Inactivation of expressed and conducting rCx46 hemichannels by phosphorylation

Received: 13 March 1998 / Received after revision and accepted: 18 June 1998

Abstract Hemichannels of rat connexin 46 (rCx46) were expressed in *Xenopus laevis* oocytes and analysed by two-electrode voltage-clamp experiments. It is established that rCx46 hemichannels can be activated at low external Ca^{2+} and positive membrane potentials. Upon larger depolarizations, the hemichannels of oocytes activate in a time-dependent manner, occasionally followed by a spontaneous inactivation. We found that, in the absence of inactivation, treatment of oocytes with 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), an activator of protein kinase C (PKC), reversibly reduced the amplitude of the rCx46-mediated current and, after an incubation time of about 30 min, induced inactivation of the voltage-dependent current. After wash-out of OAG the corresponding membrane conductance increased and the inactivation behaviour disappeared. The OAG-induced inactivation, as well as the spontaneous inactivation, could be removed by application of the specific PKC inhibitor calphostin C and also by phloretin. The data provide evidence that the activation and inhibition of PKC affect the rCx46-mediated membrane conductance as well as the voltage-dependent current inactivation in an inverse manner.

Key words Connexin46 expression · Hemichannel · Inactivation · 1-Oleoyl-2-acetyl-*sn*-glycerol (OAG) · Phosphorylation · Protein kinases · *Xenopus* oocytes

Introduction

Connexins in rodents are members of a family of at least 13 homologous proteins which oligomerize to form cell-type-specific hexameric hemichannels [1]. Plasma membranes of adjacent eukaryotic cells can form gap junction channels by head to head association between hemichannels. At least two different connexin genes are expressed in all cell types studied to date. The correlation between the functional plasticity of gap junction coupling and connexin diversity is not known at present [8]. Therefore, only knowledge of the properties of hemichannels comprised of a single type of connexin will allow to elucidate the function of the different connexins contributing to the hemichannels within a cell.

The expression of cloned connexin DNAs in paired *Xenopus* oocytes that exhibit distinct transjunctional voltage sensitivities and cell-to-cell channel conductances of functional homotypic connexin channels was first reported 1987 by Dahl (for review see [1, 8]). Recently, the elucidation of the complex structure-function relationship of cell-to-cell channels has become even more simple through the analysis of single cells with expressed hemichannels. In general the unapposed hemichannels stay in their closed configuration under physiological incubation conditions. A low external [Ca^{2+}] and depolarization have been identified as keys to the opening of rCx46 hemichannels expressed in *Xenopus* oocytes, which allows the analysis of voltage gating behaviour [2, 5, 7]. Further more, a decrease in the conductance was observed at large depolarizing voltages [7] which we attribute to voltage-dependent current inactivation. For the lens fibre protein rCx46 a posttranslational phosphorylation has been identified as an additional gating mechanism by immunoprecipitation [3].

In the present study voltage-jump current-relaxations were performed using rCx46 hemichannels expressed in *Xenopus* oocytes. To examine the role of protein kinases in the modulation of membrane conductance and voltage-dependent inactivation, rCx46 hemichannels were analysed as a function of the phosphorylation state. The effect of a specific phosphorylating treatment was examined by application of a protein kinase C (PKC) activator (1-oleoyl-2-acetyl-*sn*-glycerol, OAG), a specific PKC inhibitor (calphostin C) and a protein kinase inhibitor (phloretin).

Materials and methods

Expression of rCx46 cRNA in *Xenopus* oocytes

The *Escherichia coli* strain TOPF' (Invitrogen) was used to host the plasmid and connexin gene. Strains were cultivated with plasmids and bacteria were grown at 37°C in Luria Bertani medium (LB) containing ampicillin/methicillin at concentrations of 20/80 μg ml^{-1}.

A. Ngezahayo · C. Zeilinger · I. Todt · I. Marten · H.-A. Kolb (✉)
Institut für Biophysik, Universität Hannover,
Herrenhäuser Strasse 2, D-30419 Hannover, Germany
e-mail: kolb@mbox.biophysik.uni-hannover.de
Tel.: +49-511-7622608, Fax: +49-511-7622606

