

Regulation of Connexin Hemichannels by Monovalent Cations

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Opening of connexin hemichannels in the plasma membrane is highly regulated. Generally, depolarization and reduced extracellular Ca^{2+} promote hemichannel opening. Here we show that hemichannels formed of Cx50, a principal lens connexin, exhibit a novel form of regulation characterized by extraordinary sensitivity to extracellular monovalent cations. Replacement of extracellular Na^+ with K^+ , while maintaining extracellular Ca^{2+} constant, resulted in >10-fold potentiation of Cx50 hemichannel currents, which reversed upon returning to Na^+ . External Cs^+ , Rb^+ , NH_4^+ , but not Li^+ , choline, or TEA, exhibited a similar effect. The magnitude of potentiation of Cx50 hemichannel currents depended on the concentration of extracellular Ca^{2+} , progressively decreasing as external Ca^{2+} was reduced. The primary effect of K^+ appears to be a reduction in the ability of Ca^{2+} , as well as other divalent cations, to close Cx50 hemichannels. Cx46 hemichannels exhibited a modest increase upon substituting Na^+ with K^+ . Analyses of reciprocal chimeric hemichannels that swap NH_2 - and COOH -terminal halves of Cx46 and Cx50 demonstrate that the difference in regulation by monovalent ions in these connexins resides in the NH_2 -terminal half. Connexin hemichannels have been implicated in physiological roles, e.g., release of ATP and NAD^+ and in pathological roles, e.g., cell death through loss or entry of ions and signaling molecules. Our results demonstrate a new, robust means of regulating hemichannels through a combination of extracellular monovalent and divalent cations, principally Na^+ , K^+ , and Ca^{2+} .

INTRODUCTION

Gap junction channels, encoded by the gene family of connexins, are intercellular channels that directly mediate signaling between neighboring cells. These cell–cell channels are formed by the docking of two hemichannels or connexons, one contributed by each of two contacting cells. Hemichannels, while recognized as precursors to cell–cell channels, are also known to form functional channels themselves outside regions of cell–cell contact (Bennett et al., 2003; Goodenough and Paul, 2003). Thus, connexins can participate in mediating signaling across the plasma membrane as well as between cells.

In the simplest view, an unapposed or undocked hemichannel is half of a cell–cell channel, and in many ways it seems to be just that. Unitary conductance of a hemichannel is usually nearly twice that of the corresponding cell–cell channel (Trexler et al., 2000; Valiunas and Weingart, 2000; Srinivas et al., 2005), and hemichannel voltage and chemical gating characteristics, when combined in series, can often predict the behaviors of cell–cell channels (Trexler et al., 1996; Beahm and Hall, 2002; Contreras et al., 2003; Valiunas et al., 2004; Srinivas et al., 2005; for review see Bukauskas and Verselis, 2004). However, unapposed hemichannels possess a strong sensitivity to extracellular Ca^{2+} and other divalent cations not seen in cell–cell channels,

perhaps due to exposure of a regulatory site in the undocked hemichannel configuration. Hemichannels are expected to be highly regulated because when open in the plasma membrane, they would produce large membrane conductances and potentially serve as pathways for loss of cellular content and entry of extracellular ions detrimental to cell survival.

For all connexins shown to function as unapposed hemichannels, lowering extracellular Ca^{2+} , as well as other divalent cations, promotes hemichannel opening. Here we report a novel form of hemichannel regulation by monovalent cations. While examining the effects of extracellular Ca^{2+} on Cx46 and Cx50 hemichannels, we found that replacement of external Na^+ with K^+ caused a robust increase in the amplitude of Cx50-, but not Cx46-mediated hemichannel currents. External Cs^+ , Rb^+ , NH_4^+ , but not Li^+ , choline, or TEA exhibited similar effects, suggesting a binding site for alkali cations. The primary effect of K^+ appears to be a reduction in the ability of Ca^{2+} , as well as other divalent cations, to close hemichannels. These results suggest a mechanism of hemichannel regulation that is connexin specific and that acts synergistically through a combination of changes in the concentrations of external divalent and monovalent cations.

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Abbreviations used in this paper: CL, cytoplasmic loop; NT, NH_2 terminus.

MATERIALS AND METHODS

Expression of Cx46 and Cx50 in *Xenopus* Oocytes

Cx46 DNA was cloned from rat genomic DNA using PCR amplification with primers corresponding to NH₂- and COOH-terminal sequences as described previously (Trexler et al., 1996). The Cx50 coding sequence was subcloned into the SP64T transcription vector (provided by Dr. Thomas White, SUNY Stony Brook, Stony Brook, NY). Synthesis of RNA and preparation and injection of oocytes have been described previously (Trexler et al., 1996, 2000). Injected oocytes were kept at 18°C in a modified ND96 solution containing (in mM) 88 NaCl, 1 KCl, 1 MgCl₂, 1.8 CaCl₂, 5 glucose, 5 HEPES, 5 pyruvate, pH 7.6. We constructed two chimeras in which the NH₂ terminus (NT) through the cytoplasmic loop (CL) domains of Cx46 and Cx50 were swapped, designated as Cx46*Cx50NT-CL and Cx50*Cx46NT-CL. The chimeras were made using PshAI and BamHI as cloning sites for the respective gel purified inserts and vectors. The borders of the domains of the two connexins are based on accepted sequence alignment (Bennett et al., 1991) and are Met1–Val156 for NT-CL of Cx46 and Met1–Val159 for NT-CL of Cx50. Chimeras were verified by sequencing.

