Connexin32 gap junction channels in stably transfected cells
Equilibrium and kinetic properties

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ABSTRACT Communication-deficient cells (the SKHep1 cell line) were stably transfected with a plasmid containing cDNA which encodes the major gap junction protein of rat liver, connexin32. Application of the dual whole-cell voltage clamp technique with patch electrodes to pairs of transfected SKHep1 cells revealed strong sensitivity of junctional conductance (g_j) to transjunctional voltages (V_js) of either polarity, with the ratio of minimal to maximal g_j (g_{min}/g_{max}) being ~0.1 at the highest V_js. Steady-state g_j values as a function of voltages of either polarity were well fit by the Boltzmann equation. V_0, the voltage at which g_j was reduced by 50%, was ~25–30 mV; A, the Boltzmann parameter describing voltage dependence, was ~0.06 (corresponding to an energy difference between states of ~1 kCal/mol and to ~2 gating charges moving through the field). The kinetics of the transjunctional voltage dependence were slow ($\tau > 5$ s at 20–40 mV, $\tau = 2$ s at and beyond 70 mV). Voltage sensitivity of the opening rate constant (α) was ~30% lower than that of the closing rate constant (β) over the V_j range 0–70 mV; at higher voltages, voltage sensitivity of α and β saturated. The kinetic response of g_j to a paradigm in which g_j was first rendered low by a prepulse of opposite polarity indicated that the voltage sensors are likely to be arranged in series. Transitions between open and closed states in response to transjunctional voltages of either polarity are single order processes; transitions from one closed state to the other involve passage through the open state.

INTRODUCTION

Gap junctions are aggregates of intercellular channels that serve to interconnect, or couple, adjacent cells, allowing passage of ions and second messenger molecules between cells of most tissues (see Bennett and Spray, 1985; Hertzberg and Johnson, 1988; Bennett et al., 1991). As is the case for other channels in biological membranes, gap junction channels transit between conducting (open) and nonconducting (closed) states, and the equilibrium between open and closed states determines conductance of the junctional membrane. Treatments that alter junctional conductance include changes of intracellular pH and perhaps Ca, cAMP and tumor-promoting phorbol esters, possibly acting through phosphorylation of connexin molecules, lipophilic molecules including heptanol, octanol, arachidonic acid and halothane, and voltage applied either transjunctionally or from inside to outside the cell (see Spray and Bennett, 1985; Spray and Burt, 1990).

Voltage-dependent junctional conductance has been characterized in salivary gland cells of insects (Obaid et al., 1983; Verselis et al., 1991), rectifying synapses of crayfish (Furshpan and Potter, 1959; Giaume and Korn, 1983; Jaslove and Brink, 1986), certain leech neurons (Baylor and Nichols, 1969), hatchetfish pectoral motoneurons (Auerbach and Bennett, 1969), and in cleavage stage embryonic cells of fish, amphibia, tunicate, and squid (reviewed in Spray et al., 1991). Although cardiac gap junctions may exhibit voltage sensitivity when transjunctional voltage is large (Rook et al., 1988; Veenstra, 1990), significant dependence of junctional conductance on voltages considered physiologically relevant (~ ± 50 mV) has not been reported previously for cells comprising any mammalian tissue. Technical difficulties may have contributed to the failure to find voltage-dependent junctions between mammalian cells. The small cell sizes, in contrast to enormous cells of insect salivary gland and early embryo, have necessitated the use of whole-cell recording techniques to measure junctional conductance (g_j), where each cell is voltage clamped with a single patch pipette. Through uncompensated series resistance, in cases where g_j is high, this technique can introduce large errors in estimation of voltage actually imposed across the junctional membrane.

Previous studies on cells dissociated from rat liver, where connexin32 is the dominant gap junction protein, found the junctional membrane to be insensitive to large apparent transjunctional voltages (Spray et al., 1986; Riverdin and Weingart, 1988). However, in a study on liver membranes incorporated into planar lipid bilayers, a small voltage-dependent component was detected (Young et al., 1987). We have recently confirmed this finding on bilayers and have shown that pairs of liver cells voltage clamped with independent two-electrode

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0006-3495/91/11/1267/11 $2.00

voltage clamp circuits display a similar voltage dependence, as do pairs of communication incompetent cells stably transfected with a vector containing connexin32 cDNA (Campos de Carvalho et al., 1991; Moreno et al., 1991*a*). Furthermore, recent studies on *Xenopus* oocytes demonstrate significant transjunctional voltage dependence, although kinetics and steady state values differ substantially from those reported here (Barrio et al., 1991).

The present paper reports the use of the stably transfected SKHep1 cell line to quantify the steady state and kinetic properties of gap junction channels formed of connexin32. Our findings indicate, as is the case for the gap junction channels between early amphibian embryonic cells (consisting of *Xenopus* connexin38: Ebihara et al., 1989), connexin32 gap junction channels can be modeled as possessing two voltage dependent gates in series, each with first order rate constants describing transitions from open to closed states (Spray et al., 1981; Harris et al., 1981; see also Spray et al., 1991). Thus far, rat connexin32, *Xenopus* connexin38, and *Drosophila* salivary gland gap junctions are the only voltage-dependent channels in which first order kinetics have been demonstrated. Understanding how voltage induces the conformational change in the molecules leading to channel opening and closing may provide general insight into the nature and underlying structural correlates of channel gating processes.

METHODS

Cell line

The parental cell line, SKHep1, is derived from a highly metastatic human hepatoma (Fogh, 1977; Doerr et al., 1989). Transfection, selection and maintenance of these cells have been described in detail previously (Eghbali et al., 1990).

Electrophysiological methods

The dual whole-cell voltage clamp technique was used with patch-type electrodes, as described previously (Moreno et al., 1991*b*). To obtain the relation between g_j and transjunctional voltage, holding potentials of both cells were maintained at 0 mV. At ≥ 30 s intervals, command pulses (10–20 s duration) were applied to one cell while the other cell's voltage was held constant. Successive pulses of increasing magnitude (± 10 to ± 80 mV) were applied and junctional currents were recorded. In some experiments other pulse protocols were chosen, often with superimposed brief test pulses during the long transjunctional command step. These are described in more detail in the text and relevant figure legends.

Data analysis

To calculate the values for time constants of decline of junctional current, the experimental results were digitized with a Neurocorder (Neurodata Instruments Corp., New York), and recorded on a VCR (model FVH-840; Fisher Corp., Chatsworth, CA). This system has a cutoff frequency of 0.8 MHz. Using the Neurocorder digitizer in the play mode, and an AD/DA converter (model TL1 TecMar; Axon Instruments, Inc., Foster City, CA) with Assystant acquisition software (Assyst Software Technologies, Rochester, NY), we retrieved the information previously stored in VCR cassettes into a 386 computer. All data were analyzed using Sigmaplot 4.0 (Janadel Scientific, Corta Madera, CA), which is capable of curve fitting by least squares or Gaussian iteration with any explicit user function.

RESULTS

Connexin32 gap junction channels are voltage sensitive.

