Intracellular Cysteine 346 Is Essentially Involved in Regulating Panx1 Channel Activity*

Received for publication, January 5, 2010, and in revised form, September 7, 2010. Published, JBC Papers in Press, September 9, 2010, DOI 10.1074/jbc.M110.101014

Stefanie Bunse, Matthias Schmidt, Nora Prochnow, Georg Zoidl, and Rolf Dermietzel

From the Departments of 8Neuroanatomy and Molecular Brain Research and 6Cytology, Ruhr-University Bochum, 44780 Bochum, Germany

Pannexins constitute a family of proteins exhibiting predominantly hemichannel activity. Pannexin channels have been suggested to participate in a wide spectrum of biological functions such as propagation of calcium waves, release of IL-1β, and responses to ischemic conditions. At present, the molecular mechanisms regulating pannexin hemichannel activity are essentially unknown. Because cysteines have been shown to constitute key elements in regulating hemichannel properties of the connexin-type we performed site-directed mutagenesis of intracellular cysteine residues of Panx1. Cysteine to serine exchange (Cys → Ser) at the C-terminal position amino acid 346 led to a constitutively leaky hemichannel and subsequently to cell death. Increased channel activity was demonstrated by dye uptake and electrophysiological profiling in injected Xenopus laevis oocytes and transfected N2A cells. Mutations of the remaining intracellular cysteines did not result in major changes of Panx1 channel properties. From these data we conclude that the Cys-346 residue is important for proper functioning of the Panx1 channel.

In addition to connexins, a second family of proteins, termed pannexins (Panx)3 (1), is suggested to form hemichannels. The pannexin channel family consists of three members: Panx1, which is abundantly expressed in different organs of the vertebrate organism, Panx2, which is found mainly in the central nervous system, and Panx3, which is primarily expressed in skin and cartilage (2–8). Panx1 is capable of forming gap junctions and hemichannels in the Xenopus laevis oocytes expression system (9, 10), but it is still a matter of debate whether pannexin-formed gap junctions do exist under in vivo conditions. Apparently, Panx1 primarily forms hemichannels (9, 11, 12). Among others, a role of Panx1 hemichannels was suggested in the initiation and propagation of calcium waves (13), the release of interleukin-1β due to interaction with the P2X7 receptor (14–16), neuronal cell death after ischemia (17, 18), and in activation of inflammasomes (19). Panx1 hemichannels open in response to a rise in intracellular Ca2+, to depolarization to membrane potentials above −20 mV, and to mechanical stretch (10, 11, 20).

Even though there is no direct sequence homology between pannexins and connexins, both protein types possess the same topology with four transmembrane domains and intracellularly located N and C termini (for review, see Ref. 21). Significant differences between the two protein families are the number of extracellular cysteines and the fact that Panx1 is glycosylated at the second extracellular loop (9, 22). Deglycosylation with N-glycosidase F enhanced gap junctional coupling, indicating that glycosylation might be a regulatory key element to distinguish between gap junctional coupling and hemichannel activity (23).

Besides the importance of extracellular cysteines in gap junction channel formation of connexins (24), intracellular cysteine residues have been suggested to be responsible for sensitivity of Cx43 and Cx46 hemichannels to changes in redox potentials (25, 26).

Recently, we reported that Panx1 channels are sensitive to reducing agents such as Tris(2-carboxyethyl) phosphine (TCEP) or dithiothreitol (27). These findings are of particular interest as redox regulation plays an important role in the biology of cells under physiological and pathological conditions such as hypoxia (17). In this case, Panx1 hemichannels have been found to open under oxygen and glucose deprivation serving a kind of large death pore that leads to neuronal degeneration (17). Under ischemic conditions changes in redox potential occur, which were proposed to play an essential role for Panx1 hemichannel activity (18). Furthermore, intracellular cysteines of Cx43 are likely to be important for regulation of hemichannel gating in response to metabolic inhibition (25). An essential prerequisite for an understanding of Panx1 hemichannel operation is the molecular mapping of amino acid residues that are required for hemichannel activity. Therefore, we performed site-directed mutagenesis of the intracellular cysteines at positions Cys-136, Cys-346, and Cys-426 (supplemental Fig. 1) and studied the cellular localization of mutated hemichannels as well as their physiological properties. Surprisingly, expression of the C-terminal mutant Panx1-C346S induced rapid cell death, whereas the other mutants showed only subtle changes in their physiological properties. Dye uptake experiments and electrophysiological studies strongly indicated that the mutation C346S led to a constitutively leaky hemichannel, suggesting that the Cys-346 residue constitutes a key element of Panx1 channel gating.

*This work was supported by a grant from the Graduiertenkolleg GRK 736 “Development and Plasticity of the Nervous System: Molecular, Synaptic and Cellular mechanisms” (to S. B.) and by Deutsche Forschungsgemeinschaft Grant 292/11-4 (to R. D. and G. Z.).

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–5.

‡ Joint senior authors.

§ To whom correspondence should be addressed: Universitätsstrasse 150, MA, 6/159, 44780 Bochum, Germany. Tel.: 0049-234-3225003; Fax: 0049-234-32124655; E-mail: Rolf.Dermietzel@rub.de.

†† The abbreviations used are: Panx, pannexin; Cbx, carbenoxolone; ConA, concanavalin A; Cx, connexin; LY, Lucifer yellow; NFR, normal frog Ringer’s; TCEP, Tris(2-carboxyethyl) phosphine.

