J Membrane Biol (2015) 248:285–294
DOI 10.1007/s00232-014-9767-4

Effects on Channel Properties and Induction of Cell Death Induced by C-terminal Truncations of Pannexin1 Depend on Domain Length

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Received: 26 August 2014 / Accepted: 22 December 2014 / Published online: 8 January 2015

Abstract Pannexin1 (Panx1) is an integral membrane protein and known to form multifunctional hexameric channels. Recently, Panx1 was identified to be responsible for the release of ATP and UTP from apoptotic cells after site-specific proteolysis by caspases 3/7. Cleavage at the carboxy-terminal (CT) position aa 376–379 irreversibly opens human Panx1 channels and leads to the release of the respective nucleotides resulting in recruitment of macrophages and in subsequent activation of the immunologic response. The fact that cleavage of the CT at this particular residues terminates in a permanently open channel raised the issue of functional relevance of the CT of Panx1 for regulating channel properties. To analyze the impact of the CT on channel gating, we generated 14 truncated versions of rat Panx1 cleaved at different positions in the C-terminus. This allowed elaboration of the influence of defined residues on channel formation, voltage-dependent gating, execution of cell mortality, and susceptibility to the Panx1 inhibitor carbenoxolone. We demonstrate that expression of Panx1 proteins, which were truncated to lengths between 370 and 393 residues, induces differential effects after expression in *Xenopus laevis* oocytes as well as in Neuro2A cells with strongest impact downstream the caspase 3/7 cleavage site.

Keywords Membrane proteins · Hemichannel · Pannexin · Channel activity

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Abbreviation

Cbx	Carbenoxolone
CT	C-terminus
Ct-trunc	Carboxy-terminally truncated
FACS	Fluorescence-activated cell sorting
Panx1	Pannexin1
SCAM	Substituted cysteine accessibility method
TEVC	Two-electrode voltage clamp

Introduction

Panchin et al. (2000) described pannexins as a new family of proteins displaying structural similarities to the well-characterized connexins and sequence homology to the invertebrate innexins.

The pannexin protein family consists of three integral, non-junctional putative channel forming membrane glycoproteins (Panchin et al. 2000; Dahl and Locovei 2006). While data for Panx2 and Panx3 are still unsatisfactory, at least Panx1 has been shown to be involved in transmembrane signal transmission (Harris 2007; MacVicar and Thompson 2010; Scemes et al. 2009; Sosinsky et al. 2011; Bond and Naus 2014). Recent studies focusing on the structure of the channel pore indicated an average diameter of ~140 Å of the Panx1 channel. The actual pore diameter was calculated as ~19 Å on average (Wang and Dahl 2010). Attempts to define the transmembrane topology of Panx1 by substituted cysteine accessibility method (SCAM) indicated that portions of the external part of the first transmembrane domain and the first extracellular loop line the outer part of the pore of Panx1 channels, while the inner part of the pore is lined, at least partly, by the

C-terminus (CT) of the protein (Wang and Dahl 2010). The abundance of consensus sequences at the CT including PDZ domains and phosphorylation sites (Barbe et al. 2006; Penuela et al. 2013) suggested an important role of this part of Panx1 in channel functioning. Distinct mechanisms identified to open Panx1 channels include mechanical stress (Bao et al. 2004), high extracellular K+ (Jackson et al. 2014), and activation of purinergic receptors (Qiu and Dahl 2009).

Convincing experimental evidence indicated that Panx1 channels are apt to release ATP and UTP after initial stimulation of macrophages by external apoptotic stimuli (Chekeni et al. 2010). By means of sequence analyses, two putative caspase cleavage sites were identified in the CT of Panx1. The active site exhibits a canonical caspase cleavage sequence at position aa 376–379 in human Panx1, with a typical tetrapeptide motif DxxD which is highly conserved among different species during evolution.

Panx1 cleavage by caspase 3/7 at this particular site leads to an irreversible opening of the channel. As a consequence, ATP and UTP are released, which both function as "find-me" signals for recruitment of phagocytes (Chekeni et al. 2010; Sandilos et al. 2012). The initial ability of apoptotic cells to influence their extracellular environment by the release of nucleotides for recruitment of phagocytes is believed to be essential for the ongoing apoptotic cascade (Elliott et al. 2009).

Structural analyses of the CT by Spagnol et al. (2014) explored the secondary structure of this domain and provided a mechanistic model for the opening event of Panx1 upon apoptosis. Also, multifunctional behavior of the Panx1 has been shown to depend on the presence of K^+ (Wang et al. 2014), where the high-conductance state, which is responsible for ATP release, depends largely on a terminal cysteine residue. These current data have brought the CT of Panx1 into focus for extended functional studies. In this context, we considered it important to elaborate on the molecular embedding of the caspase 3/7 cleavage site with respect to its implications on cellular functioning.

For this purpose, we generated several carboxy-terminally truncated versions (Ct-trunc) of Panx1, which allowed us to analyze the influence of the CT down to a few residues on channel formation, voltage-dependent gating, development and extent of cell mortality, and sensitivity to the potent Panx1 inhibitor carbenoxolone (Cbx).

In particular, we aimed to address the following questions:

(i) Does partial cleavage of the C-tail impair Panx1, and if so, where is the crucial segment responsible for the deleterious effect?
(ii) Does truncation of C-terminus influence channel gating?
(iii) Is sensitivity of Panx1 toward the blocking agent Cbx domain-specific?

