Permeant-specific gating of connexin 30 hemichannels

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Abstract

Gap junctions confer interconnectivity of the cytoplasm in neighboring cells via docking of two connexons expressed in each of the adjacent membranes. Undocked connexons, referred to as hemichannels, may open and connect the cytoplasm with the extracellular fluid. The hemichannel configuration of connexins (Cxs) displays isoform-specific permeability profiles that are not directly determined by the size and charge of the permeant. To further explore Ca**+**-mediated gating and permeability features of connexin hemichannels, we heterologously expressed Cx30 hemichannels in Xenopus laevis oocytes. The sensitivity toward divalent cation-mediated gating differed between small atomic ions (current) and fluorescent dye permeants, indicating that these features are distinctly gated.

Three aspartate residues in Cx30 (Asp-50, Asp-172, and Asp-179) have been implicated previously in the Ca**+** sensitivity of other hemichannel isoforms. Although the aspartate at position Asp-50 was indispensable for divalent cation-dependent gating of Cx30 hemichannels, substitutions of the other two residues had no significant effect on gating, illustrating differences in the gating mechanisms between connexin isoforms. Using the substituted cysteine accessibility method (SCAM), we evaluated the role of possible pore-lining residues in the permeation of ions and ethidium through Cx30 hemichannels. Of the cysteine-substituted residues, interaction of a proposed pore-lining cysteine at position 37 with the positively charged compound [2-(trimethylammonium)ethyl] methane thiosulfonate bromide (MTSET) increased Cx30-mediated currents with unperturbed ethidium permeability. In summary, our results demonstrate that the permeability of hemichannels is regulated in a permeant-specific manner and underscores that hemichannels are selective rather than non-discriminating and freely diffusible pores.

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Figure 1. Activity of Cx30 hemichannels at physiological divalent cation concentrations. A, membrane currents were recorded by application of 100-ms voltage steps from +60 to −140 mV in steps of 20 mV from a holding potential of −50 mV in oocytes exposed to control solution (top panels) and after 1-min exposure to control solution containing 50 μM Gd3+ (bottom panels). Uninj, uninjected. B, summarized I/V curves for Cx30-expressing oocytes (n = 9) and uninjected oocytes (n = 9, inset). C, histogram of currents obtained at −80 mV in control solution (n = 9) and control solution containing 50 μM Gd3+ (n = 9). D, ethidium uptake recorded after 60-min exposure to control solution (n = 5) or control solution containing 50 μM Gd3+ (n = 5). Data in I/V curves and in the bar graphs are presented as mean ± S.E. The individual data points are shown by overlaid scatterplots in bar graphs. Statistical significance was tested using repeated measures two-way ANOVA with Bonferroni post hoc test and marked by asterisks above the horizontal lines when comparing between the two groups. ***, p < 0.001; ns, not significant.

Results

The gating of Cx30 hemichannels by divalent cations depends on the permeant

To determine whether the Cx30-dependent permeation of atomic ions (current) and fluorescent dye responds proportionately to gating by divalent cations, we expressed Cx30 in Xenopus oocytes. This heterologous expression system has the inherent advantage of low expression of endogenous proteins with overlapping activation, inhibition, and/or permeability profiles. In control solution (which, for frog Ringer’s solution, contains 1 mM Ca2+ and 1 mM Mg2+), the membrane current was 3-fold larger in oocytes expressing Cx30 than that observed in uninjected oocytes (representative current traces, Fig. 1A; I/V curves, Fig. 1B; membrane currents obtained at Vm = −80 mV, Fig. 1C). The connexin hemichannel inhibitor gadolinium (Gd3+) had no effect on membrane currents of uninjected control oocytes (Fig. 1, A, B (inset), and C) but decreased membrane currents in oocytes expressing Cx30 to close to that of uninjected oocytes (Fig. 1, A–C). Cx30-expressing oocytes thus permit Gd3+-sensitive membrane currents in the presence of 1 mM each of the divalent cations. In contrast, batch-matched oocytes kept in control solution displayed no Cx30-mediated Gd3+-sensitive ethidium uptake (Fig. 1D), indicating that the basal concentration of divalent cations (1 mM) suffices to prevent ethidium permeability through Cx30 even though it does not fully block ionic conductance.