3 The abbreviations used are: Panx, pannexin; Cbx, carbenoxolone; ConA, concanavalin A; Cx, connexin; LY, Lucifer yellow; NFR, normal frog Ringer’s; TCEP, Tris(2-carboxyethyl) phosphine.
EXPERIMENTAL PROCEDURES

Plasmids and Site-directed Mutagenesis—The full-length mouse Panx1 cDNA (GI: 86262133; nucleotides 442–1722) was cloned into the pEGFP-N3 vector (Clontech) and into the pCS2+ vector. Mutageneses of single intracellular cysteines of Panx1 were performed using the TransformerTM Site-directed Mutagenesis kit (Clontech) according to the manufacturer’s protocol. Following mutagenesis primers were used: C136S (5'-ggacgttccacacctctCtctacagcttaaggtc-3'), C346S (5'-gcatacaagctcagacctgaag-3'), and C426S (5'-gaactgctccagctgaattcg-3'). For mutagenesis of the pCS2+ Panx1 plasmid a selection primer mutating the BamHI restriction site was used (5'-gctacgctttttgacagTctccatcgtaccg-3'), whereas for the pEGFP-N3-Panx1 the NotI restriction site was mutated (5'-gtacaagtagctcagacctgaag-3'). The coding regions of Panx1 WT and of Panx1-C346S were cloned into the pIRE2-EGFP vector (Clontech).

Synthesis of cRNA—The pCS2+-Panx1 plasmid and its mutated variants were used for in vitro transcription as templates. The plasmids were linearized with NotI, purified using the PCR Purification kit (Qiagen), and transcribed using the mMESSAGE mMACHINE SP6 kit (Ambion; Applied Biosystems) according to the manufacturer’s guidelines. The quantity of cRNAs was determined using 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—Defolliculated oocytes from X. laevis were obtained from EcoCyte Bioscience. Injections were performed using the Roboocyte® automated oocyte injection and recording system (Multichannel Systems). Oocytes were injected with approximately 50 nl of cRNA solution (1 μg/μl) and kept in Barth’s solution (EcoCyte Bioscience) containing 88 mM NaCl, 2.4 mM NaHCO3, 1 mM KCl, 0.33 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 5 mM Tris, pH 7.4, supplemented with 50 mg/liter Gentamicin.

Cell Culture and Transfection—N2A cells were cultured as described previously (29). 10,000 cells were seeded on coverslips placed in 24-well plates and transfected 1 day after plating using 200 ng of DNA/expression plasmid and the transfection reagent Effectene® (Qiagen) according to the manufacturer’s protocol.

Dye Uptake Oocytes—Oocytes were injected with cRNA either for Panx1 WT or for Panx1-C346S. One half of each set of oocytes was incubated in regular Barth’s solution, and the other half was incubated in Barth’s solution containing 10 μM carbeneoxolone (Cbx). Two days after injection, intact oocytes were placed in normal frog Ringer’s (NFR; EcoCyte Bioscience) solution containing Lucifer yellow (LY, 1 mg/ml; Sigma) for 45 min. The oocytes were washed extensively with PBS and fixed with 2% paraformaldehyde for 10 min. After washing with PBS, photographs were taken using an Axiohot fluorescence microscope (Zeiss).

Immunocytochemistry of Injected Oocytes—Two to 3 days after injection oocytes were fixed with 2% paraformaldehyde for 10 min and subsequently washed in PBS. Oocytes were frozen in tissue freezing medium (Jung) at −60 °C. 12-μm-thick sections were cut using a Leica CM 3050 cryostat and mounted on superfrost microscope slides (Menzel-Gläser; Thermo Fisher Scientific). Immunocytochemistry was performed as described previously (30) using the anti-Panx1 4515 antibody from chicken (13). The goat antibody against chicken IgY coupled to Alexa Fluor 488 (Invitrogen) was used as secondary antibody.

Pulldown of Concanavalin A (ConA)-labeled Membrane Proteins from Injected Oocytes—Two to 3 days after injection approximately 10–20 injected oocytes were incubated in 10 μM Biotinyl-ConA (Sigma) for 30 min. Oocytes were washed extensively with NFR solution. Then, 20 μl of lysis buffer (100 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, protease inhibitor mixture (Sigma) 1:1000, 1 mM PMSF) per oocyte was added and samples homogenized. The lysate was shaken at 4 °C for 1 h and centrifuged at 16,000 × g for 15 min. The supernatant was centrifuged again, and a 20-μl aliquot was taken for total fraction. 40 μl of streptavidin-agarose (Sigma) was centrifuged, washed four times with lysis buffer, and resuspended in lysis buffer to restore the initial volume. It was then added to the oocyte-lysate, and the probe was incubated on a rotating wheel at 4 °C overnight. Subsequently, the probe was centrifuged at 16,000 × g for 15 min, the supernatant was removed, and the agarose beads were washed five times with 1.5 ml of lysis buffer. The pellets were resuspended in 20 μl of Laemmli buffer and heated to 95 °C for 5 min. Finally, the samples were centrifuged at 16,000 × g for 2 min, the supernatant was separated by SDS-PAGE gel, and Western blotting was performed. The IgG-purified anti-Panx1 4515 antibody from chicken (13) was used as primary antibody and a goat polyclonal antibody to chicken tagged by Cy5.5 (Abcam) as secondary antibody. The Odyssey® Infrared Imaging System (Licor) was applied for immunodetection.

Western Blot Analysis—Total protein lysates of transfected N2A cells or injected oocytes were prepared at the time points indicated using the lysis buffer described above. Lysates (30 μg) were separated by SDS-PAGE and Western blotting performed using the IgG-purified anti-Panx1 4515 antibody from chicken (13) and experimental conditions as described above.