Previous studies on hepatocyte pairs, which express connexin32, failed to detect a major voltage sensitive component of junctional conductance (Spray et al., 1986; Riverdin and Weingart, 1988). The transfected cells used in the present study are more appropriate for dual whole-cell voltage clamp study due to their relatively lower junctional and nonjunctional conductances. In our initial experiments on these cells, we noted that junctional conductance (g_j) appeared to be voltage dependent when coupling was reversibly reduced by application of halothane, but as g_j recovered, its voltage sensitivity disappeared (data not shown).

To determine whether voltage sensitivity was induced by the uncoupling procedure or was an inherent property of the junctional channels, voltage dependence was compared in cells where g_j ranged from 5–10 nS using patch pipettes with <5 Mohm or >5 Mohm resistances. An example is shown in Fig. 1, which shows a striking relaxation of g_j when patch pipette resistance was low and a much smaller degree of voltage dependence when pipette resistance was higher. These data suggest that

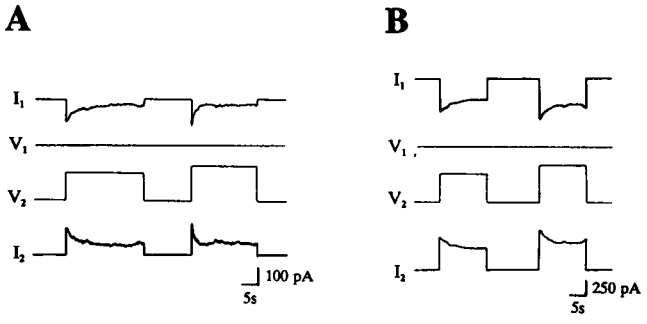

FIGURE 1 Apparent voltage dependence of junctional currents depends on electrode and junctional resistances. (*A*) With low resistance patch electrodes (5 Mohm) and low g_j (5 nS), junctional current (I_j) declines markedly in response to transjunctional voltages (V_j) of 47 and 60 mV. (*B*) With high resistance patch electrodes (11 Mohm) and high g_j (12 nS), I_j is less responsive to V_j steps (here, 50, 65 mV).

the ratio between pipette and junctional resistances may present a major obstacle to evaluation of voltage dependent g_j when the dual whole-cell voltage clamp technique is used.

Transjunctional voltage dependence of junctional current between transfected cells: steady-state properties.

When a command pulse was applied to one cell of a pair while the other cell's voltage was held constant, the current recorded in the second cell was very similar in magnitude, but opposite in sign, to the current flowing through the junctional membrane (I_j). For small transjunctional voltages (V_j), I_j was constant for even very long duration command pulses (Fig. 2, traces for V_j = 12 mV). For larger V_j values, I_j relaxed during the pulse, reaching steady-state levels within 10–15 s for small V_js and within much shorter times at higher V_js (Fig. 2, compare −25, −50, and −82 mV pulses). For all but the largest pulses, the decline in I_j followed a single exponential time course (see below). The relaxation in I_j was mirrored by a relaxation in the current trace of the pulsed cell ($I_2 = I_{nj} + I_j$, where I_2 is input current of the pulsed cell, I_{nj} is nonjunctional current of that cell and I_j, the current recorded in the other cell, is $-I_1$). In this case, typical of the records obtained on connexin32-transfected SKHep1 cells, the current traces I_1 and I_2 are mirror images, with I_2 differing in magnitude by the presence of only a small ohmic nonjunctional conductance. Thus, I_{nj} is a small fraction of I_j and does not display pronounced voltage dependence.

For the purpose of comparing junctional conductance immediately after the command pulse was applied to that reached under steady-state conditions, junctional currents were measured at the onset of the command pulse (I_0) and at the end of the pulse (I_∞). Plots of I_0 and I_∞ as a function of voltage are shown for a representative experiment in Fig. 3. In the range of ± 50 mV, I_0 is a linear function of applied V_j, whereas I_∞ shows rectification in both quadrants, deviating from linearity at ~ ± 25 mV. Thus, the initial conductance (I_0/V_j) is relatively insensitive to V_j, whereas the steady-state junctional conductance (g_∞) displays marked voltage sensitivity.

The apparently exponential relaxation of I_j in response to V_j pulses suggested that the underlying transition of gap junctional channels from open to closed

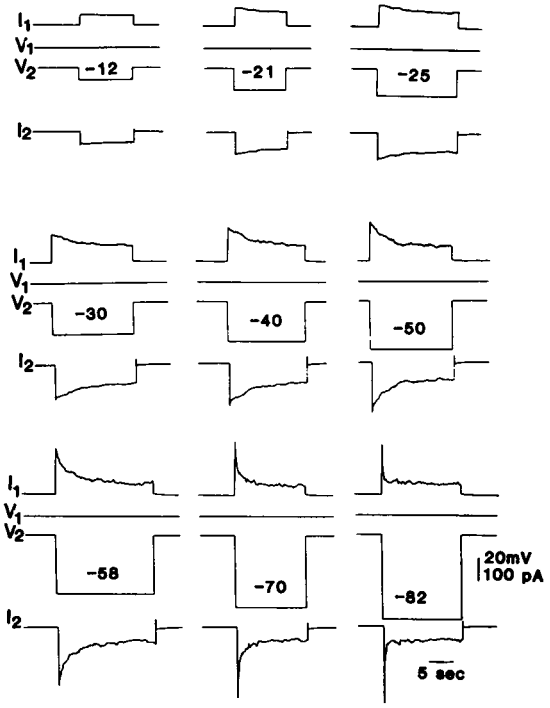

FIGURE 2 Transjunctional voltage dependence of gap junction conductance between transfected SKHep1 cell pairs. Families of curves were generated in which V_1 was held constant at 0 mV, whereas voltage steps of varying amplitudes were applied to cell 2 (V_2, *third traces in all records;* magnitude of each voltage step is indicated above each pulse). The upper and lowest traces correspond to the currents injected (I_1, I_2) from each amplifier in order to keep the holding potential of each cell constant in response to voltage commands delivered to cell 2. For each pulse, the kinetics of the relaxation of I_1 and I_2 are similar, although the changes are of opposite polarity, indicating that nonjunctional conductance is low. Relaxation of I_1 is to a lower level for larger pulses, and the shape of the relaxation is exponential.