Electrophysiological Recordings

In macroscopic recordings of hemichannel currents, *Xenopus* oocytes were placed in a polycarbonate RC-1Z recording chamber (Warner Instruments) with a slot-shaped bath connecting inflow and outflow compartments to allow for rapid perfusion. A suction tube was placed in the outflow compartment, and a separate reservoir connected to the main chamber with an agar bridge was used for grounding. Bath volume was ~0.3 ml and total volume exchange was achieved in 5–10 s by application of solutions to the inflow compartment. Flow rates in all experiments were consistent. At the start of each experiment, oocytes were bathed in the modified ND96 solution. Perfusion solutions consisted of (in mM) 100 NaCl, 1 MgCl₂, and 10 HEPES, pH 7.6; CaCl₂ was added to adjust Ca²⁺ concentration to levels between 0.05 and 2 mM. The effectiveness of Ni²⁺ and Co²⁺ were examined by replacing CaCl₂ with NiCl₂ or CoCl₂ at desired concentrations. In monovalent ion substitution experiments, NaCl was replaced with the chloride salt of the monovalent ion to be tested and pH was adjusted with the corresponding base. In Cx50- or Cx46-expressing oocytes, the magnitude of the leak current was typically –20 to –50 nA at –40 mV in modified ND96 solutions containing 1.8 mM Ca²⁺ and switching from Na⁺ to K⁺ or Cs⁺ solutions caused only a small increase in the magnitude of the leak current (–20 to –40 nA). The reversal potentials, E_{rev}, of the hemichannel currents were measured in Na⁺ and K⁺ by holding cells at –40 mV and applying a 0.2 mM Ca²⁺ solution to activate the hemichannels. Repeated 200 ms voltage ramps from +20 to –40 mV were applied over a 30-s interval as the hemichannels activated; stepping to +20 mV does not induce substantial closure of the V_j gate (Srinivas et al., 2005). E_{rev} was determined from the voltage at which the currents from the ramps intersected and typically ranged from –10 to –15 mV. Cells bathed in K⁺ showed a small positive shift in E_{rev} of a few millivolts compared with those in Na⁺. These small changes contribute little to the potentiation of the current observed. Thus, the data for Cx50 are plotted as the “fold change in current compared to Na⁺,” which represents the ratio of the leak-subtracted value of the holding current at –40 mV in Na⁺ and the holding current at –40 mV in the test ion. For Cx46, currents were evaluated at the ends of steps to +50 mV as there was little activation at inside negative voltages.

Ca²⁺ concentration–response curves in Na⁺- and K⁺-containing solutions were determined by exposing the same oocytes to Ca²⁺ concentrations of 0.05, 0.10, 0.25, 0.6, 1.0, 1.5, and 2.0 mM. After exposure to each concentration for 1 min, oocytes were perfused with the modified ND96 solution. This protocol en-

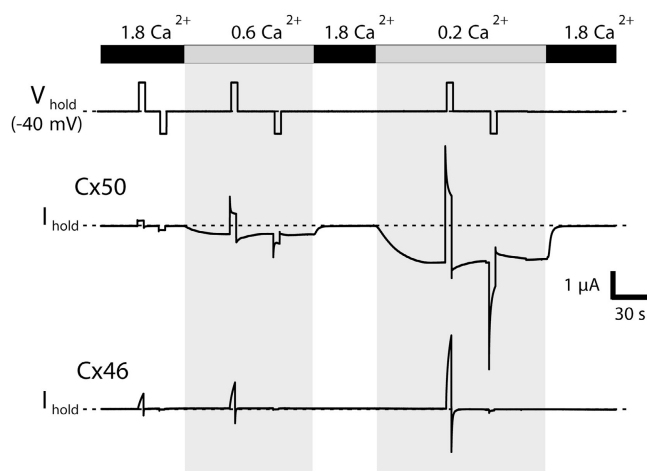


Figure 1. Membrane currents in *Xenopus* oocytes expressing Cx50 (middle) and Cx46 (bottom) respond differently to changes in external calcium. MND96 solutions with external Ca²⁺ concentrations of 1.8, 0.6, and 0.2 mM were applied to oocytes voltage clamped at –40 mV (V_{hold}). A pair of 5-s voltage steps, one depolarizing to +50 mV and one hyperpolarizing to –110 mV were applied at each Ca²⁺ concentration. Reducing external Ca²⁺ led to the development of a progressively larger inward current at V_{hold} in Cx50-expressing oocytes that was accompanied by progressively larger currents in response to both polarities of applied voltage steps. In contrast, little or no change in current at V_{hold} or in response to hyperpolarizing voltage steps were observed in Cx46-expressing oocytes upon reducing external Ca²⁺. However, currents elicited by the depolarizing steps were progressively larger as Ca²⁺ was reduced, consistent with the effects of Ca²⁺ previously described (Ebihara and Steiner, 1993).

ured that the current returned to baseline levels. Recordings of hemichannel currents were obtained with a GeneClamp 500 two-electrode voltage clamp (Axon Instruments). Both current-passing and voltage-recording pipettes contained 2 M KCl.

For patch clamp recordings of hemichannel currents, *Xenopus* oocytes were manually devitellinized in a hypertonic solution consisting of (in mM) 220 Na Aspartate, 10 KCl, 2 MgCl₂, 10 HEPES, and then placed in ND96 solution for recovery. Oocytes were then individually moved to the recording chamber containing the patch pipette solution (IPS), which consisted of (in mM) 140 KCl, 1 MgCl₂, 5 HEPES, 1 CaCl₂, 5 EGTA, and pH adjusted to 7.6 with KOH. The smaller bath compartment was connected via a 3 M agar bridge to a ground compartment. Hemichannel currents were recorded in cell-attached and excised-patch configurations using an Axopatch 1D amplifier (Axon Instruments). Single hemichannel I–V curves were obtained by applying 8-s voltage ramps from –70 to +70 mV. Single channel records from voltage steps and ramps were leak subtracted by measuring leak conductance of a given patch from full closing transitions and extrapolating linearly with voltage. Currents were filtered at 1 kHz and data were acquired at 10 kHz.

