# Voltage-dependent gap junction channels are formed by connexin32, the major gap junction protein of rat liver

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ABSTRACT We report here experiments undertaken in pairs of hepatocytes that demonstrate a marked voltage sensitivity of junctional conductance and, thus, contradict earlier findings reported by this laboratory (Spray, D. C., R. D. Ginzberg, E. A. Morales, Z. Gatmaitan, and I. M. Arias. 1986. *J. Cell Biol.* 101:135–144; Spray, D. C., R. L. White, A. C. Campos de Carvalho, and M. V. L. Bennett. 1984. *Biophys. J.* 45:219–230) and by others (Dahl, G., T. Moller, D. Paul, R. Voellmy, and R. Werner. 1987. *Science [Wash. DC.]* 236:1290–1293; Riverdin, E. C., and R. Weingart. 1988. *Am. J. Physiol.* 254:C226–C234). Expression in exogenous systems, lipid bilayers in which fragments of isolated gap junction membranes were incorporated (Young, J. D.-E., Z. Cohn, and N. B. Gilula. 1987. *Cell.* 48:733–743.) and noncommunicating cells transfected with connexin32 cDNA (Eghbali, B., J. A. Kessler, and D. C. Spray. 1990. *Proc. Natl. Acad. Sci. USA.* 87:1328–1331), support these findings and indicate that the voltage-dependent channel is composed of connexin32, the major gap junction protein of rat liver (Paul, D. 1986. *J. Cell Biol.* 103:123–134).

## INTRODUCTION

The abundance of gap junctions between hepatocytes has made the liver an ideal preparation for isolation and characterization of gap junction proteins and cloning of cDNA encoding two gap junction proteins ($M_r$ 21 kDa and 27 kDa, newly termed connexins 26 and 32 [25, 30, 49]). Connexins isolated from other tissues have been found to be highly homologous (5, 16). Initial electrophysiological studies on pairs of rat hepatocytes using the dual whole-cell voltage clamp technique with patch pipettes (37) detected little or no voltage sensitivity of junctional conductance ($g_j$). This finding was subsequently confirmed by another laboratory using the same technique (32). In the *Xenopus* expression system, $g_j$ between oocytes paired after injection with liver mRNA or with RNA transcribed from connexin32 cDNA was also reported not to be voltage dependent (9, 44). Initial studies on isolated rat liver junctional membranes incorporated into lipid bilayers at the tips of patch pipettes also reported conductances that were insensitive to voltage (42). However, in experiments from another laboratory in which liver gap junction membranes treated with detergents were incorporated in planar bilayers, the total conductance between chambers was shown to exhibit a voltage-dependent component which was $< 25\%$ of the total conductance (48). As in cells from cleavage stage amphibian embryos, where voltage dependence of junctional membranes has been analyzed extensively (18, 19, 38, 39), the conductance of the incorporated membranes was reduced by voltages of either sign applied to either compartment and the most rapid closing occurred within a few seconds. We have reevaluated the issue of whether and to what extent gap junctions between hepatocytes are voltage dependent in several ways: first, pairs of hepatocytes have been voltage clamped using the dual voltage clamp technique with two microelectrodes rather than one patch pipette in each cell, in order to avoid possible difficulties with series resistance of the patch electrodes.[1] Second, liver junctional membranes have been incorporated into lipid bilayers, using detergent treatment (48). Finally, we have compared these findings with recordings obtained from pairs of cells transfected with connexin32 cDNA (12).

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[1] In freshly dissociated hepatocyte pairs, $g_j$ can exceed 1 μS and nonjunctional conductance may be as high as 10 μS (34), which presents a case that is less than ideal for voltage clamp using the whole-cell technique with patch electrodes. Operationally, the series resistance of a patch pipette after the seal is broken may be 5–20 MΩ, which can be reduced by feeding back an amplified command pulse or subtracting part of the voltage measured at the summing junction of the voltage clamp; the error introduced by incomplete compensation can be quite high. Another source of inadequate voltage control might arise from access resistance to the junctional membrane as has recently been proposed by Jongsma and colleagues (23). Either of these resistances (and, in the case of liver, the high nonjunctional conductance) may result in substantial voltage drop not associated with the junctional membrane and could explain why voltage dependence appears in several preparations only when $g_j$ is lowered experimentally (e.g., 4, 26, 33).