Materials and Methods

Truncation of Panx1

Rat Panx1 (rPanx1, accession number P60570) was amplified from isolated cDNA and cloned into the plasmid vector pCS2+. In rat, Panx1 has a length of 426 aa (Fig. 1). Here, the conserved motive DxxD of the caspase cleavage site is located at position aa 375–378.

Truncations were performed by use of specific PCR primers. The plasmid pCS2+ was used as vector backbone for all constructs to allow expression in mammalian cells as well as in vitro transcription for the generation of cRNA for injection into *Xenopus laevis* (*X. laevis*) oocytes. rPanx1 was truncated to the denoted lengths of 307, 327, 347, 367, 370, 374, 377, 380, 383, 387, 389, 393, 397, and 407 aa (Fig. 1).

Xenopus laevis Oocyte Injection and Two-Electrode Voltage Clamp (TEVC) Analysis

Xenopus laevis oocytes were obtained from EcoCyte Bioscience (Castrop-Rauxel, Germany) defolliculated and plated in 96-well plates ready to use. Injection of cRNA was

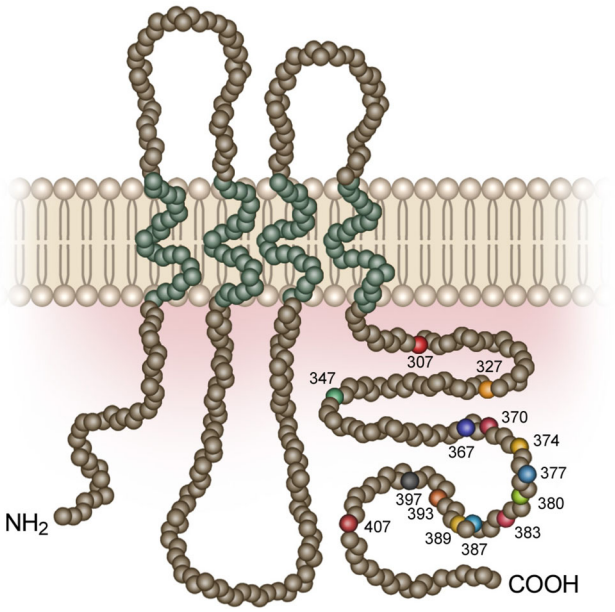

Fig. 1 Position of the amino acids chosen for truncation of Panx1. The graph depicts the topology of rPanx1. The amino acids forming the transmembrane domains are marked in *green*. The amino acids marking the truncation sites are depicted in different *colors*

performed by use of the Roboocyte® (Multichannel Systems, Reutlingen, Germany) automated oocyte injection and recording system. Synthesis of cRNA for the injection into *X. laevis* oocytes was made by the mMESSAGE mMACHINE Kit (Ambion, Applied Biosystems, Foster City, CA, USA) according to manufacturer's guidelines. A total of 50 nl cRNA solution with a concentration of 1 µg/µl were injected into the cells' ooplasm. Before TEVC recordings, an incubation time of approximately 60 h was maintained, the oocytes were kept at 17 °C in normal frog Ringer (NFR, in mM: NaCl 90, KCl 2, $CaCl_2$ 2, $MgCl_2$ 1, and HEPES 5, at pH 7.4). TEVC recordings were performed from oocytes with the Roobocyte® system and Roobocyte® TEVC measuring heads. Electrodes were filled with 2.5 M potassium acetate providing electrode impedances of ~1 MΩ. During measurements, oocytes were superfused with NFR (3–4 ml/min). The Panx1 channel blocker Cbx was diluted in NFR and applied by use of a gravity-based 8-channel perfusion system. Recordings always took place at RT. In TEVC recordings, oocytes were clamped to a voltage of −70 mV, and voltage steps were applied from −100 to 60 mV in 20 mV increments with a duration of 2 s/step.

Assessment of Mortality Rate of *X. laevis* Oocytes

Oocytes were incubated in NFR or NFR containing 50 µM Cbx at 17 °C and were inspected every 3 h by means of a binocular microscope. Formation of protrusions of ooplasm from the oocyte was taken as a common indicator for oocytes' cell death (Bunse et al. 2011).

Determination of Mortality in N2A Cells with FACS Analysis

Neuro2A cells were co-transfected with constructs encoding the truncated Panx1 versions and pEGFP, referring to Panx1-wt and pEGFP-transfected cells as positive control and pEGFP-transfected cells as negative control to adjust background. 24 h post transfection, cells were collected and suspended in PBS. The fluorescent dye 7-aminoactinomycin (7-Aad) was added to the cell suspension to stain dead cells, and cells were then analyzed in a FACS flow cytometer (FACSCalicur; Becton–Dickinson, Mountain View, CA). Cells, which were neither positive for EGFP nor for 7-Aad, were neglected. Data were analyzed using CellQuest Pro software (version 4.0.1; Becton–Dickinson).

