J Membrane Biol (2011) 244:21–33
DOI 10.1007/s00232-011-9393-3

Single Cysteines in the Extracellular and Transmembrane Regions Modulate Pannexin 1 Channel Function

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Received: 3 June 2011 / Accepted: 26 August 2011 / Published online: 21 September 2011

Abstract Pannexins form high-conductance ion channels in the membranes of many vertebrate cells. Functionally, they have been associated with multiple functional pathways like the propagation of calcium waves, ATP release, responses to ischemic conditions and apoptosis. In contrast to accumulating details which uncovered their functions, the molecular mechanisms for pannexin channel regulation and activation are hardly understood. To further elucidate regulatory mechanisms, we substituted cysteine residues, expected key elements for channel function, in extracellular and transmembrane regions of Pannexin 1 (Panx1). Most apparently, substitution of the transmembrane cysteine C40 resulted in constitutively open channels with profoundly increased activity. Hence, *Xenopus laevis* oocytes injected with corresponding cRNA showed strongly impaired viability, anomalous dye uptake and greatly increased whole-cell conductivity. All changes induced by C40 substitution were significantly reduced by the Panx1 channel blocker carbenoxolone, indicating that channel activity of the mutated Panx1 had been affected. In contrast, no changes occurred after substitution of the two other transmembrane cysteines, C215 and C227, in terms of channel conductivity. Finally, substitution of any of the four extracellular cysteines resulted in complete loss of channel function in both *X. laevis* oocytes and transfected N2A cells. From this, we conclude that cysteine residues of Panx1 reveal differential functional profiles for channel activation and drug sensitivity.

Keywords Membrane protein · Site-directed mutagenesis · Hemichannel · Pannexin · Channel activity

Introduction

Proteins from the pannexin family (Panx1–3) form membrane channels that allow permeation of ions and small molecules in many vertebrate cells (Panchin 2005; Barbe et al. 2006; Dahl and Locovei 2006; D'hondt et al. 2009; MacVicar and Thompson 2010). Although they are capable of forming gap junctions when experimentally expressed in *Xenopus laevis* oocytes, Panx channels seem to remain unpaired in vivo (Bruzzone et al. 2003; Locovei et al. 2006a; Zoidl et al. 2007; Sosinsky et al. 2011).

Studies to elucidate Panx channel function have mainly focused on Panx1. From these studies, it has been concluded that Panx1 channels are involved in the initiation and propagation of Ca^{2+} waves (Locovei et al. 2006b) and serve as ATP release channels (Bao et al. 2004a). Panx1 function is also discussed in the context of apoptosis (Chekeni et al. 2010), neuronal cell death after ischemia (Thompson et al. 2006; Zhang et al. 2008), activation of the inflammasome (Silverman et al. 2009) or tumor suppression (Lai et al. 2007). In heterologous expression systems, Panx1 channels are inactive under resting conditions (Bruzzone et al. 2003, 2005). Channel opening can be

Electronic supplementary material The online version of this article (doi:10.1007/s00232-011-9393-3) contains supplementary material, which is available to authorized users.

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induced by intracellular depolarization (>+20 mV), rise of the intracellular Ca^{2+} concentration, mechanical stress during osmotic shock, activation of purinergic receptors (Locovei et al. 2006a) and truncation of part of the C terminus (Chekeni et al. 2010).

Redox regulation has been proposed to be one key element for the modulation of Panx1 channel activity that associates Panx1 channels with neuronal cell death under ischemic conditions (Thompson et al. 2006; Zhang et al. 2008). Because the regulation of channel gating in response to metabolic inhibition critically involves intracellular cysteines in Cx43 hemichannels (Retamal et al. 2006), similar mechanisms may be expected in pannexins. In a previous study, we demonstrated that replacement of intracellular cysteine 346 in Panx1 by serine leads to a constitutively active channel that induces rapid cell degeneration when expressed in *X. laevis* oocytes or mammalian N2A cells. In contrast, after similar cysteine-to-serine exchanges of intracellular cysteine C136 or C426, electrophysiological properties or cell viability remained unaltered (Bunse et al. 2010). This result, which was confirmed recently (Wang and Dahl 2010), indicates that Panx1 channel function critically depends on some, but not all, cysteine residues, a result that strongly supports in-depth mapping of cysteine residues, which we addressed in the present study.

Besides the three intracellular cysteines, Panx1 contains seven more cysteines, three in the transmembrane domains (C40, C215 and C227) and two in each extracellular loop (C66, C84, C245 and C264; Suppl. Fig. 1). In connexins, extracellular cysteines play a critical role in the formation of gap junctions (Dahl et al. 1991, 1992). Since Panx1 channels do not form gap junctions in vivo, other functions must be assumed for the extracellular cysteines in Panx1. Thus, one might speculate that the extracellular and/or the transmembrane cysteine residues may act as target sites for channel modulation by redox signaling or alternatively may be important for channel gating.

In a recent study aimed to identify the Panx1 pore-lining domains using the substituted cysteine accessibility method in *X. laevis* oocytes, the effects of replacement of the endogenous cysteine residues on Panx1 channel function were briefly mentioned (Wang and Dahl 2010). Thus, it was reported that oocytes expressing the transmembrane mutant Panx1-C40S showed reduced viability and that replacement of the extracellular cysteines resulted in nonfunctional channels.

To expand the insight into the significance of cysteine residues for Panx1 function, we studied membrane insertion and conduction properties of Panx1 mutant channels. We replaced all four extracellular and three transmembrane cysteine residues by serine using site-directed mutagenesis and ectopically expressed the mutants in N2A cells in addition to *X. laevis* oocytes. In both expression systems, we observed distinct changes of Panx1 channel function. The transmembrane mutant C40S induced a constitutively open Panx1 channel similar to the intracellular mutant C346S described earlier (Bunse et al. 2010; Wang and Dahl 2010). Mutation of all four extracellular cysteines resulted in nonfunctional channels, as revealed from a lack of conductivity. Finally, the transmembrane mutants Panx1-C215S and -C227S were not notably different from wild type (wt) in terms of conductivity and their behavior toward the reducing agent Tris(2-carboxyethyl) phosphine (TCEP).

Materials and Methods

Plasmids and Site-Directed Mutagenesis

For site-directed single-cysteine mutagenesis of Panx1, the previously described plasmids pEGFP-N3-Panx1 and pCS2+-Panx1 (Bunse et al. 2010) were used as target plasmids. The Transformer Site-Directed Mutagenesis Kit (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) was applied according to the manufacturer's protocol, and the following mutagenesis primers were used: C40S (5′-ggacaagatggtcacat**C**tattgccgtggg-3′), C66S (5′-ggtaccca gataagc**A**gcttctccccgagttc-3′), C84S (5′-cctttgtggattcatac**A**g ctgggctgctgtacag-3′), C215S (5′-catgaaatacattagct**C**ccggct ggtgacatttgtgg-3′), C227S (5′-ggttatactgttggcat**C**tatctacttga gctattacttcagc-3′), C245S (5′-ctcggacgagtttctg**A**gcagcatcaa atcaggcg-3′) and C264S (5′-ccccgatcgcttccag**A**gcaagctcatc gccgtgg-3′). To enable selection of mutated pCS2+-Panx1 plasmids, a selection primer mutating the *Bam*HI restriction site was used (5′-gctacttgttctttttgcagg**T**tcccatcgattcg-3′), while the *Not*I restriction site was mutated (5′-gtacaagtaaag cggc**G**gcgactctagatc-3′) in the pEGFP-N3-Panx1 plasmid.

