Connexin32 gap junction channels in stably transfected cells: Unitary conductance

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ABSTRACT Pairs of SKHep1 cells, which are derived from a highly metastatic human hepatoma, were studied using the whole cell voltage clamp technique with patch-type electrodes containing CsCl as the major ionic species. In 12 of 81 cell pairs, current flow through junctional membranes was detectable; in the remaining 69 cell pairs, junctional conductance was less than the noise limit of our recording apparatus (worst case:10 pS). Macroscopic junctional conductance (g_j) in the small percentage of pairs where it was detectable ranged from 100 to 600 pS. Unitary junctional conductance (γ_j) determined in the lowest conductance pairs or after reducing conductance with a short exposure to the uncoupling agent halothane was 25–35 pS. To study properties of gap junction channels formed of connexin32, the parental SKHep1 cell line was stably transfected with a plasmid containing cDNA that encodes connexin32, the major gap junction protein of rat liver cells. In 85 of 98 pairs of voltage clamped connexin32-transfected SKHep1 cells, macroscopic g_j was >1 nS; g_j increased with time after dissociation (from 1.8 ± 0.6 [mean ± SE; $n = 7$] nS at 2 h after plating to 9.3 ± 2.2 [$n = 9$] nS, the maximal value, at 24 h). Unitary conductance of gap junction channels between pairs of transfected SKHep1 cells was measured in low conductance pairs and after reducing g_j by exposure to halothane or heptanol. Histograms of γ_j values in transfected cells, in 10 experiments where >100 transitions were measurable, displayed two peaks: 120–130 pS and 25–35 pS. The smaller size corresponded to channels that were occasionally detected in the parental cells. We therefore conclude that connexin32 forms gap junctions channels of the 120–130 pS size class.

INTRODUCTION

Gap junction channels span the membranes of adjacent cells and thereby provide a direct pathway for communication between cells in almost all animal tissues (see Bennett and Spray, 1985; Hertzberg and Johnson, 1988; Robards et al., 1990; Bennett et al., 1991). High resolution ultrastructural imaging techniques (Unwin, 1989; Makowski, 1988) have revealed that each gap junction channel is a dodecamer of protein subunits termed connexins (Beyer et al., 1987), half of which are located in the membrane of each cell forming hemichannels or connexons. The channel pore is believed to extend through the center of both hemichannels, thus maintaining cytoplasmic continuity between the cells, but excluding contact of cytoplasmic fluid with the extracellular medium.

The property of gap junctions that is essential to their function in various tissues is their permeability to large ions and small molecules. Second messenger molecules readily diffuse between coupled cells (e.g., Tsien and Weingart, 1976; Saez et al., 1989), as do current carrying ions responsible for electrotonic conduction in excitable cells (see Spray and Burt, 1990). The electrical equivalent of permeability is conductance. For the gap junction channels thus far studied, permeability and conductance are linearly related, so that measurement of conductance reliably reports the number of open gap junction channels (see Verselis et al., 1986; Zimmerman and Rose, 1985; Spray and Burt, 1990).

Rat and mouse liver have provided the sources of isolated gap junctions used for high resolution imaging studies, and these tissues express two gap junction proteins, connexins 32 and 26 (Paul, 1986; Kumar and Gilula, 1986; Zhang and Nicholson, 1989). Previous studies on rat liver gap junction membranes incorporated into exogenous lipids at patch pipette tips (Spray et al., 1986) or in planar films (Young et al., 1987; Campos de Carvalho et al., 1991) therefore used preparations in which both proteins were presumably present. In these biophysical studies there was probably contamination by nonjunctional channels as well, and channels of multiple sizes were detected.

The unitary conductance (γ_j) of individual gap junction channels has been measured in a variety of mammalian tissues, ranging from ~20 pS in fibroblasts (Rook et al., 1989), ~50 pS in cardiac myocytes, astrocytes, and leptomeningeal cells (Burt and Spray, 1988; Dermietzel et al., 1991; Rook et al., 1988; Spray et al., 1991*b*) to 120–150 pS in lacrimal and acinar gland cells and in isolated rat liver junctional membranes (Neyton and Trautmann, 1985; Spray et al., 1986; Young et al., 1987; Somogyi and Kolb, 1988). Even larger unitary conduc-

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tances have been recorded in chick embryonic heart (Veenstra and DeHaan, 1986) and from lens membranes incorporated into bilayers (Zampighi et al., 1985). The tissues which express the 50 pS channel all express the cardiac gap junction protein, connexin43. The tissues in which 120–150 pS channels have been observed express connexin32 as well as connexin26.

We have sought to determine the channel size of gap junctions in which connexin32 was the sole junctional protein. To this end, we have stably transfected a communication-deficient cell line. SKHep1 cells, with a vector containing connexin32 cDNA (Eghbali et al., 1990) and here report the unitary conductances of resulting gap junction channels. We also characterize an infrequently observed small junctional channel ($\gamma_j \leq 30$ pS) in the parental cell line. This channel presumably corresponds to a novel connexin, which has not yet been cloned and sequenced, although unitary conductances of approximately the same size were noted previously in studies on other cell types (Somogyi and Kolb, 1988; Rook et al., 1989; Spray et al., 1991*a*; Moore et al., 1991). A preliminary report of some of these findings has appeared (Eghbali et al., 1990).