Macroscopic and single hemichannel data were acquired using an AT-MIO-16X D/A board from National Instruments using our own acquisition software. All chemicals were purchased from Sigma-Aldrich.

RESULTS

Cx46 and Cx50 hemichannel currents are strongly modulated by external Ca²⁺, but the response to Ca²⁺

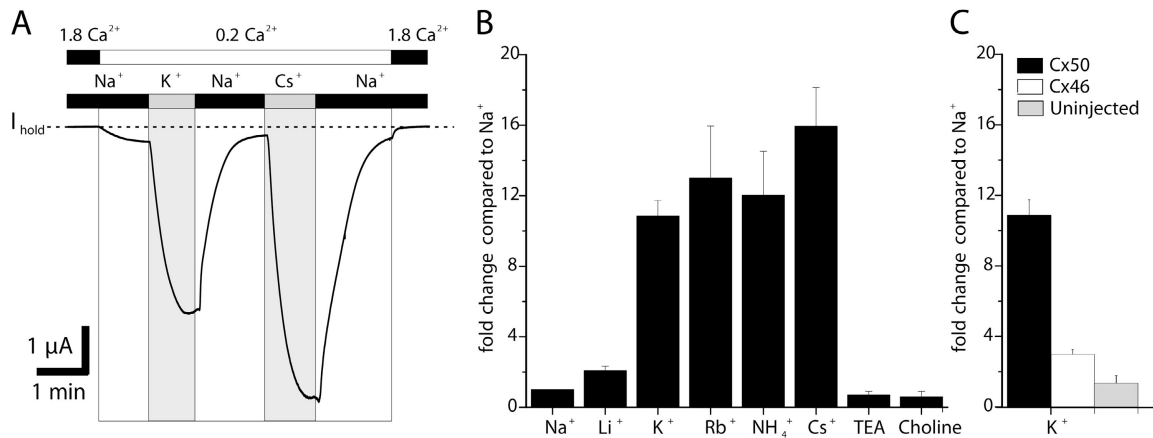


Figure 2. External monovalent cations strongly potentiate Cx50, but not Cx46, hemichannel currents. (A) Membrane currents were monitored in a Cx50-expressing oocyte voltage clamped at -40 mV. Reduction of Ca^{2+} from 1.8 mM to 0.2 mM in a Na^+ solution promoted opening of Cx50 hemichannels evident by the development of an inward current. Equimolar replacement of Na^+ with K^+ or Cs^+ (gray bars) in the continued presence of 0.2 mM Ca^{2+} caused a large increase in the amplitude of the inward current at -40 mV, an effect that was reversible upon switching back to Na^+ . (B) Bar graph summarizing the effect of replacing external Na^+ with a variety of monovalent cations on Cx50 hemichannel currents. K^+ , Rb^+ , NH_4^+ , and Cs^+ , but not TEA^+ , Li^+ , and choline, caused a robust potentiation in current. Bars represent the means \pm SEM of the fold change caused by each monovalent cation (n ranged from 4 to 15). Mean values in K^+ and Cs^+ were significantly different ($P < 0.05$). (C) Cx46 hemichannels were only moderately affected by replacing external Na^+ with K^+ . Bar graph shows a comparison of the increase in Cx46 (white bars) and Cx50 (solid bars) hemichannel currents upon replacing external Na^+ with K^+ . Because Cx46 activates only at positive voltages, current amplitudes were measured at the ends of 5-s depolarizing voltage steps to $+50$ mV. Such changes were not observed in uninjected oocytes (gray bars). Each bar represents the mean \pm SEM of six oocytes.

differs in these two closely related connexins. Fig. 1 shows recordings from *Xenopus* oocytes expressing Cx46 or Cx50, during which external Ca^{2+} was reduced from 1.8 to 0.6 mM and to 0.2 mM. The oocytes were voltage clamped to a holding potential of -40 mV, and a pair of voltage steps, one depolarizing to $+50$ mV and one hyperpolarizing to -110 mV, was applied at each Ca^{2+} concentration to examine the characteristics of the hemichannel currents. Cx50-expressing oocytes in 1.8 mM Ca^{2+} typically showed little evidence of current activation in response to hyperpolarizing or depolarizing voltage steps. In contrast, *Xenopus* oocytes expressing Cx46 exhibited a polarity-dependent activation with a slowly rising outward current evident upon depolarization to $+50$ mV. Cx46 currents deactivated completely when the membrane potential was stepped back to -40 mV and remained deactivated when hyperpolarized to -110 mV. Upon reducing external Ca^{2+} to 0.6 mM, an inward current developed at the holding potential in Cx50-expressing oocytes. Application of voltage steps to $+50$ and -110 mV at this reduced external Ca^{2+} concentration produced large outward and inward currents, respectively, that declined from an initial peak to a nonzero steady-state value, characteristic of Cx50 hemichannel currents as previously described (Zampighi et al., 1999; Beahm and Hall, 2002; Srinivas et al., 2005). Upon lowering Ca^{2+} to 0.2 mM, the magnitude of the inward current at the holding potential increased further as did the currents in response to the

$+50$ and -110 mV voltage steps. In contrast, reducing external Ca^{2+} to 0.6 mM or 0.2 mM Ca^{2+} for Cx46-expressing oocytes caused little change in current at the holding potential and upon stepping to -110 mV. The amplitude of the outward current observed at $+50$ mV, however, was higher in 0.6 mM Ca^{2+} and higher still in 0.2 mM Ca^{2+} , consistent with potentiation of Cx46 hemichannel currents by reduced external Ca^{2+} as originally reported (Ebihara and Steiner, 1993).

