Molecular analysis of voltage dependence of heterotypic gap junctions formed by connexins 26 and 32

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ABSTRACT Heterotypic gap junctions formed by pairing *Xenopus* oocytes expressing hemichannels formed of Cx32 with those expressing hemichannels formed of Cx26 displayed novel transjunctional voltage (V_j) dependence not predicted by the behavior of these connexins in homotypic configurations. Rectification of initial and steady-state currents was observed. Relative positivity and negativity on the Cx26 side of the junction resulted in increased and decreased initial conductance (g_{j0}), respectively. Only relative positivity on the Cx26 decreased steady-state conductance ($g_{j\infty}$). This behavior suggested that interactions between hemichannels influences gap junction gating. The role of the first extracellular loop (E1) in these interactions was examined by pairing Cx32 and Cx26 with a chimeric connexin in which Cx32 E1 was replaced with Cx26 E1 (Cx32*26E1). Both junctions rectified with g_{j0}/V_j relations that were less steep than that observed for Cx32/Cx26. Decreases in $g_{j\infty}$ occurred for either polarity V_j in the Cx32/Cx32*26E1 junction. Mutation of two amino acids in Cx26 E1 increased the steepness of both the g_{j0}/V_j and $g_{j\infty}/V_j$ relations. These data demonstrate that fast rectification can arise from mismatched E1 domains and that E1 may contribute to the voltage sensing mechanisms underlying both fast and slow V_j-dependent processes.

INTRODUCTION

Molecular genetic studies of ion channels have generated new insights into the mechanisms of ion channel gating and permeability. Through the identification of domains whose activities are critical determinants of protein function, the molecular basis of these functions becomes addressable. Some properties of proteins appear to be determined by discrete domains that confer these characteristics even when part of chimeric constructions (1–3). Others (4) and those discussed here, seem to arise from the interactions of multiple domains.

Gap junction channels comprise a unique family of ion channels, some members of which have proven to be voltage dependent (5). They show no sequence homology with the superfamily of sodium, potassium, and calcium channels or ligand-gated channels (6). A gap junction channel forms by the association of two hemichannels, one contributed by each of two coupled cells. Each hemichannel is a hexamer of molecules termed connexins. The connexins are a multigene family with at least 11 mammalian members (for three of these amphibian homologues are known; Bennett, M. V. L. manuscript in preparation). Multiple connexins can be expressed by a single cell type and as demonstrated for Cx26 and Cx32 in hepatocytes, more than one connexin can be localized to the same gap junction plaque (7). The topology of connexins, predicted from hydropathy plots (8) and in the cases of Cx32 and Cx43, verified by protease and antibody studies (9–11), includes four transmembrane domains (M1–M4), a cytoplasmic loop, cytoplasmic amino and carboxy termini and two extracellular loops (E1 and E2). Presumably the association between hemichannels occurs by contact between extracellular loops which creates a continuous, insulated channel between the coupled cells (Fig. 1 *A*).

By virtue of spanning two cell membranes and the intervening gap between them, gap junctions can be subjected to two different kinds of voltage stimuli (Fig. 1 *B*). A difference between the potentials in the interiors of two coupled cells constitutes a transjunctional potential (V_j) and a difference between the potential in the interior of cells and the extracellular space is an inside-outside potential ($V_{i\text{-}o}$). $V_{i\text{-}o}$ will be developed largely across the channel wall because the access resistance through the intercellular gap is small compared to the leakage resistance of the wall (12). $V_{i\text{-}o}$ can be changed without changing V_j by simultaneously applying equal polarizations to the coupled cells. When V_j is changed by applying a voltage step to one of two coupled cells, $V_{i\text{-}o}$ is changed in the stepped cell as well as along the channel. Many gap junctions are sensitive to V_j with no sensitivity to $V_{i\text{-}o}$. A few have been found that are sensitive to both (13, 14). The responsiveness of gap junctions to applied voltage is most likely the result of electrical work done in changing the conformation of the channel protein rather than due to blockade by ions at sites within the channel. Single channel recordings show transitions between open and closed states that are abrupt, with no evidence

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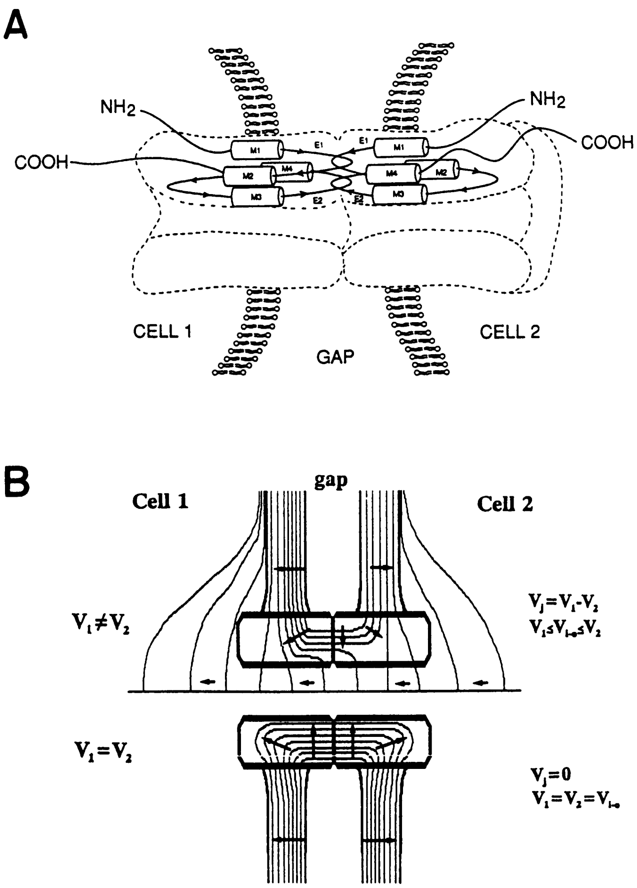


FIGURE 1 (*A*) Schematic representation of a gap junction channel between two cells. The dotted lines indicate the channel boundaries and the superimposed solid lines diagram the position of single connexin molecules in each of the paired hemichannels. The transmembrane domains are labeled M1–M4, the extracellular loop between M1 and M2 is indicated as E1 and the extracellular loop between M3 and M4 is labeled E2; the amino and carboxy termini are labeled NH_2 and COOH, respectively. (*B*) Diagram (from reference 13) illustrating the presumed isopotential lines in the presence of a $V_{i\text{-}o}$ and a V_j (*top half*) and a $V_{i\text{-}o}$ in the absence of a V_j (*bottom half*). Arrows indicate field direction and suggest relative magnitude.

of flicker (6). Opening rate constants, derived from macroscopic currents, are relatively insensitive to voltage (15) and the size of the channel and its permeability to large organic ions (16) all argue against blockade as the basis for voltage dependent decreases in gap junctional conductance. In addition, voltage dependent gating has been observed in perfused and reconstituted preparations without impermeant ions in the bathing media (17).