To determine the individual gating by the two divalent cations (Ca2+ and Mg2+) on Cx30-mediated conductance and ethidium uptake, we exposed the oocytes to solutions free of Mg2+ (MgFS), free of Ca2+ (CaFS), or free of both divalent cations (DCFS). Membrane currents in uninjected control oocytes were not significantly affected by removal of either of the divalent cations or both divalent cations (Fig. 2, A, B, and D). In Cx30-expressing oocytes, selective removal of Mg2+ had no effect on Cx30-mediated membrane currents, whereas selective removal of Ca2+ caused a 4-fold increase in membrane currents (Fig. 2, A, representative current traces; C, I/V curves; and D, summarized data at −80 mV). This current was further augmented (to 10-fold of control) by removal of both divalent cations (Fig. 2, A, D). In comparison, selective removal of Ca2+ did not significantly affect Cx30-mediated ethidium uptake, and ethidium uptake was only increased (by 5-fold) over that of the control situation when both divalent cations were removed, in a Gd3+-sensitive manner (Fig. 2E). As expected, ethidium uptake in uninjected oocytes was unaffected by the extracellular concentration of divalent cations and insensitive to Gd3+ (Fig. 2E). These results suggest that the Ca2+ sensitivity differs between Cx30-mediated current and ethidium permeability.

The Ca2+ and Mg2+ sensitivity of the hemichannel current and ethidium uptake was more rigorously examined by determination of the IC50 for each divalent cation in the complete absence of the other for both ethidium uptake and membrane currents. The IC50 for Ca2+ was 4.5-fold lower for ethidium uptake than for the membrane currents (49 ± 8 μM, n = 5 for ethidium uptake versus 228 ± 37 μM, n = 9 for membrane current, p < 0.01) (Fig. 3A). Similarly, the IC50 for Mg2+ was 3.5-fold lower for ethidium uptake than for the membrane currents (179 ± 26 μM, n = 5 for ethidium uptake versus 635 ± 123
μM, n = 12 for membrane current, p < 0.05) (Fig. 3B). Given that all measurements occurred within the linear response range of the system for detection of both dye and current, these differences indicate that Ca\(^{2+}\) and Mg\(^{2+}\) have distinguishable effects on the permeability of Cx30 hemichannels to current and larger permeants.

**Amino acids important for calcium sensitivity**

The Ca\(^{2+}\) sensitivity of connexin hemichannels are proposed to be conferred via extracellular aspartate residues, which bind Ca\(^{2+}\) with high affinity (30). Three aspartate residues, implicated in regulating the calcium sensitivity of other connexin hemichannels (31–34), correspond to Asp-50, Asp-172, and Asp-179 in Cx30 (Fig. 4A). The role of these aspartate residues in calcium sensitivity of Cx30 hemichannels was tested by individually mutating each to asparagine, which is similar in size but lacks the negative charge of the aspartates and, thus, the ability to coordinate calcium binding. Oocytes expressing the Cx30-D50N mutation consistently lysed (Fig. 4B), even when stored in solution containing 10 mM Ca\(^{2+}\) (data not shown), indicating lack of Ca\(^{2+}\)-dependent gating and, thus, continuous ion leakage through the hemichannel pore. We observed a sizeable ATP release from the Cx30-D50N–expressing oocytes when investigated <24 h after microinjection of the RNA encoding this channel (Fig. 4C). Although this was prior to visible cell lysis, the degree to which the membrane may have been compromised during this initial period could not be assessed. Membrane currents and ethidium uptake were not significantly affected by the removal of divalent cations from uninjected oocytes (Fig. 4, D, H, and I) but were both readily observed in DCFS in oocytes expressing the WT (Fig. 4, E, H, and J) and the mutant Cx30 constructs Cx30-D172N (Fig. 4, F, H, and I) or Cx30-D179N (Fig. 4, G–I). For all constructs, the membrane current activity and ethidium uptake were nearly abolished by 1 mM and 5 mM Ca\(^{2+}\) in the test solutions (Fig. 4, H and I). Despite consistent observation of DCFS-sensitive current and dye uptake in oocytes expressing WT Cx30 and the mutant versions (D172N and D179N), no DCFS-dependent ATP release was detected using a luciferase assay (Fig. 4J). The functionality of the assay was confirmed by measurement of total ATP content by oocyte lysis at the termination of the experiment (Fig. 4, C and J). Thus, aspartate in position 50 is required for Ca\(^{2+}\)-dependent gating of Cx30 hemichannels, whereas the aspartate residues at position 172 and 179 are of minor, if any, importance for divalent cation–dependent gating of both current and ethidium uptake through Cx30. Furthermore, data show that, although removal of divalent cations opens Cx30 to ethidium and current, it does not, under our experimental conditions, confer detectable permeability to ATP, as has been reported for other hemichannels.