Potentiation of Cx50 Hemichannel Currents by External Monovalent Ions

Studies intended to examine the effects of Ca^{2+} on Cx50 yielded an unexpected result. The amplitude of the Cx50 hemichannel current was extraordinarily sensitive to the composition of monovalent cations in the extracellular solution. This effect is illustrated in Fig. 2 A, in which an oocyte expressing Cx50 was perfused with solutions in which external NaCl was replaced with KCl or with CsCl . While holding the oocyte at -40 mV, Ca^{2+} was reduced to 0.2 mM to promote opening of Cx50 hemichannels, evident by the development of an inward current. Equimolar replacement of extracellular Na^+ with K^+ in the continued presence of 0.2 mM Ca^{2+} caused a further, robust increase in current. In the illustrated example, the current at the holding potential of -40 mV increased from -0.3 μA in Na^+ to -3.8 μA in K^+ , corresponding to an ~ 13 -fold increase. This effect was completely reversible upon

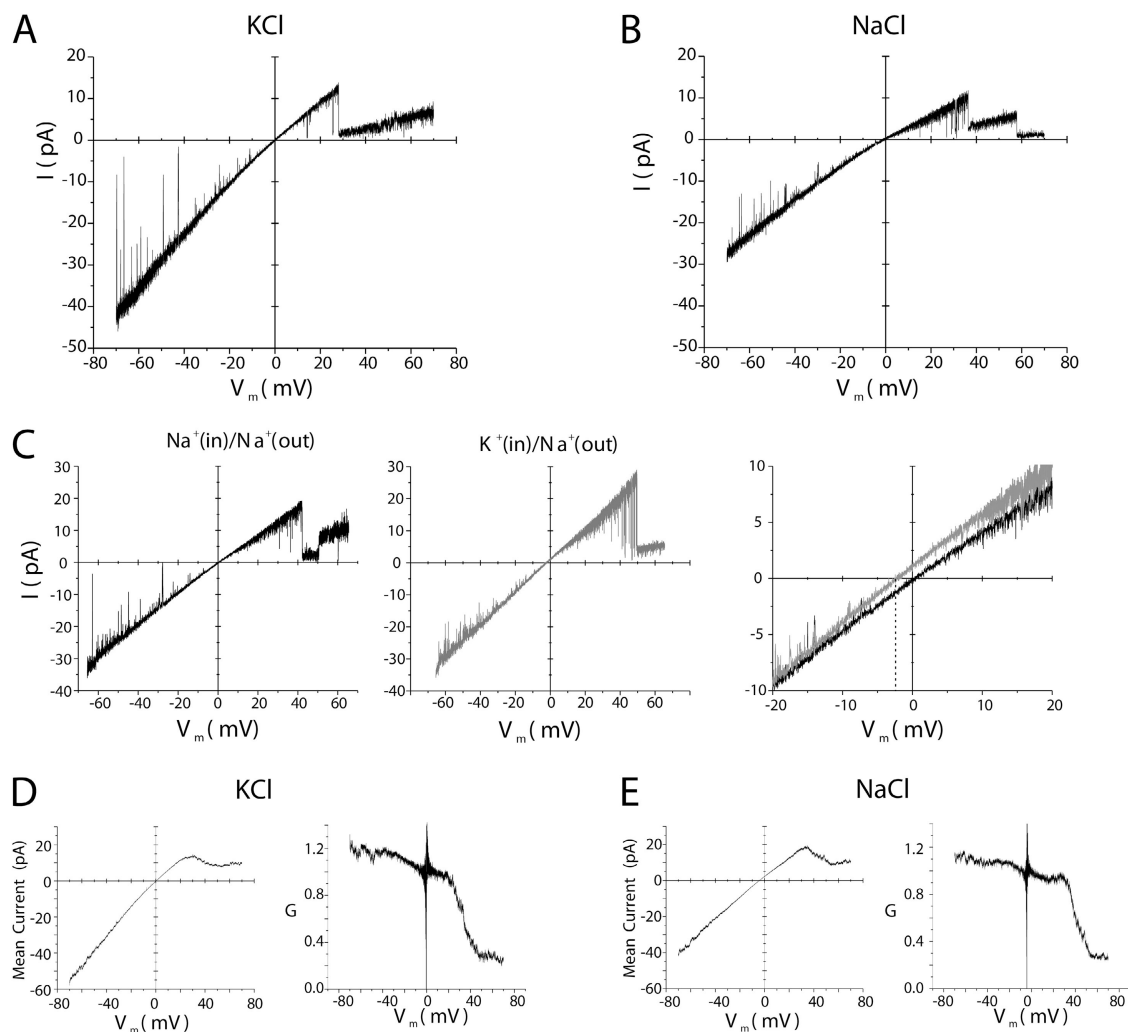


Figure 3. Unitary conductance and gating properties of Cx50 hemichannels do not differ in NaCl or KCl solutions in the absence of Ca^{2+} . (A and B) Single Cx50 hemichannel currents obtained with 8-s voltage ramps from -70 to 70 mV applied to excised patches in symmetric KCl (A) or NaCl (B) solutions each containing 1 mM Ca^{2+} / 5 mM EGTA to maintain the free Ca^{2+} concentration $<10^{-7}$ M. (C) Single Cx50 hemichannel currents obtained with 8-s voltage ramps from -65 to 65 mV applied to excised inside-out patches in symmetric NaCl solutions (left) or asymmetric KCl(in)/NaCl(out) solutions (center). Recordings are from the same patch. Segments of the I-V relationships are expanded to show the small shift in $E_{\text{rev}} = -2.6$ mV (right). (D and E) Conductance-voltage (G-V) relationships of Cx50 hemichannels (shown on right in D and E) constructed from ensemble ($n = 20$) averaged currents (shown on left in D and E) in response to ± 70 mV, 8-s voltage ramps applied to cell attached patches. Pipette solutions containing KCl (D) or NaCl (E) were the same as in A and B.

switching back to the Na^+ solution, indicating that the large increase in K^+ was not due to recruitment of new hemichannels to the plasma membrane. The mean increase in current caused by replacement of Na^+ with K^+ in Cx50 was 10.9 ± 0.9 ($n = 15$; Fig. 2 B) and was observed in all Cx50-expressing oocytes tested. Such changes were not observed in uninjected oocytes (Fig. 2 C).