Studies of voltage dependence of gap junctions formed by cloned connexins have been conducted primarily on homotypic junctions, those formed by the association of identical hemichannels (18–22). With the exception of Cx26, these junctions have been shown to be dependent only on V_j with changes in conductance that are symmetric about $V_j = 0$ mV, i.e., identical for hyperpolarization or depolarization of either cell. Junctions formed by Cx26 possess a small degree of sensitivity to $V_{i\text{-}o}$ in addition to sensitivity to V_j. The presence of $V_{i\text{-}o}$ and V_j-dependence results in an asymmetry in the G_j/V_j relationship of Cx26 about $V_j = 0$ mV (18).

In the nervous system, gap junctions serve as electrical synapses. Some of these synapses are not significantly voltage dependent (5, 23), whereas others, such as the giant motor synapse of crayfish nerve cord (24, 25), hatchetfish medulla (26), and lamprey spinal cord (27) display asymmetric V_j-dependence in which depolarization on the presynaptic side rapidly increases junctional conductance. The time course of these changes in conductance is in the submillisecond range and thus are much more rapid than the changes in conductance in response to V_j observed for homotypic gap junctions. The connexins that form these fast rectifying electrical junctions have not been identified but insight into how electrical asymmetry in gap junctions may arise has recently been offered by Barrio et al. (18). They found, by expression of exogenous RNAs in *Xenopus* oocytes, that the heterotypic junctions formed between an oocyte that expresses only Cx26 and another that expresses only Cx32, display a fast V_j-dependence not present for these connexins in their homotypic configurations. These results imply that gating by hemichannels can differ depending on the identity of the hemichannels to which they are joined. In contrast, the Cx38/Cx43 heterotypic junction described by Swenson et al. (28) behaved as a composite of Cx38 and Cx43 homotypic junction behavior. These hemichannels appeared to operate identically regardless of whether they were paired with Cx38 or Cx43; and the hemichannel interactions that occurred in Cx38/Cx38, Cx43/Cx43, and Cx38/Cx43 appeared to be functionally equivalent.

We developed a novel technique for the creation of gene chimeras and used to it explore the basis for the difference in voltage dependence in homotypic and heterotypic junctions of Cx26 and Cx32 (28a). Because the interaction between hemichannels is likely to be mediated by contacts between the extracellular loops, we examined the possibility that the unpredicted electrical asymmetry of the heterotypic junctions arose from mismatching of the extracellular loops. Our initial approach was to make a chimera in which the first extracellular loop of Cx32 was replaced with the first extracellular loop of Cx26, and to pair it with Cx26 and Cx32. We found that mismatching the first extracellular loops in a heterotypic junction could create rectifying behavior but that it did not fully account for all of the properties of the Cx32/Cx26 junction.

MATERIALS AND METHODS

Construction of chimeric connexins

The first extracellular loop (E1) of Cx32 encoded by nucleotides 152–256 (8) was replaced by the corresponding sequence, nucleotides 121–225 of Cx26 (29) by the procedure described by Rubin et al. (28a) (Fig. 2). Briefly, the first extracellular loop of Cx26 was amplified by the polymerase chain reaction (PCR) using two bifunctional oligonucleotide primers: 5′-CTG GTG GTG GCT GCA AAG GAG GTG TGG GGA-3′ and 5′-TTG CAG GGA CCA CAG CCG GAT GTG AGA GAT-3′. The underlined portions of these oligonucleotides were complementary to sequences of Cx26 at 5′ and 3′ boundaries of E1 and served as "forward" and "reverse" primers for the amplification of the intervening Cx26 DNA by PCR. The remaining sequences at the 5′ ends of both primers were complementary to the regions of Cx32 that were on either side of the borders of the E1 domain. PCR product was phosphorylated and served as a primer for the in vitro mutagenesis of single stranded Cx32 in standard methods of mutagenesis using a kit supplied by Amersham, (Arlington Heights, Illinois) with modifications as described (28a). The sequence of these clones was confirmed by dsDNA sequencing using Sequenase (United States Biochemical Corp., Cleveland, Ohio). The first two amino acid residues, Lys 41 and Glu 42, of Cx26 E1 were replaced with the amino acids Glu 41 and Ser 42 of Cx32 using a 30 base oligonucleotide primer and the standard in vitro mutagenesis kit.

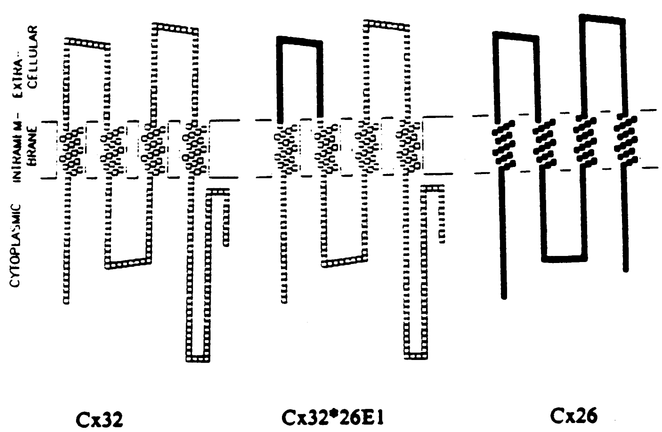
Preparation of RNA

RNA was transcribed from linearized plasmids using T7 RNA polymerase. Briefly, synthesis was performed in the presence of the cap analogue $m^7G(5')ppp(5')G$ (Boehringer-Mannheim, Indianapolis, Indiana) at a 10:1 ratio to added rGTP for 2 h at 37°C under standard reaction conditions followed by an additional 5 min synthesis in the presence of equimolar rGTP to ensure full length transcription of initiated capped transcripts. Integrity of the synthesized RNA was determined by electrophoresis through a 1% agarose gel.