**Probing of the Cx30 pore with cysteine-interacting thiol reagents**

To evaluate the role of possible pore-lining residues in permeation of atomic ions *versus* ethidium through Cx30 hemichannels, we employed the substituted cysteine accessibility method (SCAM). This is an established experimental approach to determine pore-lining residues (35, 36) where individual residues are mutated to cysteines. Their accessibility to the aqueous environment (e.g. by exposure to the conductive pore) is then measured by reactivity with membrane-impermeable thiol reagents added from the extracellular medium, which can affect the permeation pathway through the pore. Select amino acids in Cx30, identified to be pore-lining in other connexin isoforms (35–42), were substituted with cysteine to generate: Cx30-V37C, E42C, V84C, Y136C, I145C, and F150C (Fig. 5A). The equivalent positions to Val-37 and Glu-42 in M1/E1 are in the pore in the X-ray structure of Cx26 (37), and Glu-42 was reactive in SCAM analysis of Cx46 hemichannels (35, 39).
Val-84 in M2 and Tyr-136, Ile-145, and Phe-150 in M3 were all mapped to the Cx32 gap junction pore by SCAM (36, 42).

These mutated forms of Cx30 all expressed robustly at the plasma membrane fraction of cRNA-injected *Xenopus* oocytes (Fig. 5B). However, only two of the Cx30 mutants (Cx30-V37C and Cx30-I145C) displayed functional hemichannel activity, as recorded by Gd3+-sensitive DCFS-induced membrane currents (Fig. 5, C, representative current traces; D and E, summarized I/V curves; and F, currents obtained at -80 mV) and ethidium uptake (Fig. 5G). Prior to thiol reactivity testing, we verified that WT Cx30 hemichannel activity and its sensitivity to removal of divalent cations were unaffected by addition of the cationic thiol reagent MTS-ET (molecular mass, 278 Da) (Fig. 6, A and D). Ethidium uptake in Cx30-expressing oocytes was also not affected by addition of MTS-ET and remained sensitive to removal of divalent cations (Fig. 6E). Thus, any effects of MTS-ET on a cysteine mutant can be reliably assigned to an interaction with the mutated residue.

Of the two functional cysteine mutants tested, MTS-ET did not affect currents of Cx30-I145C (Fig. 6, C and D) but more than doubled the current through Cx30-V37C in the presence of divalent cations (Fig. 6, B and D). Both mutant constructs retained their DCFS-induced enhancement of the current after treatment with MTS-ET (Fig. 6, B–D), which also remained Gd3+-sensitive (data not shown). Time control experiments without MTS-ET addition verified that the altered current observed in Cx30-V37C–expressing oocytes was in fact due to addition of the MTS-ET reagent and did not occur as a simple function of time (n = 10, data not shown). In contrast, ethidium uptake in oocytes expressing Cx30-V37C or Cx30-I145C was unaffected by MTS-ET treatment, although both mutant versions of Cx30 remained sensitive to DCFS after MTS-ET treatment (Fig. 6E). In summary, residue 145 was either inaccessible to the MTS-ET reagent or the reaction did not affect pore permeability to ethidium or ions. In contrast, the MTS-ET reaction at residue 37, although not preventing opening of the pore in the absence of extracellular divalent cations, significantly increased current in the presence of divalent cations, with no concomitant change in ethidium permeability.