Data Analysis

Data acquired in TEVC recordings were analyzed with the Roboocyte® software, Microsoft Excel, and GraphPad Prism5 (GraphPad Software, La Jolla, CA). The Roboocyte® software was used to analyze maximum currents at +60 mV in IV curves, for calculation of the inhibitory effects of pharmaceutics and for readout of the resting potential. Statistical analyses were performed by use of the GraphPad Prism5 software by One-way ANOVA (Kruskal–Wallis-test) without assuming Gaussian distributions. Uninjected cells served as an internal control. For each recording day, the mean current amplitude obtained from uninjected cells was calculated and subtracted from average current amplitudes obtained from injected oocytes to uncover the fraction of current induced by Panx1 channels.

Results

Truncation of the Panx1 C-terminal Domain

Truncation of a protein is a legitimate means to gain insights into the function of specific peptide domains (Tobler et al. 1999).

Referring to the irreversible channel opening by cleavage of human Panx1 through Casp3/7 at position aa 376–379 (Chekeni et al. 2010), the goal of this study was to elaborate whether a specific domain of the CT is crucial for this dominant negative effect in rat also and subjected diverse CT-truncations to a scrutinizing electrophysiological study. Regarding caspase cleavage and the function of Panx1 in apoptosis, truncation of the protein can be considered an analog of the naturally occurring process mimicking cleavage by effector caspases.

Electrophysiological Characterization of Ct-trunc Panx1 in *X. laevis* Oocytes

After injection of cRNA encoding Ct-trunc Panx1, oocytes expressing some of the truncated versions showed signs of severe cell degeneration, accompanied by protrusions of ooplasm, which did not allow further electrophysiological characterization (Table 1). Hence, only those oocytes, which did not show signs of degeneration, were chosen for TEVC recordings.

Maximum current amplitudes recorded from oocytes expressing Ct-trunc Panx1, which did not impair cellular survival, revealed significant differences (Fig. 2a). In oocytes expressing Panx1-wt, average current amplitudes were significantly higher than currents recorded from non-injected cells on each experimental day. The average current amplitudes at +60 mV holding potential in Panx1-wt expressing oocytes were 972.9 ± 145.2 nA, when normalized to non-injected (ni) cells. In oocytes, which expressed Panx1-307, average maximum currents were 5742.6 ± 948.2 nA, which was significantly higher than Panx1-wt mediated currents indicating a gain of function for this juxtamembrane mutation. Interestingly, the expression of

Table 1 List of constructs leading to breakdown of cellular homeostasis in *X. laevis* oocytes

Construct	% dead oocytes/96 h
Panx1-347	40.6
Panx1-370	66.0
Panx1-374	25.6
Panx1-377	34.0
Panx1-380	95.8
Panx1-383	85.0
Panx1-387	77.2
Panx1-389	71.7
Panx1-393	54.5

Constructs leading to ≥25 % cell death are listed

Panx1-327 with an extension of the carboxy-terminus by only 20 aa reduced maximum current amplitudes by an order of magnitude (Panx1-327, 512.1 ± 117.6 nA) compared to the Panx1-307 mutant. Panx1-347 and -367 evoked maximum currents comparable in size (Panx1-347: 1513.6 ± 938.4 nA, and Panx1-367: 1342.6 ± 340.7 nA). Oocytes expressing Panx1-truncated versions larger than 367 aa and smaller than 397 aa (i.e., Panx1-370, -374, -377, -380, -383, -387, -389, -393, -393) could not be measured since cells were not viable. Notably, the caspase 3/7 cleavage site responsible for the initiation of ATP and UTP release during ongoing apoptosis (Chekeni et al. 2010) resides within this segment. In Panx1-380, the whole caspase cleavage site is included. Extension of the carboxy-terminus by further 20 aa, i.e., to Panx1-397, led to a gain of function by a 3-fold increase of evoked currents compared to Panx1-wt (2652.9 ± 886.9 nA). The addition of another 10 aa, i.e., to obtain Panx1-407, further increased maximum current amplitudes to the highest values of all truncated versions (6878.6 ± 522.1 nA). In terms of length, completion of the carboxy-terminus by 19 aa to its full length, i.e., Panx1-426 (or Panx1-wt), accounts for a concurrent reduction in current amplitude by more than 80 %.

This remarkable impact of the C-terminus length on Panx1 current flow clearly indicates that the signature of the carboxy-terminus is essential for gating properties of the Panx1 channel.

With respect to Cbx sensitivity, it turned out that three out of six Ct-trunc Panx1 versions proved to be Cbx sensitive (Fig. 2b). Inhibition of currents in oocytes expressing Panx1-347, -397, or -407 in the presence of 10 μM Cbx was considerably stronger than in Panx1-wt expressing oocytes (average reduction, Panx1-347: 46.2 %, Panx1-397: 68.2 % and Panx1-407: 52.7 % vs. Panx1-wt: 28.8 %). Of note is the fact that the intermediate mutant (Panx1-367) appeared Cbx insensitive, while the upstream (Panx1-347) and