Expression of junctional currents in pairs of *Xenopus* oocytes

Adult female *Xenopus laevis* frogs were purchased from Xenopus I, (Ann Arbor, Michigan) and maintained at 18°C in a 12 h L/D cycle. Defolliculated oocytes were placed in ND96 medium containing 1.8 mM $CaCl_2$, allowed to recover overnight and then coinjected with 50 nl of an aqueous solution containing approximately 1 μg/μl RNA and 0.25 μg/μl of two antisense oligonucleotides 5′-G CTT TAG TAA TTC CCA TCC TGC CAT GTT TC-3′ and 5′-TTC CTA AGG CAC TCC AGT CAC CCA TGC TCA-3′ that are complementary to endogenous *Xenopus* Cx38 (commencing at nt −5 in the sequence reported in reference 19) and Cx43 (commencing at nt 190 in the sequence reported in reference 30) mRNA, respectively. These antisense oligonucleotides block all endogenous junctional communication within 48 h of pairing (28a; see also reference 18). 24 h postinjection, oocytes were devitellinized manually in hypertonic medium (200 mM K-aspartate, 20 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.6, see reference 31) and paired in ND96 medium containing calcium. Junctional currents produced by exogenous RNA were evident 6–8 h after pairing at room temperature.

Junctional conductance was measured with a dual voltage clamp as described by Verselis et al. (13). Data were collected with a PC-AT computer using pCLAMP software (Axon Instruments Inc., Foster



THE FIRST EXTRACELLULAR LOOP - 35 amino acids - 71% Identity

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Cx26:  K E |V W G D E| Q A D |F| V |C N T L Q P G C| K N |V C Y D H| Y |F P P I S H| I |R|
       * *            *   *                          *
Cx32:  E S |V W G D E| K S S |F| I |C N T L Q P G C| N S |V C Y D H| F |F P P I S H| V |R|
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FIGURE 2 Topology of Cx26, Cx32, and the chimera Cx32*26E1 and comparison of the amino acid sequences of the first extracellular loops of Cx26 and Cx32. Cx32 residues are represented by open boxes and Cx26 residues by solid boxes. Sequences involved in the domain replacement which correspond to amino acids 41–75 of Cx32 and Cx26 are shown below. Changes in charged residues that occurred as a result of the replacement are indicated by asterisks. The domain replacement results in no net change in the overall charge of this domain.

City, California) and a LABMASTER/TL-1 interface combination. Junctional currents were filtered at 1–2 kHz with an eight-pole Bessel filter (AP Circuit Corp., New York, NY). Initial and steady-state g_j (g_{j0} and $g_{j\infty}$), were obtained by extrapolating exponential fits of responses to step changes in V_j. The steady-state conductance, $g_{j\infty}$ was fit to the Boltzmann relation of the form

$$g_{j\infty} = \{g_{jmax} - g_{jmin})/(1 + \exp[A(V_j - V_0)]\} + g_{jmin}, \quad (1)$$

where g_{jmax} is the maximal conductance, g_{jmin} is the residual conductance approached at large values of V_j, V_0 is the voltage at which $g_{j\infty} = (g_{jmax} + g_{jmin})/2$, and $A = zq/kT$ is a constant expressing voltage sensitivity in terms of the number of equivalent gating charges, z, moving through the entire applied field, where q is the electron charge, and k and T have their usual meanings. The heterotypic junctions display an initial fast V_j-dependence of g_j, the time course of which was not resolved by the voltage clamp. These initial values of g_j, g_{j0} were fit by the Boltzmann relation of Eq. 1, but with different parameters. For analysis of the subsequent slow changes in g_j, it was assumed that the fast and slow processes operated independently and in series. Thus, Eq. 1 was fit to the ratio $g_{j\infty}/g_{j0}$. Both g_{j0} and $g_{j\infty}$ changed with V_j in the heterotypic pairs. The results are plotted as G_{j0} and $G_{j\infty}$, values of g_j normalized to g_j at $V_j = 0$ to allow comparison of the conductance-voltage relations of different junctions.

RESULTS

Voltage dependence of junctions formed with Cx26 and Cx32

The steady-state conductance-voltage ($G_{j\infty}/V_j$) relations for homotypic and heterotypic junctions of Cx32 and Cx26, as determined in the *Xenopus* oocyte expression system, have been reported (18, 28a) and are presented for reference in Fig. 3, *A* and *B*. The smooth curves are fits to the Boltzmann relation described by Eq. 1 and are based on data from Table 1. Also shown, in Fig. 3 *C*, is a hypothetical $G_{j\infty}/V_j$ relation of heterotypic junctions formed by Cx32 and Cx26. Homotypic junctions formed from Cx32 (Fig. 3 *A*) display symmetric reduction in steady-state junctional conductance about $V_j = 0$ mV. Homotypic junctions formed by Cx26 are characterized by a more complex voltage dependence (Fig. 3 *B*). In addition to slow decreases in $G_{j\infty}$ apparent for large values of V_j, of either sign, initial currents display a small degree of fast rectification whose time course cannot be resolved. This fast rectification increases G_{j0} for depolarization and decreases G_{j0} for hyperpolarization of either cell and thus depends on V_{i-o}. For polarizations of ± 100 mV this fast V_{i-o}-dependence results in conductance changes of $\sim \pm 10\%$ of the value for G_j at $V_j = 0$ mV (18). The asymmetry of the $G_{j\infty}/V_j$ relation also indicates a small degree of V_{i-o} dependence shown in Fig. 3 *B*. The hypothetical curve shown in Fig. 3 *C* was generated with the assumption that the component hemichannels would operate independently and would retain characteristics determined for each connexin in homotypic junctions. The small degree of V_{i-o} dependence of the Cx26 hemichannel was ommitted. It was further assumed that, like Cx38 (28, 32, 33), Cx26, and Cx32 hemichannels would close for relative positivity imposed on their side of the junctions.