To assess whether there is a possible charge dependence of the thiol reagent, a parallel experimental series was carried out with the anionic membrane-impermeable reagent MTS-ES (molecular mass, 242 Da). MTS-ES also had no effect on WT Cx30 hemichannel-mediated currents or ethidium uptake in control solution, and the hemichannels remained sensitive to DCFS (Fig. 7, A, D, and E). In contrast to the cationic thiol, MTE-ES also had no effect on any of the tested transport parameters for either of the mutant constructs (Fig. 7, B–E), indicating that either the anionic thiol does not gain access to the hemichannel pore, is unreactive with the tested sites, or did not affect the pore properties upon reaction.

**Discussion**

This study demonstrates that the divalent cation–mediated gating of the Cx30 hemichannel pore depends on the nature of the tested permeant. The results support the notion that open connexin hemichannels do not represent freely permeable non-selective pores and that the specific permeability profile of each hemichannel can be modulated by external factors in such a manner that a permeability profile determined in one setting may not apply to another setting.

For reasons of technical ease, connexin hemichannel activity is commonly determined by use of fluorescent dye permeability (14, 43). Connexin hemichannel–mediated dye uptake/release has typically been inferred to represent uptake/release of other molecules of similar or smaller molecular weight. In recent comprehensive biophysical studies, we established that connexin hemichannels are permeable to select permeants in an isoform-specific manner and that fluorescent dye uptake cannot be used as an indicator of permeability to other smaller solutes (Refs. 20, 28; for a review, see Ref. 45). Although Cx26 and Cx43 in their hemichannel configuration were rather restrictive in their permeability profile, Cx30 hemichannels were permeable to both atomic ions and ethidium upon

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**Figure 3. Cx30 hemichannel gating of currents versus ethidium uptake by divalent cations.** A and B, membrane currents were recorded in Cx30-expressing oocytes by application of 100-ms voltage steps from +60 to −140 mV in steps of 20 mV from a holding potential of −50 mV. The currents recorded at −80 mV were used for further analysis. The membrane currents were recorded after exposure to different concentrations of Ca2+ (A, n = 9) or Mg2+ (B, n = 12) in random order for 1 min. Between each test solution, the oocytes were exposed to control solution until the current returned to baseline. After exposure to eight different concentrations of Ca2+ (A) or Mg2+ (B), the membrane currents were recorded after 1-min exposure to 5 mM Ca2+ (A) or Mg2+ (B) containing 50 μM Gd3+. Ethidium (Eth) uptake was determined after 60-min exposure to (A) different concentrations of Ca2+ or 5 mM Ca2+ containing 50 μM Gd3+ or (B) different concentrations of Mg2+ or 5 mM Mg2+ containing 50 μM Gd3+ (n = 5). For both membrane currents and ethidium uptake, 100% activity was determined in divalent cation-free solution, and 0% activity was determined in 5 mM Ca2+ (A) or Mg2+ (B) containing 50 μM Gd3+. The normalized data were fitted by nonlinear regression analysis with a variable slope Y = 100/(1 + 10n Ki) × Hill slope), where X is the log of the concentration, to obtain the IC50 and statistical significance was tested with unpaired t test. Data in I/V curves are presented as mean ± S.E.
removal of divalent cations from the test solution (28). The latter isoform therefore served as an ideal candidate to compare how gating affects different permeants. Differential regulation of ionic and metabolite permeability may have physiological significance. The studies were carried out with heterologous expression in *Xenopus* oocytes to obtain an experimental scenario in which the Cx30 hemichannels could be studied in isolation. *Xenopus* oocytes efficiently express ectopic proteins such as connexins and generally produce functional membrane channels with properties similar to those expressed by mammalian cells. However, we cannot exclude that certain binding partners are missing, that the lipid bilayer composition varies from that of mammalian cells and could modify properties, or that the phosphorylation pattern is altered in a manner required to close the hemichannel depends on the permeant. These data support the initial observation that Ca$^{2+}$ is the more potent stabilizer of the closed state of the connexin hemichannel, as also reported in the analysis of Cx26-mediated peak tail currents (20), we can exclude that saturation of one or the other underlies the permeant-specific shifts in IC$_{50}$. These data support the initial observation that Ca$^{2+}$ is the more potent stabilizer of the closed state of the connexin hemichannel, as also reported in the analysis of Cx26-mediated peak tail currents (20), we can exclude that saturation of one or the other underlies the permeant-specific shifts in IC$_{50}$.