Synthesis of cRNA

For in vitro transcription of the pCS2+-Panx1 plasmid and its mutated variants, plasmid DNA was linearized with *Not*I and purified using the PCR Purification Kit (Qiagen, Hilden, Germany). cRNA was synthesized using the mMESSAGE mMACHINE SP6 Kit (Ambion, Applied Biosystems, Austin, TX) according to the manufacturer's instructions. The quantity of cRNAs was determined by UV absorbance (260 nm), and the quality was checked by agarose gel electrophoresis. The concentration was adjusted to 1 μg/μl with water.

Oocyte Injection

The automated oocyte injection and recording system Roboocyte (Multichannel Systems, Reutlingen, Germany) was

used for injection of cRNA (approximately 50 nl, 1 μg/μl) into defolliculated *X. laevis* oocytes obtained from EcoCyte Bioscience (Castrop-Rauxel, Germany). After injection, oocytes were kept at 17°C in Barth's solution (EcoCyte Bioscience) containing (in mM) NaCl 88, $NaHCO_3$ 2.4, KCl 1, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, $MgSO_4$ 0.82 and Tris 5 (pH 7.4) supplemented with 50 mg/l gentamicin.

Cell Culture and Transfection

Culture conditions of N2A cells were described previously (Zoidl et al. 2002). In brief, 10,000 cells were seeded on coverslips placed in 24-well plates and transfected 1 day after plating using 200 ng DNA per plasmid and the transfection reagent Effectene (Qiagen) according to the manufacturer's protocol. Medium was changed 1 day after transfection.

Dye Uptake Oocytes

Oocytes were injected with cRNA either for Panx1 wt or for Panx1-C40S. One-half of each set of oocytes was incubated in regular Barth's solution and the other half in Barth's solution containing 50 μM carbenoxolone (Cbx). Uptake of a fluorescent dye to monitor channel activity was performed as described previously (Bunse et al. 2010). Dye uptake was quantified by measuring the mean fluorescence intensity of each oocyte. Regions of interest covering the whole vegetale pole but excluding the equator were drawn in ImageJ (http://rsb.info.nih.gov/ij), and the mean gray-scale intensity was determined.

Immunocytochemistry of Injected Oocytes

Oocytes were fixed with 2% paraformaldehyde for 10 min 2–3 days after injection. After washes in PBS, oocytes were frozen in tissue freezing medium (Jung, Nussloch, Germany) at −60°C. Using a Leica (Heidelberg, Germany) CM 3050 cryostat, 12-μm-thick sections were cut and subsequently mounted on superfrost microscope slides (Menzel-Gläser; Thermo Fisher Scientific, Braunschweig, Germany). Immunoreactivity for Panx1 was analyzed with an anti-Panx1 4515 antibody from chicken (Locovei et al. 2006b) and visualized by a goat-anti-chicken IgY coupled to alexa488 (Molecular Probes, Invitrogen, Carlsbad, CA).

Pull-Down of Glycosylated Membrane Proteins from Injected Oocytes

Pull-downs of membrane proteins labeled with concanavalin A were carried out as described previously (Bunse et al. 2010). The generated probes were analyzed by Western blots, and immunodetection was performed using the chicken anti-Panx1 4515 antibody (Locovei et al. 2006b) as primary antibody and a polyclonal goat anti-chicken antibody tagged with Cy5.5 (Abcam, Cambridge, UK) as secondary antibody.

Confocal Microscopy

Images of transfected N2A cells and immunostained sections of injected oocytes were obtained using a confocal microscope (LSM 510 Meta system; Zeiss, Göttingen, Germany).

Oocyte Recordings

Two-electrode voltage-clamp recordings from injected oocytes were carried out using the Roboocyte system 2–3 days after RNA injection. Recording pipettes were filled with 2.5 M potassium acetate, and oocytes were continuously superfused with normal frog Ringer solution (EcoCyte Bioscience) containing (in mM) NaCl 90, KCl 2, $CaCl_2$ 2, $MgCl_2$ 1 and HEPES 5 (pH 7.4). Oocytes were voltage-clamped to −70 mV and currents monitored in response to 2-s voltage steps (from −100 to +60 mV, in 20-mV increments). Drugs were applied using the Roboocyte gravity-based eight-channel perfusion system. Acquisition, display and storage of data were controlled by the Roboocyte system.

N2A Cell Recordings

Transfected N2A cells were continuously superfused with oxygenated external solution containing (in mM) NaCl 125, KCl 2.5, NaH_2PO_4 1.23, $NaHCO_3$ 25, $MgCl_2$ 1.28, $CaCl_2$ 2, ascorbate 0.4 and glucose 12.5 at a rate of 2 ml/min. Recording pipettes were filled with internal solution containing (in mM) NaCl 147, HEPES 10, EGTA 10 and $MgCl_2$ 3. Whole-cell patch-clamp recordings were carried out using an Axopatch 200B amplifier (Molecular Devices, Wokingham, UK). Signals were filtered by a Humbug noise eliminator (Digitimer, Welwyn Garden City, UK) and digitized at a sampling rate of 10 kHz with WinWCP software (Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, UK).

N2A cells were voltage-clamped to −30 mV, and currents in response to 500-ms voltage steps (−50 to +60 mV, in 10-mV increments) or to a 200-ms voltage ramp (−100 to +80 mV) were monitored. Drugs were bath-applied via the superfusion system.

Data Analysis

Current–voltage relationships were calculated from the mean current amplitude during the last 250 or 25 ms of the

voltage steps for oocytes and N2A cells, respectively. To achieve drug effects, mean current amplitudes at +60 mV (oocytes) or +100 mV (N2A cells) were compared to control values before drug application. Statistical significance of the observed changes was tested either by Student's *t* test or by the Mann-Whitney rank sum test using SigmaStat (SysStat Software, San Jose, CA). All results are shown as mean ± SD.

Results

Panx1-C40S Causes Oocyte Degeneration

cRNAs encoding the four extracellular cysteine mutants C66S, C84S, C245S and C264S and the three transmembrane cysteine mutants C40S, C215S and C227S of Panx1 were injected into *X. laevis* oocytes. Two days after injection, oocytes expressing Panx1-C40S showed typical signs of degeneration, as indicated by a complete loss of pigmentation and by ooplasm protruding from the cell membranes (Fig. 1a). All other oocytes, whether expressing Panx1 wt or any of the other mutants, as well as non-injected oocytes did not undergo similar degeneration. To test whether the reduced viability of oocytes expressing Panx1-C40S was caused by modified Panx1 channel properties, we compared the temporal profile of oocyte degeneration under control conditions and in the presence of the Panx1 channel blocker Cbx. While oocytes expressing Panx1-wt remained viable up to 96 h postinjection in both conditions, degeneration of Panx1-C40S RNA-injected oocytes started about 40 h after injection, and complete loss of oocytes was observed 24 h later. This degeneration was delayed by approximately 30 h in the presence of 50 µM Cbx (Fig. 1b). Nonlinear fits to survival curves revealed 50% survival at 50.2 and 79.5 h postinjection for Panx1-C40S-expressing oocytes under control conditions and in the presence of Cbx, respectively.