METHODS

Cell line

The parental cell line, SKHep1, is derived from a highly metastatic human hepatoma (Fogh, 1977; Doerr et al., 1989). Parental cells are not coupled with respect to the dye Lucifer Yellow; immunofluorescence staining and Northern blots with antibody and cDNA probes for connexins 43, 32, and 26 are negative (Eghbali et al., 1990; Fishman et al., 1990). The parental line and transfectants are grown in RPMI medium containing 5% fetal calf serum (GIBCO, Grand Island, NY).

Transfection procedure

Transfection of SKHep1 cells with the vector pcEXV-3 containing connexin32 cDNA has been described in detail previously (Eghbali et al., 1990). In brief, plasmids (pcEXV-3 containing connexin32 cDNA, and pSV2-neo) were introduced into these cells using the $CaPO_4$ precipitation technique. Colonies surviving selection for 3 wk during exposure to the antibiotic G418 were screened with Lucifer Yellow injection; those showing extensive dye coupling were picked and subcloned. Subclones stably express dye coupling, and immunoreactivity to connexin32 antibody for at least 20 passages (Eghbali et al., 1990).

Preparation

Early passages of parental and transfected SKHep1 cells were kept frozen at −80 to −90°C until use and were then thawed and cultured to confluence. On the day of each experiment (or occasionally, the day before), confluent cultures were split at low density on glass coverslips and used between 2 and 24 hours thereafter, at which time they had adhered strongly to the substrate.

Voltage clamp methods

The dual whole cell voltage clamp technique (Spray et al., 1979) was used with patch-type electrodes (White et al., 1985; Neyton and Trautman, 1985). Patch pipettes (2.5–7 MOhms) were pulled and polished on a Brown-Flaming puller and were filled with solution at pH = 7.2 containing (in millimolar) CsCl, 135; $CaCl_2$, 0.5; Na_2ATP, 2; MgATP, 3; Hepes, 10; EGTA, 10. Seals to cell surfaces were achieved with suction and were monitored by applying simultaneously through both electrodes 14 ms, 10 mV pulses at 10 Hz. During suction, the current from the pipette was measured in the search mode of the voltage clamp system (model List Patch Clamp L/M-EPC7; Medical Systems Inc., NY). When seal resistance was in the gigaohm range, brief strong pressure was applied to achieve access to the cell interior. During the experiments, the external solution was superfused using a peristaltic pump/dispenser (Wiz pump; ISCO, Lincoln, NE). The external solution generally contained (in millimolar): CsCl, 7; $CaCl_2$, 0.1; NaCl, 160; Hepes; 10; $MgSO_4$, 0.6; pH 7.2. A programmable stimulator (model A-65 Timer; Winston Electronics Co., San Francisco, CA) was used to deliver voltage pulses. Junctional conductance (g_j) was monitored with the application of alternate 200 mS, 10 mV pulses to each of the cells in the pair. Within a pair of cells where each is voltage clamped to the same holding potential, a voltage command pulse delivered to one cell (V_j) results in an incremental current required to maintain the voltage of that cell. This current (I_1) is divided into junctional (I_j) and nonjunctional (I_{nj}) components. (Note that the bath is grounded, so that no current flows from cell to cell via extracellular space.) In cases where nonjunctional conductance (g_{nj}) is much less than junctional conductance (g_j), most current will flow to the second cell and the voltage clamp on the second cell will record a current (I_2) that is equal in magnitude and opposite in sign to I_j. Junctional current (I_j) divided by the amplitude of the transjunctional voltage (V_j) gives g_j directly (Spray et al., 1981).

Currents and voltages during the experiments were recorded on video tape after pulse code modulation (model Neuro-corder DR-484; Neurodata Corp, NY) using a 100 kHz bandwidth filter. The voltages and currents were also recorded on a four-channel chart recorder (model Gould Brush 4000; Gould Inc., Cleveland, Ohio)

Measurement of single gap junction channel events

Several conditions are necessary to observe single channel currents, one of the most crucial of which is the ratio between junctional and input (parallel sum of seal and nonjunctional) resistances. When this ratio is < 10 and the number of junctional channels is small, detection of single events is favored. High seal resistance was achieved by using freshly polished glass pipettes for each experiment. In cases where g_j was initially high, it was decreased by treatment with either heptanol (0.5 mM, dissolved from 20 mM stock in DMSO) or halothane (2 mM, diluted from saturated stock saline solution: see Burt and Spray, 1989). After application of the uncoupling agents, g_j decreased to zero conductance and the cells were then rinsed with external solution to obtain channel reopening. When g_j was near zero, single channel events were observed and measured (note that these measurements are obtained while conductance is increasing or decreasing and are therefore not at steady state). Because halothane is volatile and its effect is rapidly reversible, it was used more often than heptanol. Currents through single gap junction channels are recognizable as step changes recorded in currents of both amplifiers occurring simultaneously but of opposite polarities. This criterion of simultaneity excludes events due to current flowing through nonjunctional channels, which are observable as currents recorded only in the circuit clamping that cell.