Several possibilities exist for how the gating with respect to larger dyes may require lower concentrations of divalent cations. It is possible that there are two stages in channel gating as divalent cation concentration increases, initially to a residual conductance and ethidium uptake. The IC$_{50}$ for conductance was ~4-fold higher than that for ethidium uptake for both Ca$^{2+}$ and Mg$^{2+}$. The IC$_{50}$ values were ~3-fold higher for Mg$^{2+}$ than for Ca$^{2+}$, whether tested for Cx30-mediated current or ethidium uptake. As the Cx30-mediated currents are well within the detection range for the two-electrode voltage clamp, and the ethidium uptake was measured in the linear part of the time-dependent flux (20), we can exclude that saturation of one or the other underlies the permeant-specific shifts in IC$_{50}$. These data support the initial observation that Ca$^{2+}$ is the more potent stabilizer of the closed state of the connexin hemichannel, as also reported in the analysis of Cx26-mediated peak tail currents (46), and that the concentration of divalent cations required to close the hemichannel depends on the permeant.

Several possibilities exist for how the gating with respect to larger dyes may require lower concentrations of divalent cations. It is possible that there are two stages in channel gating as divalent cation concentration increases, initially to a residual state that prevents dye permeability but allows ion passage and, subsequently, to a fully closed state. However, this has not been specifically seen in single-channel recordings of Ca$^{2+}$-mediated gating of other hemichannels. An alternative possibility is suggested by the recent structure of the Cx26 channel in Ca$^{2+}$-bound and free states, which concludes that, rather than chan-
nel constriction, Ca\(^{2+}\) induces a change in the pore lining from a mix of anionic and cationic domains to a fully positive potential across the whole length of the pore (47). This is likely to have a much greater inhibitory effect on the passage of larger cations than smaller atomic cations through the pore. In addition, our preliminary studies indicate that Cx30 channels are not very ion-selective, so some current could still be carried by anions even with the highly positive potentials in the pore induced by Ca\(^{2+}\).

In terms of the Ca\(^{2+}\) gating sites in Cx30, Asp-50 appears to be indispensable for Ca\(^{2+}\)-dependent closure of Cx30 hemichannels, as the oocytes expressing the mutated construct, Cx30-D50N, lysed even in the presence of high concentrations of Ca\(^{2+}\) in the incubation medium. This repeatedly observed cell lysis indicates that the mutated channel is unable to bind Ca\(^{2+}\) and, therefore, is constitutively open, with an ensuing continuous ion flux across the plasma membrane. We observed a robust ATP release from the Cx30-D50N-expressing oocytes, although it is unresolved whether this ATP exits through the pore of the mutated connexin or whether it is the result of the early stages of oocyte lysis. The equivalent mutation in Cx26 leads to keratitis ichthyosis deafness (KID) syndrome (48), although, in this case, Ca\(^{2+}\) sensitivity was simply reduced (49, 50), as high Ca\(^{2+}\) concentration in the incubation medium rescued oocytes expressing the Cx26-D50N mutant (31). The amino acids homologous to Cx30–Asp-172 and Cx30–Asp-179 in Cx32 have also been implicated in binding Ca\(^{2+}\) (34): mutation of either abolished the majority of the Ca\(^{2+}\)-dependent gating in Cx32 (34), and mutation of the latter site is associated with X-linked Charcot–Marie–Tooth disease (34, 51). However, in Cx30, mutation of these sites had no effect on the Ca\(^{2+}\)-mediated gating, indicating that the amino acid residues coordinating the Ca\(^{2+}\) ions in the different connexin isoforms may not be identical and that this isoform-specific manner of gating is yet another molecular differentiation between the connexin isoforms. Mutations may, however, be misleading, as they could influence any part of the gating pathway. In fact, Cx26-D50 has been implicated by molecular modeling, supported by cycle mutagenesis, to be more important for stabilization of a salt bridge with Lys-61 (49), whereas Glu-42, Glu-47, and the carbonyl of Gly-45 are implicated as the Ca\(^{2+}\)-binding site in the recent structure of the Ca\(^{2+}\)-bound form of Cx26 (47).