The rCx46 transcript was supplied in the SP64T vector for RNA transcription. SP64T contains 250 bp of the non-coding sequence from *Xenopus* β-globin including a poly-A tract that increases translational efficiency. RNA was prepared by using a synthesis kit containing SP6 RNA polymerase and CAP analogue purchased from Ambion (Austin, USA). rCx46 was linearized with *Xba*I for RNA transcription. Transcript concentration was estimated spectrophotometrically and analysed on agarose gels. Stage V and stage VI *Xenopus* oocytes were isolated and defolliculated by collagenase treatment (5 mg ml^{-1}, 355 U mg^{-1}, 1.5 h; Worthington, Type 2) in Ca^{2+}-free ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, Na-HEPES pH 7.4 and adjusted with sorbitol to 240 mosmol/l). Oocytes were injected with 26 nl (Nanoliter Injector, World Precision Instruments) of RNA solution containing 2.5 ng transcript and incubated at 16°C in ND96 supplemented with 1 mM $CaCl_2$ and antibiotics (100 U ml^{-1} penicillin/streptomycin). For control experiments the RNA solution was injected together with 24 nl of DNA antisense to the endogenous *Xen*Cx38 oligo 5′-gCT gTG AAA CAT ggC Agg Atg (8 pmol/µl) (Tib Molbiol).

Electrophysiological recordings

Voltage-clamp recordings of macroscopic currents from single *Xenopus* oocytes were performed 1–5 days after RNA injection. Both voltage- and current-recording micropipettes were filled with 3 M KCl (tip resistance, 1–1.5 MΩ). The control bath solution was nominally Ca^{2+}-free and contained 100 mM KCl, 20 mM sorbitol, 2 mM $MgCl_2$ 10 mM MES/Tris adjusted to pH 7.7. Currents were measured using an amplifier (Turbo TEC 10 CD (NPI)) Voltage protocols were applied by using a Pentium 100 MHz computer coupled to an ITC-16 interface (Instrutech). Currents were filtered at 1 kHz and sampled at 0.5 kHz. Data acquisition and analysis were performed by Pulse and Pulsefit (Heka).

Results and discussion

Voltage-jump current-relaxations of rCx46 hemichannels expressed in *Xenopus laevis* are shown in Fig. 1. At holding potentials negative to –65 mV the hemichannels stay in the closed configuration, but activate at more positive voltages (Fig. 1A). Occasionally, current inactivation was observed at high depolarizing voltages (>50 mV) as shown in Fig. 1A. However, for a selected oocyte the occurrence of inactivation could not be predicted. The presence or absence of voltage-dependent inactivation of expressed rCx46 hemichannels was found to be a stable phenomenon during recording times of more than 1 h. The large currents of Fig. 1A are not seen in non-injected oocytes or in oocytes injected only with DNA antisense to the endogenous oocyte connexin *Xen*Cx38. No significant change of the current recordings was found by co-injecting rCx46 cRNA and *Xen*Cx38 antisense.

To study the underlying mechanism of inactivation oocytes that showed spontaneously voltage-dependent current inactivation at high positive voltages were selected. When 200 nM calphostin C was added to the bath the current amplitude increased and the inactivating behaviour disappeared (Fig. 1B). All of the selected oocytes (n=5) responded similarly to the application of calphostin C. The minimal time lag after which 200 nM calphostin C induced complete suppression of the spontaneously occurring voltage-dependent inactivation was about 45 min at 70 mV. The lowest effective calphostin C concentration was found to be about 100 nM. After wash-out of calphostin C the current and the corresponding membrane conductance remained unchanged within the experimental error (see also