We tested a series of other monovalent cations and found similar large increases in Cx50 hemichannel currents when Na^+ was replaced with Cs^+ , Rb^+ , and NH_4^+ (Fig. 2 B). Of the ions tested, Cs^+ caused the largest increase in current (15.9 ± 2.2 , $n = 10$). The effects of

Rb^+ and NH_4^+ were also robust and slightly greater in magnitude than that of K^+ . In contrast, Li^+ , TEA, and choline were ineffective at increasing Cx50 hemichannel currents. In fact, replacing Na^+ with TEA or choline produced a reduction in current, likely attributable to the reduced mobilities of these ions compared with Na^+ .

We next determined to what extent Cx46 hemichannel currents were sensitive to the extracellular cation composition. Comparison of mean changes observed in Cx46- and Cx50-expressing oocytes upon substitution of external Na^+ with K^+ (Fig. 2 C) shows a substantially smaller effect on Cx46 hemichannels (approx-

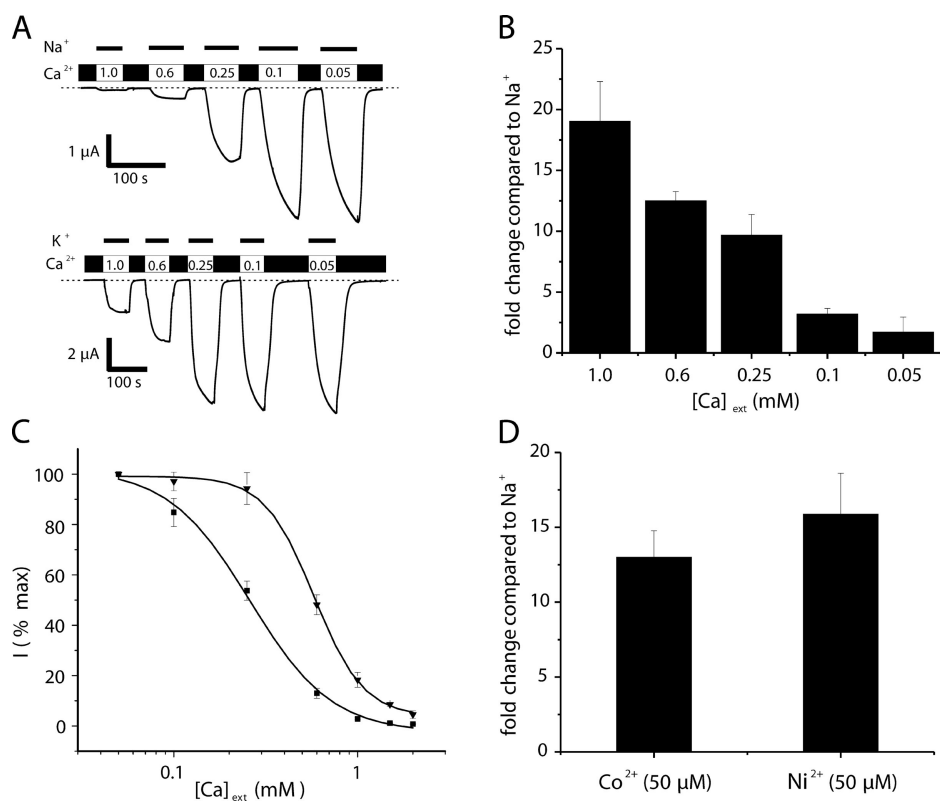


Figure 4. Potentiation by K^+ is dependent on external Ca^{2+} . (A) Example of recordings of membrane currents in a Cx50-expressing oocyte voltage clamped at -40 mV over a range of Ca^{2+} concentrations in NaCl and KCl. For each series, the oocyte was placed in 100 mM NaCl containing 2 mM Ca^{2+} and sequentially exposed to 100 mM NaCl (top panel, dashes) or 100 mM KCl (bottom panel, dashes) containing Ca^{2+} concentrations of (in mM) 1.0, 0.6, 0.25, 0.1, and 0.05 (open bars). Between each exposure, oocytes were returned to 100 mM NaCl containing 2 mM Ca^{2+} (filled bars). Saturation of the effect of Ca^{2+} was achieved at a higher external Ca^{2+} concentration in K^+ . (B) Bar graph showing the magnitude of potentiation of Cx50 hemichannel currents upon replacement of external Na^+ with K^+ at different Ca^{2+} concentrations. The effect of external K^+ was markedly reduced at the lower end of the Ca^{2+} concentration range examined. Current magnitudes due to the differences in mobility between Na^+ and K^+ are not corrected. Each bar represents the mean \pm

SEM of 6–15 oocytes. (C) Concentration response curves for Ca^{2+} inhibition of Cx50 hemichannel currents in external Na^+ (squares) and K^+ (inverted triangles) solutions. For Na^+ and K^+ , current amplitudes at each Ca^{2+} concentration were normalized to the responses obtained at 0.05 mM Ca^{2+} in Na^+ and K^+ , respectively. Solid lines are fits of the data to the Hill equation of the form Normalized $I = K_d^n / (K_d^n + [Ca^{2+}]^n)$. K_d and n values were 259 μ M and 2.2 in Na^+ and 580 μ M and 3.2 in K^+ . (D) Bar graph showing that the inhibition of Cx50 hemichannel currents by other divalent cations, Ni^{2+} and Co^{2+} , is also affected by replacement of Na^+ with K^+ . Each bar represents the mean \pm SEM of three to four oocytes.

mately threefold), which represents a modest increase beyond that ascribable to the ~ 1.5 -fold increase expected due to differences in cationic mobility. Similar modest effects on Cx46 hemichannels were observed upon replacing Na^+ with Cs^+ (unpublished data). These results suggest that connexin hemichannels are regulated by monovalent ions with effectiveness, in the case of Cx50, following the sequence $Cs^+ > Rb^+ \cong K^+ \cong NH_4^+ \gg Li^+ \cong Na^+$.