Injection into *Xenopus* oocytes of antisense oligonucleotides directed against the endogenous connexins, Cx38 and Cx43, blocked the formation of endogenous gap junctions between paired oocytes and permitted the unambiguous characterization of macroscopic currents produced by injection of exogenous RNAs (see also reference 18). Heterotypic junctions formed by pairing an oocyte injected with Cx26 RNA with one injected with Cx32 RNA exhibited voltage dependent behavior (Fig. 4 *A*) that was markedly different from that of homotypic junctions composed of these connexins and the predicted behavior illustrated in Fig. 3 *C*. G_{j0} decreased substantially on hyperpolarization and increased on depolarization of the cell expressing Cx26 producing a somewhat sigmoidal G_{j0}/V_j relation. The same relation could be obtained by equal and opposite polarizations of the cell expressing Cx32 indicating dependence only on V_j. We term this process fast V_j-dependent rectification. The absence of V_{i-o} dependence was confirmed by equal simultaneous polarizations of both cells (data not shown). The G_{j0}/V_j relation was fit by the Boltzmann relation (Eq. 1) although in another paper (18) a linear fit was satisfactory. Slow changes in $G_{j\infty}$ in response to V_j were also present and were also asymmetric. The effective gating charge of the $G_{j\infty}/V_j$ relation for Cx32/Cx26 junctions with V_j relatively positive on the Cx26 side was close to that of homotypic Cx26/Cx26 junctions, but the V_0 was decreased by 25 mV (Table 1). We observed no slow decrease in G_j for voltages that made the Cx32 side relatively positive. The asymmetry in the $G_{j\infty}/V_j$ relation of heterotypic junctions results from the apparent loss of slow V_j-dependence in one of the opposed hemichannels. If Cx32 and Cx26 hemichannels are closed by relative positivity on their side as are Cx38 hemichannels, it would be the slow gating mechanism of the Cx32 hemichannel that was not seen and that of the Cx26 hemichannel that was preserved. The maximum slope of the G_{j0}/V_j relation was less than that of the $G_{j\infty}/V_j$ relation for relative positivity on the Cx26 side implying a smaller gating charge. However, because of uncertainty about the asymptotes of the G_{j0}/V_j relation, the values of z and V_0 for this process could not be accurately determined. The gating charge estimated for the fast process is in the range of 0.6 equivalent charges. These data are in general agreement with those presented recently by Barrio et al. (18).

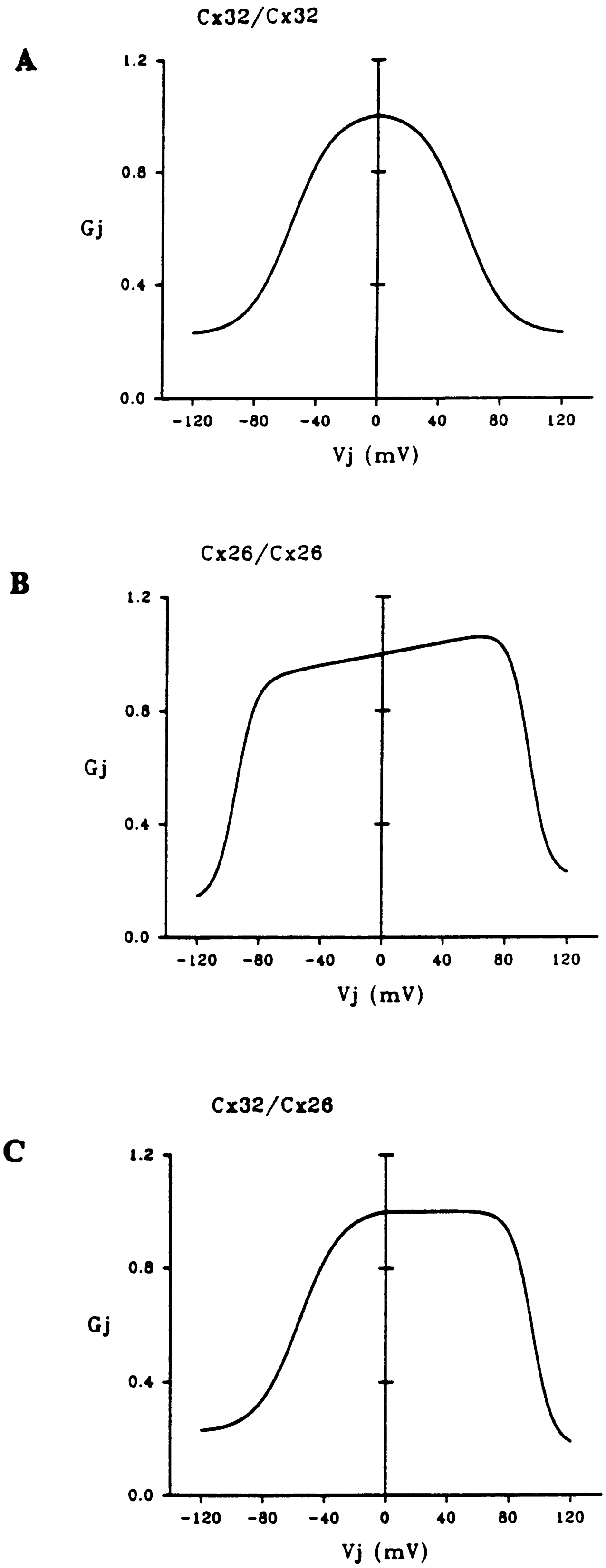

FIGURE 3 Conductance-voltage relations for (*A*) homotypic Cx32, (*B*) homotypic Cx26 junctions, and (*C*) the predicted conductance-voltage relation for heterotypic Cx32/Cx26 junctions. Smooth curves are fitted Boltzmann relations of the form described in the materials and methods. Values for the parameters are those calculated (28a), and are presented in Table 1. The theoretical curve for the conductance-voltage relationship of heterotypic junctions formed by Cx32 and Cx26, *C* is the relation that would obtain from independent hemichannel gating if, as was shown for Cx38, Cx26, and Cx32 hemichannels close in response to relative positivity on their side of the junction. The small contribution to the conductance-voltage relation that could be made by the inside-outside voltage dependence of the single Cx26 hemichannel has been omitted.