Current steps observed simultaneously in the two cells' current

traces were measured in that trace where the signal to noise ratio was larger and unitary junctional conductance (γ_j) was calculated by dividing the current steps by the transjunctional voltage (V_j), defined as the difference between the holding potentials of the cells of a pair. Generally, one cell's voltage was held at zero and the other at negative potentials, between −30 and −60 mV.

Calculated unitary junctional conductances (γ_j's) were grouped in 5 or 10 pS bins and plotted in event histograms. The events grouped this way showed normal distributions and were fitted to Gaussian curves using the function:

$$F(x) = \frac{1}{\sigma\sqrt{2\pi}} e^{-1/2\left(\frac{\gamma_j - \overline{\gamma_j}}{\sigma\sqrt{2\pi}}\right)^2}$$

where σ corresponds to the standard deviation and $\overline{\gamma_j}$ to the mean of the distribution. The best fit was achieved using a Gaussian-Newton approximation algorithm supplied by Assystant Software System (Asyst Software Technologies, Rochester, NY). Data sets in which multiple peaks were prominent in the event histograms were analyzed by assuming that each population was independent. Using these equations in Sigmaplot 4.0 (Jandel Scientific, Corta Madera, CA), best Gaussian fit was obtained for the amplitude distribution corresponding to the smallest events and then this population of events was subtracted and populations consisting of progressively larger events were treated similarly. Statistical comparisons were obtained applying the Student's *t*-test to means and standard deviations of best fits of Gaussian distributions determined iteratively. Digitization of the single channel currents was done as follows: each original current trace was recorded on videotape and then played back through a 100 Hz filter; this represents the frequency limit cited in the text as the "acquisition frequency." Acquisition of data into the computer was done at 1 kHz, corresponding to the number of samples/seconds stored in the computer from the previously filtered traces.

RESULTS

Junctional conductance in cell pairs from the parental cell line

SKHep1 cells are not coupled with respect to transfer of intracellularly injected Lucifer Yellow, and this absence of coupling facilitates selection of well coupled transfectants (Eghbali et al., 1990). To establish whether *any* gap junction channels were *ever* present in the parental cell line, we tested cell pairs using the highly sensitive technique of dual whole cell recording, which allows detection of currents through single gap junction channels.

Macroscopic junctional conductance

Using the double voltage clamp technique, in cases where detection was optimal, we were able to measure junctional conductances (g_j) in only 12 of 81 pairs of the SKHep1 parental cell line. In the remaining 69 cell pairs, g_j was < 10 pS, the worst case limit of detection. Thus, < 15% of these cell pairs exhibited *any* endogenous gap junction channel activity, and we term this cell line "communication-deficient." An example from one of the most strongly coupled pairs of the parental cell line is shown in Fig. 1 (*inset*). Values of junctional conductance in the cell pairs that were coupled were between 100 and 600 pS; a histogram of these values is shown in Fig. 1. Mean junctional conductance for the 15% of the cells that were coupled was ~400 pS.

Microscopic junctional conductance

In two of the 12 coupled cell pairs from the parental cell line we added 2 mM halothane in order to optimize detection of single channel events. With few channels open, simultaneous and mirrorlike changes in the currents of the two cells were observed (Fig. 2, *inset*). These events satisfy the criteria for junctional channels, occurring simultaneously and with identical sizes in both current traces. The conductance corresponding to each change of state was calculated, and a histogram summarizing these measurements is shown in Fig. 2. In this histogram, single channel events from two experiments on the parental cell line are combined with those recorded in a poorly coupled transfected pair (see below) which did not display larger channels. This histogram indicates that the unitary conductance of the junctional channels present in the parental cell line is ~30 pS [the curve superimposing the data in Fig. 2 represents a Gaussian distribution with a mean of 29.6 ± 9.3 pS (SD)]. The 15% of the coupled cell pairs in the parental population are therefore coupled by an average of ~13 open gap junction channels.

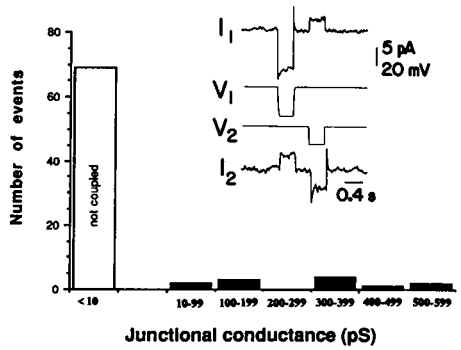

FIGURE 1 Histogram of junctional conductances (g_j) measured between pairs of SKHep1 cells. g_j was calculated from I_1, I_2, V_1, and V_2 traces (as shown in *inset*) obtained while recording in the dual whole cell voltage clamp configuration; g_j is calculated as the current in the postsynaptic cell (upward current records in *inset*) divided by the voltage step ($g_j = -I_j/\Delta V$). In most cases (69 pairs) g_j was < 10 pS (first column in *histogram*); g_j values between the 12 coupled pairs are displayed in 100 pS bins. Note that the highest g_j values recorded were < 600 pS.