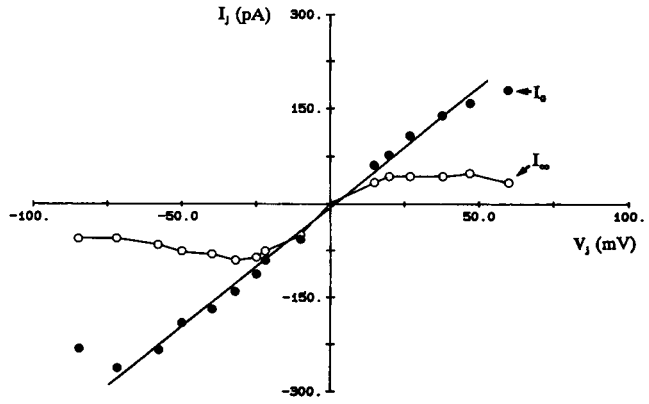

FIGURE 3 Relation between junctional current (I_j) and transjunctional voltage (V_j) in a connnexin32 transfected cell pair. For each V_j applied as in Fig. 2, junctional currents were measured at the beginning of the command (I_0, *solid circles*) and at its end (I_∞, *open circles*). For V_j values ±50 mV, the I_0-V_j relation is linear with a slope, determined by linear regression, of 3.8 nS. At V_j values > ±20–30 mV, I_∞ deviated from linearity, with g_j values at ±50 mV < 0.5 nS.

states might be modeled by a Boltzmann relation, where the transition between states is assumed to be reversible and first order. The Boltzmann equation defines V_0 (the voltage at which the voltage dependent component of g_j is reduced by 50%) and A (a parameter quantifying voltage sensitivity) according to the following equation (see Spray et al., 1981):

$$g_\infty = (g_{max} - g_{min})/[1 + \exp[A(V - V_0)]] + g_{min}. \quad (1)$$

Rearranging gives:

$$\exp[A(V - V_0)] = (g_{max} - g_\infty)/(g_\infty - g_{min}). \quad (2)$$

To quantify the voltage dependence, steady-state g_j values (g_∞) were determined for each voltage. Minimum conductance (g_{min}) was measured as the g_j value attained at the end of the largest V_j applied (50–70 mV); maximal conductance (g_{max}) was g_j obtained at the smallest V_j value or at the onset of a moderate V_j pulse. For eleven experiments, g_∞ values at 10 mV increments were normalized to g_{max} and ln $(g_{max} - g_\infty)/(g_\infty - g_{min})$ was plotted as a function of V_j (Fig. 4 *B*). Values obtained by regression (Fig. 4 *B*) were then used to draw the Boltzmann curves for each polarity of V_j (Fig. 4 *A*, solid lines). The Boltzmann parameters defining these points are $V_0 = 25$ mV, $A = 0.06$, and $g_{min} = 0.1$. For comparison to other voltage dependent processes, the energy differences between states can be calculated as $\Delta U = AV_0kT$ and is ~1 kcal/mol (compared to 2 kcal/mol for amphibian embryonic junctional channels [Harris et al., 1981]). The equivalent number of charges moving through the membrane is ~1.75, corresponding to a limiting slope of twofold change in the ratio of open to closed channels for every 10 mV.

Transjunctional voltage dependence of g_j between transfected cells: kinetic aspects

The relaxation of junctional currents in response to long transjunctional voltages appeared to be an exponential function (Fig. 2). To evaluate the time constants of these declines, data were digitized and plotted semilogarithmically. Slopes were determined by regression analysis as is illustrated in Fig. 5. These studies confirmed that at low to moderate V_j values, relaxation of I_j is well described by a single exponential. At higher V_j values regression coefficients become smaller, and the initial phase of decline did not extrapolate to convergence, suggesting that a faster process may also contribute a minor component to channel closing.

Time constants of the decline in I_j during command potentials were plotted as a function of V_j in order to determine the effect of V_j on the transition between states. These data are shown in Fig. 6 *A*. At V_j values

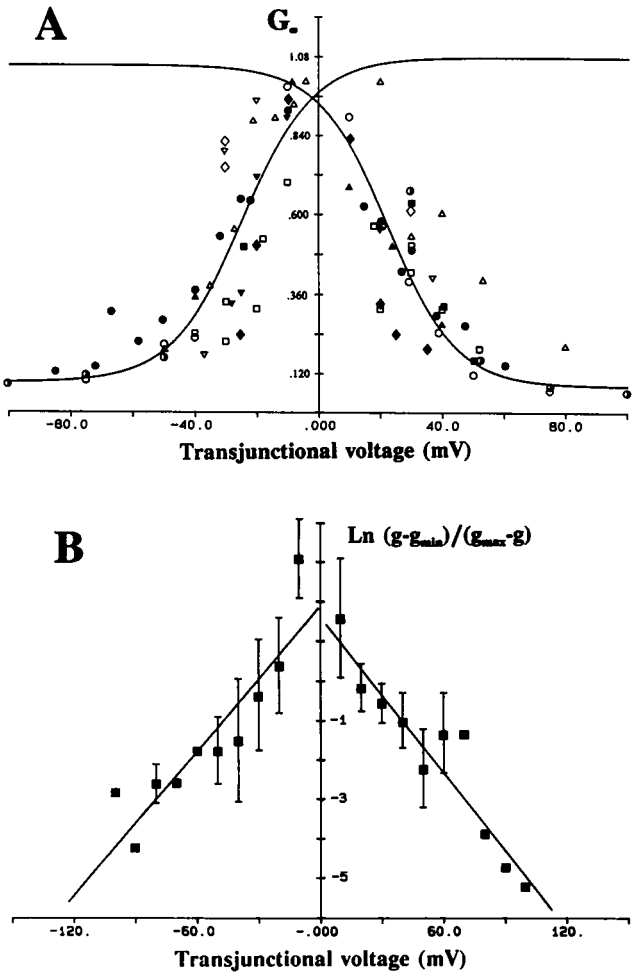

FIGURE 4 Relation between the normalized steady-state junctional conductance (G_∞) and the transjunctional voltage. (*A*) Plot of normalized junctional conductance (G_j) values obtained as a function of transjunctional voltage in the nine experiments (each indicated by a separate symbol) used for curve fitting in *B*. The superimposed smooth curves represent the Boltzmann equations describing the transitions in each polarity. (*B*) The semilogarithmic plot of steady-state junctional conductance of nine different experiments, after rearranging terms in the Boltzmann equation (see text), plotted as a function of the transjunctional voltage. Each point corresponds to the mean values in different experiments ($1 < n < 6$) grouped in bins of 10 mV. Best fits ($0.9 < r < 0.95$) were determined by linear regression of values obtained with positive and negative voltages. The values for voltage dependent parameters obtained from the intercepts are $V_0 = 25$ and 26 mV with $A = 0.065$. Boltzmann equations with these parameters are drawn atop the data in *A*.

~ ±20–40 mV, τ was longest (~6 s). At V_j values >30 mV, τ declined, reaching minimal values (2–3 s) at the highest voltages.