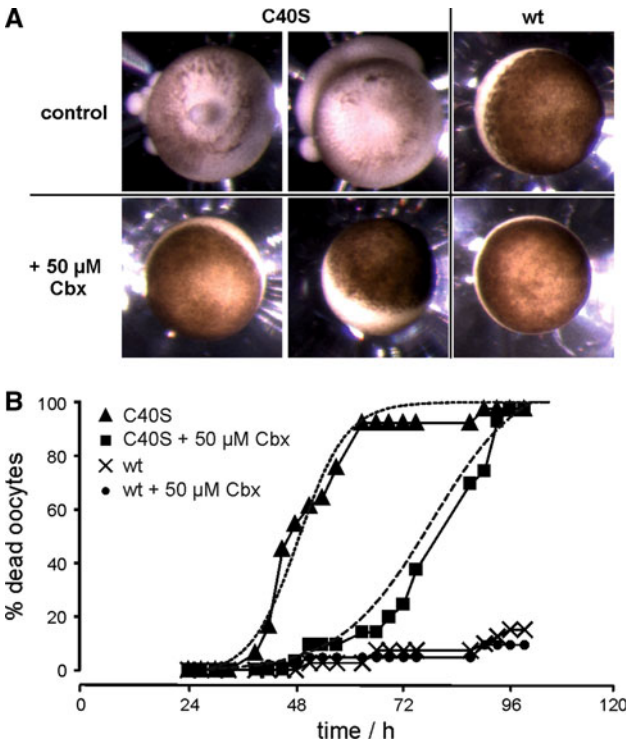

Fig. 1 Induction of *X. laevis* oocyte degeneration by expression of Panx1-C40S and partial rescue by Cbx. **a** Panx1 wt- or Panx1-C40S-expressing oocytes were incubated either in normal Barth's solution or in Barth's solution containing 50 µM Cbx. **b** Oocyte viability was determined by counting dead oocytes at different time points starting 24 h after injection. Degeneration was indicated by yolk protruding from the oocyte. *Dotted lines* indicate nonlinear fits, from which half-life was calculated. Numbers of oocytes analyzed in two different experiments: Panx1 wt, 42; Panx1 wt + 50 µM Cbx, 44; Panx1-C40S, 42; Panx1-C40S + 50 µM Cbx, 46

Panx1-C40S Causes Dye Uptake

Mutation of the intracellular cysteine C346 leads to constitutively active Panx1 channels and is likely to cause oocyte degeneration (Bunse et al. 2010; Wang and Dahl 2010). To examine whether degeneration of Panx1-C40S-expressing oocytes was caused by a similar change in channel activity, we incubated oocytes with the dye lucifer yellow (LY). Because Panx1 channels are normally closed at resting membrane potential, oocytes expressing Panx1 wt did not show dye uptake (Fig. 2a). In contrast, oocytes expressing Panx1-C40S accumulated LY intracellularly, indicating the presence of open Panx1 channels. This dye uptake was considerably reduced in the presence of Cbx. Quantification of the dye uptake revealed that the intracellular LY intensity was reduced in the presence of Cbx by 31% (56.4 ± 22.5 vs. 39.1 ± 16.2, $P = 0.02$) in Panx1-C40S-expressing oocytes, while it remained unchanged in oocytes expressing Panx1 wt (23.7 ± 3.0 vs. 26.7 ± 10.5, $P = 0.20$) (Fig. 2b).

Cysteine Replacement Causes Distinct Electrophysiological Properties in *X. laevis* Oocytes

If the substitution of C40 allows LY to pass the membrane, the electrical conductivity of the mutated Panx1 channel might also change. Therefore, we investigated the electrophysiological properties of the mutant Panx1 channels in *X. laevis* oocytes. Current responses to hyperpolarizing and depolarizing voltage steps were recorded in oocytes injected with cRNAs for Panx1 wt and the different mutants. Because Panx1 wt channels are closed at the oocyte resting membrane potential and open in response to intracellular depolarization, the current–voltage relationship is characterized by a strongly increasing slope at

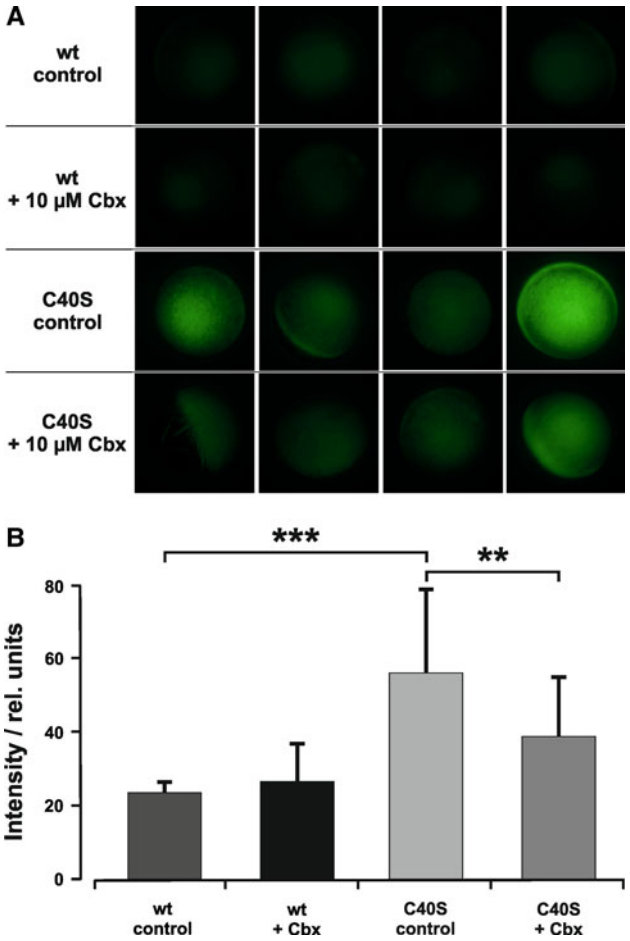

Fig. 2 Dye uptake of *X. laevis* oocytes expressing Panx1 wt or Panx1-C40S. Four individual examples of oocytes incubated either in normal Barth's solution containing 1 mg/ml LY or in Barth's solution containing 10 µM Cbx and 1 mg/ml LY are given (**a**). Uptake of LY was quantified by measuring the mean gray-scale intensity of the vegetale pole for each oocyte (**b**). Oocytes from two independent experiments were pooled. Number of oocytes: wt control, 10; wt + Cbx, 10; C40S control, 11; C40S + Cbx, 11

potentials above +20 mV, which can be significantly reduced by 10 µM Cbx (Fig. 3).

Maximum currents at +60 mV were larger by an order of magnitude in oocytes expressing Panx1-C40S (21,300 ± 8,094 nA, $P < 0.001$) (Fig. 4a) compared to Panx1 wt (1,597 ± 938 nA) without any obvious difference in current kinetics (Suppl. Fig. 2). While maximum currents in oocytes expressing Panx1-C215S (2,092 ± 899 nA, $P < 0.001$) were significantly larger compared to Panx1 wt, no significant difference to Panx1 wt was found in oocytes expressing Panx1-C227S (1,423 ± 893 nA, $P = 0.12$).

Surprisingly, in *I–V* curves from oocytes expressing any of the extracellular cysteine mutants the slope was constant over the whole voltage range and no increase appeared at positive membrane potentials. Therefore, maximum currents at +60 mV were significantly smaller in Panx1-C66S- (432 ± 351 nA, $P < 0.001$) (Fig. 4a), -C84S- (537 ± 357 nA, $P < 0.001$), -C245S- (262 ± 225 nA, $P < 0.001$) and -C264S- (448 ± 339 nA, $P < 0.001$) expressing oocytes compared to oocytes expressing Panx1 wt but similar to currents in noninjected oocytes (328 ± 187 nA).

Application of the blocker Cbx significantly reduced Panx1 channel-mediated currents (Figs. 3, 4b) to 51.5% (±13.1%, $P < 0.01$) of the control in oocytes expressing Panx1 wt, to 41.1% (±10.1%, $P < 0.001$) in C40S, to 51.1% (±13.9%, $P < 0.001$) in C215S and to 60.1% (±12.5%, $P < 0.001$) in C227S-expressing oocytes. Currents recorded from oocytes expressing the mutants C66S, C84S, C245S and C264S were Cbx-insensitive (Fig. 3c–f); and the remaining current amplitudes in the presence of Cbx were in the same range as in noninjected oocytes (C66S, 85.0 ± 13.7%; C84S, 91.0 ± 11.0%; C245S, 87.9 ± 9.4%; C264S, 88.1 ± 8.2%; ni, 90.1 ± 9.8%).

