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.

Abbreviations used in this paper: CL, cytoplasmic loop; NT, NH\(_2\) terminus.
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 PshAl 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 for use in perfusion. 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 ~20–50 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 (~20–40 nA). The reversal potentials, Erev, 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 30s interval as the hemichannels activated; stepping to +20 mV does not induce substantial closure of the Vg gate (Srinivas et al., 2005). Erev 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 Erev 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 ensured 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 8s 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. 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 (Vhold). A pair of 5s 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 Vhold 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 Vhold or in response to hyperpolarization 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).

**RESULTS**

Cx46 and Cx50 hemichannel currents are strongly modulated by external Ca2+, but the response to Ca2+...
differ 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 hyperpolarization or depolarization 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

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**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 ± 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 ± SEM of six oocytes.
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 (approxi-
Kdn/(Kdn and B). The single hemichannel currents are shown in Fig. 3 (A ramps, 5 patches) in symmetric 140 mM KCl. Examples of the mean slope conductance measured at ramps applied to excised patches containing single NaCl and KCl salts. In response to compared single hemichannel currents recorded in /H11001ction of Cx50 macroscopic currents by external K.

To examine the underlying basis for the large potentia-
tion of Cx50, following the sequence Cs/NaCl on the outside and KCl on the inside, only produced an Erev of −2.6 mV. Similar small changes in reversal potential were observed in macroscopic recordings upon switching between NaCl and KCl solutions containing 0.2 or 0.7 mM Ca2+(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 pi-
pettes 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.

Figure 4. Potentiation by K+ is dependent on external Ca2+. (A) Example of recordings of membrane currents in a Cx50-expressing oocyte voltage clamped at −40 mV over a range of Ca2+ concentrations in NaCl and KCl. For each series, the oocyte was placed in 100 mM NaCl containing 2 mM Ca2+ and sequentially exposed to 100 mM NaCl (top panel, dashes) or 100 mM KCl (bottom panel, dashes) containing Ca2+ 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 NaCl containing 2 mM Ca2+ (filled bars). Saturation of the effect of Ca2+ was achieved at a higher external Ca2+ concentration in K+. (B) Bar graph showing the magnitude of potentiation of Cx50 hemichannel currents upon replacement of external Na+ with K+ at different Ca2+ concentrations. The effect of external K+ was markedly reduced at the lower end of the Ca2+ concentration range examined. Current magnitudes due to the differences in mobility between Na+ and K+ are not corrected. Each bar represents the mean ± SEM of three to four oocytes.

Potentiation by Monovalent Cations Is Dependent on External Divalent Cations

To examine the underlying basis for the large potentia-
tion 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 Vm = 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 mo-
bility 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 se-
lect 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 Erev of −2.6 mV. Similar small changes in reversal potential were observed in macro-
scopic recordings upon switching between NaCl and K+ solutions containing 0.2 or 0.7 mM Ca2+ (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.

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+ ≈ K+ ≈ NH4+ ≫ Li+ ≈ Na+. 

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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 monovalent cations characteristic of Cx50, we constructed chimeric hemichannels that consisted of Cx50 from the NH\(_2\)-terminal half (NT) through the CL domain, the remainder being Cx46 sequence. Oocytes were voltage clamped at −40 mV. Currents in oocytes expressing Cx46*50NT-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. 

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 constructed chimeric hemichannels. Oocytes were voltage clamped at −40 mV. 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 NH$_2$-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 Ca$^{2+}$, 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 Ca$^{2+}$ concentration was reduced. Second, recordings of single hemichannels in Ca$^{2+}$/EGTA solutions in which free Ca$^{2+}$ 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 Ca$^{2+}$ (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 Ca$^{2+}$, as well as the small shifts in $E_{\text{rev}}$ observed at the single hemichannel level under bi-ionic conditions in the absence of Ca$^{2+}$, are consistent with no change in selectivity properties of Cx50 hemichannels in NaCl and KCl regardless of Ca$^{2+}$ 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 Ca$^{2+}$ 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 Ca$^{2+}$ 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 NH$_4^+$ 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 Ca$^{2+}$, 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 Ca$^{2+}$ action itself. In Cx46 hemichannels, Ca$^{2+}$ 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 Ca$^{2+}$ able to close Cx46 hemichannels from either side of the membrane, but substantially reduced in effectiveness from the cytoplasmic side, suggestive of a Ca$^{2+}$ binding site in or near the external mouth of the hemichannel pore (Pfahl and Dahl, 1999). A recent study of Cx37 hemichannel currents proposed that polyanion blocking is achieved in a voltage-dependent manner by traversing the length of electric field from the

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indicating that the residues responsible for the divergent chimeric hemichannel (Cx50*46NT-CL) is not, related by monovalent ions much like Cx50 and the concomitant half of Cx50 (Cx46*50NT-CL) is strongly regulated by 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.

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+ 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 NH2-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 NH2-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 Ca2+ 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 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 Ca2+ regulates opening of hemichannels, this study demonstrates that extracellular K+ ions can potentially serve as potent modulators that change the sensitivity of hemichannels to Ca2+.

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