Confocal Microscopy—Pictures of transfected N2A cells and immunostained sections of injected oocytes were taken with the confocal microscope LSM 510 Meta system (Zeiss) as described (12).

Oocyte Recordings—Two-electrode voltage clamp recordings from injected oocytes were carried out with the Roboocyte® system 2–3 days after injection. Recording pipettes were filled with 2.5 M potassium acetate resulting in an electrode impedance of approximately 1 megohm. For recording, oocytes were continuously superfused with NFR containing 90 mM NaCl, 2 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES, at pH 7.4. To characterize hemichannel activity, oocytes were voltage-clamped to −70 mV and the currents monitored in response to 2-s voltage steps (from −100 mV to +60 mV, in 20-mV increments).

Drugs were diluted to their final concentration in NFR and were applied using the Roboocyte gravity-based 8-channel perfusion system, which allowed a fast change between NFR and...
Mutation of Cys-346 of Panx1 Affects Hemichannel Activity

Panx1 contains 10 cysteines: 2 in each extracellular loop, 3 in the transmembrane domains, 1 in the intracellular loop and 2 in the C-terminal domain (supplemental Fig. 1). We performed site-directed mutagenesis of the three intracellular cysteines C136S, C346S, and C426S. cRNAs, *in vitro* transcribed from the mutated plasmids, were injected into *X. laevis* oocytes to perform two-electrode voltage clamp measurements. Two days after injection, the majority of Panx1-C346S-expressing oocytes were degenerated, whereas those injected with cRNA for the Panx1 WT or the other two mutants displayed no signs of cell degeneration (Fig. 1A, *left panel*). To test whether the C346S mutation affects channel properties of Panx1 and could thus be responsible for the rapid cell death, we incubated Panx1-C346S and Panx1 WT-expressing oocytes either in normal Barth’s solution or in Barth’s solution containing 10 μM Cbx. Fig. 1A shows examples of single oocytes for each condition 48 h after injection. Oocytes injected with cRNA for Panx1-C346S and incubated in normal Barth’s solution revealed typical signs of cell death-like yolk protruding from the ooplasm. Oocytes injected with cRNA for Panx1 WT were healthy under both conditions. The exit of yolk from the oocyte was defined as a visual marker for cell death, and the number of oocytes matching this criterion in each group was determined at different time points after injection. Degeneration of Panx1-C346S-expressing oocytes was delayed significantly when treated with 10 μM Cbx compared with untreated cells (Fig. 1B). Increasing the Cbx concentration to 50 μM further delayed degeneration. Nonlinear fits to survival curves of nontreated Panx1-C346S-expressing oocytes in two independent experiments revealed a 50% survival 62 and 58 h after injection. In the presence of 50 μM Cbx, this time point was shifted to 94 and 92 h. In contrast, oocytes injected with either Panx1-C136S or C426S did not show signs of degeneration when all C346S oocytes had deteriorated (supplemental Fig. 2, A and B). The biphasic form of the survival rate of C346S after Cbx exposure with baseline survival levels during the initial phase (40 h after 10 μM treatment) and accelerated degeneration thereafter, as well as 72 h after 50 μM Cbx of treatment can be explained by accruing toxic effects of intracellular accumulation of mutant proteins, which is not influenced by hemichannel blocking (Fig. 1B and supplemental Fig. 2A).

Dye uptake from the external medium offers an indirect, but reliable method to analyze properties of hemichannel function (31). Fig. 2 illustrates the results of a typical dye uptake experiment using LY fluorescent dye. Whereas oocytes expressing Panx1 WT were impermeable to LY, oocytes injected with Panx1-C346S RNA (but not C136S or C426S) (supplemental Fig. 3) accumulated the dye under control conditions. This uptake was reduced in the presence of 10 μM Cbx, indicating that sufficient amounts of this mutant were incorporated into the cell membrane. However, the lack of a similar phenotype of mutants C136S and C426S raises the possibility that impaired trafficking might have obscured changes in hemichannel activity of these two mutants. Therefore, we looked for the presence of these mutants in cell membranes of injected oocytes. Because Panx1 has been described to be glycosylated and glycosylation has been shown to be important for its trafficking to the plasma membrane (9, 22), we applied cell surface biotinylation assays. Western blots of ConA pulldown fractions from oocyte lysates demonstrate that in particular the intermediate glycosylated variant (GLY1) of Panx1 is pulled down in case of the WT and of all mutants (Fig. 3A). Preferred binding of ConA to the high mannose-type intermediate GLY1 isoform explains the dominance of the intermediate band under pulldown conditions.
Mutation of Cys-346 of Panx1 Affects Hemichannel Activity