TABLE 1 Boltzmann parameters for homotypic and heterotypic junctions

Connexins	z	A	$-V_0$	G_{min}	z	A	$+V_0$	G_{min}
Cx32/Cx32‡	1.8	0.073 ± 0.018	53.8 ± 6.4	0.22 ± 0.06	1.9	0.075 ± 0.020	60.3 ± 7.6	0.22 ± 0.07
Cx26/Cx26‡	4.0	0.160 ± 0.029	93.1 ± 6.1	0.15 ± 0.04	3.8	0.150 ± 0.270	98.8 ± 4.5	0.19 ± 0.03
Cx32/Cx26		No slow V_j			3.3	0.132 ± 0.009	75.7 ± 3.3	0.16 ± 0.02
Cx32*26E1/Cx32*26E1‡§	≥1.5	≥0.061	≤96.6	≥0	≥1.4	≥0.056	≤101.5	≥0
Cx32/Cx32*26E1§	≥0.9	≥0.037	≤113.8	≥0	1.9	0.076 ± 0.009	88.8 ± 3.3	0.19 ± 0.02
Cx32*26E1/Cx26		No slow V_j			2.8	0.112 ± 0.011	76.8 ± 2.8	0.13 ± 0.05
Cx32/Cx26*32ES		No slow V_j			5.6	0.222 ± 0.002	66.1 ± 4.0	0.20 ± 0.04

Values for V_0 are absolute values for polarity indicated; ‡Values determined (28a); §$G_{j\infty} - V_j$ relation did not approach a asymptote G_{min} for V_j as large as ± 120 mV. Values are presented as lower limits for z and upper limits for V_0 with $G_{min} = 0$.

Voltage dependence of junctions formed with chimeric connexins

The electrical asymmetry observed in heterotypic junctions is likely to arise from interactions of the extracellular loops as these are the only regions of the connexins that are expected to be in contact. We examined the contribution of the first extracellular loop to the voltage dependence of gap junctions by producing a chimeric connexin in which the first extracellular loop of Cx32 was replaced with the corresponding domain of Cx26. This chimeric connexin is designated Cx32*26E1 or precisely Cx32 (amino acids 1–40)/Cx26 (41–75)/Cx32 (76–283).

Heterotypic pairing of Cx32*26E1 with either Cx26 or Cx32 resulted in electrically asymmetric junctions with both fast and slow processes and G_j/V_j relations similar in form to those of Cx32/Cx26 junctions. (We write these junctions as Cx32/Cx32*26E1 and Cx32*26E1/Cx26 where for clarity, the connexin on the right hand side of the pairing designation is relatively positive for positive V_j in the G_j/V_j plots). The fast V_j-dependence of the heterotypic Cx32/Cx32*26E1 junctions increased G_{j0} for depolarization on the Cx32*26E1 side (or hyperpolarization on the Cx32 side), but resulted in a G_{j0}/V_j relation that was less steep than those of all other heterotypic junctions examined (Fig. 4 *B*). The slow V_j-dependence of these junctions decreased $G_{j\infty}$ markedly with depolarization of the Cx32*26E1 side, but unlike the other heterotypic junctions also decreased $G_{j\infty}$ upon hyperpolarization of the cell expressing Cx32*26E1. The gating charge and V_0 of the slow V_j-dependence for relative positivity on the Cx32*26E1 side were close to those reported for homotypic Cx32*26E1/Cx32*26E1 junctions (Table 1). The gating charge and V_0 of the slow V_j-dependence for relative positivity on the Cx32 side could not be precisely calculated as G_{min} was not achieved for this polarity within the 120 mV range of applied transjunctional voltages (Table 1). The values given in Table 1 for this polarity are limits determined by assuming $G_{min} = 0$. Regardless of the uncertainty in G_{min}, V_0 for slow V_j-dependence in response to relative positivity on the Cx32 side of these junctions was increased by at least 35 mV compared to that reported for the Cx32 homotypic junction (Table 1). With $G_{min} = 0$, the gating charge of the slow V_j-dependence in response to relative positivity on the Cx32 side would be 0.9. This would represent a significant reduction compared to the gating charge of either component hemichannel in homotypic junctions. A G_{min} of 0.25, the conductance measured at 120 mV for this polarity of V_j, would correspond to a gating charge of 1.9. If 0.25 was close to the true G_{min}, there was no change in gating charge, compared to the homotypic values.

In heterotypic Cx32*26E1/Cx26 junctions, fast V_j-dependent increases in G_{j0} and slow V_j-dependent decreases in $G_{j\infty}$ were observed upon depolarization of the cell expressing Cx26 (or hyperpolarization of the cell expressing Cx32*26E1) (Fig. 4 *C*). The fast V_j-dependent rectification was less steep than for Cx32/Cx26 junctions but steeper than for Cx32/Cx32*26E1 junctions (compare Fig. 4, *A–C*). The fit of the slow V_j-dependence to the Boltzmann equation was close to that obtained for Cx32/Cx26 junctions (Table 1). As with Cx32/Cx26 junctions, no slow V_j-dependence was observed when the cell expressing Cx26 was hyperpolarized by as much as 120 mV.

These results indicate that asymmetrical E1 pairing can cause electrical asymmetry, as the G_j/V_j relation for Cx32/Cx32*26E1 junctions were similar in form to that of Cx32/Cx26 junctions. However, similar electrical asymmetry was seen in Cx32*26E1/Cx26 junctions in which only the E1 domain was the same in both hemichannels. The slow decrease in G_j was attenuated when V_j was opposite to that causing the fast increase in all pairings, but the reduction was only partial with the Cx32/Cx32*26E1 junctions.