For a process involving only two states (open and closed), opening and closing rate constants (α and β) at each voltage can be calculated from the corresponding

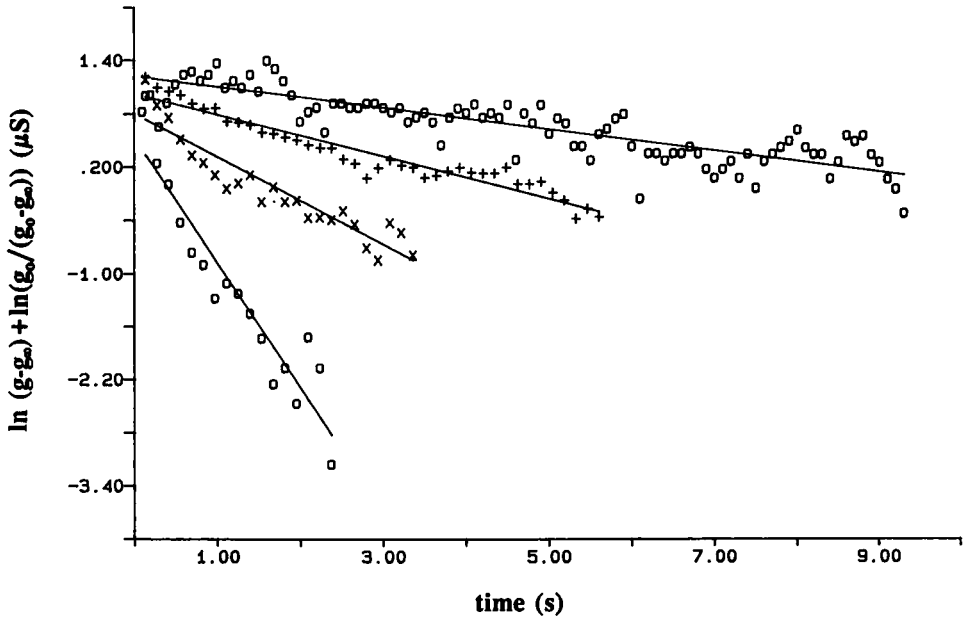

FIGURE 5 Exponential time course of relaxation of junctional current. Currents were digitized and plotted semilogarithmically. Four currents, corresponding top to bottom to V_j commands of 12, 48, 72, and 85 mV are illustrated in this figure. Best fits were determined by linear regression, as indicated by the solid lines superimposed on the data. Note that the time course for relaxation is shorter for larger V_j.

time constant according to the equations:

$$\alpha = G_\infty/\tau \quad (3)$$

$$\beta = (1 - G_\infty)/\tau, \quad (4)$$

where G_∞ is the normalized steady-state junctional conductance. The rate constants α and β were calculated for each of the experiments shown in Fig. 6 *A* and are plotted as a function of voltage in Fig. 6 *B*. At the lowest V_j values, the time constant is governed by the opening rate constant, α. At the highest voltages β dominates, being about ten times as large as α. The data were fit to the equation:

$$\alpha = \lambda \exp[-A_\alpha(V - V_0)] \quad (5)$$

and

$$\beta = \lambda \exp[A_\beta(V - V_0)]. \quad (6)$$

Where A_α and A_β are voltage sensitivities of the rates, λ is a constant (the rate at which $\alpha = \beta$, reached at $V_j = V_0$), and V_0 is as defined earlier. The curves best fitting the data appear as solid lines in Fig. 6 *B*. Best fit to experimental data occurred when $A_\alpha = 0.23 \pm 0.002$, $A_\beta = 0.034 \pm 0.002$ (SD, $n = 5$), and $\lambda = 0.1$; the closing rate is thus 50% more voltage sensitive than the rate of opening over the entire range of V_j tested.

The opening and closing rate constants over a range of voltages calculated for macroscopic kinetic data allow us to predict the shape of current relaxations obtained in experiments where few channels are active. An example of such an experiment is illustrated in Fig. 7. In the recording in Fig. 7 *A* voltage sensitivity is manifested as steplike decreases in junctional current that are mirrored by steps in the other cell's current record. The steplike changes in currents in these records represent openings and closings of individual gap junction channels as described previously (Eghbali et al., 1990; Moreno et al., 1991*b*). The same V_j pulse was presented repeatedly in this experiment, producing the junctional currents shown in Fig. 7 *B*. Averaging these records led to the record shown in Fig. 7 *C*. The solid line superimposed on the recording shows the relaxation predicted from the analysis of macroscopic data presented above.

This analysis also allows us to predict the time course of recovery of I_j at the end of voltage pulse. In Fig. 8 an experiment is illustrated in which test pulses were repeatedly applied during and after the application of command potentials. In Fig. 8 *A*, the command pulse is 30 mV and the time constant of decline in I_j during the pulse is ~4.8 s. In Fig. 8 *B*, V_j is 50 mV, and I_j declines with $\tau = 3.8$ s. For both pulses, the time constant for recovery is fit by $\tau = 4.5$ s. As predicted by the model, the initial voltage dependent relaxation is more rapid for the larger voltage step, whereas the time course of recovery is fit by the same exponential in the two records.

Finally, the predicted time and voltage sensitivities of

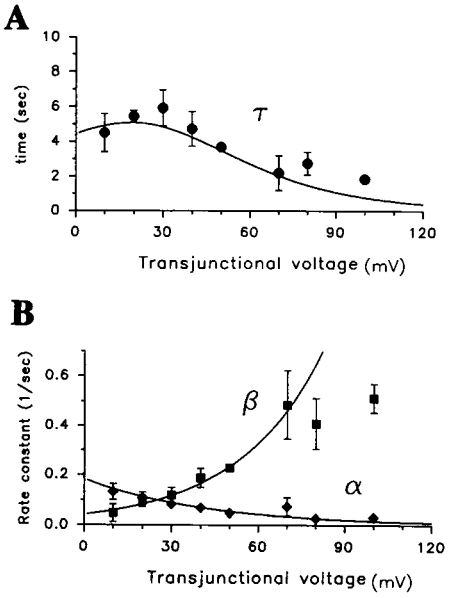

FIGURE 6 Voltage dependence of time constant (τ) and rate constants (α and β). (*A*) Dependence of time constant on voltage. The time constant of decline in g_j for each trace shown in Fig. 5, as well as for four other experiments where pulses applied also with opposite polarity, was calculated by least square fitting to a semilogarithmic relation and means ± SD were plotted as a function of V_j for negative and positive pulses. The solid line is derived from rate constant data in 6 *B*. (*B*) Voltage dependence of rate constants (α and β; where $\tau = 1/(\alpha + \beta)$. Following the model proposed by Harris et al. (1981), rate constants for transitions between open and closed states were obtained (see Eqs. 3 and 4 in text) using values for τ shown in *A*. Each continuous curve is an exponential obtained using linear regression with Eqs. 5 and 6 in text, where $V_0 = 25$ mV, $\lambda = 0.1$, $A_\alpha = 0.023 \pm 0.002$ and $A_\beta = 0.034 \pm 0.002$ (SD). At low V_j values, the opening rate constant, α, dominates, $\alpha = \beta$ at V_0, and at higher V_j values, the reaction is dominated by the closing rate constant β. Note that the rates saturate $\gtrsim 70$ mV.

the rate constants can be used to determine whether voltage gating of the connexin32 gap junction channel is adequately described by a two-state reaction scheme for closing by pulses of each polarity in each direction. This model is depicted in Fig. 9, and consists of two gates in series where either would be closed by transjunctional voltage of appropriate polarity. In such a scheme, prior closure of the channel by a voltage of one polarity would necessitate passage through an open state before it could be closed by a pulse of opposite polarity.