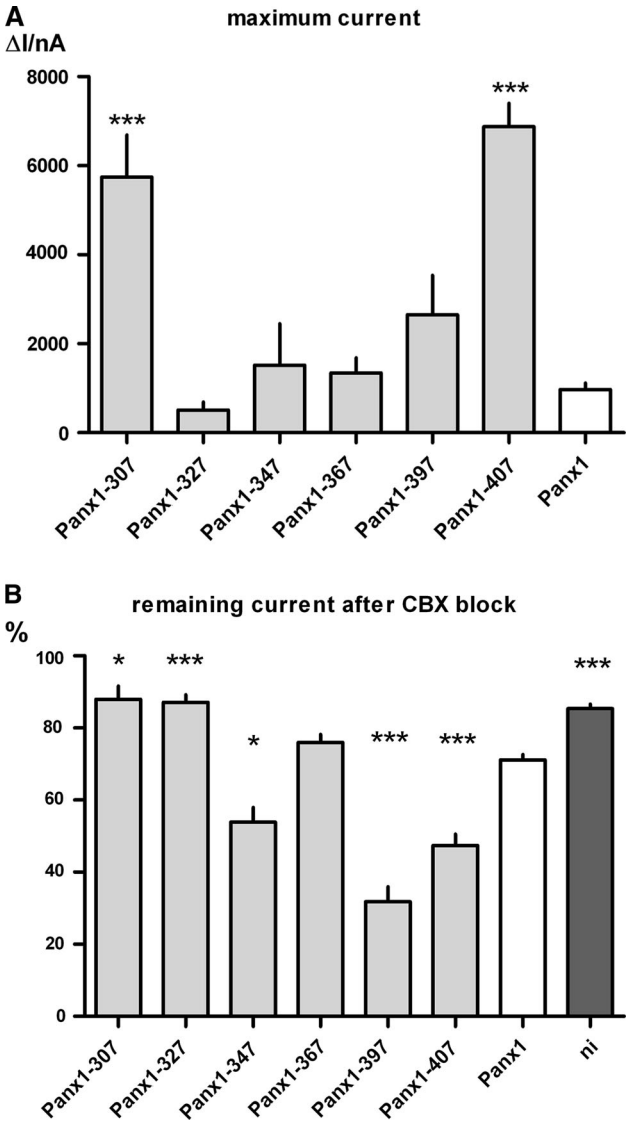

Fig. 2 Comparison of the electrophysiological properties of truncated versions of Panx1 in *X. laevis* oocytes. Whole cell membrane currents from cRNA-injected *X. laevis* oocytes were recorded using the TEVC technique. Oocytes were clamped to −70 mV, voltage steps starting from −10 to +60 mV membrane potential were applied subsequently. IV curves were calculated from corresponding current responses. *ni* not injected. **a** Quantitative analysis of the maximum current measured at a membrane potential of +60 mV. The maximum currents show a high variation in comparison with Panx1 expressing oocytes. **b** Quantitative analysis of the remaining current after inhibition of channel activity with Cbx (in % of control). Cbx sensitivity was investigated in oocytes expressing Panx1-347, Panx1-397, and Panx1-407. Panx1 $n = 232$, Panx1-307 $n = 15$, Panx1-327 $n = 41$, Panx1-347 $n = 27$, Panx1-367 $n = 43$, Panx1-397 $n = 17$, Panx1-407 $n = 37$, *ni* $n = 106$. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, SEM, significance vs. Panx1-wt

downstream mutants (Panx1-397) were still responsive, although with reduced sensitivity (see above). The juxtamembrane mutants (Panx1-307, -327) again were non-responsive. This finding is of particular interest since it indicates that specific domains within the carboxy-terminus

of Panx1 are critical for the interaction with this potent Panx1 inhibitor.

The Impact of Panx1 Truncation on Cell Survival in *X. laevis* Oocytes

Oocytes expressing Ct-trunc Panx1 with sequences longer than 367 aa and shorter than 397 aa either showed spontaneous degeneration after incubation for 60 h or were severely damaged when penetrated with the recording electrode to perform TEVC measurements, indicating increased fragility of the plasma membrane. In order to evaluate the impact of the length of the carboxy-terminus on cellular survival, Ct-trunc Panx1 with various lengths spanning the indicated range was expressed in *X. laevis* oocytes, and oocytes' viability was continuously monitored during the first 96 h after the cRNA injection by visual inspection. Figure 3 shows oocytes expressing either Panx1-wt or Panx1-387 as one of the prime examples for truncation-induced cell degeneration. In oocytes expressing Panx1-387, 72 h after cRNA injection, yolk was found to protrude from the ooplasm. This was regarded as an indicator for cell death in the survival curves (Fig. 4).

The survival curves (Figs. 4, 5) of the Ct-trunc Panx1 reveal significant differences regarding oocyte viability and Cbx sensitivity.

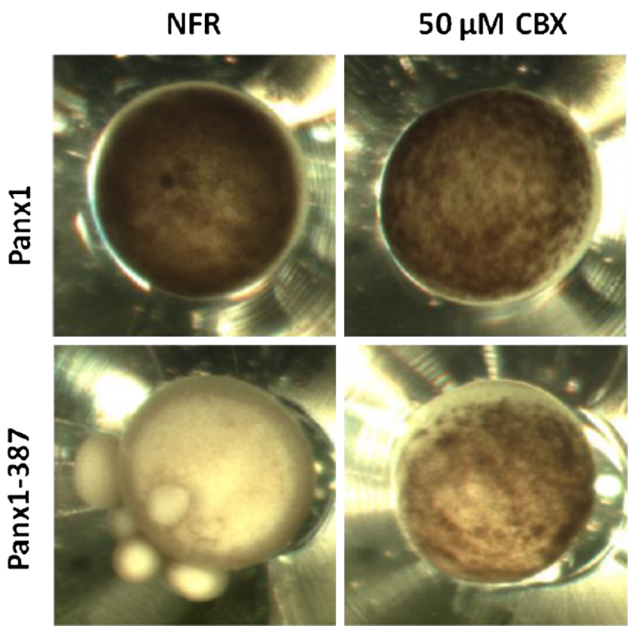

Fig. 3 Morphological appearance of *X. laevis* oocytes after expression of Panx1-truncated versions. Panx1-wt or Panx1-387 expressing oocytes, incubated either in Normal Frog Ringer (NFR, no treatment) or in NFR solution containing 50 µM Cbx 72 h after injection. An oocyte was considered dead, when yolk was protruding from the ooplasm, as depicted in the lower *left panel*