FIGURE 3. Expression analyses of mPanx1 mutants with modified intracellular cysteine residues in injected oocytes. A, glycosylated membrane proteins of cRNA-injected oocytes were labeled with biotinyl-ConA and then lysed (left panel). The lanes marked total display a fraction of the total lysates before the pulldown was performed. The lanes marked PD show the fractions that were obtained after a pulldown with streptavidin-agarose. As a negative control, a pulldown was performed on Panx1 WT (wt)-injected oocytes omitting the ConA labeling (ø). The Western blot analysis was performed using a Panx1 antibody. Uninjected oocytes did not show any immunoreactivity, whereas all injected oocytes displayed a strong signal specific antibody. Uninjected oocytes did not show any immunoreactivity, whereas all injected oocytes displayed a strong signal at the plasma membrane and only little signal in the ooplasm. The expression and localization of the WT and the mutant proteins were also studied in a mammalian expression system. Western blot analyses were performed using lysates derived from N2A cells, which were either transfected with plasmids encoding for Panx1 WT, C136S, C346S, or C426S, and from untransfected cells. The WT Panx1 was further confirmed using N2A cells transfected with expression constructs for C-terminal-tagged EGFP fusion proteins of Panx1 WT and the mutants C136S and C426S, which has been described for Panx1 (9, 22), representing its glycosylated isoform (GLY2). Faulty glycosylation and processing of Panx1 in Xenopus oocytes as has been described for a number of exogenously expressed proteins (32, 33) may account for this effect. Because recent studies showed that full glycosylation might not prevent transport of Panx1 to and insertion into the cell membrane (34, 35), we confirmed the membrane localization of the mutants and WT Panx1 by immunocytochemistry. Fig. 3C shows sections of oocytes injected with cRNA for Panx1 WT, C136S, C346S, or C426S, and from untransfected control oocytes, stained with a Panx1-specific antibody. Uninjected oocytes did not show any immunoreactivity, whereas all injected oocytes displayed a strong signal at the plasma membrane and only little signal in the ooplasm. The expression and localization of the WT and the mutant proteins were also studied in a mammalian expression system. Western blot analyses were performed using lysates derived from N2A cells, which were either transfected with plasmids encoding for Panx1 WT, C136S, C346S, or C426S, and from untransfected cells. The WT Panx1 was further confirmed using N2A cells transfected with expression constructs for C-terminal-tagged EGFP fusion proteins of Panx1 WT and the mutants C136S and C426S, which has been described for Panx1 (9, 22), representing its glycosylated isoforms. The cellular distribution of the mutants versus the WT Panx1 was further confirmed using N2A cells transfected with expression constructs for C-terminal-tagged EGFP fusion proteins of Panx1 WT and the mutants C136S and C426S, which has been described for Panx1 (9, 22), representing its glycosylated isoforms. The cellular distribution of the mutants versus the WT Panx1 was further confirmed using N2A cells transfected with expression constructs for C-terminal-tagged EGFP fusion proteins of Panx1 WT and the mutants C136S and C426S, which has been described for Panx1 (9, 22), representing its glycosylated isoforms. The cellular distribution of the mutants versus the WT Panx1 was further confirmed using N2A cells transfected with expression constructs for C-terminal-tagged EGFP fusion proteins of Panx1 WT and the mutants C136S and C426S, which has been described for Panx1 (9, 22), representing its glycosylated isoforms. The cellular distribution of the mutants versus the WT Panx1 was further confirmed using N2A cells transfected with expression constructs for C-terminal-tagged EGFP fusion proteins of Panx1 WT and the mutants C136S and C426S, which has been described for Panx1 (9, 22), representing its glycosylated isoforms.
Mutation of Cys-346 of Panx1 Affects Hemichannel Activity

**FIGURE 5.** Electrophysiological characterization by the two-electrode voltage clamp technique indicates increased hemichannel activity of Panx1-C346S. A, average I–V curve from un.injected oocytes under control conditions and after application of 10 μM Cbx. B, average I–V curve from Panx1 WT-expressing oocytes under control conditions and after application of 10 μM Cbx. For voltages greater than −20 mV, an exponential current increase can be observed. Application of Cbx significantly decreased the slope of the curve at positive membrane potentials compared with the control. C, average I–V curve from oocytes expressing Panx1-C346S. Application of Cbx decreases current amplitudes for voltages lower than −20 mV as well indicating increased hemichannel activity at resting conditions. Error bars in A–C indicate S.D. D, quantitative analyses of oocytes expressing the WT and mutant forms of Panx1 reveal a significantly reduced negative resting potential in the Panx1-C136S mutant. E, Panx1-C346S-expressing oocytes showing significantly smaller input resistance than WT-expressing oocytes. F, expression of Panx1-C346S leading to a significant decrease in input resistance compared with the WT. G, quantitative analysis of the effect of Cbx on maximum current measured at +60 mV. For clarity, significant differences between the WT-expressing oocytes and the mutants or un injected oocytes (n) are indicated only. For all parameters presented in D–F, differences between uninjected oocytes and all other groups were highly significant (***, p < 0.001). Numbers of oocytes: D–F: n, 99; Panx1 WT, 182; Panx1-C136S, 78; Panx1-C346S, 29; Panx1-C426S, 85; G: n, 49; Panx1 WT, 56; Panx1-C136S, 42; C346S, 17; C426S, 20.

cRNAs for Panx1 WT and the mutants were characterized by two-electrode voltage clamp experiments. In addition, we investigated whether the inhibiting effect of the reducing agent TCEP (27) can be related to any of the three intracellular cysteines. Current-voltage relationships (I–V curves) were obtained by clamping the oocytes to −70 mV and applying voltage steps ranging from −100 mV to +60 mV in 20-mV increments. Fig. 5, A–C, shows examples of averaged control I–V curves and I–V curves in the presence of 10 μM Cbx for un injected oocytes as well as Panx1 WT and cytes. Consistent with other reports (10, 15, 37), a low concentration of the gap junction blocker Cbx is sufficient to block WT Panx1-mediated currents at depolarizing voltages. In oocytes expressing Panx1-C346S, Cbx also blocked currents evoked at hyperpolarizing voltages. This feature further indicates atypical hemichannel activity of this mutant at resting conditions. In oocytes expressing Panx1 WT or the mutant proteins C136S or C426S, hemichannel activity was never observed either at resting membrane potential or when oocytes were hyperpolarized (supplemental Fig. 5).