Oocytes expressing Panx1-C40S also showed significantly reduced input resistances (0.03 ± 0.03 MΩ, $P < 0.001$) (Fig. 4c) compared to oocytes expressing either Panx1 wt (0.49 ± 0.31 MΩ), -C215S (0.43 ± 0.39 MΩ) or -C227S (0.35 ± 0.21 MΩ). Moreover, oocytes expressing Panx1-C66S (0.87 ± 0.49 MΩ), -C84S (0.69 ± 0.60 MΩ), -C245S (1.09 ± 0.45 MΩ) or -C264S (0.69 ± 0.42 MΩ) had input resistances similar to noninjected oocytes (0.68 ± 0.41 MΩ).

Membrane Localization of Mutant Panx1 Proteins is not Impaired in Oocytes

Results from oocyte recordings indicated that substitution of extracellular cysteines resulted in nonfunctional Panx1 channels. Since the lack of function could be caused by lack of transport and/or insertion into the cell membrane, we next focused on the localization of the extracellular cysteine mutants. To verify their presence in the oocyte cell membrane, Panx1 channel localization was first assessed by immunocytochemistry in sections from noninjected control oocytes and from oocytes expressing wt or mutant Panx1 (Fig. 5). Only mutant C40S had to be excluded since oocytes did not survive up to the time point of analysis. Noninjected oocytes did not show any immunoreactivity, while all injected oocytes displayed a strong signal at the plasma membrane and only little signal in the ooplasm.

Additional evidence for a membrane localization of the extracellular cysteine Panx1 mutants was derived from cell surface biotinylation assays. Western blots of concanavalin A-pull-down fractions from oocyte lysates demonstrate that particularly the intermediate glycosylated variant of Panx1 is pulled down in case of the wt and mutants analyzed (Fig. 6). This results from the preferential binding of

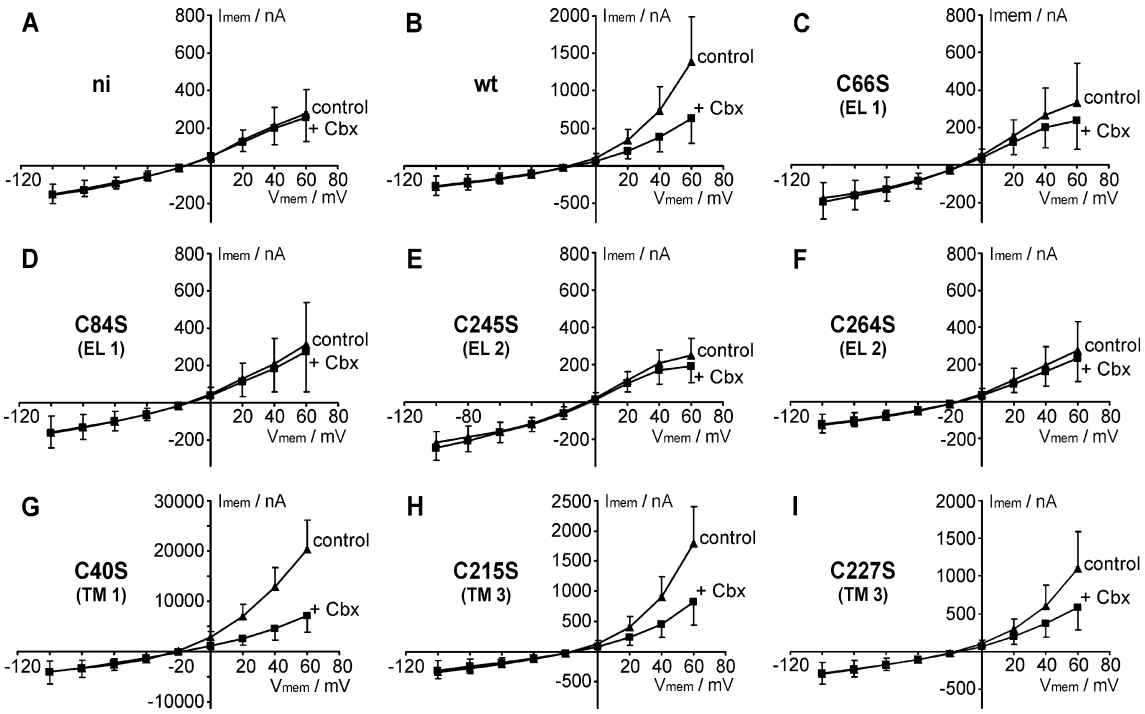

Fig. 3 Electrophysiological characterization of extracellular and transmembrane cysteine mutants in *X. laevis* oocytes. Averaged *I–V* curves from uninjected oocytes (**a**) and oocytes expressing either Panx1 wt (**b**), one of the four extracellular mutants (**c–f**) or one of the three transmembrane mutants (**g–i**) are shown under control conditions and in the presence of 10 µM Cbx. Positions of the mutated cysteines are indicated in parentheses. *EL1*, first extracellular loop; *EL2*, second extracellular loop; *TM1*, first transmembrane domain; *TM3*, third transmembrane domain; *wt* wild type; *ni* not injected. Number of oocytes: ni, 30; wt, 50; C66S, 27; C84S, 41; C245S, 32; C264S, 35; C40S, 34, C215S, 45; C227S, 38

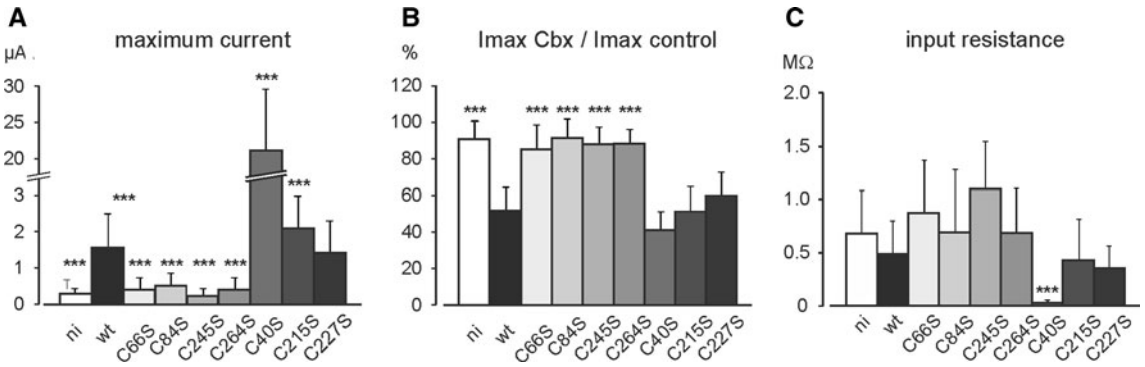

Fig. 4 Electrophysiological characterization of cysteine mutants in *X. laevis* oocytes. **a** Average maximum currents for a voltage step from −70 to +60 mV. **b** Effect of 10 µM Cbx on the maximum current at +60 mV. **c** Input resistance at resting membrane potential. Numbers of oocytes: ni, 96; Panx1 wt, 185; Panx1-C66S, 40; Panx1-C84S, 68; Panx1-C245S, 31; Panx1-C264S, 61; Panx1-C40S, 35; Panx1-C215S, 71; Panx1-C264S, 109

concanavalin A to mannosyl groups, which form the side chains in case of the intermediate glycosylated Panx1 (GLY1) species (Boassa et al. 2007). Thus, our results suggest that trafficking to the plasma membrane was possible for all four extracellular cysteine mutants in *X. laevis* oocytes and that the lack of functional channels is not simply a result of their absence from the membrane.