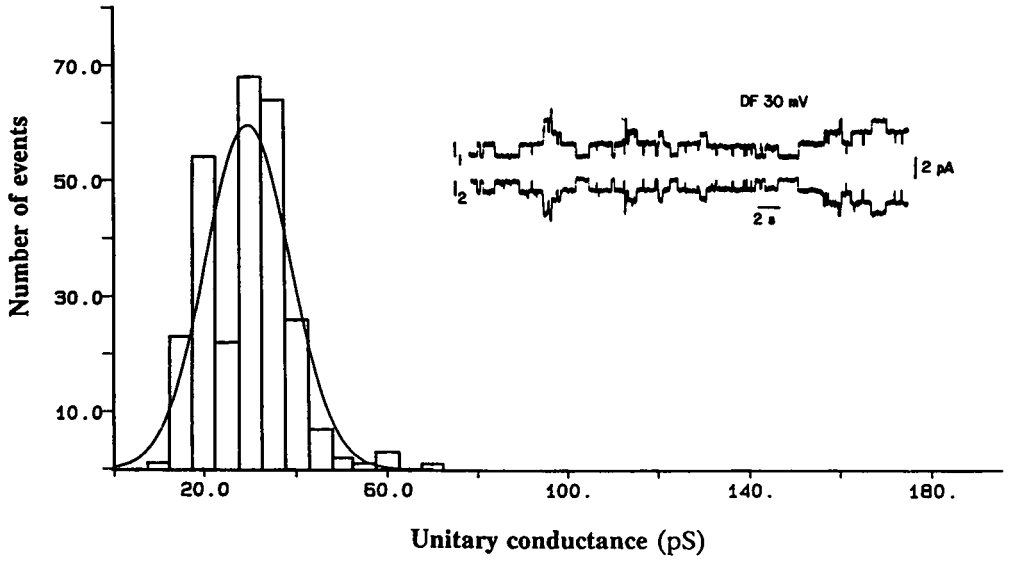

FIGURE 2 Amplitudes of unitary gap junctional conductances recorded between SKHep1 cell pairs. (*inset*) Original record obtained from a nontransfected cell pair that shows the changes of state (openings are upward in the *upper trace*) of endogenous gap junction channels. The current was recorded through a 10 Hz filter. The histogram presents the unitary conductances, grouped in 5 pS bins, calculated in three separate experiments, two from nontransfected cell pairs and one from a transfected pair which did not display any larger channels. The solid line is the best fit to a Gaussian distribution, with mean = 29.6 ± 9.2 (SD) pS.

Properties of gap junction channels between connexin32-transfected SKHep1 cells

Pairs of SKHep1 cells stably expressing connexin32 were voltage clamped in order to evaluate properties of the junctional membrane and to compare these properties with those of the parental cell line.

Macroscopic conductance of transfected cells

Junctional conductance (g_j) measured between cell pairs stably transfected with connexin32 cDNA was >1 nS in 85 of 98 cases. (This criterion level of 1 nS was set arbitrarily to definitively exclude overlap with the endogenous coupling present in the parental population.) The incidence of these g_j measurements is shown in a histogram in Fig. 3 *A* and a recording of macroscopic junctional currents from representative cell pair is shown in Fig. 4 *B*, first panel. The incidence of poorly coupled pairs in this histogram is possibly biased quite high due to our selection of cell pairs with small contact areas in order to optimize recordings of single channel currents. Furthermore, these recordings were obtained from cell pairs at various times after plating.

After splitting the transfected cells, more than 2 h elapse while they adhere to the substrate, and during this time high resistance seals were not easily obtained. At 2 h after plating, g_j averaged 2 nS ($\pm 0.5, n = 5$); mean values of g_j in cell pairs recorded at various times are shown in Fig. 3 *B*. After 24 h of plating g_j was maximal (~ 10 nS; $n = 14$); similar g_j values were obtained for the next three days or longer. Experiments on the same culture dish after three days became more difficult because cells became confluent and flattened and isolated pairs were not easily located or voltage clamped.

Although these findings indicate that g_j increases rapidly during the first 24 h in culture, the origin of the coupling present at 2 h after plating remains unresolved. Some cells may remain paired due to incomplete dissociation; however, the rapid turnover time of connexin32 in hepatocytes (2–3 h half-life: e.g., Traub et al., 1989) could also allow such rapid reexpression between completely dissociated SKHep1 pairs; alternatively, there may be a pool of connexin precursors which can assemble without protein synthesis (see Bennett et al., 1991).