Next, the basic electrophysiological properties of WT and mutants were compared. Panx1-C346S-expressing oocytes revealed a significantly more positive resting membrane potential (−10.2 ± 3.4 mV) compared with the other groups (non injected, −21.6 ± 7.0 mV; Panx1 WT, −20.2 ± 7.7 mV; Panx1-C136S, −21.8 ± 7.6 mV; Panx1-C426S, −23.4 ± 5.8 mV) (Fig. 5D). Most likely, the shift in resting membrane potential is caused by an increased conductance of the Panx1-C346S mutant channel. Fig. 5E shows a comparison of the average input resistances of injected and un injected oocytes. Panx1-C346S-expressing oocytes had a significantly smaller input resistance (0.03 ± 0.02 meohm; p < 0.001) compared with Panx1 WT (0.53 ± 0.47 meohm) and the other two mutants (Panx1-C136S, 0.42 ± 0.2 meohm; Panx1-C426S, 0.34 ± 0.15 meohm), also reflecting the increased channel activity of this mutant. In addition, Panx1-C426S showed a significantly (p < 0.001) increased conductance compared with Panx1 WT. Fig. 5F compares mean current amplitudes induced by a voltage step from −70 mV to +60 mV for oocytes injected with WT and mutant cRNAs as well as for noninjected oocytes. As expected, the average currents of the WT (1,610 ± 1,411 nA) and all mutants were significantly higher than those derived from un injected control oocytes (426 ± 415 nA; p < 0.001). In addition, the currents derived from the mutant Panx1-C346S (11,326 ± 4,474 nA; p < 0.001) were much larger than currents...
The ability of TCEP to partially block Panx1-mediated currents (27) was also tested on the WT and the mutants. Control $I\!-\!V$ curves were compared with $I\!-\!V$ curves after application of 10 mM TCEP. To verify that the oocytes expressed Panx1, $I\!-\!V$ curves were also recorded in the presence of 10 μM Cbx to demonstrate the Cbx-induced decrease in current after recovery from the TCEP application. Fig. 6, A and B, shows average $I\!-\!V$ curves for uninjected and WT-expressing oocytes. Application of 10 mM TCEP reduced current amplitudes to 73.3% (±6.3%) of the control for Panx1 WT. Likewise, current amplitudes were also reduced in the case of the three mutants (Panx1-C136S, 70.8% ± 13.3%; Panx1-C346S, 68.5% ± 12.5%; Panx1-C426S, 61.8% ± 8.3%) (Fig. 6C). Furthermore, the value for uninjected oocytes (93.6% ± 14.7%; $p < 0.001$) was significantly higher compared with all other groups. Obviously, the inhibitory effect of TCEP was not abolished for any of the mutants, as they all showed a significant decrease of maximum current amplitudes after application of TCEP compared with the control.

We further wanted to examine whether the abnormal hemichannel activity of Panx1-C346S can also be found in a mammalian expression system or whether it is linked to the *Xenopus* oocyte expression system. Therefore, the coding regions of Panx1 WT and Panx1-C346S were cloned into a pIRE2-EGFP vector. Current-voltage relationships were recorded using the whole cell patch clamp technique. Cells were clamped to −30 mV, and currents measured in response to voltage steps ranging from −50 mV to +60 mV in 10-mV increments. Average control $I\!-\!V$ curves as well as $I\!-\!V$ curves after application of 20 μM Cbx from pIRE2-EGFP-, pIRE2-EGFP-Panx1 WT-, and pIRE2-EGFP-Panx1-C346S-transfected cells are summarized in Fig. 7, A–C, respectively. In contrast to vector-only transfected cells, the slope of the curve from Panx1 WT- and Panx1-C346S-transfected cells increases exponentially for positive potentials and is reduced after application of Cbx.

A quantitative comparison of the maximum current amplitude measured for the voltage step from −30 mV to +60 mV is shown in Fig. 7D. Control cells transfected with pIRE2-EGFP showed significantly smaller amplitudes (162.5 pA ± 117.2 pA) than cells transfected with pIRE2-EGFP-Panx1 (292.1 pA ± 245.3 pA; $p = 0.017$) or pIRE2-EGFP-Panx1-C346S (806.6 pA ± 496.3 pA; $p < 0.001$). Consistent with the data obtained from WT and the other mutants (Panx1-C136S, 1,055 ± 696 nA; Panx1-C426, 1,708 ± 815 nA). This is in line with the very low input resistance and the results obtained from the dye uptake and the survival studies. It also confirms that the C346S hemichannel is constitutively leaky, leading to a high conductivity of the whole cell. A quantitative analysis of the Cbx effect on currents derived from injected and uninjected oocytes is given in Fig. 5G. Current amplitudes remaining after application of 10 μM Cbx were determined for voltage steps from −70 mV to +60 mV. The amplitude in the presence of Cbx for uninjected oocytes was 90.5% (±9.4%) of the control and differed significantly ($p < 0.001$) from the WT as well as from all mutants. Although no significant difference between the C136S (59.9 ± 11.1%) and C426S (49.3 ± 13.9%) and the WT (57.5 ± 16.9%) was observed, the remaining current amplitude percentage was significantly smaller in the Panx1-C346S mutant (38.4 ± 13.7%; $p < 0.001$). Most likely, this is caused by the large currents of the Panx1-C346S-expressing oocytes, in which the contribution of currents mediated by the mutant channel to the overall current is substantially increased. Thus, a larger fraction of the total current can be blocked by Cbx.
Mutation of Cys-346 of Panx1 Affects Hemichannel Activity