Potentiation by Monovalent Cations Is Dependent on External Divalent Cations

To examine the underlying basis for the large potentiation of Cx50 macroscopic currents by external K^+ , we compared single hemichannel currents recorded in NaCl and KCl salts. In response to ± 70 mV voltage ramps applied to excised patches containing single hemichannels, mean slope conductance measured at $V_m = 0$ was 292 ± 11 pS in symmetric 140 mM NaCl ($n = 21$ ramps, 3 patches) and 469 ± 33 pS ($n = 33$ ramps, 5 patches) in symmetric 140 mM KCl. Examples of single hemichannel currents are shown in Fig. 3 (A and B). The ~ 1.5 -fold higher unitary conductance in

KCl corresponds to the ~ 1.5 -fold higher aqueous mobility of K^+ compared with Na^+ , and does not explain the ~ 11 -fold mean increase in hemichannel current observed macroscopically when substituting Na^+ with K^+ . We also found that Cx50 hemichannels do not select between the two monovalent cations, as shown in Fig. 3 C, where exposure of excised patches to a bi-ionic condition, NaCl on the outside and KCl on the inside, only produced an E_{rev} of -2.6 mV. Similar small changes in reversal potential were observed in macroscopic recordings upon switching between Na^+ and K^+ solutions containing 0.2 or 0.7 mM Ca^{2+} (see MATERIALS AND METHODS). G-V relationships constructed from ensemble I-V curves obtained from cell-attached patches in which ± 70 mV, 8 s voltage ramps were applied, were also essentially similar except for a small shift in voltage sensitivity at positive voltages when pipettes were filled with KCl or with NaCl (Fig. 3, D and E). Thus, there do not appear to be any substantial differences in unitary conductance, permeability, or gating properties at the single hemichannel level upon substituting external Na^+ with K^+ that could account for the potentiation observed macroscopically.

Since single hemichannel recordings were obtained in solutions containing Ca^{2+} /EGTA that maintained free Ca^{2+} concentrations $<10^{-7}$ M, we reasoned that external K^+ may potentiate Cx50 hemichannel currents by modulating the effects of Ca^{2+} . Therefore, we examined macroscopic currents in Na^+ and K^+ solutions at different external Ca^{2+} concentrations ranging from 0.05 to 2.0 mM (Fig. 4). In both Na^+ and K^+ solutions, lowering external Ca^{2+} caused an increase in current in a concentration-dependent manner (Fig. 4 A). At each Ca^{2+} concentration, comparison of the current amplitudes in Na^+ or K^+ salts showed that the effect of external K^+ was markedly reduced at the lower end of the Ca^{2+} concentration range examined. At external Ca^{2+} concentrations of 0.25, 0.6, and 1.0 mM, replacement of external Na^+ with K^+ increased Cx50 hemichannel current by 9.7-, 12.5-, and 19-fold, respectively, whereas at Ca^{2+} concentrations of 0.1 and 0.05 mM, the increase was only 3.1- and 1.7-fold, respectively (Fig. 4 B).

Concentration response curves (Fig. 4 C) showed that the increase in current saturated at Ca^{2+} concentrations between 0.1 and 0.05 mM when the external solution consisted of NaCl . Upon replacement of NaCl with KCl , saturation was achieved at a higher external Ca^{2+} concentration of ~ 0.25 mM, evident as a shift in the Ca^{2+} concentration–response curve and an increase in the apparent K_d from 259 to 580 μM . These data suggest that external K^+ reduces the apparent binding affinity of Ca^{2+} in Cx50 hemichannels.

Since divalent cations other than Ca^{2+} have been reported to close connexin hemichannels (Ebihara and Steiner, 1993), we examined whether regulation of Cx50 hemichannel currents by other divalent cations was similarly affected by replacement of Na^+ with K^+ . Thus, we replaced Ca^{2+} in the external solution with Co^{2+} and Ni^{2+} , both potent modulators of Cx46 and Cx50 hemichannels. Using Co^{2+} or Ni^{2+} at a concentration of 50 μM , Cx50 hemichannel currents were comparable to those in 0.2 mM Ca^{2+} . As shown in Fig. 4 D, Cx50 hemichannel currents were substantially potentiated in the presence of Co^{2+} or Ni^{2+} when external Na^+ was replaced by K^+ , consistent with Ni^{2+} or Co^{2+} binding to the same site as Ca^{2+} and with modulation by these divalent cations being sensitive to the composition of external monovalent cations. Effects of K^+ substitution on regulation by Mg^{2+} were not examined because Cx50 hemichannels exhibit weak sensitivity to extracellular Mg^{2+} , requiring concentrations in excess of 10 mM to produce substantial reductions in hemichannel currents.

The Different Sensitivities of Cx50 and Cx46 to K^+ Is Contained to the NH_2 -terminal Half

Since Cx46 hemichannels lack the robust sensitivity to monovalent cations characteristic of Cx50, we con-