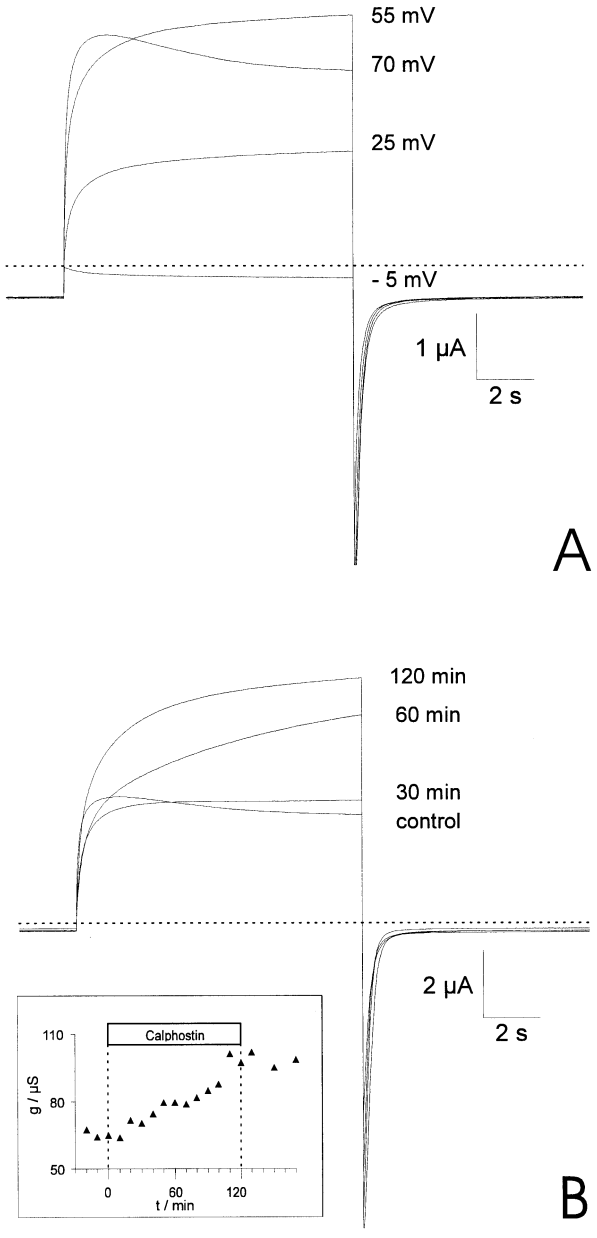

Fig. 1A–C Representative voltage-dependent current-relaxations recorded from *Xenopus* oocytes expressing rat connexin 46 (*rCx46*) hemichannels. **A** Macroscopic currents evoked by test potentials in the range from –110 to 70 mV from a holding potential of –90 mV in 15-mV increments. For clearer presentation the current response to –5 mV, 25 mV, 55 mV and 70 mV are presented as indicated. The holding and test potentials were applied for 10 s. Repolarization to –90 mV produces inward tail currents. **B** Time sequence of current relaxation after addition of 200 nM calphostin C to the bath. For clearer presentation only the voltage-jump current-relaxation after a step to 70 mV is given. The recording time is indicated in minutes. The *inset* shows the corresponding time course of membrane conductance (g) in the presence and absence of 200 nM calphostin C. For estimation of the membrane conductance the quasi-stationary current values were taken from the corresponding set of voltage-jump current-relaxations (see Fig. 1A) yielding a membrane current/voltage (*I/V*) relation. The membrane conductance was derived from the linear part of this *I/V* relation at positive voltages and corrected for the leak conductance. For clearer presentation the peak of some tail currents was cut off

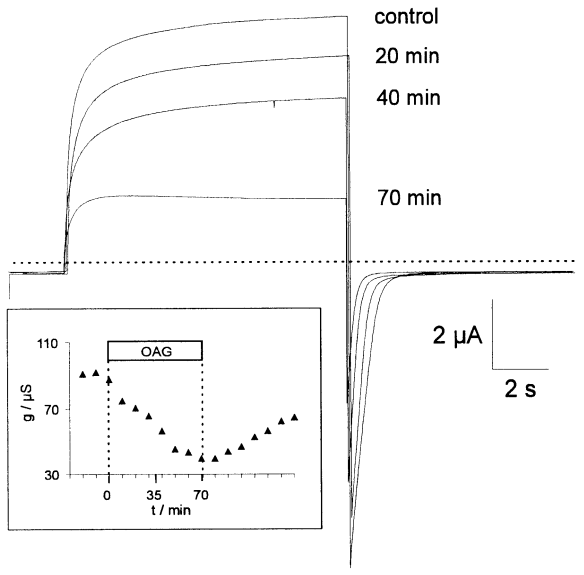

Fig. 2 Effect of 1-oleoyl-2-acetyl-*sn*-glycerol (*OAG*) on the voltage-jump current-relaxation. For clearer presentation the current trace recorded at a test potential of 70 mV was selected from the measured sequence of voltage-jump current relaxations (see Fig. 1A). The current relaxation evoked in the absence OAG is denoted as "control". The recording times after application of OAG are given in minutes. The corresponding time course of membrane conductance (*g*) is given in the *inset*. The oocyte was incubated for 70 min with 200 µM OAG as indicated and washed-out thereafter by superfusion with control bath. The membrane conductance was derived as described in the legend of Fig. 1B. The experiments were performed on one oocyte. The variability of tail currents will be examined in a forthcoming paper

inset of Fig. 1B). Non-injected oocytes did not respond to application of 200 nM calphostin C. Addition of 200 nM of the non-specific protein kinase inhibitor phloretin caused a similar effect as calphostin C (data not shown). It is noteworthy that in the absence of a spontaneously occurring inactivating behaviour neither calphostin C nor phloretin affected the shape and amplitude of current relaxations.