Substitution of Extracellular Cysteines Results in Nonfunctional Channels in N2A Cells

The effects of single-cysteine residue replacement on Panx1 channels expressed in *Xenopus* oocytes correlated well with results briefly mentioned in an earlier report (Wang and Dahl 2010). However, to make sure that the

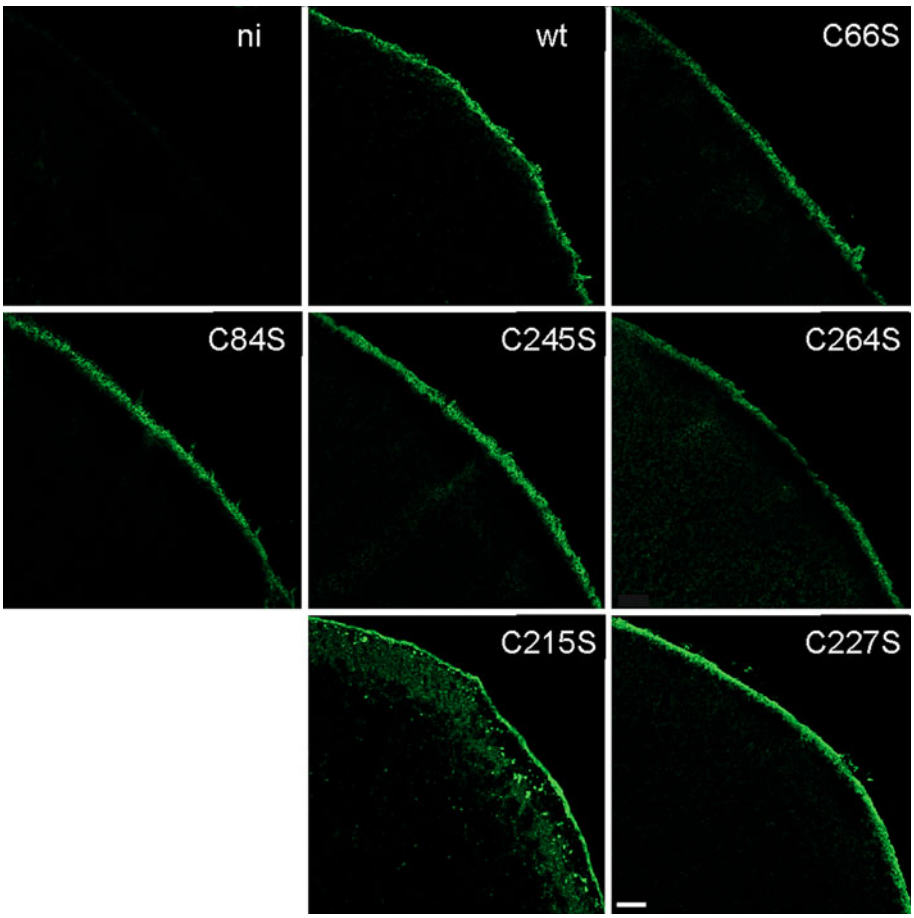

Fig. 5 Expression of Panx1 mutants with modified extracellular and transmembrane cysteine residues in *X. laevis* oocytes. Immunocytochemistry was performed on sections of cRNA-injected oocytes, and confocal images were taken. *Bar* 20 µm

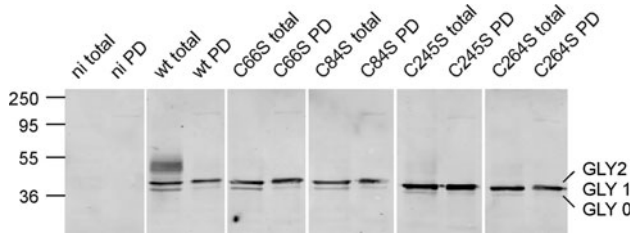

Fig. 6 Expression analysis of extracellular cysteine mutants in *X. laevis* oocytes. Biotinyl-ConA was used to label glycosylated membrane proteins of injected oocytes. After cell lysis, labeled proteins were pulled down with streptavidin agarose. Total-cell lysates ("total") were used as positive control. Pull-down fractions are labeled with *PD*. A Panx1 antibody was used for Western blot analysis. Number of oocytes: ni, 37; wt, 29; C66S, 15; C84S, 21; C245S, 15; C264S, 17. *ni* not injected

loss of channel function of the Panx1 extracellular cysteine mutants was not a peculiarity of the oocyte expression system, we also analyzed these mutants in N2A cells as a different expression system. When expressed in N2A cells, the mutants Panx1-C84S and -C264S were present in the cell membrane, similar to *Xenopus* oocytes (Fig. 7). Substitution of these two extracellular cysteines does not seem to affect Panx1 channel trafficking in this expression system because the localization appeared comparable to Panx1 wt. Membrane localization was impaired for the mutants Panx1-C66S and -C245S (Fig. 7b), and this was paralleled by reduced presence of the fully glycosylated (GLY2), but not the incompletely glycosylated (GLY0 and GLY1), protein (Fig. 7a). In addition, intracellular expression appeared stronger for Panx1-C66S, -C84S and -C245S than for Panx1 wt 48 h after transfection. Thus, a trafficking malfunction of these mutants, particularly of Panx1-C66S and -C245S, in N2A cells cannot be excluded.

Next, we performed whole-cell patch-clamp recordings from N2A cells expressing either Panx1 wt or one of the four extracellular cysteine mutants (Fig. 8). N2A cells expressing Panx1 wt exhibited current–voltage relationships similar to those observed in oocytes; i.e., current amplitudes increased in a nonlinear fashion with increasing intracellular depolarization. Furthermore, this nonlinear current increase was strongly sensitive to Cbx application. In contrast, N2A cells that expressed Panx1-C66S, -C84S, -C245S or -C264S, as well as vector only-transfected N2A cells, were characterized by linear *I–V* curves and a lack of Cbx sensitivity (Fig. 8a–f). As a consequence, mean current amplitudes at +60 mV (Fig. 8g) recorded from Panx1 wt-expressing N2A cells (436.0 ± 225.8 pA, $P < 0.001$) were significantly larger than currents from cells

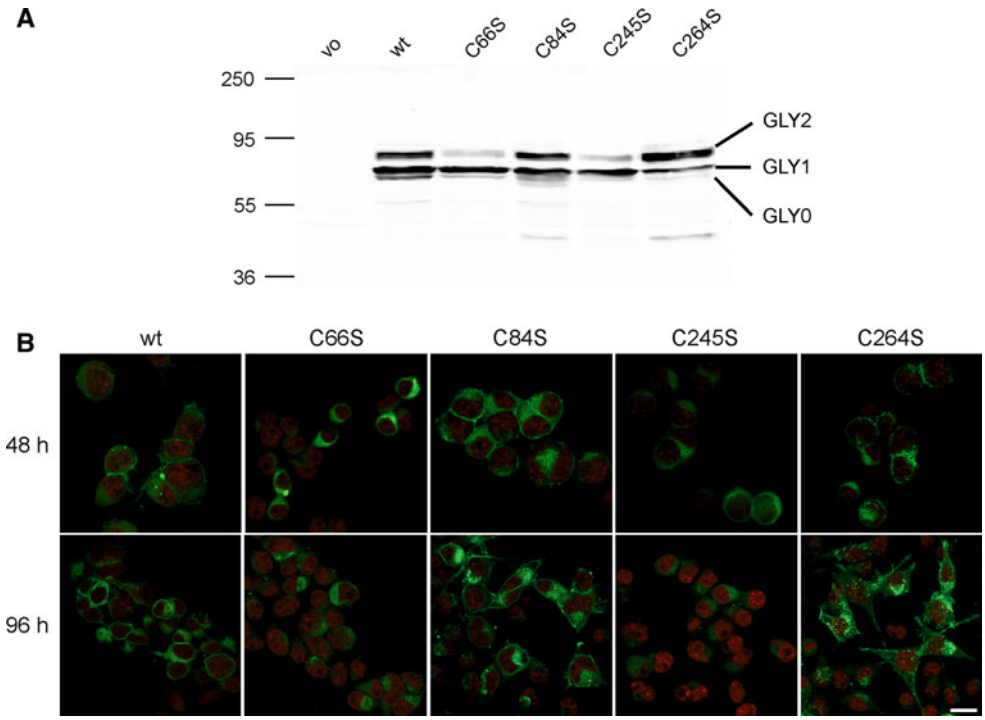

Fig. 7 Expression analyses of Panx1 mutants with modified extracellular cysteine residues in N2A cells. **a** Western blot analysis of N2A cells 48 h after transfection with expression constructs for C-terminal EGFP fusion proteins for Panx1 wt and the mutants Panx1-C66S, -C84S, -C245S and -C264S or with vector only (*vo*). **b** Confocal images of N2A cells transfected with the same constructs as in (**a**) and fixed either 48 or 96 h after transfection with 4% PFA. Cells were counterstained with DAPI to label cell nuclei. *Bar* 10 µm