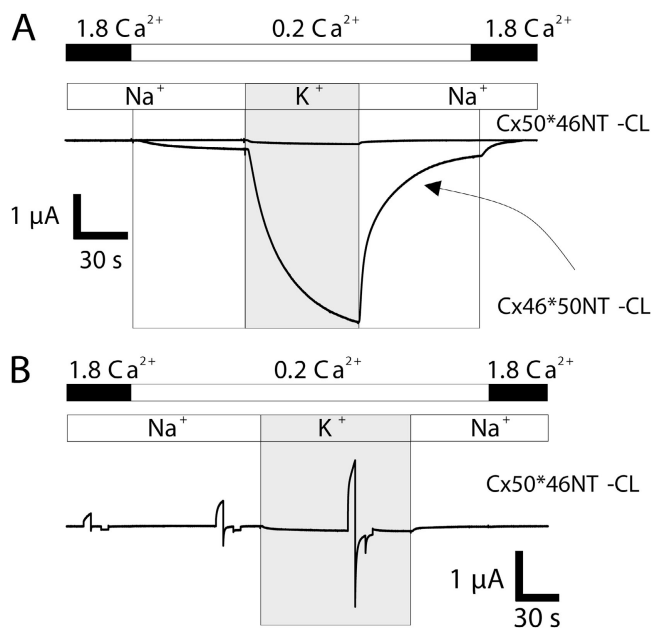


Figure 5. The difference in the sensitivity of Cx46 and Cx50 hemichannels to external K^+ resides in the NH_2 -terminal half. (A) Shown are hemichannel currents upon replacing external Na^+ with K^+ in oocytes expressing Cx46*50NT-CL and Cx50*46NT-CL chimeric hemichannels. Oocytes were voltage clamped at -40 mV. Currents in oocytes expressing Cx46*50NT-CL, in which Cx46 sequence from the NT through the CL was replaced with Cx50, were strongly potentiated, much like Cx50, when Na^+ was replaced with K^+ . The reciprocal chimera, Cx50*46NT-CL, was similar to Cx46 in being largely insensitive to external K^+ , except for a small shift in the baseline current that was also observed in uninjected oocytes. (B) Hemichannel currents in oocytes expressing Cx50*46NT-CL in response to hyperpolarizing and depolarizing pulses to -110 and $+50$ mV, respectively, applied from holding potential of -40 mV in Na^+ - and K^+ -containing external solutions. Membrane currents in Cx50*46NT-CL-expressing oocytes qualitatively resemble those in Cx46-expressing oocytes and show an increase in external K^+ similar to that of Cx46 hemichannels.

structed chimeric hemichannels that consisted of Cx50 from the NH_2 terminus (NT) through the cytoplasmic loop (CL) domain, the remainder being Cx46 sequence, Cx46*50NT-CL, and the reciprocal chimera, Cx50*46NT-CL. Both chimeric hemichannels produced membrane currents when injected into *Xenopus* oocytes, indicative that hemichannel function was maintained. Currents in oocytes expressing Cx46*50NT-CL showed a robust and reversible increase (14.3 ± 4.1 , $n = 6$) upon replacement of external Na^+ with K^+ . In contrast, currents in oocytes expressing Cx50*46NT-CL were weakly affected by replacement of external Na^+ with K^+ , much like Cx46 (Fig. 5). Since we did not observe robust potentiation by K^+ in Cx50*46NT-CL oocytes, we examined whether this chimera retained sensitivity to external Ca^{2+} . Voltage steps to $+50$ and -110 mV from a holding potential of -40 mV showed activation only upon depolarization to $+50$ mV, and lowering Ca^{2+} from 1.8 to 0.2

mM showed potentiation of the outward current at +50 mV, much like Cx46 (Fig. 5 B). Substitution of Na⁺ with K⁺ showed two- to threefold further potentiation, not the robust (>10-fold) potentiation characteristic of Cx50. These data suggest that the difference in the abilities of Cx50 and Cx46 hemichannels to respond to monovalent cations is contained within the NH₂-terminal half.

DISCUSSION

In this study, we demonstrate that Cx50 macroscopic currents are remarkably sensitive to external monovalent cations. Replacement of Na⁺ with K⁺ in the bathing media causes a robust increase in current. Other alkali cations, specifically Cs⁺ and Rb⁺, but not Li⁺, were also able to cause a robust increase in current. Our data suggest that potentiating monovalent ions produce their effect by reducing the ability of divalent cations, most importantly Ca²⁺, to close Cx50 hemichannels. Several lines of evidence support such a mechanism. First, potentiation of macroscopic currents by substitution of Na⁺ with K⁺, Cs⁺, or Rb⁺ progressively decreased as the external Ca²⁺ concentration was reduced. Second, recordings of single hemichannels in Ca²⁺/EGTA solutions in which free Ca²⁺ concentration was <10⁻⁷ M showed no obvious differences in gating properties, unitary conductance, or selectivity in Na⁺- and K⁺-containing solutions, other than that attributable to the difference in ionic mobilities of these cations. Third, modulation by other divalent cations, which are thought to bind to the same site as Ca²⁺ (Ebihara and Steiner, 1993; Ebihara et al., 2003) was similarly affected by replacing external Na⁺ with K⁺.

The increase in current upon switching from Na⁺ to K⁺ solutions was reversible, indicating that potentiation by K⁺ did not result from recruitment of hemichannels to the plasma membrane. Also, the small shifts in reversal potential observed macroscopically when substituting between K⁺ and Na⁺ solutions in the presence of Ca²⁺, as well as the small shifts in E_{rev} observed at the single hemichannel level under bi-ionic conditions in the absence of Ca²⁺, are consistent with no change in selectivity properties of Cx50 hemichannels in NaCl and KCl regardless of Ca²⁺ concentration. Furthermore, most of the experiments that showed potentiation were obtained at a holding potential of -40 mV, where Cx50 hemichannels show no evidence of voltage dependence. Thus, the effect of substituting K⁺ appears to be an increase in hemichannel open probability, separate from voltage-dependent gating. Single channel studies would provide direct evidence for a change in open probability, but Cx50 hemichannels have proven to be somewhat unstable in patch clamp recordings, often exhibiting rundown in excised or cell-attached recordings, so that we have been unable

to obtain sufficiently long stable recordings to assess open probabilities as a function of Ca²⁺ in Na⁺- and K⁺-containing salts. Instability may result from loose aggregation or association with other membrane proteins and/or cytoskeletal components that are disrupted by distortion of the membrane upon patching. Cx46 has been shown to be mechanosensitive (Bao et al., 2004), suggesting that connexin hemichannels may indeed be sensitive to membrane deformation.