0006-3495/91/04/920/06 $2.00

## MATERIALS AND METHODS

### Hepatocyte pairs

Cells were obtained as previously described (37) and were used within 3 h of plating. During recordings, cells were maintained at room temperature on the stage of a diavert microscope (Nikon Inc., Garden City, NY) in Dulbecco's phosphate-buffered saline solution (PBS; Gibco Laboratories, St. Lawrence, MA). Dual voltage clamp was performed as described previously (39) using homemade two-electrode amplifiers with separate voltage and current electrodes in each cell. Electrodes (5–10 MOhms) contained 300 mM KGlutamate, buffered to pH 7.0 with 1 mM Hepes. In this method, both cells were voltage clamped to the same holding potential (generally −40 mV) and one cell's voltage ($V_j$) was stepped to the command transjunctional potential. The current ($I_j$) supplied by the other cell's voltage clamp is equal in magnitude and opposite in sign to current flowing through the junctional membrane and junctional conductance ($g_j$) is calculated as $-I_j/V_j$ (38).

### Gap junction membranes

Membranes were isolated from rat hepatocytes by a detergent-independent procedure as previously described (17). These membranes were incubated at 4°C in the presence of 1% Triton X-100 for from one day to two weeks before use (48). Lipid bilayers were formed by painting the 300 μm hole in the polystyrene wall separating the two compartments of the bilayer chambers with asolectin in the decane (30 mg/ml). 1–5 μl of the detergent treated gap junction membrane suspension was added to the CIS compartment with constant stirring until conductance increased above baseline levels (generally 10 pS or less). At that time stirring ceased and recordings began.

### Transfection

The human hepatoma cell line SKHep1 was stably transfected with the vector pcEXV-2 containing the full length cDNA encoding connexin32 as previously described (12). In brief, SKHep1 cells were cotransfected with plasmids containing connexin32 and g418 resistance DNA using $CaPO_4$ precipitation techniques and selected using antibiotic resistance and Lucifer Yellow injection. Cell pairs were obtained from the stably transfected cell line by dissociation and were voltage clamped using the dual voltage clamp method with patch type electrodes (3–6 MOhms) containing in mM 120 CsCl, 10 EGTA, 10 Hepes at pH 7.2. Holding potentials were generally 0 mV. External solution contained (in millimolar): CsCl, 7; $CaCl_2$, 0.1; NaCl, 160; Hepes, 10; $MgSO_4$, 0.6; pH 7.2.

## RESULTS AND DISCUSSION

The opening or closing of many types of channels in biological membranes is sensitive to membrane potential, and this property is responsible for excitability, the mechanism by which action potentials are generated (8, 20, 21). Although some gap junction channels are also voltage sensitive, those gap junctions which serve to couple adult mammalian cells generally have been regarded as insensitive to voltage applied across either the junctional or nonjunctional membranes (e.g., reference 35).

The junctional conductance ($g_j$) between hepatocytes was indeed found to be voltage dependent. In response to sustained depolarizing or hyperpolarizing transjunctional command voltages, current through junctional membrane declined, and the relaxation was more rapid and to lower steady-state values with larger pulses (Fig. 1 *A*). The minimal junctional conductance obtained at the highest voltages ($g_{min}$) was ~20% of the maximal conductance ($g_{max}$), obtained at the lowest voltages (Table 1). The steady-state $g_j$-voltage relation is shown after subtraction of $g_{min}$ in Fig. 2. In some cell pairs, the $g_j$-voltage relation was less steep for depolarizing potentials applied in either cell, suggesting that depolarization may increase $g_j$. The magnitude of this reponse, however, was variable among cell pairs and was not quantified. Polarity reversal experiments with voltage steps to one cell, although potentially complicated by dependence of $g_j$ on membrane potential, indicated delays in closing channels by one polarity of voltage after prior closure with pulses of the opposite polarity (data not shown). These findings are consistent with the contingent gating model described previously for voltage dependence of $g_j$ in amphibian blastomeres (18).

We also examined voltage dependence using isolated liver gap junction membranes incorporated into lipid bilayers under the conditions described by Young et al. (48), which included the addition of Triton X-100 to enhance incorporation. Voltage dependence was also present in bilayers after channel incorporation (Fig. 1 *B*). Under favorable conditions (few channels between compartments) larger channels could be detected that were largely closed at higher voltages, whereas smaller channels continued to open and close; in some cases the closure of the smaller channels brought the membrane very close to lipid bilayer conductance (Fig. 1 *B*, open arrows). These experiments suggest that the voltage-insensitive component of $g_j$ seen in hepatocytes might be ascribable to the presence of lower conductance, voltage-insensitive channels. The larger channels would account for the voltage dependence of the conductance. Unitary currents of the larger channels correspond to conductances of ~150 pS when corrected to 100 mM salt concentration. This value is quite similar to that previously reported for connexin32 channels expressed in transfected cells (12). Thus, we have confirmed the presence of a voltage-dependent component of conductance in junctional membranes isolated from rat liver (48).