from oocytes, currents from cells transfected with pIRES2-EGFP-Panx1-C346S were significantly larger (p < 0.001) than those from WT transfected cells, suggesting a high conductivity of cells transfected with the C346S mutant. The difference for the maximum currents between the WT and C346S was not of the same magnitude in the N2A cells as it was in the oocytes. This was probably due to a higher vulnerability of the N2A cells that biased the selection of recorded cells toward those with low expression strength. To determine the contribution of currents deriving from functional Panx1 hemichannels, 20 μM Cbx was applied, and the remaining maximum current amplitude was determined. For vector only-transfected cells the maximum current amplitude in the presence of Cbx was significantly higher (84.6% ± 14.2%) than for Panx1 WT (60% ± 15%; p < 0.001) and Panx1-C346S (56% ± 16%; p < 0.001) (Fig. 7E). In contrast to the oocyte system, the difference between Panx1 WT and Panx1-C346S was not significant. Most likely, this is caused again by preferential recording from low Panx1-C346S-expressing cells, which survive longer after transfection than cells with high expression levels.

DISCUSSION

Cysteine residues are involved in the regulation of protein activity by redox events (chemical reduction or oxidation) and chelation of transition metals or S-nitrosylation (38). Examples of ion channels, which were found to be regulated by modulation of cysteines, are the olfactory cyclic nucleotide-gated channel (39, 40), the ryanodine receptor type 1 (41, 42), Na+ channels (43), and the NMDA receptor (44, 45). To uncover putative mechanisms of hemichannel regulation of Panx1, we investigated the role of single intracellular cysteine residues.

Site-directed mutagenesis of the intracellular cysteines Cys-136, Cys-346, and Cys-426 were performed replacing cysteine by serine. Expression of the mutant Panx1-C346S in the X. laevis oocyte expression system induced early cell death, whereas the expression of the WT or the other two mutants did not affect oocyte survival. Incubation of oocytes expressing Panx1-C346S in Cbx prolonged survival time, suggesting that initial cell death was due to hemichannel leakiness. Signs of cell degeneration also occurred in transfected N2A cells, demonstrating that the effect was not specific for the oocyte system. Dye uptake experiments in injected oocytes and electrophysiological recordings from both injected oocytes as well as transfected N2A cells clearly demonstrate that the substitution of the residue C346 by serine results in a constitutively leaky hemichannel that is conductive at resting membrane potential. This finding stresses the importance of the C-terminal cysteine in regulating Panx1 channel properties. Because Cbx treatment could not completely prevent oocytes from degeneration, we assume an additional negative dominant (toxic) effect of the mutant C346S, which explains the biphasic form of the survival and growth course. In contrast to the mutants C136S and C426S, the C346S mutant is essentially hypoglycosylated lacking the GLY2 isoform similar to a glycosylation site mutation (N337Q) described by Boassa et al. (9). Variant from the N337Q mutant we found reasonable trafficking of the immaturely glycosylated GLY1 form to the plasma membrane, as indicated by our electrophysiology and immunolabeling. This is in agreement with observations reported by Penuela et al. (22), which also describe subfractions of glycosylation deficient Panx1 protein in the plasma membrane. The Cbx effect on cell survival can be explained by effective blocking of the membrane-associated fraction of C346S mutated Panx1 hemichannels. However, accruing restriction and exhausted sorting of the mutant may exert a toxic effect on the cell physiology, which ultimately leads to accelerated cell death. Apparently, the cysteine at the C-terminal position Cys-346, unlike the C136S and C426S mutants, seems to be crucial for proper processing and post-translation modification of Panx1. Interdependence of glycosylation and proper folding for interaction with chaperones and cysteine-modifying enzymes has been described frequently (for review, see Ref. 46). Lack of a cysteine at this particular position could compromise glycosylation capability of this protein. The survival curve (Fig. 1B) and the growth curve (supplemental Fig. 3B) speak in favor of this interpretation. During the initial phase of C346S expression, oocytes death increases by only 10% over the first 24–36 h, presumably due to hemichannel leakiness (Fig. 1B), as this effect is completely blocked by Cbx and can be delayed by increase of Cbx concentrations. Around 40 h, when expression of the protein is maximal, both untreated and Cbx-treated mutants show a dramatic increase of cell degeneration, although with different steepness because of sustaining impact of Cbx on hemichannel activity. The growth curves of transfected N2A cells are also in accordance with this biphasic event. Although cell growth of C346S transfectants is not changed during the first 12–14 h compared with WT, cell number becomes significantly impaired with increasing expression times (>15 h). However, we consider the observed reduction in cell number a result of cell death rather than growth inhibition, as samples set up in parallel to the growth curve experiments showed significant signs of cellular degeneration (supplemental Fig. 3C). Deviation from the C346S mutant transfection with C136S and C436 mutants effectively increased cell growth compared with WT-transfected cells. Because the void EGFP vector revealed the same growth curve profile the most likely interpretation is that Panx1 by itself exerts an inhibitory effect on cell proliferation. Such an antiproliferative component of Panx1 has recently been described (36) in C6 glioma cells and was suggested to mediate tumor-suppressive function. It is feasible that the C136S and C436 mutants lose some biological properties with the consequence of impairment of interaction with pathways involved in growth control.