To establish that, in contrast, activation of phosphorylation induces an inactivation behaviour of rCx46 hemichannels, we selected oocytes that did not exhibit voltage-dependent inactivation and added 200 µM OAG, a known activator of PKC, to the bath. The current amplitude (Fig. 2) and the corresponding membrane conductance (see inset of Fig. 2) decreased and after about 30 min voltage-dependent inactivating behaviour became visible. After application of OAG for about 40 min a stationary current/voltage relationship was obtained. This time span is in the same range as that observed for changes of gap junction conductance in coupled cells by OAG-dependent phosphorylation [6]. The threshold potential of 55 mV, above which a significant voltage-dependent inactivation appeared, was similar to that observed during spontaneously occurring inactivation (Figs. 1A, 2). The OAG-mediated effect is reversible after wash-out (see inset of Fig. 2). Voltage-dependent current inactivation could not be induced solely by long incubation times of up to 1 h in control bath solution. After wash-out of OAG the observed increase in the corresponding membrane conductance resembles the finding observed after suppression of PKC activation (see insets of Figs. 1, 2). Such an increase in conductance could be caused by a rise of the total number of rCx46 hemichannels available for activation, the single-channel conductance and/or of the absolute channel open probability. Both the OAG-mediated as well as the calphostin C- or phloretin-induced change of voltage-jump current-relaxations were independent of the extracellular pH in the range of 6.6 to 7.7.

The OAG-induced decrease of membrane conductance and voltage-dependent current inactivation could be explained by assuming that unapposed rCx46 hemichannels adopt various subconductance states and/or a reduced channel open probability depending on their phosphorylation state. It is known that cell-to-cell channels composed of different members of the connexin family [1] react differently upon phosphorylation treatments. Various conductance states related to various phosphorylation treatments have been observed for cell-to-cell channels of Cx43. Activation of PKC decreases their permeability, whereas the corresponding membrane conductance increases [4]. The latter result is opposite to our finding for rCx46 hemichannels. In line with the presented observations we found previously that the OAG treatment of isolated pancreatic acinar cell pairs which contain Cx32 as major connexin population OAG reduced the gap junction conductance [6].

The presented data provide evidence that the phosphorylation state of *Xenopus* oocytes affects the conductance and voltage-dependent inactivation of expressed and unopposed rCx46 hemichannels. Further experiments at the single hemichannel level are necessary to elucidate the corresponding regulatory pathways of specific phosphorylation as well as their target.

Acknowledgements The authors would like to thank Dr. D. Paul (Harvard Medical School, Boston) for the generous supply of pSP64T and M. Kraus for her excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (Ko 626/8–1).

References

1. Bruzzone R, White TW, Paul DL (1996) Connections with connexins: the molecular basis of direct intercellular signaling. Eur J Biochem 238:1–27
2. Ebihara L, Berthoud VM, Beyer EC (1995) Distinct behaviour of Connexin56 and Connexin46 gap junctional channels can be predicted from their behaviour of their hemi-gap-junctional channels. Biophys J 68:1796–1803
3. Jiang JX, Paul DL, Goodenough DA (1993) Posttranslational phosphorylation of lens fiber connexin46: a slow occurrence. Invest Opththalmol Vis Sci 34:3558–3565
4. Kwak BR, Van Veen TAB, Analbers LJS, Jongsma HJ (1995) TPA increases conductance but decreases permeability in neonatal rat cardiomyocyte gap junction channels. Expl Cell Res 220: 456–463
5. Paul DL, Ebihara L, Takemoto LK, Swenson KI, Goodenough DA (1991) Connexin 46, a novel lens gap junction protein, induces voltage-gated currents in nonjunctional plasma membrane of *Xenopus* oocytes. J Cell Biol 115:1077–1089
6. Somogyi R, Batzer A, Kolb HA (1989) Inhibition of electrical coupling in pairs of murine pancreatic cells by OAG and isolated protein kinase C. J Membr Biol 108:273–282
7. Trexler EB, Bennett MVL, Bargiello TA, Verselis VK (1996) Voltage gating and permeation in a gap junction hemichannel. Proc Natl Acad Sci USA 93:5836–5841
8. Willecke K, Haubrich S (1996) Connexin expression systems: to which extend do they reflect the situation in the animal? J Bioenerg Biomembr 28:319–326