We were unable to detect DCFS-mediated ATP release by oocytes expressing Cx30 WT or the D172N or D179N mutant constructs with a luciferase assay. We have previously detected...
release of microinjected radioactively labeled ATP in Cx30-expressing oocytes (20), which may be a more sensitive approach than the luciferase assay. However, this lack of detectable DCFS-mediated ATP release, despite parallel demonstration of conductance and dye uptake in batch-matched oocytes, indicates that the Cx30 hemichannel-mediated ATP release is quantitatively minor compared with conductance and dye flux. It certainly reinforces the point that the latter should not be used to infer the former for any connexin channel. It remains possible that connexin-dependent ATP release from some cell types/under certain experimental conditions may not occur directly via the hemichannel pore.
Differential regulation of the permeability of ionic currents and larger molecules (i.e., fluorescent probes, purines, etc.) have been described for connexin channels in the gap junctional configuration (3, 4, 6, 7, 11, 29, 52–54). A possible explanation for the differences in relative permeabilities may link to the existence of different channel substates. Along these lines, Kwak et al. (29) showed that PKC activation increased electrical coupling while reducing intercellular dye transfer (29). This change was associated with a relative shift from large to small single-channel events, suggesting that only the larger conductance states permitted dye diffusion. These findings were supported by later studies of current versus permeability in relation to phosphorylation (11) and voltage-gating (52). Although such a mechanism may explain some scenarios, including the differential gating seen here, others remain unaccounted for (e.g., dye permeation in Cx36 and Cx43 hemichannels in the absence of current under similar experimental conditions (28)).

For both gap-junctional and hemichannel configurations, the underlying molecular pore-lining structural determinants of the permeation profile have remained elusive. Some guidance has been provided by the 3.5-Å structure of Cx26 (37, 47), but at this relatively low resolution, the specific positions of amino acid side chains within the pore rely predominantly on modeling. Direct empirical probing of the pore has been performed on both gap junction and hemichannel configurations by SCAM, where pore-lining residues are individually mutated to cysteine and tested for accessibility to aqueously soluble thiol reagents by assaying for changes in channel conductance. These analyses have yielded quite distinct patterns of reactivity when conducted on hemichannel and gap junction configurations, with the former implicating M1, and the latter M3 and part of M2, as pore lining (35, 36, 38, 39, 41, 42). The X-ray structure supports the M1 assignment, consistent with the crystal likely being derived from solubilized hemichannels.

In an attempt to probe residues that might differentially influence dye and ionic permeation, we conducted SCAM analyses on six Cx30 residues: Val-37 (M1 pore-lining in the Cx26 crystal structure (37) and involved in KID and Clouston syndromes when mutated to a glutamate in Cx30 (55, 56)), Glu-42 (E1 pore-lining in Cx46 and Cx50 hemichannels (35, 38–40)), and Val-84 (M2), Tyr-136, Ile-145, and Phe-150 (M3), all pore-lining in Cx32 and Cx50 gap junctions (36, 42). The X-ray structure supports the M1 assignment, consistent with the crystal likely being derived from solubilized hemichannels.