Our observations on Panx1-C346S are in line with reports on mutated connexins. Similar to the Panx1-C346S mutation, but unlike most other hemichannels formed by connexins or Panx1 WT, human Cx26 hemichannels open at resting membrane potential. This also seems to depend on a single amino acid residue, because rat Cx26 hemichannels remained closed under the same conditions, but could be “rescued” by mutating asparagine 159 to aspartate (47). Furthermore, enhanced hemichannel activity was reported for the oculodentodigital dysplasia-associated Cx43 mutations I31M, G138R, and G143S. For Cx32, the mutation F235C was shown to result in an increased number of Cx32 hemichannels open at resting potential, which leads to cell death. This mutation is linked to an unusually severe neuropathy in a case of X-linked Charcot-Marie-Tooth
disease (48). The mutation G45E in Cx26 resulted in increased apoptosis and cell death of transfected HEK293 cells within 24 h of transfection and is associated with nonsyndromic hereditary hearing impairments (49). All of these examples show that single amino acid exchanges can result in serious physiological effects, which can ultimately lead to cell death and often associate with pathological conditions.

Mutations of single amino acids are also prone to impairment of trafficking and stability of proteins (28). To ensure that the functionally proper mutants Panx1-C136S and Panx1-C426S were trafficked correctly, we performed several control experiments. Western blot analyses as well as pulldown studies did not reveal detectable difference in their cellular localization. Transfection of N2A cells with fluorescent fusion constructs revealed subtle differences for the mutant Panx1-C426S including a pronounced membrane staining. We suggest that this more pronounced fluorescence could be explained by an increased number of channels at the cell surface. Our electrophysiological data support this interpretation. The significantly increased number of channels at the cell surface. In contrast, expression of Panx1-C136S seemed to be decreased compared with the WT.

In addition to the general electrophysiological properties, we also tested whether one of the mutated cysteines is involved in the inactivation mechanism of Panx1 hemichannels following application of reducing agents. Significant decreases of the maximal current amplitude after application of TCEP were observed in all mutants, indicating that the inactivating effect of TCEP does not involve any of the intracellular cysteine residues. This result provides clear evidence that Panx1 hemichannels are sensitive to reducing agents, even after mutation of the cytoplasmic cysteine residues. Whether any of the three cysteines in the transmembrane regions or one of the four extracellular cysteines is responsible for the observed reduction in current amplitude by TCEP and other reducing agents must be addressed in future studies.

Acknowledgments—We thank Gerhard Dahl for the Panx1 antibody and Sabine Peuckert, Christiane Zoidl, and Hans-Werner Habbes for excellent technical assistance with the molecular biology and immunochemistry.