Interpretation of the experiments on hepatocyte pairs and on hepatocyte gap junction membranes incorporated into lipid bilayers are compromised by the expected coexpression of at least two types of gap junction protein (connexins 26 and 32) in the membranes tested (28, 45, 49). This is even more problematic in the case of bilayers where even minute contamination of hepatocyte

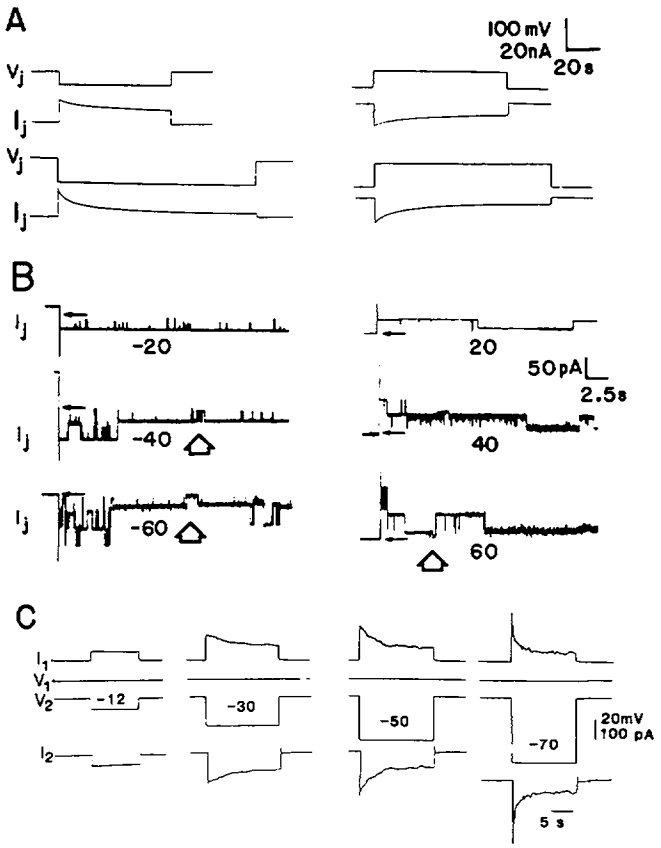


FIGURE 1 Voltage dependence in preparations containing connexin32. (*A*) In hepatocyte cell pairs where each cell was voltage clamped by a separate two electrode voltage clamp circuit, junctional current ($I_j$) decayed in response to long hyperpolarizing (*left*) and depolarizing (*right*) transjunctional voltages ($V_j$). At longer and larger voltages (*lower traces*) the decline was more rapid and to a lower level. Junctional conductance ($g_j$) is $-I_j/V_j$. (*B*) In hepatocyte gap junction membranes incorporated into lipid bilayers, currents ($I_j$) were recorded between compartments in response to imposed voltages of indicated amplitudes. Both large and smaller (*open arrows*) unitary events were detected (most marked in the −40 and −60 mV traces), with the larger channel being the most voltage sensitive. At −20 mV and +20 mV, channel transitions between open and closed states were infrequent, and at higher voltages the larger channels closed rapidly. Arrows indicate zero current; in all but the −40 mV trace, steps were given from zero transmembrane potential. The experiment illustrated was performed in 300 mM $K_2SO_4$ solutions, at pH 7.0, in both compartments. (*C*) In cell pairs normally communication-incompetent stably transfected with connexin32, junctional current ($I_j$) was also voltage dependent. For hyperpolarizing pulses ($V_2$) applied to one cell of a voltage-clamped pair, relaxation of $I_j$ was barely detectable at −12 mV, was to ~50% of initial value at −30 mV, and at higher voltages the decrease was more rapid and to a lower steady-state level. Traces show the voltage command applied to cell 2 ($V_2$) and the input and junctional currents ($I_2$, $I_1$, respectively), as well as the voltage of cell 1 ($V_1$). For *A–C* note the presence of a component of junctional conductance that remains at the highest transjunctional voltages; this is the voltage-insensitive component, corresponding to $g_{min}$.