After 48 h, degeneration started to progress differently for particular Ct-trunc Panx1. In most oocytes, vital impairment was decelerated by incubation with 50 µM Cbx (Figs. 4, 5). However, degeneration was not rescued by Cbx treatment in some of the constructs (Panx1-307, -327, -367, -370, -380, and -383) as expected from the Cbx sensitivity studies (see above).

Cumulative curves displaying oocyte degeneration when expressing truncated Panx1 channels are given in Fig. 4, and the percentage of oocytes, which did not survive Ct-trunc Panx1 expression up to 96 h after injection, is given in Table 1. Noteworthy, viability of oocytes not simply correlates with the length of the C-terminus, but seems to be domain-dependent. The most critical segment is a domain between Panx1-380 and -383, where truncation leads to a mortality rate of 95.8 and 85.0 %, respectively. It represents the segment, which also proved insensitive to rescue by Cbx treatment. Expression of Panx1-377 leads to an induction of cell death of 34.0 %. A reasonable explanation for this effect is that Panx1-380 includes the entire cleavage site, as the caspase hydrolyses the protein behind the second aspartate in the sequence DxxD, which is positioned at aa 378. Consecutive addition of amino acids leads to stepwise decrease in mortality (Panx1-383: 85.0 % decreasing to Panx1-393: 54.5 %). A total rescue of *X. laevis* oocytes is given at expression of Panx1-397 (Table 1).

The Role of Panx1 Truncation for Cellular Survival in Neuro2A Cells

From a functional point of view, it is of relevance to study the effect of Panx1 truncation also in a mammalian cell system. The differences observed by expression of the Panx1 mutants in *X. laevis* oocytes provided an important insight into the domain-dependent behavior of the Ct-trunc Panx1 in terms of cell viability. However, *X. laevis* oocytes comprise a cell system with special attributes, which cannot be extrapolated a priori to other cell systems, in particular to mammalian cells. We therefore decided to express the Ct-trunc Panx1 in Neuro2A cells in order to explore whether expression of truncated Panx1 proteins leads to comparable effects regarding cellular survival.

For this reason, Neuro2A cells co-expressing Ct-trunc Panx1 and EGFP were analyzed by FACS flow cytometry (Fig. 6). The measurement of GFP and 7-Aad provides a condition to separate living from dead cells. The FACS analysis revealed an increase in cell mortality with a peak between Panx1-380 and -387, and decaying from that point with extending length of the CT. Although the maximum mortality rate is achieved at position Panx1-387 in the N2A cell system, the critical segment is close to the one found in the *Xenopus* system (see above).