In conclusion, the distinct and selective permeability of individual connexin hemichannels may serve a physiological function. Although Cx30-mediated hemichannel activity could be beneficial in certain settings (57), one can well imagine that the Cx43-expressing myocardium would be compromised by opening of large-pore, non-selective ion channels. This study demonstrates, however, that, under narrow ranges of extracellular divalent cations, one can prevent leak of larger molecules while still allowing low levels of ionic conductance. This may be of particular importance for Cx30 in the ear, where it is exposed to a wide range of different extracellular Ca2⁺ concentrations. This could be amplified further if the larger molecules were of different charge, as would be the case for molecules such as ATP. Thus, connexin-specific gating and permeability characteristics (both in gap junction and hemichannel configurations) may provide cells with the molecular means to differentially control its membrane permeability and intercellular communication based on the connexin isoform expressed and the specific sensitivities to gating signals like divalent cations.

**Materials and methods**

**In vitro transcription**

cDNA encoding mouse Cx30 (mCx30, obtained from Klaus Willecke, Bonn University) was subcloned into the expression vector pXOOM, and its sequence was verified. Point mutations...
were introduced into Cx30 by the QuikChange site-directed mutagenesis kit (Stratagene, Santa Clara, CA) and verified by DNA sequencing. The cDNAs encoding WT and mutant versions of Cx30 were linearized downstream from the poly(A) segment and subsequently transcribed with T7 mMessage Machine according to the instructions of the manufacturer (Ambion, Austin, TX). The cRNA was extracted with MEGAclear (Ambion) prior to microinjection into defolliculated *X. laevis* oocytes (10 ng of RNA/oocyte).

**Oocyte preparation**

Oocytes were surgically removed from *X. laevis* frogs (Nasco or National Center for Scientific Research) according to European Community guidelines for the use of experimental animals and under a license issued for the use of experimental animals by the Danish Ministry of Justice (Dyreforsonstilskynet). The oocytes were prepared as described previously (58) and kept for ∼24 h at 18 °C in Kulori medium (90 mM NaCl, 1 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM HEPES, and ∼4 mM Tris (HOCH₂)₃CNH₂ (pH 7.4)) prior to cRNA injection. The microinjected oocytes were kept in Kulori medium at 18 °C for 2–4 days before the experiments were performed (except for ATP release in Cx30-D50N-expressing oocytes, which was carried out <24 h after microinjection). XeCx38 has been reported previously to be expressed by *Xenopus* oocytes (59, 60) although we have been unable to detect any Ca²⁺- or Gd³⁺-sensitive dye uptake or current in uninjected control oocytes under our experimental conditions (regardless of whether siRNA targeting Cx38 was microinjected into the oocytes (20, 45)). Cx38, at its endogenous expression level, is therefore unlikely to interfere with our data on the over-expressed Cx30.

**Solutions**

The control solution contained 100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and ∼4 mM Tris ((HOCH₂)₃CNH₂ (pH 7.4). Solutions depleted for one or both divalent cations used equiosmolar NaCl. Specifically, MgFS contained 101.5 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 10 mM HEPES, and ∼4 mM Tris (pH 7.4). CaFS contained 101.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 10 mM HEPES, and ∼4 mM Tris (pH 7.4). DCFS contained 103 mM NaCl, 2 mM KCl, 10 mM HEPES, and ∼4 mM Tris (pH 7.4). The solutions employed for the Ca²⁺ and Mg²⁺ inhibition curves were obtained by diluting solutions containing 5 mM of either Ca²⁺ or Mg²⁺ (95.5 mM NaCl, 2 mM KCl, 5 mM CaCl₂/MgCl₂, 10 mM HEPES, and ∼4 mM Tris (pH 7.4) with an equiosmolar divalent cation-free solution.

**Electrophysiology**

The two-electrode voltage clamp recordings were performed at room temperature with a Dagan Clampator interfaced to a personal computer with a Digidata 1320 A/D converter and pCLAMP 9.2 (both Axon instruments, Molecular Devices, CA). Electrodes were pulled from borosilicate glass capillaries to a resistance of 1–4 megohm when filled with 1 M KCl. Currents were low-pass-filtered at 500 Hz and sampled at 2 kHz. Current/voltage (I/V) curves were obtained from the steady-state current levels and recorded in control solution or after 60-s exposure to a test solution from a holding potential of ∼30 or ∼50 mV by application of 100- to 200-ms voltage steps from +60 to −140 mV in increments of 20 mV, as indicated in the figure legends.