Single channel recordings from transfected pairs

Following the same protocol used with the parental cell line, it was possible to observe currents through single channels in the connexin32-transfectants that represent transitions between closed and open states. In a few pairs of transfected cells, g_j was low enough that single channel events could be detected without the application of uncoupling treatments. A representative record

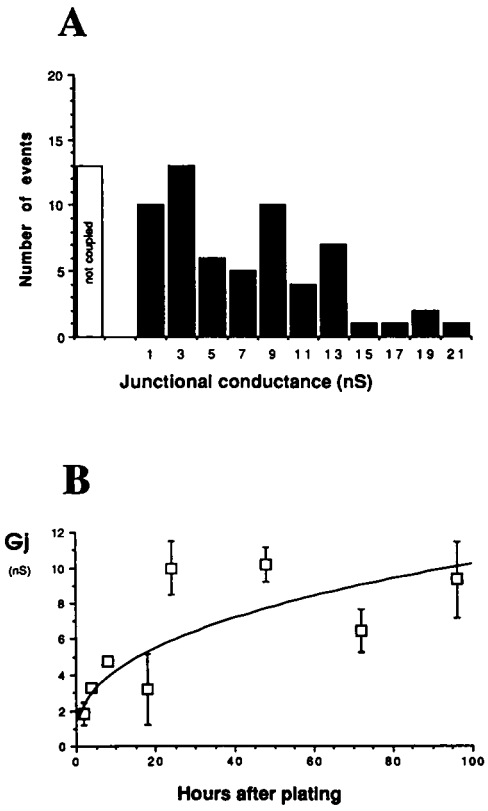

FIGURE 3 Junctional conductance (g_j) measured between connexin32-transfected SKHep1 cell pairs. Measurements as in Fig. 1, *inset*. (*A*) Histogram showing g_j values obtained from 98 cell pairs grouped in bins of 2 nS. Median g_j was 7 nS; 13 pairs were not coupled ($g_j \leq$ nS). (*B*) Distribution of g_j values shown in Fig. 3*A* as a function of time after plating. Smooth curve is linear least squares best fit assuming logarithmic acquisition of g_j over time.

is shown in Fig. 4*A*, in which small junctional currents corresponding to the sizes seen in the parental cells (Fig. 4*A*, small arrows) coexist with much larger unitary junctional currents (Fig. 4*A*, larger, open arrows). Nonjunctional channels (Fig. 4*A*, asterisk) were also apparent as transitions occurring only in one of the cell's current records.

Unitary conductances could also be resolved by reducing g_j between well coupled cell pairs. Halothane (2 mM) applied to pairs of such cells reversibly reduced g_j as illustrated in Fig. 4*B*. During the uncoupling and then during the recovery after halothane was washed away (Fig. 4*B*, upper records), single channel events could be recorded. In Fig. 4*B* (lower traces), the opening of a single gap junction channel during the application of halothane is shown. In this case, the 25–35 pS channels endogenous to the parental cell line are not evident. However, in other experiments it was common to observe both channel types (Figs. 4*A*, 5, and 6). To compare statistically the unitary conductances of gap junction channels, previous studies have used (*a*) dwell time histograms, in which the number of digitized points corresponding to each current amplitude is obtained, or (*b*) event or transition histograms in which the frequencies of the current amplitudes corresponding to opening or closing transitions are displayed. We have analyzed data obtained in pairs of connexin32-transfected SKHep1 cells using both approaches. In Fig. 5 (inset) we illustrate consecutive opening and closing of those channels found between a pair of transfected cells. In this particular case, we used that part of the trace which was digitized and stored previously in a VCR tape. With the Assystant software (version 5.0) data were acquired at 100 Hz and stored in spreadsheet computer files. Digitized data are shown in Fig. 5 (inset). Using this technique we achieved statistical determination of the channel's unitary conductances, as has been done for other cell types (e.g., Veenstra and DeHaan, 1986; Somogyi and Kolb, 1988; Spray et al., 1991*a*). This method of analysis clearly discriminates both smaller and larger unitary conductance events present in our records, but it is most unlikely that the small conductance level would remain detectable after several minutes of analysis due to drift of the basal current level. Nevertheless, application of this method to only a few of the many transitions we obtained in 10 separate experiments clearly shows that the small transitions (1.5–3.5 pA depending on transjunctional driving force) are substantially above our noise level (Fig. 5).

Event histograms were constructed from longer term recordings from cell pairs in which g_j was maintained low or repeatedly reduced and allowed to recover using halothane application. Fig. 6*A–C* illustrate channel reappearance during washout of halothane in three separate experiments. Note that in the first two current traces (Fig. 6*A*), the larger arrows indicate the opening of two large conductance junctional channels (which may or may not be the same channel), and the small arrows (to the right) indicate openings of small conductance junctional channels. Dotted lines indicate conductances of ~130 and 30 pS. The asterisk (Fig. 6, top trace) shows the opening of a nonjunctional channel occurring in the upper trace cell. The other two records (Fig. 6, *B* and *C*) show junctional currents recorded when channel activity was higher. In the second pair of traces, junctional conductance occasionally jumps to a level consistent with the simultaneous opening of two large channels, and in the bottom pair of traces there are brief openings to a level corresponding to that of the large channels.