To test the predictive validity of this reaction scheme, relaxation of I_j was recorded in response to the same V_j pulse before (Fig. 10 *A*) and after the application of a V_j pulse of the same magnitude but opposite sign (Fig. 10 *B*). Brief test pulses were continuously delivered in order to evaluate g_j at each time point. Contingent gating, in which one gate must open before the V_j of opposite sign is sensed, is indicated by the overshoot of I_j in response to reversal as shown in Fig. 10 *B* and by the transiently increased I_j seen in response to the brief test pulses. These portions of the records are enlarged and superimposed in Fig. 10 *C* (short pulses are deleted for clarity). For comparison, the prediction of the contingency model is shown in Fig. 10 *D*, where voltage and time sensitivities of rate constants are defined by the decline in macroscopic I_j as described above. Note the essential similarity of the shapes of the observed and predicted kinetic responses (Fig. 10, smooth curves from *D* are superimposed on the data in *C*).

DISCUSSION

Voltage dependence

We report here that in cells stably transfected with cDNA encoding connexin32, the major gap junction protein of rat liver, junctional conductance (g_j) displays striking voltage dependence. In previous studies on pairs of hepatocytes (Spray et al., 1986; Riverdin and Weingart, 1988), failure to detect voltage sensitivity could have been due to the ratio between the junctional resistance and the resistances of the two micropipettes. In those studies, g_j ranged from 27 to 67 nS (~15–40 Mohm). With those g_j values, and using electrodes of 5–10 Mohm, whose resistance could easily double in the process of seal formation and membrane rupture (Marty and Neher, 1983), the potential drop can be as high as 40% across the electrode tip and only 60% across the junctional membrane.

In our experiments, we compared results obtained using pipettes of two different resistances. As shown in Fig. 1, when resistances of the pipettes were high, the sensitivity to transjunctional voltage was low; when the resistance of the pipettes was 3–5 Mohm, voltage dependence was clearly observed. Our finding of voltage sensitivity under optimal recording conditions is consistent with voltage dependence appearing when g_j is reduced (Neyton and Trautmann, 1985; Spray et al., 1984) and also with recent reports of voltage sensitivity appearing across cardiac gap junctional membranes in response to large V_j values when g_j is low (Rook et al., 1988; Veenstra, 1990).

Implications for channel structure

We report here that the channels formed of connexin32 exhibit first order kinetics for opening and closure and that the steady-state g_j–V_j data are well fit in each polarity by the Boltzmann relation, which assumes the existence of two states with first order transitions between them. In pairs of early embryonic cells of amphibia, g_j also displays kinetics of closing that are well

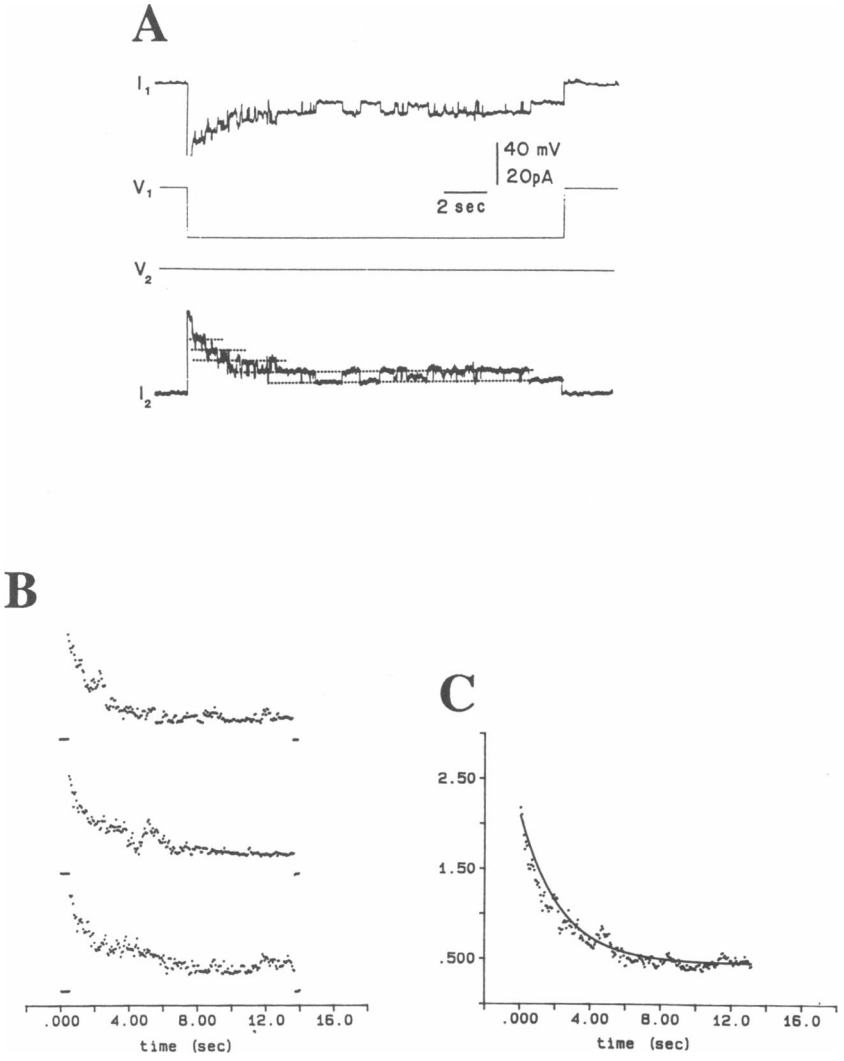

FIGURE 7 Voltage dependence visualized as an ensemble of behaviors of single gap junction channels. In a low g_j pair, where single channel events could be recorded, both cells were held at 0 mV (at the beginning of V_1 and V_2 traces) and then a voltage pulse of −40 mV was applied to cell 1 and the currents of both cells were recorded. (*A*) Single channel closings are clearly seen (*dotted lines in I_2 trace*) until a steady state is reached. Before and after the voltage pulse, no events are seen. (*B*) Three repetitions of the pulse shown in *A*, illustrated as records digitized at 30 Hz. (*C*) Average of the three I_j records shown in *B*, with superimposed kinetics of relaxation for this voltage as predicted from rate constants calculated in Fig. 6 *B*.

described as single exponential relaxations, and the Boltzmann relation is also symmetric (Spray et al., 1979, 1981; Harris et al., 1981). These findings indicate that these gap junction channels (formed of rat connexin32 and *Xenopus* connexin38) behave as if there were symmetric voltage sensors located within each hemichannel, each sensing either positive or negative voltages. Because the process is first order, closure by a voltage of one polarity would be envisioned to involve a single gate, and the gates would be symmetrically placed. If the molecular domain of the voltage sensor is optimally situated to sense the entire transmembrane field, the charge separation comprising the sensor would extend from the cytoplasmic aspect of the channel to the region of the union of the hemichannels.