**Ethidium uptake**

The ethidium uptake was performed essentially as described earlier (61), with six oocytes per experimental condition. In brief, oocytes were washed in the relevant test solution and subsequently agitated mildly at room temperature in 500 μl of test solution with 50 μM ethidium bromide for 1 h. We have shown previously that ethidium uptake is a linear function of time within this time frame (20, 61). The oocytes were washed twice in control solution, individually lysed in 50 μl of distilled H₂O, and the fluorescence was determined with a Synergy HD plate reader (BioTek) and Gen5 software (BioTek) with 340/11 nm and 590/35 nm filters for excitation and emission, respectively. The obtained fluorescence is given in arbitrary units or normalized.

**ATP release**

The oocytes (six per condition) were washed in the relevant test solution and then incubated in 250 μl of the same test solution at room temperature. After 1 h, 10 μl of each experimental solution was collected and assayed according to the ATP determination kit protocol (Molecular Probes, A22066). The bioluminescence was determined with a Synergy HD plate reader (BioTek) and Gen5 software (BioTek) prior to subtraction of background luminescence. The ATP concentration was calculated from the standard curve obtained in parallel with each experiment.

**Membrane purification and Western blotting**

Purified plasma membranes were obtained from oocytes expressing the mutant constructs (10 oocytes of each), as described in detail previously (44). Briefly, to obtain the pure plasma membrane fraction, the oocyte vitelline envelope was partly digested with subtilisin A and subsequently polymerized to the plasma membrane with Ludox and polyacrylic acid (all from Sigma-Aldrich). The oocytes were homogenized and, following a series of centrifugation steps in the presence of proteinase inhibitors, the pure fraction of plasma membrane/vitelline envelope leaflets was obtained. The samples were analyzed by SDS-PAGE (12% precast gels) and Western blotting using anti-Cx30 (1:125, Invitrogen, 71-2200), followed by anti-rabbit HRP secondary antibody (1:3000, Bio-Rad, 170-6515). Protein staining was visualized by chemiluminescence (SuperSignal West Pico, Thermo Scientific; BioSpectrumAC Imaging System, UV) and quantification in the linear range of exposure.

**Chemicals**

Gd³⁺ (50 μM final concentration, 100 mM stock in H₂O) and ethidium bromide (50 μM final concentration, 25 mM stock in H₂O) were obtained from Sigma-Aldrich. [2-(trimethylammonium)ethyl] methane thioulsolinate bromide (MTS-ET) and sodium (2-sulfonatoethyl) methane thiosulfonate (MTS-ES) were from Anatrace (Kem-En-Tec Nordic). Aliquots of dry
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MTS-ES and MTS-ET powder were placed in small tubes and stored at −20 °C. On the day of use, the powder was dissolved in water to a stock concentration of 200 mM and kept on ice for a maximum of 4 h before it was further dissolved in the test solution to a concentration of 2 mM maximum 3 min before use.

Statistics

All experiments were performed with oocytes from at least three different animal donors. For ethidium uptake experiments, n refers to the number of experiments (each carried out with six oocytes per experimental condition). For electrophysiological experiments, n refers to the number of oocytes. Inhibition curves were fitted in GraphPad Prism 6.0 according to the equation Y = 100/(1 + 10^((logIC50 − X) × Hill slope)), where X is the logarithm of the concentration. Statistical analysis was performed with GraphPad Prism 5–7, and the applied statistical test is indicated in the figure legends. Data are expressed as averages ± S.E., and p < 0.05 is considered statistically significant.

Author contributions—N. M., J. S. A., and B. N. contributed to the research design. J. S. A. and B. S. N. conducted the experiments, analyzed the data, and prepared the figures. N. M., J. S. A., B. S. N., and N. M. contributed to the interpretation and discussion of the data. J. S. A., B. S. N., M. S. N., B. N., and N. M. contributed to drafting the article and approved the final version of the manuscript.

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