expressing any of the mutants (-C66S, 82.2 ± 37.9 pA; -C84S, 77.8 ± 77.3 pA; -C245S, 76.0 ± 57.1 pA; -C264S, 78.1 ± 40.0 pA) as well as currents from cells transfected with the empty vector (63.1 ± 50.8 pA). Furthermore, application of Cbx significantly reduced current amplitudes only in N2A cells expressing Panx1 wt (to 30.7 ± 10.8% of the control, $P < 0.005$) but not in cells either expressing mutant Panx1 channels or transfected with the empty vector (Fig. 8h). This indicates that in N2A cells, similar to *X. laevis* oocytes, substitution of any of the extracellular cysteines resulted in loss-of-function Panx1 channels.

Redox Sensitivity of Panx1 Mutants

Redox regulation has been proposed as a potential regulatory mechanism of Panx1 channel activity. We demonstrated earlier that Panx1 channels are sensitive to the reducing agent TCEP because its application reduces the conductivity of wt Panx1 channels, leading to significantly decreased current amplitudes (Bunse et al. 2009). This effect remained unchanged when intracellular cysteine C136, C346 or C426 was mutated (Bunse et al. 2010); and we have suggested that other cysteines may contribute to redox regulation. In the present study, we tested whether mutation of any of the cysteines in the transmembrane region influences the sensitivity of Panx1 channels to TCEP. The extracellular cysteine mutants had to be excluded from the TCEP experiments as they caused a loss of Panx1 channel function.

TCEP application did not influence currents in noninjected oocytes (Fig. 9a). In contrast, maximum currents in Panx1 wt-expressing oocytes were reduced by approximately 30% in the presence of 10 mM TCEP (Fig. 9b), and a TCEP-induced reduction was also observed for the three cysteine mutants tested (Fig. 9c–e). The TCEP effect on noninjected oocytes (remaining current amplitude 93.6 ± 14.7%, $P < 0.001$) was significantly smaller compared to all injected oocytes (Fig. 9f). Compared to Panx1 wt (73.8 ± 5.1%), TCEP had a slightly yet significantly larger effect on oocytes expressing Panx1-C215S (68.0 ± 8.0%, $P = 0.006$) or Panx1-C40S (69.5 ± 5.0%, $P = 0.018$), while the effect was significantly smaller in oocytes expressing Panx1-C227S (79.1 ± 9.1%, $P = 0.007$). However, although mutations of the intramembrane cysteines Panx1-C40S, -C215S and -C227S affect the sensitivity of the channel to TECP with statistical significance, we regard these changes as being too small to be physiologically relevant. Thus, replacement of cysteine C40, C215 or C227 alone appears not to influence TCEP effects. Possibly, more than a single cysteine determines the redox sensitivity of Panx1.

Discussion

We investigated the effect of single-cysteine residue substitutions on the function of Panx1 channels by exchanging the extracellular cysteines C66, C84, C245 and C246 and the transmembrane cysteines C40, C215 and C227 with

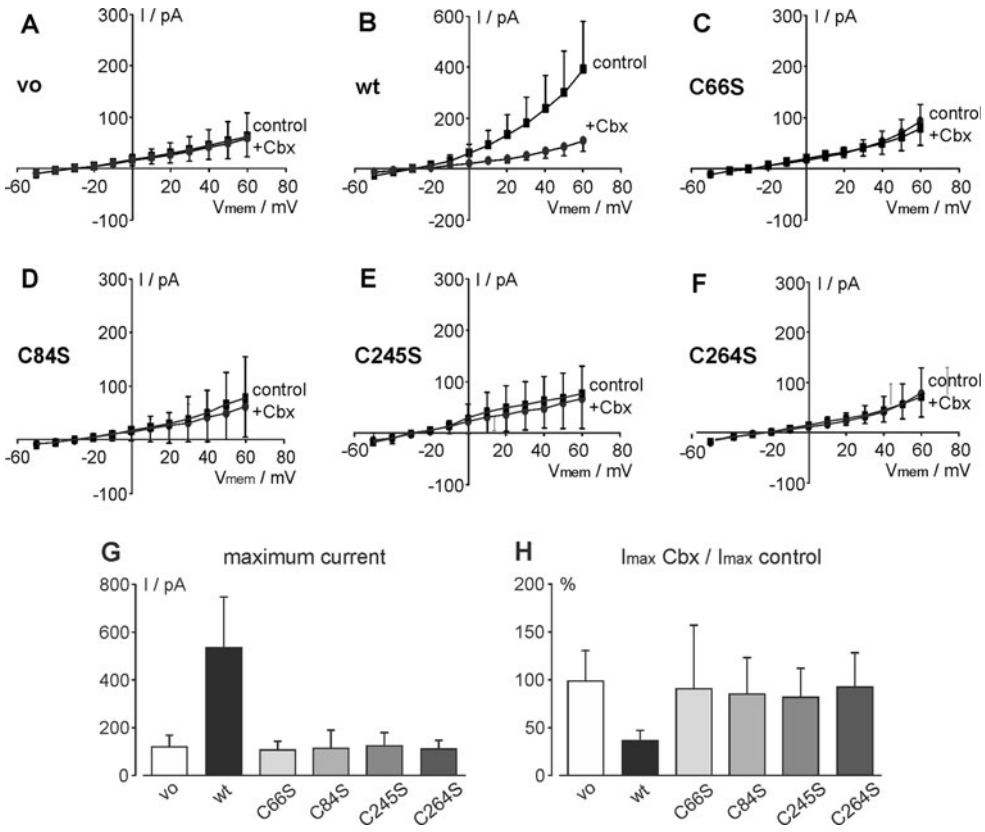

Fig. 8 Electrophysiological characterization of extracellular cysteine mutants in N2A cells. Averaged *I–V* curves of vector only (*vo*)-transfected cells (**a**) and cells expressing either Panx1 wt (**b**) or one of the four extracellular mutants (**c–f**) are shown under control conditions and in the presence of 20 μM Cbx. **g** Average maximum current for a voltage step from −50 to +60 mV. **h** Effect of 20 μM Cbx on the maximum current at +60 mV. Number of cells: vo, 10; wt, 13; C66S, 9; C84S, 10; C245S, 10; C264S, 11

serine in *X. laevis* oocytes and mammalian N2A cells. The results were considerably residue-specific and confirm what has been recently reported in brief for cysteine mutant Panx1 channels in *Xenopus* oocytes (Wang and Dahl 2010). First, mutation C40S dramatically reduced oocyte and N2A cell viability, most likely through strongly enhanced channel conductivity. Second, mutations of all four extracellular cysteines resulted in a complete loss of channel function. Third, mutations of cysteines C215 and C227 had no significant impact on Panx1 channel function. The specific functional changes of mutated Panx1 channels are not reflected by similar differences in membrane localization because all mutated Panx1 channels were detected in the cell membranes of N2A cells and oocytes.