For other membrane channels, beginning with the analysis of sodium and potassium currents in squid axon (Hodgkin and Huxley, 1952), activation of voltage sensitive currents has been interpreted as a highly cooperative interaction between gating particles, where the channel is open only if several particles are in the proper configuration. The movement of each particle has been envisioned to be governed by a first order process. With the revelation that ionic channels are formed of multile

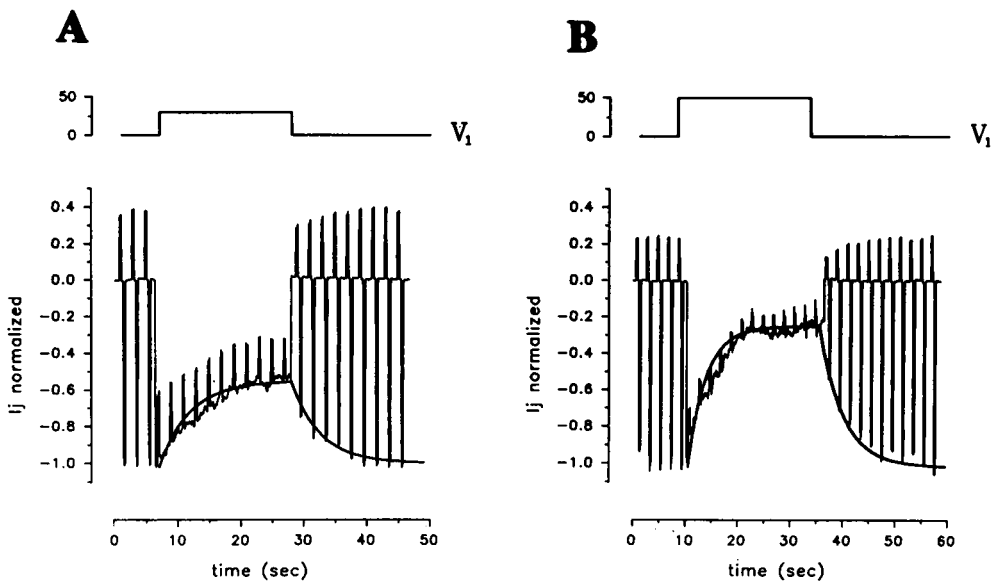

FIGURE 8 Time course of recovery of g_j after transjunctional voltage steps of a single polarity. Long (>20 s) transjunctional voltage commands were applied while superimposing brief test pulses to evaluate g_j. (*A*) In response to V_j = 30 mV, I_j relaxed with kinetics predicted from Fig. 6 *A* (τ for solid line fitting decline: 4.8 s), and recovered with a time constant of 4.5 s (predicted from the *y*-axis interacepts of α and β). (*B*) In response to V_j = 50 mV, I_j declined with a time constant of 3.8 s; at the end of the pulse, g_j recovered with the same τ as in *A*, 4.5 s. Thus, during the long command pulse, I_j decreased exponentially with a time constant that depended on the magnitude of the voltage pulse until it reached a steady-state value at the end of the pulse. Time course of recovery of the upward transient currents at the end of the command step indicate that the time course of recovery is independent of the magnitude of the command pulse. The curves superimposed on the transient currents represent predictions from rate constants determined in Fig. 6 *B*.

subunits of proteins or large proteins with homologous domains, the time course of voltage dependence has been given a molecular framework, in which each homologous protein, or each homologous domain on a large protein, may contribute one gate to the process. Opening would then require all subunits to be in the permissive configuration (e.g., Catterall, 1988).

The voltage dependence of gap junction channels is unique in that for transjunctional voltages of either polarity gating is well described by a first order process.

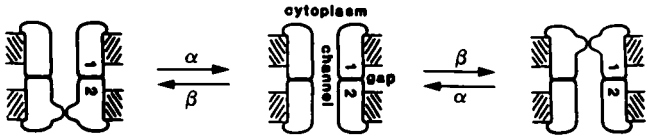

FIGURE 9 Contingency model for the action of transjunctional voltage on connexin32 gap junction channels. Each hemichannel is envisioned to possess its own gate which senses the field from cytoplasm to cytoplasm. Opening and closure are envisioned as symmetric processes governed by rate constants α and β, respectively. Transitions from one closed state (*leftmost or rightmost schematic*) to another is predicted to require transition through the open (*middle*) state.

(Na channel inactivation, which was originally modeled as showing first order voltage dependence, is now regarded as being coupled to Na channel activation, with no inherent voltage dependence [Cota and Armstrong, 1989].) The simple kinetic behavior of gap junction channels thus provides a model system in which to study the nature of the molecular mechanism responsible for this type of voltage dependence.

The gap junction channel is believed to consist of multiple copies of identical subunits, although the possibility for heterologous subunits has not been ruled out rigorously in most systems. X-ray diffraction and low angle electron microscopy have resolved six subunits (connexins) contributed by each cell, with the two connexons (hemichannels) joined across the intercellular space (Makowski, 1988; Unwin, 1989).

If the voltage dependence of the gap junction channel was conferred upon it by sensors located on each subunit, and if all subunits were required to be in the proper configuration for the channel to be either open or closed, kinetics of the voltage dependence would be expected to be at least sixth order in each polarity. At least two possibilities are consistent with the simple kinetics observed and the structural dogma. One is that