TABLE 1 **Values for voltage-dependent parameters describing junctional conductance***

| | Preparation | | |
|---|---|---|---|
| | Hepatocytes‡ | Bilayer§ | Transfectant‖ |
| $V_0$ | 39.2 ± 10.6 mV | 34.3 ± 9.6 mV | 26.5 ± 7.6 mV |
| $A$ | 0.07 ± 0.03 | 0.14 ± 0.03 | 0.06 ± 0.02 |
| $g_{min}/g_{max}$ | 0.04–0.4 | 0–0.8 | 0.15–0.24 |

*Curves were fitted to the Boltzmann relation in which the energy difference between open and closed states is assumed to be a linear function of voltage, and transitions between states are reversible and first order (see references 13, 40). The steady-state ratio of channels in open vs. closed states is therefore an exponential function of the energy difference between the states and the fraction of open channels ($p$) divided by the fraction of closed channels ($1 - p$) is given by $p/(1 - p) = (g_\infty - g_{min})/(g_{max} - g_\infty) = \exp[-A(V_j - V_0)]$, where $g_\infty$ is the steady-state junctional conductance, $g_{min}$ is $g_j$ at the highest $V_j$ values, $g_{max}$ is approximated as $g_j$ at the start of the voltage pulse or with the smallest $V_j$'s, $V_0$ is the value of $V_j$ at which half the channels are open, and $A$ is a constant expressing voltage sensitivity ($A$ can be expressed as $nq/kT$, where $n$ is the equivalent number of charges $q$ that move through the entire membrane voltage to effect the conductance changes; $k$ and $T$ have their usual meanings [13]). $A$ is therefore the negative slope of the plot of $\ln[(g_\infty - g_{min})/(g_{max} - g_\infty)]$ measured at different $V_j$ values; values for $V_0$ and $A$ in Table 1 represent regressions of data presented in Fig. 2 to the equation above. Variance is calculated as the confidence interval for the slope of the regression line and above values are expressed as standard deviations about the means (24); ‡fit from 76 points, nine experiments; §fit from 32 points, four experiments; ‖fit from 68 points, nine experiments.

gap junction membrane preparations with channels from plasma membrane may result in incorporation of the nonjunctional channels. As a definitive test for voltage dependence of the channel comprising rat liver gap junctions, we have stably transfected a normally noncommunicating cell line with a vector containing the cDNA encoding the major liver gap junction protein, connexin32 (12). Junctional conductance between these cell pairs was unmistakably voltage-dependent, decreasing at both positive and negative transjunctional voltages ($V_j$'s; Fig. 1 *C*). As in the case of hepatocyte pairs, a voltage-insensitive component ($g_{min}$) was present at the highest voltages. As is shown in Table 1, $g_{min}$ was a constant fraction of maximal conductance ($g_{max}$); this minimal junctional conductance has been interpreted previously as indicating that even at the highest voltages the channels exhibit nonzero opening probability (38, 39).

Steady-state conductances were compared in the three preparations in which we have studied the voltage dependence of connexin32 (Fig. 2). In each of the experiments from each type of preparation, $g_j$ was normalized to that obtained when the smallest $V_j$'s were applied, and normalized $g_j$ values ($G_j$'s) were plotted as means ± SD. For all three preparations, $G_j$ decreased

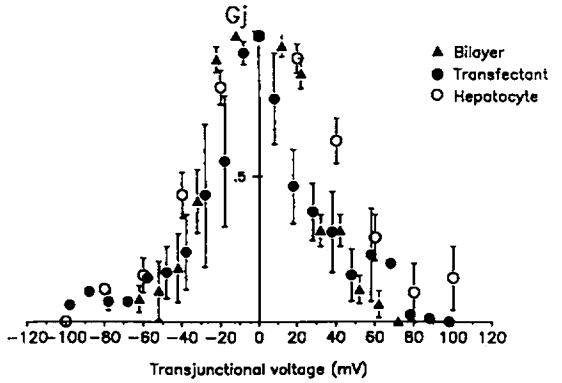


FIGURE 2 Plot of the relation between normalized steady-state junctional conductance ($G_j$) and transjunctional voltage ($V_j$, obtained by depolarizing or hyperpolarizing one cell) for hepatocyte pairs (*open triangles*), transfected cells (*closed circles*) and bilayers (*solid triangles*). $G_j$ is the normalized voltage-sensitive component of junctional conductance $[(g_\infty - g_{min})/(g_{max})]$. Values represent means and standard deviations of values from nine experiments on hepatocytes and transfectants and four experiments on bilayers. For clarity in presentation, data points for transfected cells are offset −2 mV for positive voltages along the *x*-axis and +2 mV for negative $V_j$'s; for bilayers, data points are offset by +2 mV for positive polarities and −2 mV for negative $V_j$'s. This manipulation separates the error bars for the data sets but does not affect the general shape of the curves.

noticeably with 20 mV pulses of either polarity and was reduced by 50% at $V_j$'s of 30–50 mV. The curves for bilayer experiments and for transfected cells were most similar, with the steady-state voltage dependence of the hepatocyte pairs being somewhat less steep and shifted to higher voltages for either polarity.