Although the exact mechanism by which the effect of K⁺ on hemichannels through Ca²⁺ remains to be determined, it likely involves binding of monovalent cations to the channel itself. Interestingly, the monovalent cations that are effective at potentiating hemichannel currents, i.e., K⁺, Cs⁺, Rb⁺, and NH₄⁺ but not Na⁺ and Li⁺, resemble those that are well accommodated by a K⁺ channel selectivity filter. Although the elaborate configuration of the K⁺ channel selectivity filter is not expected here, the high degree of selectivity for K⁺ over Na⁺ may signify a structure, such as an arrangement of carbonyl-like dipoles, which have been reported to be naturally optimal for K⁺ ions (Noskov et al., 2004). If this binding site is close to the divalent cation binding site, it is possible that binding of K⁺ knocks divalent cations off by speeding up dissociation via electrostatic repulsion. Similar interactions between permeant and blocking ions have been described in K⁺ channels (Bezanilla and Armstrong, 1972; Adelman and French, 1978; Yellen, 1984; Neyton and Miller, 1988). Alternatively, the binding of K⁺ to a site remote from the divalent site can lead to conformational changes that promote dissociation of divalent cations and/or render bound divalent cations less effective at closing hemichannels. At present, our results do not allow distinction among these possibilities.

Given that monovalent ions act by modulating the effects of Ca²⁺, determining the site of action of monovalent ions and whether hemichannels formed of other connexins will show sensitivity to monovalent ions is complicated by the lack of a consensus as to the mechanism of Ca²⁺ action itself. In Cx46 hemichannels, Ca²⁺ substantially shifts voltage dependence (Ebihara and Steiner, 1993), but has also been suggested to contain elements of channel block with a reported fractional electrical distance of 1.0 (Ebihara et al., 2003), suggestive of a cytoplasmic location for the blocking site. Conversely, studies at the single hemichannel level showed Ca²⁺ able to close Cx46 hemichannels from either side of the membrane, but substantially reduced in effectiveness from the cytoplasmic side, suggestive of a Ca²⁺ binding site in or near the external mouth of the hemichannel pore (Pfahnl and Dahl, 1999). A recent study of Cx37 hemichannel currents proposed that polyvalent cations block in a voltage-dependent manner by traversing the length of electric field from the

extracellular side to bind to a cytoplasmic site (Puljung et al., 2004). In Cx32, stabilization of a subconductance state was proposed as the mechanism of Ca^{2+} regulation, with a binding site localized to a ring of Glu residues in the second extracellular loop, E2 (Gomez-Hernandez et al., 2003). In Cx50 hemichannels, Ca^{2+} does not produce a substantial shift in voltage dependence (Beahm and Hall, 2002) and there is no indication as to whether Ca^{2+} binds to a cytoplasmic or extracellular location.

In our studies, K^+ added to the extracellular side is clearly effective at potentiating currents and we saw no obvious potentiation of Cx50 hemichannel currents in external NaCl at large depolarizing potentials when K^+ flux through the hemichannel is expected to be high. Thus, a K^+ binding site would not likely be in the path of permeating ions and thus may be strictly extracellular, although this has not been directly tested.

Despite the fact that Cx46 and Cx50 share a high degree of primary sequence homology, the degree of potentiation by monovalent ions differed substantially in these connexins. In Cx46 hemichannels, the mean increase in current observed upon replacement of Na^+ with K^+ was modest (approximately threefold) relative to that observed in Cx50 hemichannels (~11-fold). Cx46 hemichannels have been shown to be >10-fold more selective for K^+ over Cl^- , but essentially nonselective among monovalent alkali cations (Trexler et al., 1996). Thus, maximally half of the increase in Cx46 hemichannel current upon replacement of Na^+ with K^+ can be attributed to the higher aqueous mobility of K^+ . The modest increase beyond that attributable to mobility suggests that there is some effect of monovalent ions on Cx46 hemichannels.

The chimeric hemichannel consisting of the NH_2 -terminal half of Cx50 (Cx46*50NT-CL) is strongly regulated by monovalent ions much like Cx50 and the converse chimeric hemichannel (Cx50*46NT-CL) is not, indicating that the residues responsible for the difference in magnitude of K^+ ion regulation between Cx46 and Cx50 lie within the NH_2 -terminal half. This finding together with the likelihood that a putative K^+ binding site is extracellular leaves the E1 domain as the most probable location. Application of the substituted cysteine accessibility method (SCAM) to single Cx46 hemichannels has shown that the E1 domain contributes to the aqueous pore toward the extracellular end (Kronengold et al., 2003). Thus, an extracellular location for the divalent cation site, associated with the pore and hence E1, could provide for close proximity between divalent and monovalent cation binding sites and regulation of currents by means of electrostatic interaction. The binding site for monovalent cations, which does not appear to be in the permeation pathway, would have to be situated so that it is effectively shielded from the permeating ions.

A number of studies suggest that hemichannels can function under physiological conditions with potential roles that include release of ATP, implicated in retinal development (Pearson et al., 2005), in the spread of Ca^{2+} waves (Stout et al., 2002; Weissman et al., 2004), and as a source of current that mediates negative feedback from horizontal cells to cones in the vertebrate retina (Kamermans et al., 2001). Cx50 is abundantly expressed in lens (White et al., 1992) and has been reported to be expressed in horizontal cells in the rabbit retina along with Cx57 (Massey et al., 2003). The latter suggests a possibility that Cx50 hemichannels could participate in the feedback mechanism to photoreceptors in some vertebrate species. Although it is established that Ca^{2+} regulates opening of hemichannels, this study demonstrates that extracellular K^+ ions can potentially serve as potent modulators that change the sensitivity of hemichannels to Ca^{2+} .

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