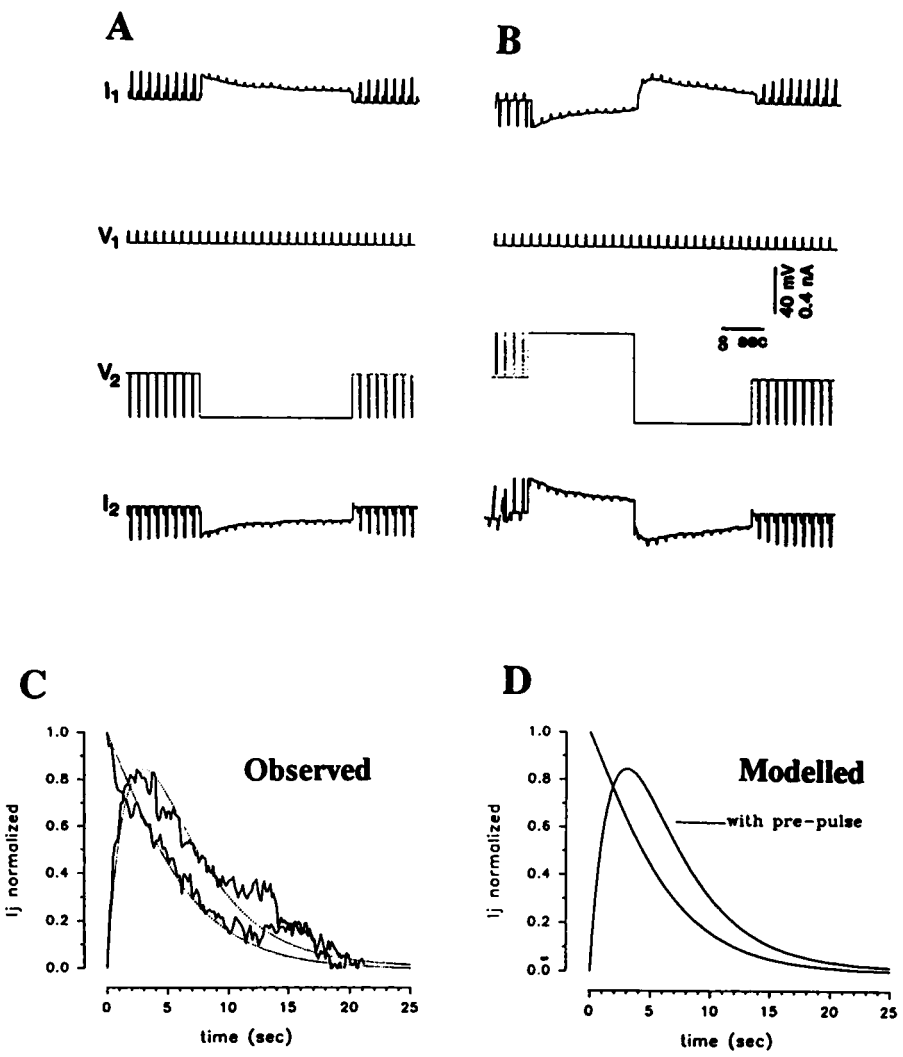

FIGURE 10 Connexin32 channels closed by voltages of one polarity must open before being closed by a voltage of the opposite polarity. (*A*) Voltage dependence of g_j during a long hyperpolarizing voltage step to cell 2. Transjunctional current (I_1) reaches it maximum value at the onset of the voltage step and decreases exponentially to a steady-state level. (*B*) Response of junctional currents to a V_j as in *A*, but preceded by a depolarizing V_j step of the same amplitude. In this case peak I_j occured 1 s after the polarity reversal and decreased thereafter. (*C*) Superposition of the initial changes in I_j with and without a prepulse (from records in *A* and *B*; test pulses removed for clarity) shows the slow increase in g_j before closure by the other polarity. Dotted curves atop records are from *D*. (*D*) Kinetics predicted from the contingency model depicted in Fig. 9 (using $\lambda = 0.1, A_\alpha = 0.023, A_\beta = 0.035, V_0 = 25$ mV). Note the essential similarity between observed (*C*) and modeled (*D*) behavior.

the voltage sensor is formed by strong interaction of the subunits so that, although there are multiple molecules, there is functionally a single sensor. Another possibility is that each molecule has its own sensor and that the conformational change causes torsion in the macromolecular complex sufficient for complete channel closing. In either possibility, the opening and closing rate constants would apply to the macromolecular assembly or only to the single subunit that was induced to move through the field.

Where is the voltage sensor on the gap junction channel?

The multiorder voltage dependence of Na^+, K^+, and Ca^{2+} channels has been attributed to S4 membrane-spanning segments, which recur four times in the sequences of these proteins. These S4 domains consist of repeated charged residues separated by three to four neutral or hydrophobic residues, and which would create an alignment of charges if arranged in α-helices.

Depolarization is envisioned to drive this helix outward, so that the charged residues are twisted upward to realign with neighboring charges and thereby each account for a single charge moved through the field.

The connexin sequences contain no domains similar to the S4 region of other voltage-sensitive channels. Each monomer of the gap junction channel possesses cytoplasmic amino- and carboxyl-termini, four membrane spanning domains, two extracellular loops that presumably bind to homologous regions of hemichannels across the gap, and a cytoplasmic loop or hinge region. The third transmembrane domain is the most amphipathic and is thus the most likely to line the water-filled pore. In this region of the molecule, charged amino acid residues align when the sequence is modeled as α-helical, and a consensual feature of all connexins is that the charged residues are flanked by bulky residues, primarily phenylalanine. If the extracellular loops are imagined to form inflexible rigid contacts and are thereby fixed at these points, gating stimuli would be expected to act by rotating connexin molecules, thereby flipping bulky groups into the channel lumen (Unwin, 1989; Bennett et al., 1991). At the single channel level, each channel appears to close completely, indicating that the conformational change is sufficient to reduce channel diameter by at least 47% (ratio of hydrated diameters of K^+, the major current-carrying ion, to Lucifer Yellow, which is near the permeability limit of the channel: unpublished data, Verselis, V. K., R. L. White, D. C. Spray, and M. V. L. Bennett). The energy required for this process can be estimated from the defining voltage dependence parameter. For amphibian embryonic cells, this energy is $\sim$2 kCal/mol and for connexin32 it is $\sim$1 kCal/mol.

Series gates on the gap junction channel are indicated by polarity reversal experiments, in which the kinetics of closure by a pulse of one polarity are compared with and without a large prepulse of the opposite polarity. Thus, sensing of a transjunctional voltage by one series gate is contingent on the other gate's being open, which provides additional evidence that the conformational changes mediating closure are virtually complete.

Origin of the voltage-insensitive component, g_{min}

Voltage dependent gap junctions are characterized by a fraction of the conductance that is not reduced further at even large transjunctional voltages. For amphibian embryonic cells, g_{min} was 5% (Spray et al., 1981). For connexin43 transfectants and cardiac and smooth muscle cells, g_{min} can be 50% or more (Fishman et al., 1991; Rook et al., 1988). In the connexin32 transfectants examined here, g_{min} is $\sim$10%. The origin of g_{min} could be incomplete closure of all channels, presence of a subpopulation of channels that are less voltage dependent, or rate constants that saturate at the highest potentials, the latter of which has been hypothesized as a dipole whose moment changes when moved (Neher and Stevens, 1979). The first possibility would require these channels to exhibit conductance substates, which was not observed. The second explanation is at least partially valid in bilayer experiments, where g_{min} can be quite variable and a separate population of small channels can be identified, but is less likely here. For transfectants, our kinetic analysis shows saturation of the rate constants at high voltages, indicating that the small voltage insensitive component is accounted for by nonzero open probability at the highest voltages [$P_0 = \alpha/(\alpha + \beta)$; at $V_j > 70$ mV, P_0 reaches a minimal value of $\sim$0.1]. During polarity reversal, the proportion of open channels would be expected to close more rapidly than those which must first open, although the deviation from predicted behavior was not detected in our experiments (Fig. 10, *C* and *D*).

The first order rate constants noted here are unique for ionic channels, most of which are formed of multimeric aggregates of subunits. These kinetics imply that each subunit either contributes partially to the channel gate or that each subunit may effectively close the channel. It is expected that future studies in which multiple connexins with different voltage sensitivities are expressed in the same cell type under experimental control may shed some light on this issue.

Supported in part by National Institutes of Health grants NS16524 and HL38449 and a Grant in Aid from the New York Chapter of the American Heart Association to David C. Spray and Program Project grant NS07512 (M. V. L. Bennett, principal investigator; subproject to David C. Spray).

Received for publication 3 February 1991 and in final form 18 July 1991.

REFERENCES

Auerbach, A. A., and M. V. L. Bennett. 1969. A rectifying synapse in the central nervous system of a vertebrate. *J. Gen. Physiol.* 53:211–237.

Barrio, L. C., T. Suchyna, T. Bargiello, L. X. Xu, R. Rojinski, M. V. L. Bennett, and B. J. Nicholson. 1991. Voltage dependence at homotypic and heterotypic rat connexin26 and connexin32 junctions expressed in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA.* In press.

Baylor, D. A., and J. G. Nicholls, 1969. Chemical and electrical synaptic connexions between cutaneous mechnoreceptor neurones in the central nervous system of the leech. *J. Physiol. (Lond.).* 203:591–609.