Junctional conductance in connexin32-transfected SKHep1 cells is strongly voltage dependent, although even at the highest voltages the open probability of these

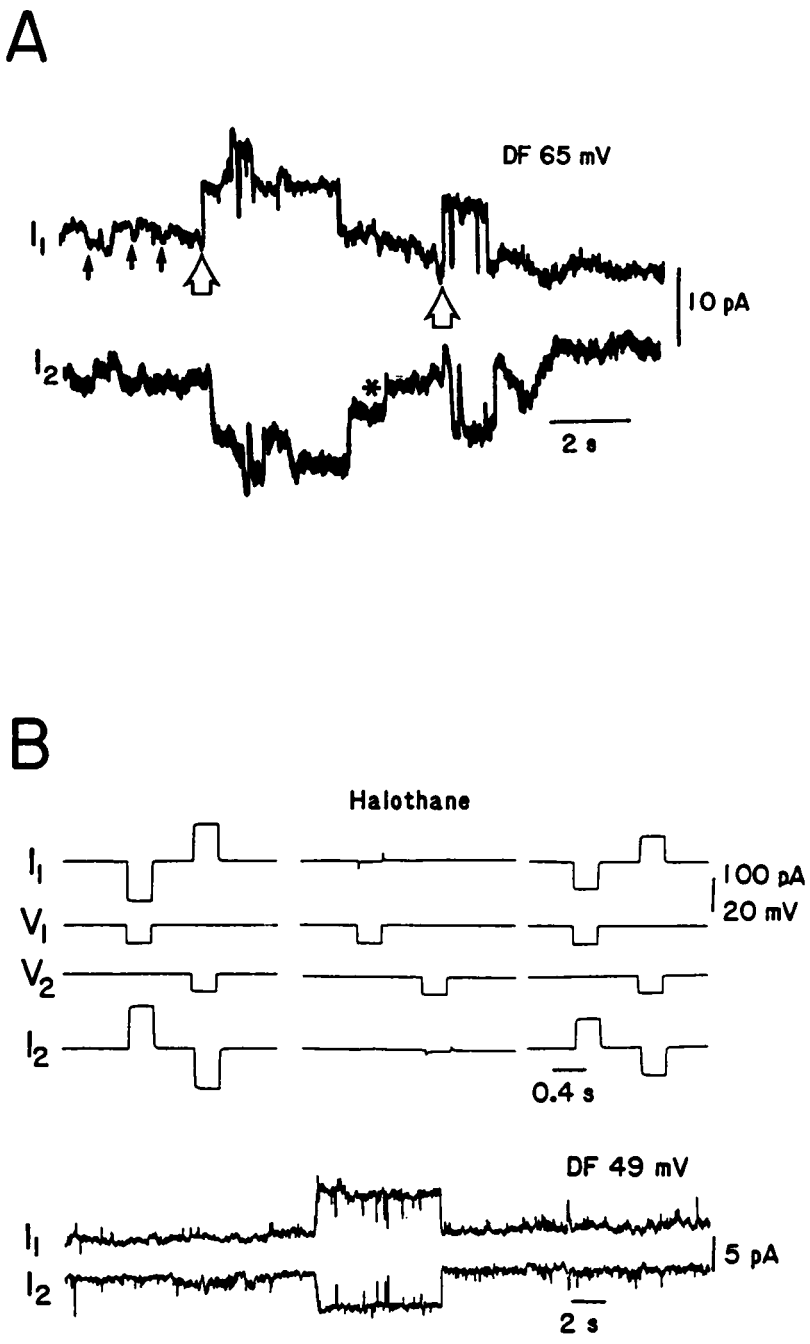

FIGURE 4 Unitary junctional conductance in connexin32-transfected SKHep 1 cells. (*A*) Single channel events in a low conductance cell pair (g_j < 500 pS). Openings of a large channel are indicated by open arrows. The closing of a few channels with much smaller unitary conductance are indicated with small solid arrows. A nonjunctional channel event in the lower cell's current trace (T_2) is indicated by an asterisk. (*B*) Single channel events in transfected SKHep1 cells during uncoupling by exposure to 2 mM halothane. (*Top series of traces*) Halothane exposure (*middle traces*) reversibly reduced junctional conductance (g_j values before, during and after exposure were ~10 nS, <0.1 and 6 nS, respectively). (*Lowest series of traces*) During halothane exposure, unitary openings and closures could be detected (opening is indicated by divergence of the current traces; $g_j = 0$ at the beginning and end of the record).

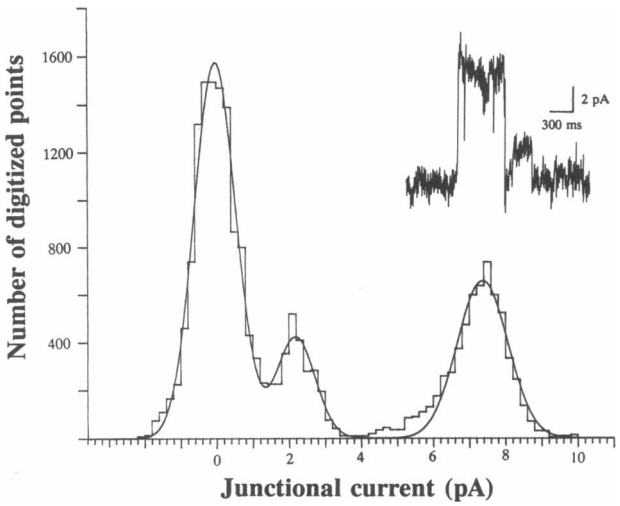

FIGURE 5 Digitization of a record containing small and large unitary events. (*Inset*) Digitized record acquired at 1 kHz from the original trace filtered at 100 Hz. (*Histogram*) Dwell time histogram showing the number of digitized points corresponding to different junctional current values shown in the inset gathered in 0.2 pA bins. The solid line atop data is the best fit to a Gaussian distribution with means of 0.0 ± 0.6, 2.2 ± 0.5, and 7.4 ± 0.7 pA. When divided by the driving force of 61 mV, these current peaks correspond to unitary conductances of 0, 36, and 120 pS, respectively.