REFERENCES

1. Panchin, Y., Kelmanison, I., Matz, M., Lukyanov, K., Usman, N., and Lukyanov, S. (2000) Curr. Biol. 10, R473–R474
2. Dvoriantchikova, G., Ivanov, D., Panchin, Y., and Steshopolov, V. I. (2006) FEBS Lett. 580, 2178–2182
3. Dvoriantchikova, G., Ivanov, D., Panchin, Y., and Steshopolov, V. (2006) Mol. Vis. 12, 1417–1426
4. Huang, Y., Grinspan, J. B., Abrams, C. K., and Scherer, S. S. (2007) Glia 55, 46–56
5. Ray, A., Zoidl, G., Weickert, S., Wahle, P., and Dermietzel, R. (2005) Eur. J. Neurosci. 21, 3277–3290
6. Vogt, A., Hormuzdi, S. G., and Monyer, H. (2005) Brain Res. Mol. Brain Res. 141, 113–120
7. Baranova, A., Ivanov, D., Petras, N., Pestova, A., Skoblov, M., Kelmanison, I., Shagin, D., Nazarevko, S., Geraymovych, E., Litvin, O., Tsiunova, A., Born, T. L., Usman, N., Staroverov, D., Lukyanov, S., and Panchin, Y. (2004) Genomics 83, 706–716
8. Zappala, A., Li Volti, G., Serapide, M. F., Pelliteri, R., Falchi, M., La Delia, F., Cricirata, V., and Cricirata, F. (2007) Neuroscience 146, 653–667
9. Boassa, D., Ambrosi, C., Qiu, F., Dahl, G., Gaietta, G., and Sosinsky, G. (2007) J. Biol. Chem. 282, 31733–31743
10. Bruzzone, R., Hormuzdi, S. G., Barbe, M. T., Herb, A., and Monyer, H. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 13644–13649
11. Locovei, S., Wang, J., and Dahl, G. (2006) FEBS Lett. 580, 239–244
12. Zoidl, G., Petrasch-Parwex, E., Ray, A., Meier, C., Bunse, S., Habbes, H. W., Dahl, G., and Dermietzel, R. (2007) Neuroscience 146, 9–16
13. Locovei, S., Bao, L., and Dahl, G. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 7655–7660
14. Locovei, S., Scemes, E., Qiu, F., Spray, D. C., and Dahl, G. (2007) FEBS Lett. 581, 483–488
15. Pleologin, P., and Surprenant, A. (2006) EMBO J. 25, 5071–5082
16. Igleias, R., Locovei, S., Roque, A.,Alberto, A. P., Dahl, G., Spray, D. C., and Scemes, E. (2008) Am. J. Physiol. Cell Physiol. 295, C752–C760
17. Thompson, R. J., Zhou, N., and MacVicar, B. A. (2006) Science 312, 924–927
18. Zhang, L., Deng, T., Sun, Y., Liu, K., Yang, Y., and Zheng, X. (2008) J. Neurosci. Res. 86, 2281–2291
19. Silverman, W. R., de Rivero Vaccari, J. P., Locovei, S., Qiu, F., Carlsson, S. K., Scemes, E., Keane, R. W., and Dahl, G. (2009) J. Biol. Chem. 284, 18143–18151
20. Bao, L., Locovei, S., and Dahl, G. (2004) FEBS Lett. 572, 65–68
21. Scemes, E., Suadicani, S. O., Dahl, G., and Spray, D. C. (2007) Neuron Glia Biol. 3, 199–208
22. Penuela, S., Bhalla, R., Gong, X. Q., Cowan, K. N., Celetti, S. J., Cowan, B. J., Bai, D., Shao, Q., and Laird, D. W. (2007) J. Cell Sci. 120, 3772–3783
23. Boassa, D., Qiu, F., Dahl, G., and Sosinsky, G. (2008) Cell Commun. Adhes. 15, 119–132
24. Dahl, G., Werner, R., Levine, E., and Rabadán-Diehl, C. (1992) Biophys. J. 62, 172–180
25. Retamal, M. A., Cortés, C. J., Reuss, L., Bennett, M. V., and Sáez, J. C. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 4475–4480
26. Retamal, M. A., Yin, S., Altenberg, G. A., and Reuss, L. (2009) Am. J. Physiol. Cell Physiol. 296, C1356–C1363
27. Bunse, S., Locovei, S., Schmidt, M., Qiu, F., Zoidl, G., Dahl, G., and Dermietzel, R. (2009) FEBS J. 276, 6258–6270
28. Matos, T. D., Caria, H., Simões-Texeira, H., Aasen, T., Dias, O., Andrea, M., Kselass, D. P., and Fallah, G. (2008) Hearing Res. 240, 87–92
29. Zoidl, G., Meier, C., Petrasch-Parwex, E., Zoidl, C., Habbes, H. W., Kremer, M., Srinivas, M., Spray, D. C., and Dermietzel, R. (2002) J. Neurosci. Res. 69, 448–465
30. Petrasch-Parwex, E., Habbes, H. W., Weickert, S., Löbbecke-Schumacher, M., Striedinger, K., Wieczorek, S., Dermietzel, R., and Epplen, J. T. (2004) J. Comp. Neurol. 479, 181–197
31. Paul, D. L., Ehihara, L., Takemoto, L. J., Swenson, K. L., and Goodenough, D. A. (1991) J. Cell Biol. 115, 1077–1089
32. Mous, J. M., Peeters, B. L., Heyns, W. J., and Rombauts, W. A. (1982) J. Biol. Chem. 257, 11822–11828
33. Goldberger, G., Arnaout, M. A., Aden, D., Kay, R., Rits, M., and Colten, H. R. (1984) J. Biol. Chem. 259, 6492–6497
34. Penuela, S., Bhalla, R., Nag, K., and Laird, D. W. (2009) Mol. Biol. Cell 20, 4313–4323
35. Prochnow, N., Hoffmann, S., Vroman, R., Klooster, J., Bunse, S., Kamermans, M., Dermietzel, R., and Zoidl, G. (2009) Neuroscience 162, 1039–1054
36. Lai, C. P., Bechberger, J. F., Thompson, R. J., MacVicar, B. A., Bruzzone, R., and Naus, C. C. (2007) Cancer Res. 67, 1545–1554
37. Wang, J., Ma, M., Locovei, S., Keane, R. W., and Dahl, G. (2007) Am. J. Physiol. Cell Physiol. 293, C1112–C1119
38. Lipton, S. A., Choi, Y. B., Takahashi, H., Zhang, D., Li, W., Godzik, A., and Bankston, L. A. (2002) Trends Neurosci. 25, 474–480
Mutation of Cys-346 of Panx1 Affects Hemichannel Activity

39. Broillet, M. C. (2000) J. Biol. Chem. 275, 15135–15141
40. Broillet, M. C., and Firestein, S. (1996) Neuron 16, 377–385
41. Aracena-Parks, P., Goonasekera, S. A., Gilman, C. P., Dirksen, R. T., Hidalgo, C., and Hamilton, S. L. (2006) J. Biol. Chem. 281, 40354–40368
42. Durham, W. J., Aracena-Parks, P., Long, C., Rossi, A. E., Goonasekera, S. A., Boncompagni, S., Galvan, D. L., Gilman, C. P., Baker, M. R., Shirokova, N., Protasi, F., Dirksen, R., and Hamilton, S. L. (2006) Cell 133, 53–65
43. Renganathan, M., Cummins, T. R., and Waxman, S. G. (2002) J. Neurophysiol. 87, 761–775
44. Choi, Y. B., Tenneti, L., Le, D. A., Ortiz, J., Bai, G., Chen, H. S., and Lipton, S. A. (2000) Nature Neurosci. 3, 15–21
45. Lei, S. Z., Pan, Z. H., Aggarwal, S. K., Chen, H. S., Hartman, J., Sucher, N. J., and Lipton, S. A. (1992) Neuron 8, 1087–1099
46. Pearse, B. R., and Hebert, D. N. (2010) Biochim. Biophys. Acta 1803, 684–693
47. González, D., Gómez-Hernández, J. M., and Barrio, L. C. (2006) FASEB J. 20, 2329–2338
48. Liang, G. S., de Miguel, M., Gómez-Hernández, J. M., Glass, J. D., Scherer, S. S., Mintz, M., Barrio, L. C., and Fischbeck, K. H. (2005) Ann. Neurol. 57, 749–754
49. Stong, B. C., Chang, Q., Ahmad, S., and Lin, X. (2006) Laryngoscope 116, 2205–2210