Additional evidence supporting a role for the first extracellular loop in asymmetry of V_j-dependence was provided by the behavior of heterotypic junctions comprised of Cx32 and a mutant of Cx26 termed Cx26*32ES in which Lys 41 (K) and Glu 42 (E) that are contained

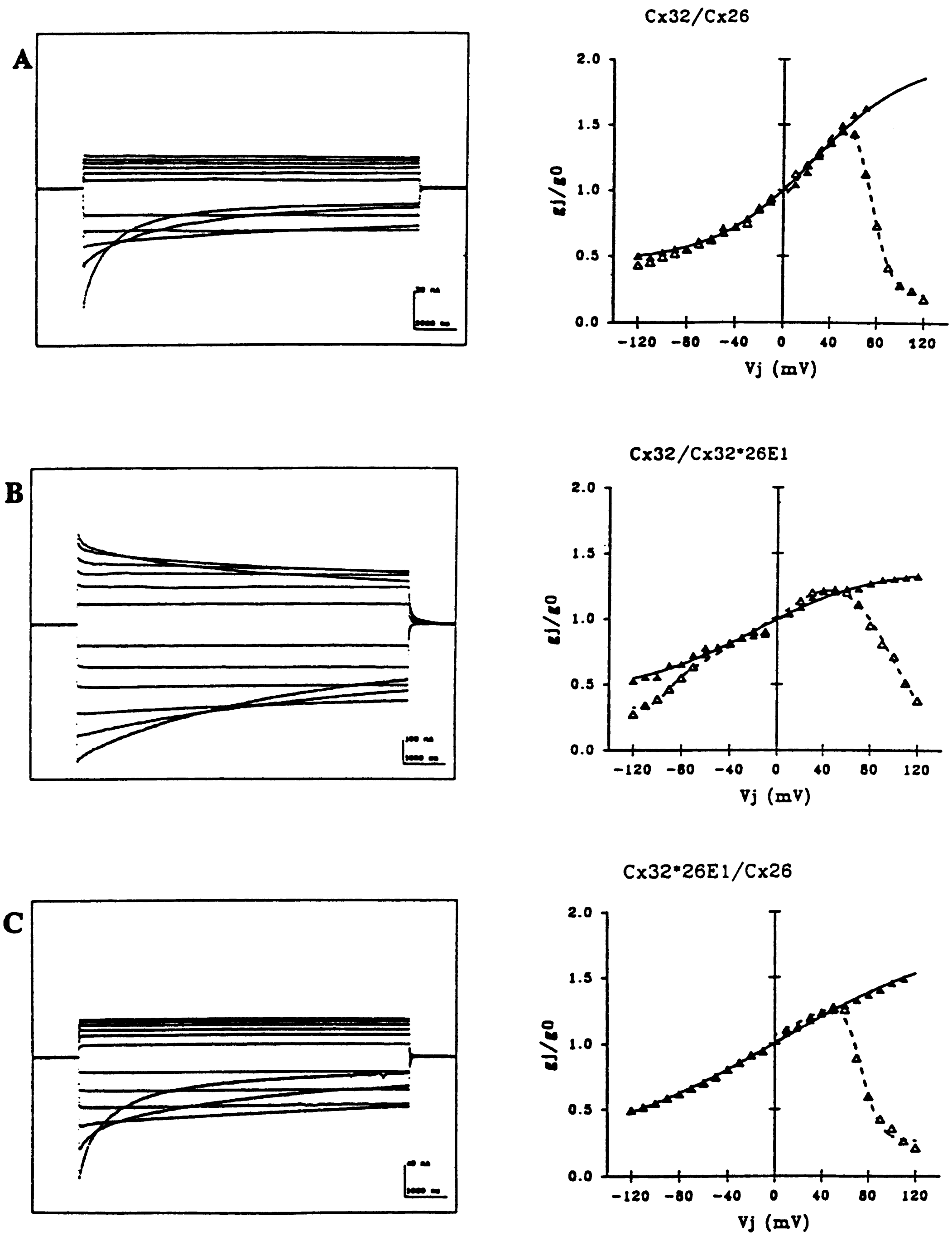

FIGURE 4 Representative junctional currents and initial and steady-state conductance voltage relations for heterotypic junctions formed by (*A*) Cx32/Cx26, (*B*) Cx32/Cx32*26E1, and (*C*) Cx32*26E1/Cx26. Junctional currents shown were elicited by ±20, 40, 60, 80, 100, and 120 mV voltage steps applied to the oocyte expressing (*A*) Cx26, (*B*) Cx32*26E1, and (*C*) Cx26, the connexins indicated on the right side of the pairing designations. Boltzmann parameters are given in Table 1. Calibration bars; (*A*) 30 nA, 1 s; (*B*) 100 nA, 1 s; (*C*) 40 nA, 1 s.

within the first extracellular loop of Cx26 were replaced by the Glu (E) and Ser (S) residues present at these positions in Cx32 and most other sequenced connexins (Fig. 5). The G_j/V_j relations were again similar in shape to those of Cx32/Cx26 junctions, but there was a marked increase in the slope of the fast V_j-dependent rectification and the slope of the slow decrease for relative positivity on the Cx26*32ES side (for $G_{j\infty}$, z = 5.6, Table

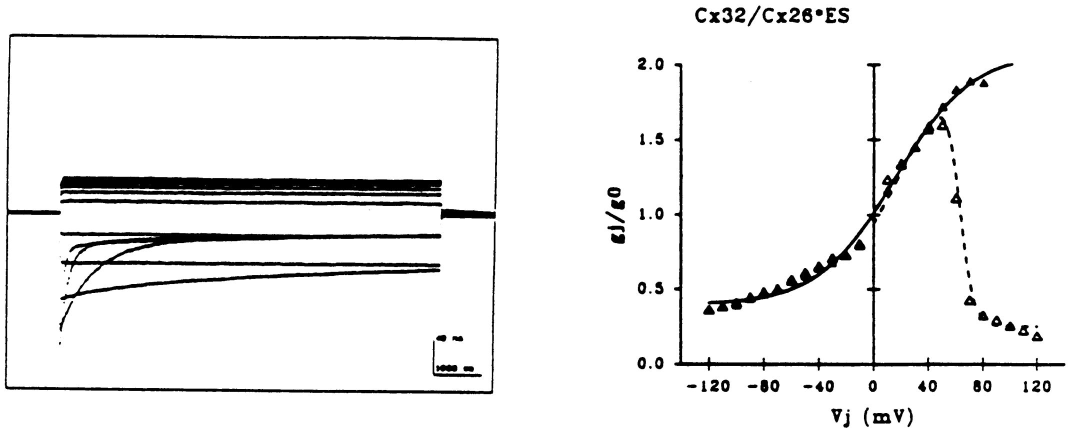

FIGURE 5 Representative junctional currents and initial and steady-state conductance voltage relations for heterotypic Cx32/Cx26*32ES junctions. Junctional currents shown were elicited by voltage steps, as in Fig. 4, applied to the cell expressing Cx26*32ES. Normalization and fits are as in Fig. 4. The Boltzmann parameters are given in Table 1. Calibration bar: 40 nA, 1 s.