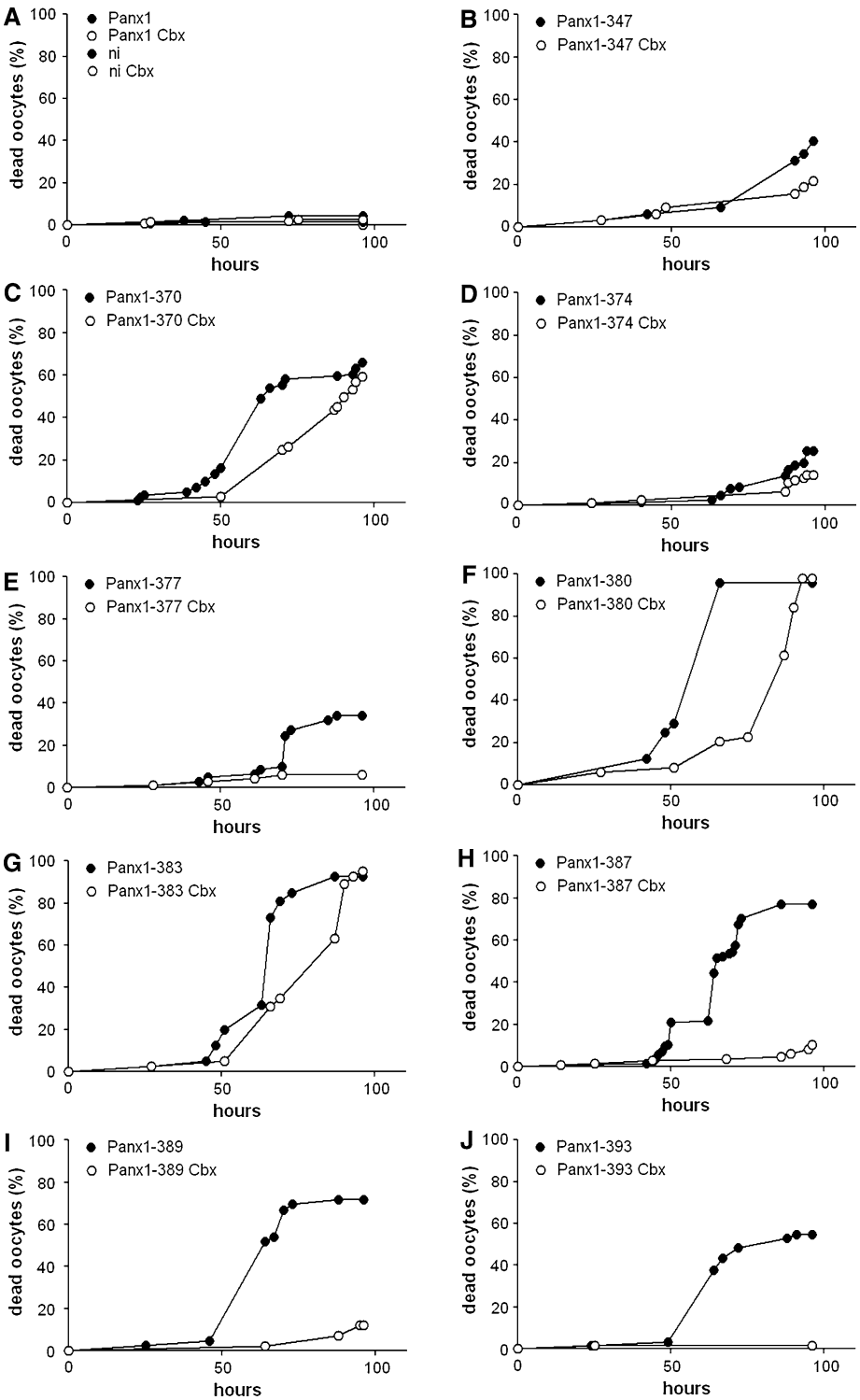

Fig. 4 Induction of cell death by expression of truncated versions of Panxl. The oocytes were examined microscopically at four time points/day, the viability was determined according to morphological criteria. An oocyte was considered dead when yolk was protruding from the ooplasm. Oocytes were either incubated in NFR (*filled circles*) or in NFR containing 50 μM Cbx (*open circles*). **a** Oocytes injected with Panx1 wt (Panx1) served as positive controls, uninjected oocytes (*ni*) served as negative controls. **b–j** Specific survival and Cbx rescue effects were revealed for different Ct-trunc Panx1

Discussion

Truncation of Panx1 Leads to Differential Effects on Channel Properties

Truncation of Panx1 by caspases 3/7 has been described to be part of an apoptotic pathway, which causes recruitment of phagocytes by sustained ATP release serving as a "find-me" signal (Chekeni et al. 2010). These initial studies indicated that the C-terminus (aa 297–426) is crucial for proper channel function, and cleavage of the C-terminus at a conserved caspase cleavage site is responsible for the leakiness of the channel to ATP. As suggested later, the C-terminal domain subject to cleavage may function as an

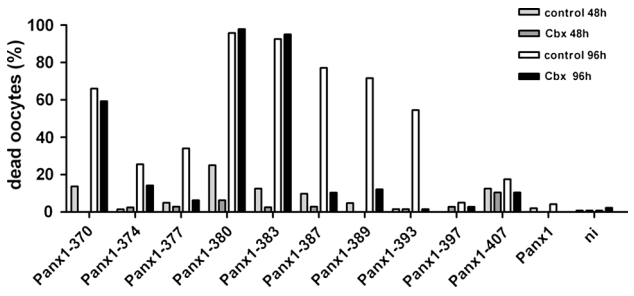

Fig. 5 Induction of cell death in *X. laevis* oocytes by expression of Panx1-truncated mutants. Cell death of oocytes expressing truncated forms of Panx1 after 48 and 96 h in NFR or NFR containing 50 µM Cbx. Panx1-347 $n = 67$, Panx1-367 $n = 54$, Panx1-370 $n = 111$, Panx1-374 $n = 127$, Panx1-377 $n = 145$, Panx1-380 $n = 74$, Panx1-383 $n = 67$, Panx1-387 $n = 266$, Panx1-389 $n = 86$, Panx1-393 $n = 88$, Panx1-397 $n = 131$, Panx1-407 $n = 68$, Panx1 $n = 96$, ni $n = 270$, results obtained in $n \geq 2$ independent experiments/construct

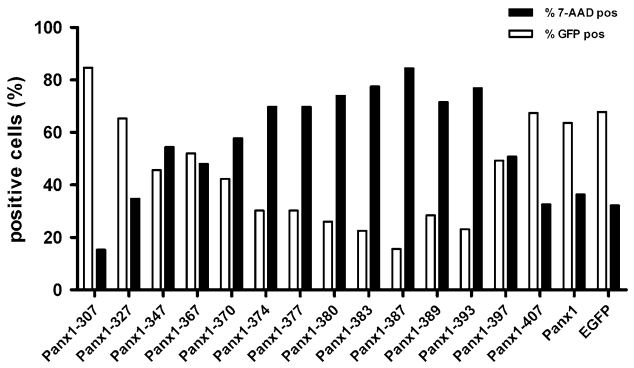

Fig. 6 Induction of cell death in the mammalian N2A cell line by expression of Panx1-truncated mutants. The percentage of cells sorted by FACS flow cytometry positive for either 7-Aminoactinomycin (7-AAD) or GFP are depicted. Cells positive for 7-AAD are considered dead, while cell populations positive for GFP are still alive. 10,000 cells counted/transfection, results obtained in two independent experiments

autoinhibitory region for Panx1 channel opening (Sandilos et al. 2012). By making use of a truncation approach, we addressed the question of a putative functional segmentation of the Panx1 CT and applied electrophysiological and pharmacological analyses.