Bennett, M. V. L., and D. C. Spray, editors. 1985. *Gap Junctions.* Cold Spring Harbor Laboratory, NY.

Bennett, M. V. L., L. Barrio, T. Bargiello, D. C. Spray, E. L. Hertzberg, and J. C. Saez. 1991. Gap junctions: New tools, new answers, new questions. *Neuron* 6:305–320.

Campos de Carvalho, A. C., E. L. Hertzberg, and D. C. Spray. 1991. Complex channel activity recorded from rat liver gap junctional membranes incorporated into lipid bilayers. *Braz. J. Biol. Med.* In press.

Catterall, W. A., 1988. Structure and function of voltage-sensitive ion channels. *Science (Wash. DC).* 242:50–61.

Cota, G., and C. M. Armstrong. 1989. Sodium channel gating in clonal pituitary cells. The inactivation step is not voltage dependent. *J. Gen. Physiol.* 9:213–232.

Doerr, R., I. Zvibel, D. Chiuten, J. D'Olimpio, and L. M. Reid. 1989. Clonal growth of tumors on tissue-specific biomatrices and correlation with organ site specificity of metastases. *Cancer Res.* 49:384–392.

Ebihara, L., E. Beyer, K. I. Swenson, D. L. Paul, and D. A. Goodenough. 1989. Cloning and expression of an *Xenopus* embryonic gap junction protein. *Science (Wash. DC)* 243:1194–1195.

Eghbali, B., J. A. Kessler, and D. C. Spray. 1990. Expression of gap junction channels in a communication incompetent cell line after transfection with connexin32 cDNA. *Proc. Natl. Acad. Sci. USA.* 87:1328–1331.

Fishman, G., A. P. Moreno, D. C. Spray, and L. A. Leinwand. 1991. Functional analysis of human cardiac gap junction channel mutants. *Proc. Natl. Acad. Sci. USA.* 88:3525–3529.

Fogh, J., W. C. Wright, and J. D. Loveless. 1977. Absence of HeLa cell contamination in 169 cell lines derived from human tumors. *J. Natl. Cancer Inst.* 68:507–517.

Furshpan, E. J., and D. D. Potter. 1959. Transmission at the giant motor synapses of the crayfish. *J. Physiol. (Lond).* 145:289–325.

Giaume, C., and H. Korn. 1983. Bidirectional transmission at the rectifying synapse: a voltage-dependent process. *Science* 220:84–87.

Harris, A. L., D. C. Spray, and M. V. L. Bennett. 1981. Kinetic properties of a voltage dependent junctional conductance. *J. Gen. Physiol.* 77:95–117.

Hodgkin, A. L., and A. F. Huxley. 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol. (Lond.).* 117:500–544.

Jaslove, S., and P. Brink. 1986. The mechanism of rectification at the electronic motor synapse of the crayfish. *Nature (Lond.).* 232:63–65.

Hertzberg, E. L., and Johnson, R. G., editors. 1988. Gap Junctions. Alan R. Liss, New York.

Makowski, L., 1988. X-Ray diffraction studies of gap junction structure. *Adv. Cell Biol.* 2:119–158.

Marty A., and E. Neher, 1983. Tight-seal whole-cell recording. *In* Single Channel Recording. B. Sakmann and E. Neher, editors. Plenum Publishing Corp., New York. 107–121.

Moreno, A. P., A. C. Campos de Carvalho, B. Eghbali, V. Verselis, and D. C. Spray. 1991*a*. Connexin 32, the major gap junctional protein of hepatocytes, forms voltage dependent channels in hepatocytes, in bilayers, and in transfected cells. *Biophys. J.* 59:920–925.

Moreno, A. P., B. Eghbali, and D. C. Spray, 1991*b*. Connexin32 gap junction channels in stably transfected cells: unitary conductance. *Biophys. J.* 60:1254–1266.

Neher, E. and C. F. Stevens. 1979. Voltage-driven conformational changes in intrinsic membrane proteins. In: *The Neuroscience: Fourth Study Program.* F. O. Schmitt and F. G. Worden, editors. The MIT Press. Cambridge, MA. 623–629.

Neyton, J. and A. Trautmann, 1985. Single channel currents of an intercellular junction. *Nature (Lond.).* 317:331–335.

Obaid, A. L., S. J. Socolar, and B. Rose. 1983. Cell-to-cell channels with two independently regulated gates in series: analysis of junctional channel modulation by membrane potential, calcium and pH. *J. Membr. Biol.* 73:69–89.

Riverdin, E. C., and R. Weingart. 1988. Electrical properties of the gap junctional membrane studied in rat liver cell pairs. *Am. J. Physiol.* 254:C226–C234.

Rook, M. B., H. J. Jongsma, and C. G. van Ginneken. 1988. Properties of single gap junctional channels between isolated neonatal heart cells. *Am. J. Physiol.* 255:H770–H782.

Spray, D. C., and M. V. L. Bennett. 1985. Physiology and pharmacology of gap junctions. *Annu. Rev. Physiol.* 47:281–303.

Spray, D. C., and J. M. Burt, 1990. Structure-activity relations of the cardiac gap junction channel. *Amer. J. Physiol.* 258:C195–C207.

Spray, D. C., A. L. Harris, and M. V. L. Bennett. 1979. Voltage dependence of junctional conductance in early amphibian embryos. *Science (Wash. DC).* 204:432–434.

Spray, D. C., A. L. Harris, and M. V. L. Bennett, 1981. Equilibrium properties of a voltage-dependent junctional conductance. *J. Gen. Physiol.* 77:77–93.

Spray, D. C., R. L. White, A. C. Campos de Carvalho, A. L. Harris, and M. V. L. Bennett. 1984. Gating of gap junctional conductance. *Biophys. J.* 45:219–230.

Spray, D. C., R. D. Ginzberg, E. A. Morales, Z. Gatmaitan, and I. M. Arias. 1986. Electrophysiological properties of gap junctions between dissociated pairs of rat hepatocytes. *J. Cell. Biol.* 101:135–144.

Spray, D. C., M. V. L. Bennett, A. C. Campos de Carvalho, B. Eghbali, A. P. Moreno, and V. Verselis. 1991. Voltage dependent gap junctional conductance. *In* Biophysics of Gap Junction Channels. C. Peracchia, editor. CRC Press, Inc., Boca Raton, FL. 97–116.

Unwin, P. N. T. 1989. The structure of ion channels in membranes of excitable cells. *Neuron.* 3:665–676.

Veenstra, R. L. 1990. Voltage-dependent gating of gap junction channels in embryonic chick ventricular cell pairs. *Am. J. Physiol.* 258:C662–C672.

Verselis, V. K., M. V. L. Bennett, and T. A. Bargiello. 1991. A voltage-dependent gap junction in *Drosophila melanogaster. Biophys. J.* 59:114–126.

Young, D. E., Z. Cohn, and N. B. Gilula. 1987. Functional assembly of gap junction conductance in lipid bilayers: demonstration that the major 27kD protein forms the junctional channel. *Cell.* 48:733–743.