It has been reported that substitution of the intracellular cysteine residue C346 leads to constitutively active Panx1 channels, while mutations of the remaining intracellular cysteines C136 and C426 do not change Panx1 channel function (Bunse et al. 2010; Wang and Dahl 2010). Together with the results from the present study, this demonstrates the importance of specific single-cysteine residues for normal Panx1 function.

Role of Extracellular Cysteines

The extracellular cysteines are of special interest because they represent a characteristic feature of pannexins. In contrast to connexins, which possess three conserved cysteines in each extracellular loop, pannexins possess only two (Panchin et al. 2000). As mentioned parenthetically earlier (Wang and Dahl 2010), mutation of any of the four extracellular cysteines resulted in nonfunctional Panx1 channels in *X. laevis* oocytes. We provide evidence for this result here because current–voltage relationships of injected oocytes were indistinguishable from noninjected control oocytes but considerably different from those expressing Panx1 wt. Furthermore, expression of the four mutant Panx1 channels in N2A cells revealed similar results because transfected cells showed the same lack of a voltage-dependent conductance increase as vector only-transfected controls.

A possible explanation for nonfunctional channels is that protein trafficking to the membrane was affected by the mutations. However, immunocytochemistry and pull-down analyses of oocytes revealed an expression pattern that disagrees with a general trafficking deficiency because all four extracellular cysteine mutants were present in the plasma membrane. Therefore, we propose that trafficking to the cell membrane of the extracellular cysteine mutants was possible in injected oocytes and that the channels were functionally impaired. In contrast to the apparently uniform expression of the extracellular cysteine mutants in the cell membrane of oocytes, trafficking of Panx1-C66S and -C245S appeared to be impaired in N2A cells. Considerably

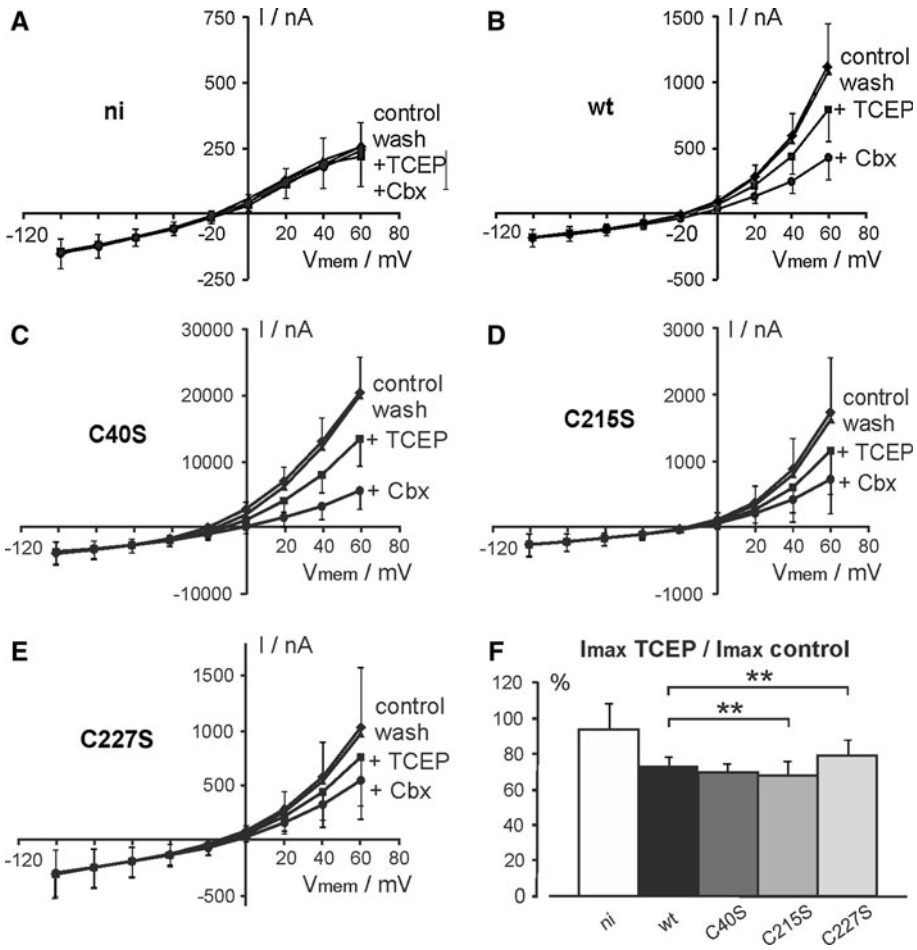

Fig. 9 Influence of TCEP on currents of extracellular and transmembrane cysteine mutants of Panx1 in *X. laevis* oocytes. Averaged *I–V* curves of uninjected oocytes (**a**) and oocytes expressing either Panx1 wt (**b**) or one of the three transmembrane mutants (**c–e**) are shown under control conditions, in the presence of 10 mM TCEP, after washout and in the presence of 10 μM Cbx. **f** Influence of 10 mM TCEP on the maximum current at +60 mV. Numbers of oocytes: ni, 23; Panx1 wt, 32; Panx1-C40S, 12; Panx1-C215S, 13; Panx1-C227S, 24. **Significant difference ($P < 0.01$)

reduced membrane localization was present 2 or 4 days after transfection compared to mutants Panx1-C84S and -C264S as well as Panx1 wt. Furthermore, the level of intracellular expression appeared higher for mutants Panx1-C66S, -C84S and -C245S than for Panx1 wt; and the signal of the fully glycosylated form (GLY2) of Panx1 obtained in Western blot analyses of transfected N2A cells was markedly decreased for Panx1-C66S and -C245S. This could explain differences in trafficking and/or modification between different expression systems that have to be taken into account when analyzing functional changes in response to single-amino acid mutations. Future studies are needed to shed light on the exact deficits in the trafficking of the mutants Panx1-C66S and -C245S.

Interestingly, the resting membrane potentials of Panx1-C66S- and -C245S-expressing oocytes were significantly more positive than those of oocytes expressing either Panx1 wt or mutants Panx1-C84S and -C264S. Possibly, mutations of C66 and C245, directly or indirectly, change the membrane conductivity, thereby influencing the membrane potential. These changes do not seem to impair survival as oocyte viability was unchanged.

In connexins, extracellular cysteines are considered essential for gap junction formation. Thus, when connexin mutants in which any of the six extracellular cysteines was substituted by serine were expressed in oocytes, functional gap junction channels were not formed (Dahl et al. 1991, 1992). Furthermore, while disulfide bonds between cysteines link the two extracellular loops within one connexin, no intermolecular disulfide bridges are formed between two connexons or within one connexon between two connexins (Dupont et al. 1989; John and Revel 1991; Rahman and Evans 1991). This, together with results from studies in which cysteines were displaced instead of being eliminated (Foote et al. 1998), suggests that extracellular cysteines in connexins are essential for the three-dimensional connexin structure that is necessary for the formation of a densely packed Cx gap junction channel.

Although mutations of the extracellular cysteines eliminate gap junction formation, hemichannel function in mutated Cx43 remains unchanged. Thus, a Cx43 mutant lacking all cysteines showed similar permeation to carboxyfluorescin as the Cx43 wt hemichannel (Bao et al. 2004b). This underlines the fundamental functional

difference of extracellular cysteines in pannexins and connexins. Therefore, it will be exciting to see whether disulfide bonds are formed between the extracellular cysteines of one pannexin molecule, thus resembling the connexins; whether intermolecular bonds are present between two pannexin molecules; or whether no disulfide bonds are formed at all. Knowledge about the structural organization of the extracellular cysteines will reveal more insight into the impact of these residues for hemi- and/or gap junction channel formation and whether similar mechanisms have coevolved in two evolutionarily distinct protein families.