Using the equations previously derived for voltage dependence of gap junctions between amphibian embryonic cells (Table 1, legend), we can compare the parameters defining voltage dependence of hepatocyte pairs, liver membranes incorporated into bilayers, and connexin32 channels expressed in the communication-deficient cell line (Table 1). This comparison reveals several differences among the preparations. $V_0$, the voltage at which $g_j$ is reduced to 50% of the maximum conductance, is shifted by ~5–10 mV when hepatocyte pairs are compared to either junctional membranes incorporated in bilayers or connexin32-transfected cells. This difference could arise in part from residual uncompensated resistance of any of the components listed in footnote 1, from a cytoplasmic factor that could modify gating characteristics, or by the existence of other types of junctional channels in hepatocytes. The difference compared to transfectants could also represent the contribution of channels in hepatocytes with hybrid voltage sensitivity, which might be expected if connexins 32 and 26 formed heteromeric channels, as has been postulated (44). A second difference among the preparations is the magnitude of the voltage insensitive component ($g_{min}/g_{max}$), which for the bilayer is a variable fraction as high as 0.8. This variability presumably reflects a variable relative incorporation of other channels to the total bilayer conductance, some of which may be other gap junction channels or even possibly nonjunctional channels isolated from the hepatocyte. Note that $g_{min}$ values observed in the transfectants are much less variable than in the other preparations. Differences in $A$, the parameter translating voltage sensitivity into work done on the molecule by the voltage field (13), indicate that the bilayer conductance is more steeply voltage dependent than the other preparations, which may reflect incorporation of nonjunctional channels that are not closed until moderately strong transbilayer potentials are applied. The average value of the product of $A$ and $V_0$ in all three preparations corresponds to an energy difference between states of ~1–2 kcal/mol, which is similar to that calculated for gap junction channels of early amphibian embryonic cells (18).

Why this modest voltage dependence has not been observed in most studies using the *Xenopus* oocyte expression system (9) remains unexplained, as voltage dependence has been found by others in connexin32-injected oocyte pairs (3). Sensitivity of voltage dependence to cytoplasmic pH ($pH_i$) as was recently suggested by Dahl and colleagues (10) remains a possibility, which would be consistent with the previously reported differences in resting $pH_i$ between *Xenopus* embryonic cells and rat hepatocytes (37, 40). The previous failure to observe voltage dependence in liver membranes incorporated into lipids at the tips of patch pipettes (42) may have been due to the presence of other channels or to the relatively short durations of the $V_j$ pulses in those experiments; alternatively, the voltage sensor of the protein might have been altered during its isolation.

Voltage-dependent junctional conductance has now been described in a variety of preparations (e.g., 2, 14, 15, 22, 27, 29, 31, 38, 39, 43, 47). We have sought but failed to find junctional voltage dependence over physiologically relevant $V_j$'s (± 50 mV) in several other mammalian preparations (e.g., pancreatic islet cells: 1; the WB cell line: 36; leptomeningeal cells: 41). In cells that express connexin 43 (heart: 5; astrocytes: 11), junctional conductance was insensitive to voltages up to ±50 mV in cells with high input resistances and low $g_j$'s, a condition which optimizes dual whole-cell recording conditions (6). At higher voltages or during junction formation, other groups have reported relaxation of junctional currents in heart cells of neonatal rat and embryonic chick, although values of voltage-dependent

parameters are much lower than reported here for connexin32 channels (7, 33, 46).

We conclude that connexin32, the major gap junction protein of hepatocytes, forms voltage-dependent channels, where conductance is reduced by sustained transjunctional potentials of either polarity. The functional significance of this gating mechanism is obscure in liver, where cells are quite strongly coupled and resting potentials are low, but could serve to uncouple hepatocytes rapidly after acute injury to the tissue (e.g., surgical or chemical partial hepatectomy, acute responses to hepatotoxic agents). Of possibly more significance, expression of connexin32 has recently been demonstrated between oligodendrocytes and between some neurons in the brain (11), where low coupling strength and high resting potentials could presumably allow transjunctional potentials of uncoupling magnitude to develop.

We thank Dr. M. V. L. Bennett for comments on an earlier draft of this manuscript.

This work was supported in part by National Institutes of Health grants HL 34479 and NS 16524 to David C. Spray, NS 07512 (P.I.: M. V. L. Bennett, subproject to David C. Spray), and a National Science Foundation Binational grant to Antonio C. Campos de Carvalho and David C. Spray.

*Received for publication 9 April 1990 and in final form 5 November 1990.*

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