1). In these junctions, the difference in primary sequence between the E1 domains was smaller than in Cx32/Cx26 junctions, but the asymmetry of the G_j/V_j relations was greater.

DISCUSSION

The voltage dependence of Cx32/Cx26 heterotypic junctions (reference 18 and Fig. 4*A*) differed qualitatively from that of all characterized homotypic junctions and heterotypic junctions formed by pairing *Xenopus* Cx38 with rat Cx43 (28). The voltage dependence of heterotypic Cx38/Cx43 junctions was consistent with the hemichannel properties inferred from homotypic Cx38/Cx38 and Cx43/Cx43 junctions. Each hemichannel appeared to act independently in that there was no indication that either hemichannel influenced the gating properties of the other. In contrast, heterotypic junctions formed by pairing Cx32 with Cx26 exhibit novel voltage dependent properties that would not have been predicted from the behavior of Cx26 and Cx32 homotypic junctions. Heterotypic Cx32/Cx26 junctions exhibited a fast V_j-dependent rectification that was not seen in either homotypic pair. Although a small degree of fast voltage dependence was described in Cx26/Cx26 junctions, it was sensitive to $V_{i\text{-}o}$. The slow V_j-dependence of Cx32/Cx26 junctions was markedly asymmetric, and was present only when the cell expressing the Cx26 hemichannel was made relatively positive. There was a conspicuous absence of slow V_j-dependence in response to transjunctional voltages that would be expected to close the Cx32 hemichannel if, as demonstrated for Cx38, it were to close in response to relative positivity on its side of the junction. The slow V_j-dependence present when the Cx26 hemichannels were made relatively positive resembled that observed for Cx26 homotypic junctions. These data suggest that the slow gating process in only the Cx26 hemichannel was operational within the range of applied transjunctional voltages in these heterotypic junctions. Unlike heterotypic Cx38/Cx43 junctions, protein interactions between the hemichannels of heterotypic Cx32/Cx26 junctions appear to alter their gating properties.

Consideration of the inferred membrane topology of gap junctions suggests that contact between the extracellular loops in the intercellular gap would mediate any interactions between the hemichannels. We used domain replacement to examine the interactions between the first extracellular loops of paired hemichannels. Heterotypic Cx32/Cx32*26E1 junctions differ from homotypic Cx32/Cx32 junctions by the presence of the first extracellular loop of Cx26 in one of the component hemichannels. The appearance of fast V_j-dependent rectification in these junctions suggests that mismatch of E1 domains contributes to the observed electrical asymmetry of Cx32/Cx26 heterotypic junctions. The steepness of the fast process in Cx32/Cx32*26E1 junctions was less than that observed for Cx32/Cx26 junctions and, as is discussed below, slow V_j-dependence was observed for both polarities of V_j indicating that mismatch of the first extracellular loops is not the sole cause of the novel behavior of Cx32/Cx26 junctions. If mismatching of E1 alone had led to complete replication of the Cx32/Cx26 properties, it would have been expected that Cx32*26E1/Cx26 junctions, in which E1 was matched, would not rectify. Such complimentary results would have implied that E1 mismatch made a modular contribution to rectification, a contribution that was not dependent upon interactions with other domains and was identical whether the E1's were part of Cx26 or Cx32. Mismatching of E1 alone, however, did not result in fully rectifying junctions and conversely, junctions formed with matched E1's, Cx32*26E1/Cx26, did have properties similar to Cx32/Cx26 junctions. Thus, it would appear that mismatching of other domains, in addition to E1, is necessary for the full expression of Cx32/Cx26 rectification. The requirement for multiple domains could signify that contributions to voltage dependence will be made by more than one domain acting additively or through interdomain interactions that are necessary to generate the appropriate protein conformations for rectification. If mismatch of E1 were responsible for the generation of rectification, then the tertiary structure of the E1 domain must have been altered by interdomain interactions when it was part of Cx32 in order to account for the fact that Cx32/Cx32*26E1 junctions were not electrically identical to Cx32/Cx26 junctions. If distortion of the Cx26 E1 structure was induced by interaction with other domains, then the rectification observed in Cx32*26E1/Cx26 junctions could have arisen by E1 mismatch.

The distinction between modular contributions to rectification and dependence upon interdomain interactions will be distinguished in future experiments in which chimeras composed of substitutions of other domains as individual units and in combination will be paired with Cx32, Cx26, and each other. Recent results with a chimera in which E2 of Cx32 has been replaced with E2 of Cx26 and another chimera in which both of the extracellular loops of Cx32 have been replaced with the extracellular loops of Cx26 have indicated that Cx32/Cx32*26E2 heterotypic junctions do not rectify and that Cx32/Cx32*26(E1 + E2) heterotypic junctions behave identically to Cx32/Cx32*26E1 junctions. Thus, rectification like that observed for Cx32/Cx26 cannot be generated by mismatching only the extracellular loops (data not shown). This result suggests that the Cx26 E1 domain can function in the same manner in the presence of Cx32 E2 or Cx26 E2 and that nonextracellular loop domains are important in rectification.

The steepness of the fast process was greater in Cx32/Cx26*32ES junctions than in the other heterotypic junctions studied. The increased steepness of the fast V_j-dependent process in these heterotypic junctions, which differs from these Cx32/Cx26 junctions by only two amino acids in the E1 domain of Cx26, further implicates this domain's involvement in the observed rectification. The mutation made the primary amino acid sequence of the E1 domain of Cx26 more like that of Cx32 but rather than reducing the degree of electrical asymmetry, the asymmetry was markedly enhanced. It remains to be determined how the mutation altered the nature of hemichannel interactions.