Role of Transmembrane Cysteines

Expression of Panx1-C40S in *X. laevis* oocytes induced rapid cell death similar to the intracellular cysteine mutant Panx1-C346S (Bunse et al. 2010; Wang and Dahl 2010). For Panx1-C346S, we have proposed that the reduced oocyte viability is caused by increased channel activity. In addition, a delayed toxic effect that is insensitive to Cbx leads to the delayed oocyte degradation in the presence of Cbx. Although we cannot exclude that variations in expression level between Panx1 wt and the mutants cause differences in whole-cell conductivity, we propose a similarly increased activity for the Panx1-C40S mutant. This assumption is supported by, first, the greatly reduced oocyte viability, which was not observed in oocytes expressing any of the other Panx1 mutants or the wt protein. Second, LY was accumulated intracellularly only by oocytes that expressed Panx1-C40S, and the uptake could be at least partially blocked by Cbx. Neither oocytes expressing Panx1 wt nor any of the other mutants showed a similar behavior. Third, oocytes expressing Panx1-C40S were characterized by significantly smaller input resistances and by currents in response to intracellular depolarization that were larger by an order of magnitude compared to all other oocytes. Neither the LY uptake nor the reduced viability mediated by Panx1-C40S can be explained if the mutant channel gating was unaltered even by a significantly increased expression level. We therefore propose that the substitution of C40 results in a Panx channel with greatly altered gating behavior. This effect seems not to be specific for mouse Panx1 since zebrafish Panx1 channels with the corresponding mutation C41S show identical changes when expressed in N2A cells (data not shown). Furthermore, expression of Panx1-C40S in N2A cells not only induces rapid cell death (Suppl. Fig. 3) but also reveals that this mutant, similar to Panx1-C346S (Bunse et al. 2010), lacks the GLY2 isoform normally present for wt Panx1 (Suppl. Fig. 4). As indicated by immunocytochemistry and electrophysiology in oocytes, trafficking of Panx1-C40S to the cell membrane leads to expression of channels with abnormal conductivity at resting membrane potential. This appears to disrupt the intracellular milieu in such a way that oocytes degenerate. Early after expression onset, cell degeneration is obstructed in the presence of Cbx; but with ongoing expression, mechanisms that are Cbx-insensitive apparently lead to delayed cell death.

While the effect of the C40S mutation on whole-cell conductivity is obvious, the reason for this increase on the single-channel level remains open. Different explanations are possible, like changes in channel opening and/or closing, shifts to higher level subconductance states, increased channel expression or half-life time or a combination of more than one. However, we cannot distinguish between different possibilities from whole-cell data so that single-channel recordings will be necessary to identify the type(s) of change induced by the mutation.

While the results greatly resemble what we reported for Panx1-C346S-expressing oocytes and N2A cells (Bunse et al. 2010), we also found some remarkable differences: oocytes expressing Panx1-C40S showed almost twice as large maximum current amplitudes and degenerated more rapidly than oocytes expressing Panx1-C346S. This could be caused either by an enhanced maintained activity of Panx1-C40S or by an increased expression level in comparison to Panx1-C346S. On the other hand, dye uptake in Panx1-C40S-expressing oocytes was less strong (on a qualitative level) compared to Panx1-C346S, and Cbx was a less effective uptake blocker. Finally, oocytes expressing Panx1-C346S, but not -C40S, showed a much more positive resting membrane potential than Panx1 wt-expressing oocytes. The reduced dye uptake and the unchanged membrane potential of Panx1-C40S-expressing oocytes may have two reasons. First, since C40 is located in the pore-lining domain TM1 (Wang and Dahl 2010), it might selectively influence ion passage depending on size or charge of the molecule, different from Panx1-C346S. Second, mutation C40S may cause a stronger increase of channel activity so that cell death occurs very quickly. As a result, the selection of oocytes for dye uptake becomes difficult because cells that express a significant amount of Panx1-C40S will degenerate rapidly. Because such oocytes will show distinct signs of degeneration early after injection, they may not have been chosen for dye uptake experiments, so selection of oocytes was biased toward low-expressing cells with generally reduced uptake.

Similar to oocytes expressing mutants of the intracellular cysteines C136 and C426 (Bunse et al. 2010), mutants C215S and C227S did not show any sign of cell degeneration and protein localization at the plasma membrane was not obviously affected. Maximum currents recorded from C215S-expressing oocytes were significantly larger than those derived from Panx1 wt-expressing oocytes. This may

have two different reasons. First, the mutation may lead to an increased number of channels at the cell surface, thereby increasing whole-cell conductivity. Protein localization at the plasma membrane was evaluated on a qualitative level, so small changes in the amount of protein at the membrane, although capable of affecting conductivity, may remain undetected. Second, single-channel conductance may be increased due to structural changes caused by the mutation, although the first transmembrane domain, together with parts of the C terminus, is regarded as the potential pore-lining region (Wang and Dahl 2010). Compared to wt, the resting membrane potential was slightly more positive in Panx1-C215S- and Panx1-C227S-expressing oocytes, although the difference between Panx1-C215S and wt was not significant. Possibly, Panx1-C227, which is also located in the third transmembrane domain, may cause the mutant channel to be slightly more leaky than the wt channel.

Effects of TCEP

Similar to the intracellular cysteine mutants (Bunse et al. 2010), the inhibitory effect of TCEP on current amplitudes was not abolished in any of the cysteine mutants in transmembrane domains. Although a pore-lining function of the third transmembrane domain appears unlikely, it is feasible that redox sensitivity and channel tuning involve this domain and that more than one cysteine residue may play a role in the underlying mechanism. A single-cysteine residue mutation may not completely uncover the contribution of the mutated cysteine because the remaining cysteines may compensate for the mutation. If the TCEP effect depends on either intracellular or transmembrane cysteine residues, however, it must be assumed that it enters the cell through open Panx1 channels because it cannot penetrate cell membranes. Alternatively, and possibly more straightforward, TCEP might predominantly or exclusively act on extracellular cysteine residues, and changes in transmembrane or intracellular cysteine residues might be ineffectual. Yet, replacement of any of the extracellular cysteine residues led to nonfunctional Panx1 channels, which made it impossible to test this hypothesis in our experiments.

Conclusion

The substitution of single cysteines in Panx1 proteins reveals the important functional role of these amino acids for the regulation of Panx1 channel activity. The changes observed after single-cysteine substitution are individually distinct and can oppositely influence channel function. In particular, the intracellular mutant C346S and the transmembrane mutant C40S appear to completely abolish proper channel gating and apparently result in constitutively open Panx1 channels. As a consequence, this disrupts the viability of *Xenopus* oocytes or mammalian N2A cells that express either of these mutants. On the contrary, substitution of extracellular cysteines results in Panx1 channels present in the cell membrane, which appear nonfunctional because their voltage gating behavior is disturbed. Such channels may, however, still be activated by other extracellular or intracellular signals. Future studies will shed light on the molecular mechanisms underlying these regulatory events and their role for Panx1 channel physiology and pathology.

Acknowledgment We thank Gerhard Dahl for the kind gift of Panx1 antibody, Matthias Tenbusch for help with the FACS analysis and Sabine Peuckert, Sabine Schreiber-Minjoli, Christiane Zoidl and Hans-Werner Habbes for excellent technical assistance regarding molecular biology and immunocytochemistry. This project was supported by grants of the GRK 736 (Development and Plasticity of the Nervous System: Molecular, Synaptic and Cellular Mechanisms) to S. B. and the DFG (292/11-4) to R. D. and G. Z.

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