channels does not go to zero (Moreno et al., 1991). To determine whether γ_j of these connexin32-containing cells was voltage dependent, junctional current was recorded while voltage steps of increasing magnitude were applied to one of the cells (transjunctional voltage between 15 and 70 mV; note that even at the highest voltages, open channel probability is not reduced to zero). Unitary conductance, calculated from the slope of the relation between transjunctional voltage and unitary current was constant, in this case about 125 pS (Fig. 7). Thus, γ_j is not appreciably voltage dependent in SKHep1 cells transfected with connexin32.

Event histograms corresponding to unitary conductances of channels recorded from nine different pairs of cells are displayed in Fig. 8, including one experiment where heptanol was superfused (Fig. 8 *I*). For each of those experiments, amplitudes of at least 100 open/closed transitions were measured and corresponding conductances were calculated. In several of these experiments (Fig. 8 *A*, *C*, *E*, and *G*) the exclusive or much more frequently occurring event corresponded to a conductance of 100 pS or greater. In other experiments (e.g., Fig. 8 *B*, *D*, *F*,and *I*) the distribution was more bimodal, with numerous small events also being detected.

Data from all the experiments performed with halothane (and the single experiment where heptanol was used as an uncoupling agent) were gathered into a summary event histogram, which shows a distinctly bimodal distribution of γ_j values (Fig. 9). Unitary conductances for each channel are ~30 and 120 pS (curves drawn atop the data represent Gaussian distributions with means of 29 and 114 pS and SD of ±6 and ±25 pS, respectively). The small conductance events represent <15% of the total. This summary histogram demonstrates the presence of two channel sizes, the smaller of which corresponds to the endogenously occuring channel of the parental cell line. The larger events thus correspond to the expression of channels resulting from transfection with connexin32, the major gap junction protein of rat liver.

DISCUSSION

Unitary conductances of gap junction channels

We demonstrate here that a channel with unitary conductance of ~120 pS is expressed after cells are stably transfected with connexin32 cDNA. Previous studies in which currents through individual gap junction channels have been measured indicate that, in various tissues and with different electrolytes within the patch pipettes, γ_j varies greatly, from <20 pS to >200 pS. For several of these preparations, the identities of the connexins that presumably form the channels have been established using antibody or cDNA probes for connexins or their RNAs. Thus, rat heart, astrocytes, and leptomeningeal cells all express connexin43, as does the WB cell line from oval cells of liver (Beyer et al., 1987; Dermietzel et al., 1989, 1991; Spray et al., 1991*a*,*b*). These cell types all express a junctional channel with γ_j about 50–60 pS when potassium glutamate is the major ionic species in the electrodes (Burt and Spray, 1988; Rook et al., 1988; Dermietzel et al., 1991; Spray et al., 1991*b*; channels of similar size have also been recorded between pairs of adult guinea pig myocytes (Rudisuli and Weingart, 1989). Human smooth muscle and endothelial cells and SKHep1 cells transfected with cDNA encoding the human cardiac gap junction protein express a connexin that is 95% identical to rat connexin43; single channel studies on these human cells reveal unitary events that are similar in size to the channels recorded in guinea pig and rat heart (Fishman et al., 1990, 1991; unpublished results with Christ G. A. and V. B. Hatcher). In addition to this channel type, leptomeningeal cells and an oval (WB) cell line exhibit other channel sizes. In leptomeningeal cells, this additional channel type may represent connexin26, which is recognized in this tissue using antibodies (Dermietzel et al., 1989; Spray et al., 1991*b*). In WB cells, the unitary