Within the scope of this study, 14 consecutive truncated versions of Panx1 were generated. Two different expression systems were used to analyze the effect of an expression of these proteins on cell viability as well as on electrophysiological channel properties.

While expression of some proteins led to cell death in mammalian cells as well as in *X. laevis* oocytes, others could be expressed in oocytes without causing degradation and proved suitable for TEVC recording. This confirms an earlier report on three different truncated forms of Panx1 (Jackson et al. 2014). In the following, aspects of the influence of the C-terminus length in our truncated Panx1 channels on channel gating properties, on cellular survival, and on Cbx sensitivity will be discussed separately. A summary of the results of this study is given in Fig. 7.

Electrophysiology of Channels Formed by Truncated Panx1 Proteins

In addition to gating under physiological conditions, i.e., by mechanical stress or high extracellular K^+, truncation of Panx1 results in specific effects on channel gating depending on the site of truncation. A stepwise reduction of the length of the C-terminus did not simply lead to an accruing loss of function. Instead, the results indicate a domain-specific profile in form of functional segmentation.

Expression of Panx1-307, the shortest form generated, resulted in large Cbx-insensitive currents. In this mutant, nearly the entire C-terminus is missing. Because oocytes expressing Panx1-307 survive without any signs of impairment, a constitutive, permanently open channel can be excluded. This finding also allows proposing the localization of a voltage sensor: since Panx1-307 channel activation is voltage dependent it is unlikely that the sensor is part of the C-terminus, which in this Ct-trunc Panx1 consists of only 10 amino acids. Therefore, it is reasonable to assume that the voltage sensor of Panx1 is located upstream residue 306, i.e., either in the N-terminal domain, the intracellular loops and/or the transmembrane segments. Interestingly, currents recorded from oocytes expressing Panx1-327, -347, and -367 did not show large differences to currents recorded from oocytes expressing the full-length Panx1 protein. This leads to the conclusion that residues 308–327 are necessary and sufficient for proper channel gating, and the addition of further 20–40 residues does not influence this function.

Stable current responses were obtained from oocytes expressing Panx1-397, while oocytes expressing Ct-trunc Panx1 with lengths between 367 and 393 aa did not survive and could not be characterized electrophysiologically. This means that residues 394–397 are sufficient for a rescue of oocyte survival. The channel can be closed again, although complete channel function is not restored. Interestingly, oocytes expressing Panx1-407 show a very high current response with an almost 10-fold larger average magnitude than Panx1-wt expressing oocytes. In fact, we expected that an addition of further amino acids would cause stabilization of channel function and result in channel properties more similar to the full-length Panx1 protein.

The difference of 10 residues between Panx1-397 and -407 results in a channel that does not cause cell death, but some conformational change must be induced that prevents proper channel gating.

The addition of further 20 residues reflecting the wild-type protein results in a functionally competent channel.

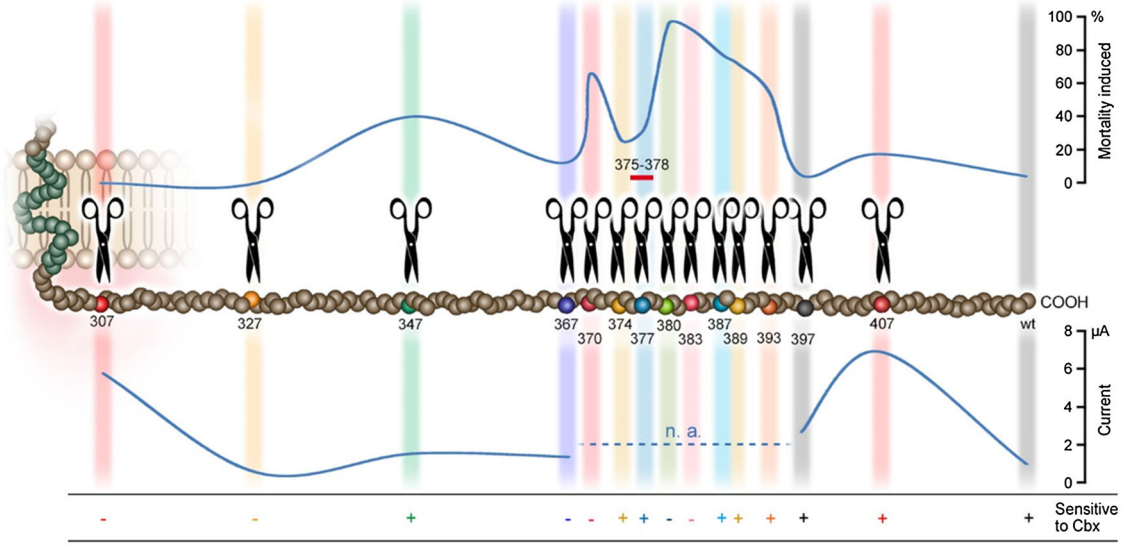

Fig. 7 The impact of C-terminal truncation on Panx1 channel properties. The influence of carboxy-terminal truncation on cell mortality, gating properties, and Cbx sensitivity is depicted according to the site of truncation. The illustrated results reveal data obtained in oocyte experiments. The *red bar* marks the position of the Casp 3/7 cleavage site. + Positive, − negative, *n.a.* not applicable

The expression of truncated versions of Panx1 with lengths ranging from 370 to 393 aa showed lethal effects both on *X. laevis* oocytes as well as Neuro2A cells (see below).