The conductance-voltage relationships of these gap junctions were fit to a Boltzmann relation to generate values for the parameters V_0, z, and G_{min}. These parameters can be used to compare the voltage dependent behavior of different connexins regardless of the complexity of the actual transitions between open and closed states. In both Cx32/Cx26 and Cx32*26E1/Cx26 heterotypic junctions, the gating charge of the slow V_j-dependence observed when the cell expressing Cx26 was made relatively positive was very similar to that observed in Cx26/Cx26 homotypic junctions (Table 1), but in both heterotypic junctions, V_0 was less by ~25 mV. When the cell expressing Cx32*26E1 in the pairs forming Cx32/Cx32*26E1 junctions was made relatively positive, the gating charge of the slow V_j-dependence was also similar to that seen in homotypic Cx32*26E1/Cx32*26E1 pairs, but unlike the two preceding cases, V_0 was also about the same. However, when the cell expressing Cx32 in Cx32/Cx32*26E1 junctions was made relatively positive (by convention, negative V_j) the V_0 of the slow decrease in $G_{j\infty}$ was increased by as much as 50–60 mV relative to the value characteristic of Cx32 homotypic junctions. These changes suggest that interactions between hemichannels can significantly shift the $G_{j\infty}/V_j$ relation along the voltage axis. One interpretation of this shift is that the interactions altered the chemical free energy difference between open and closed states without significantly affecting the gating charge. The large shifts in V_0 caused by heterotypic pairings could explain the electrical asymmetry of slow V_j-dependence in heterotypic junctions, if the V_0 for the Cx32 side positive was larger than the test pulses applied.

The fast V_j-dependence could arise from either voltage dependent gating or changes in single channel conductance. These possibilities should be distinguishable by single channel studies of the rectifying junctions. In the present study the fast changes in conductance have been treated as voltage dependent gating. The conservation of the gating charge of the unattenuated slow V_j-dependent process and the apparent differences in the steepness of the fast rectification in these junctions suggests that these two V_j-dependent processes arise by separate mechanisms. We recently demonstrated that the differences in the calculated gating charges for slow V_j-dependence in Cx26 and Cx32 cannot be accounted for by differences in the sequences of their first extracellular loops (28a). Thus, the generation of fast V_j sensitivity by first extracellular loop interactions may occur independently of their role in slow V_j-dependent gating. Interactions between the two distinct V_j-dependent processes may be reflected in the correlation between the degree of attenuation of the slow V_j-dependent process for negative V_j and the steepness of the fast rectification. The heterotypic Cx32/Cx32*26E1 junctions were the only heterotypic junction in which slow V_j-dependent gating was observed in response to V_j of either polarity, and they also exhibited the shallowest slope for the fast rectification. A correlation between the fast and slow processes is also suggested by the behavior of Cx32/Cx26*32ES junctions in which the steepness of both the fast and slow G_j/V_j relations was increased by the mutation.

We have used the domain replacement procedure to construct a chimeric connexin in which the first extracellular loop of Cx26 was substituted for the corresponding region in Cx32. The properties of junctions formed by heterotypic pairings of this chimera demonstrated that the E1 domain contributes to the fast V_j-dependent rectification of heterotypic junctions, but that other domains are involved. Substitution of two charged amino acids in Cx26 with two different residues present in other sequenced connexins had a marked effect on the steepness of the slope of both the fast and slow V_j-dependent processes in heterotypic junctions with Cx32. This result suggests that E1 may contribute to the formation or operation of the voltage sensing mechanisms underlying both fast and slow V_j-dependent processes.

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DISCUSSION

Session Chairman: Alan Finkelstein *Scribes:* Han-qing Xie and Marc J. Glucksman

OLAF ANDERSEN: What is the basis for the asymmetry of the Cx26 gating curve for the symmetrical channel? That is, why does the inside-out voltage-dependent process produce asymmetry?

JOSHUA RUBIN: There are two independent processes interacting to produce the asymmetry. The steady-state curves reflect the presence of a fast $V_{i\text{-}o}$ dependence and a slower V_j dependence.

Asymmetry is a result of a process that is dependent on the membrane potential. One oocyte is kept at a fixed potential while the other oocyte is stepped to various potentials. The current flow across the membrane is measured and indicates that there is a fast change in conductance as a result of changes in the membrane potential. Depolarization of the cell causes a fast increase in conductance and hyperpolarization causes a fast decrease in conductance. On top of this there are slower changes in conductance arising from the V_j dependence.

ANDREW HARRIS: If you include all of the data from stepping one cell then stepping the other and plot against to V_j, would you get a symmetrically shaped curve?

RUBIN: Yes.

ALAN FINKELSTEIN: Let me clarify some terms: V_j refers to the potential difference between the two cells. If for example there is a 20-mV difference between cell 1 and 2, the absolute voltage does not matter.

There is another voltage dependent factor $V_{i\text{-}o}$, the potential difference between the cell and the external medium. There is no potential difference between the cells, and you change the potential difference between the inside and outside of the cells. In some gap junctions the change in coupling between the cells is a consequence of this transmembrane potential.

ANDERSEN: Getting back to the issue of hemichannels. One cannot "tear" the gap junctions apart, we are told that they can dissociate by incubation in hypertonic solutions, which may be analogous to the two different ways you can open a zippered jacket.

(*a*) If you pull in a direction perpendicular to the zipper, you will tear the fabric.

(*b*) If you unzip from the end you will have two hemizippers.

DAVID SPRAY: We looked for that "zipper" for a long time. Many people would agree that there is no stoichiometric result in hemichannels in gap junction preparations. $V_{i\text{-}o}$ dependence is an uncommon property found only in some vertebrate connexins. So what is being measured? Is it the field across one or another extracellular loops?

RUBIN: We have exchanged both extracellular loops and have not changed the $V_{i\text{-}o}$ voltage dependence. The ES mutant also has inside-out voltage dependence. The extracellular loops may have a role in $V_{i\text{-}o}$ dependence but they certainly cannot by themselves confer this kind of voltage dependence.

JOE MINDELL: If you normalize out the fast V_{io} in the symmetric Cx26 junction, is the slow process becoming symmetrical?

JOSHUA RUBIN: Yes.

GERHARD DAHL: In connexins, there is no equivalent to the S4 segment of other voltage-gated ion channels. In fact, all connexins known today have the same set of a few charged amino acids in their transmembrane segments, while voltage sensitivity of various connexons differs considerably. Do you dare to speculate where the voltage gate could be located?

RUBIN: In our studies we are trying to identify regions of the molecule involved in voltage gating. There are biophysical data generated by Andrew Harris and David Spray that suggests that a component of the voltage sensor may reside along the channel lining sequences. They studied the time course of changes in conductance in