Sensitivity to Cbx

The mechanism by which Cbx inhibits Panx1 currents remains unknown to date. In oocytes expressing Panx1-wt, Cbx application significantly reduces evoked currents (Bunse et al. 2009). In contrast, as demonstrated by oocyte survival and the electrophysiological recordings, Cbx treatment did not affect oocytes that expressed some of the truncated Panx1 proteins. Thus, lethal effects induced by some of the Ct-trunc Panx1 were almost completely blocked by Cbx in oocytes expressing Panx1-377, -387, -389, and -393, but other mutants were either insensitive to Cbx, or Cbx application delayed the progression of cell death. The inhibitory effect of Cbx was observed for Panx1-347, -374, -397, and -407. No Cbx sensitivity was observed in truncated Panx1-307, -327, -367, -370, -380, and -383 (Fig. 7). Although Ct-trunc Panx1 that results in Cbx-insensitive cell death can conduct Cbx-sensitive currents (Jackson et al. 2014), differences in Cbx sensitivity of cell death deserve a closer look. Notably, upon truncation of Panx1 behind the fourth transmembrane domain and stepwise elongation, Cbx sensitivity first occurs after expression of Panx1-347, as indicated by the block of Panx1-mediated currents, while currents mediated through Panx1-327 were still Cbx insensitive. This observation favors a concept of a putative Cbx interaction site located in a segment of the protein, which spans a domain between residues 327 and 347. It is conceivable to assume that the proteins Panx1-367, -370, -380, and -383 undergo conformational changes, which mask the Cbx interaction site. This would explain the insensitivity of the listed Ct-trunc Panx1 and furthermore strengthens the concept of ligand/binding site interaction of Cbx and Panx1 with variable, susceptible domains at the C-terminus of the protein rather than inhibition of the channel pore by a change of general conformation. In case of a specific interaction site residing at the C-terminus, one has to acknowledge that Cbx must be able to pass or enter the channel in order to affect Panx1 function intracellularly.

Functional Implications and Conclusions

In two recent studies on the effect of substitution of single cysteins by serine in the Panx1 protein (Bunse et al. 2010, 2011), results comparable to our truncation data were achieved. Substitution of a cysteine residue in the first transmembrane loop (C40) or a cysteine residue in the carboxy-terminal domain (C346) also led to constitutively open channels, but both mutants revealed persistent sensitivity to Cbx. These data are in accordance with our proposed concept of a specific Cbx binding site in the C-terminal domain of Panx1 and is worth of further in-depth analyses on the molecular pharmacology of Cbx–Panx1 interaction. In Neuro2A cells, viability was investigated after expression of all 14 truncated versions of Panx1 24 h post transfection by FACS analysis. Remarkably, in Neuro2A cells, the length of the Ct-trunc Panx1 directly correlates with cell survival. Starting with Panx1-307, the fraction of degenerating cells continuously

increased with increasing Ct-trunc length until mortality rate peaked in cells expressing Panx1-387. Further prolongation of Panx1 constructs resulted in a steady decline of mortality until full survival was reached with the full-length protein. Not surprisingly, expression of Panx1-wt also leads to a certain population of dead cells. This most likely is caused by mechanical stress during cell collection for FACS analysis that activates Panx1 (Bao et al. 2004). The results obtained from the oocyte survival studies reveal that the critical region, i.e. the region where a putative caspase cleavage site does not lead to high mortality, is very small. In fact, only truncation at residues 367, 374, and 377 did not lead to more than 25 % degenerated oocytes. These findings unravel a highly conserved evolutionary mechanism. Obviously, the exact position of the caspase cleavage site is crucial for its function in apoptosis. If the motif DxxD was shifted just a few residues up- or downstream its actual position, cleavage would probably lead to formation of a pore with another conformation, most likely with an altered pore diameter. As a consequence, the release of the "find-me" signals ATP and UTP would be changed. Based on the results presented here, it can be postulated that the contribution of the permanently open channel to cell death would increase dramatically. However, if the cells died too fast, less nucleotides could be released to activate phagocytes, and consequently, the activation of the immune response would be considerably altered as well. Thus, the exact position for the caspase cleavage site is of great importance and may have developed under strong selective pressure during evolution of the Panx1 protein. Since Panx1 is of significant importance in a variety of clinically relevant pathologies including inflammation (Adamson and Leitinger 2014), virus infection like HIV (Orellana et al. 2013; Paoletti et al. 2013), and targeting of antibiotics where the CT of Panx1 seems to be crucial for general functioning (Poon et al. 2014; Gregory 2014), we expect important insights into translational aspects (Dahl and Keane 2012; Wang et al. 2013) when mechanistic models of the functional segmentation of the CT are taken into account.

Acknowledgments We thank 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 K.E., and the DFG (292/11-4) to R.D.

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