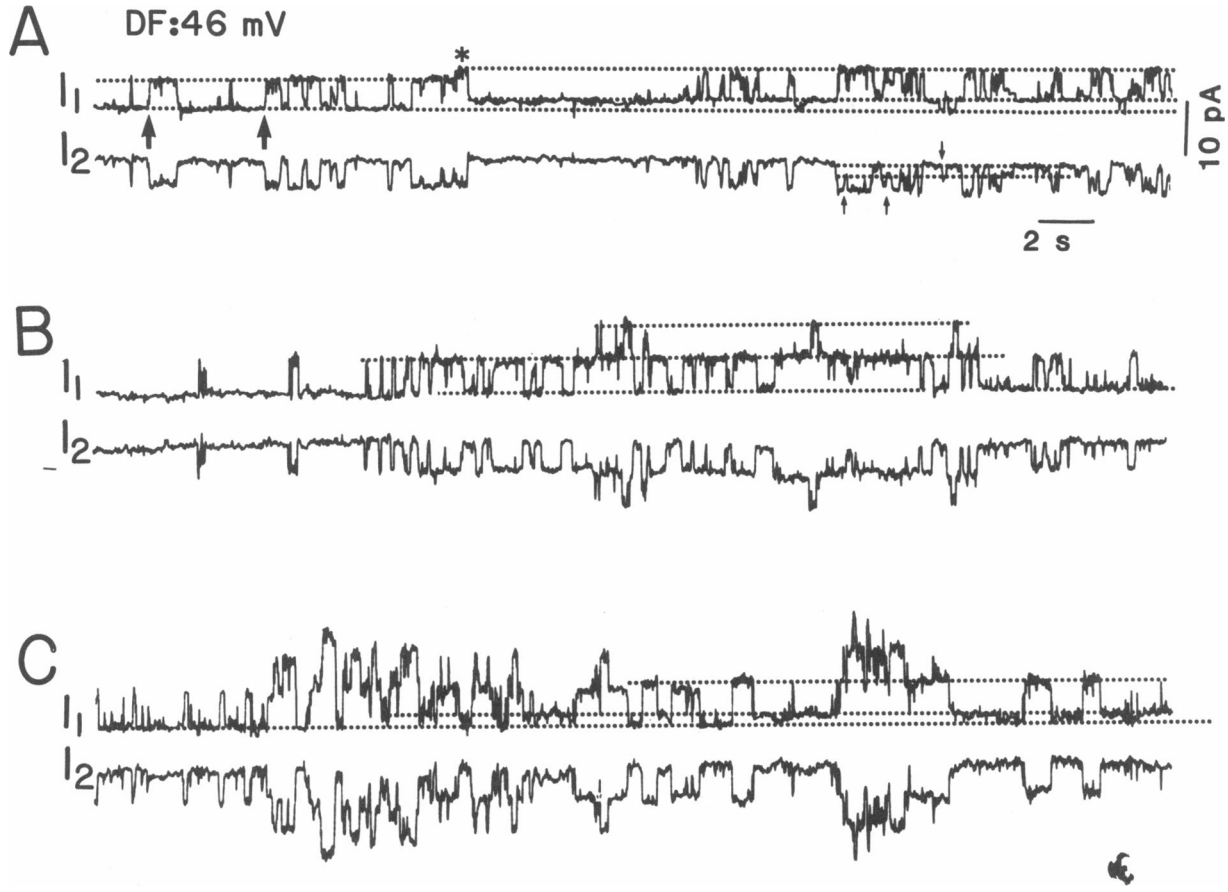

FIGURE 6 Recording of single channel transitions corresponding to connexin32 and endogenous gap junction channels in cell pairs during halothane exposure. (*A*) In this record, activity of large channels is quite low, and two of the openings of these channels to the conductance levels corresponding to the first set of dashed lines are indicated by the large arrows in the i_1 trace. The presence of a nonjunctional channel is indicated by an asterisk, after which I_2 moves to a new stable conductance level. Openings and closings of the small junctional channel are indicated by small arrows to the left in the record in trace I_2, and the levels of the transitions are marked by dotted lines. (*B*) An example in which large channels are more active in the central portion of the record, where there are occasional discrete jumps to a level corresponding to two channels being open simultaneously. (*C*) A record from a cell pair with even more activity, in which there are occasional jumps to a conductance level corresponding to simultaneous opening of three large channels. Lower pair of dotted lines in I^1 record indicate levels corresponding to activity of a nonjunctional channel. Driving force is 46 mV in all records.

conductance of the apparently coexpressed junctional channel is ~20–30 pS, and antibody prepared against an isolated brain gap junction-associated antigen shows punctate appositional immunostaining (Spray et al., 1991*a*).

Connexin32 is expressed in hepatocytes and between cells of acinar tissue in pancreas and salivary and lacrimal glands (e.g., Paul, 1986; Hertzberg et al., 1988). Single channel studies of isolated gap junction membranes incorporated into lipid bilayers indicate that there may be multiple unitary conductances, a prominent one of which is ~120–200 pS, depending on the ionic composition of the internal solution (Spray et al., 1986; Young et al., 1987; Campos de Carvalho et al., 1991; Neyton and Trautmann, 1985; Somogyi and Kolb, 1988). The data presented in this paper indicate that this larger channel is formed by connexin32. These tissues also express connexin26, and the smaller channel sizes in these tissues are possibly formed of this connexin, alone or in combination with connexin32. Expression studies on *Xenopus* oocytes indicate that heterotypic connexin26-connexin32 channels can also form (Barrio et al., 1991), and heteromeric hemichannels containing both types of subunits may also exist (Traub et al., 1989).

The cells used in this study, the human hepatoma SKHep1 line, infrequently express a small number of gap junction channels with a unitary conductance of ~30 pS. Studies using antibodies against specific domains of known rat connexins have failed to reveal the identity of the endogenous connexin. Immunofluores-

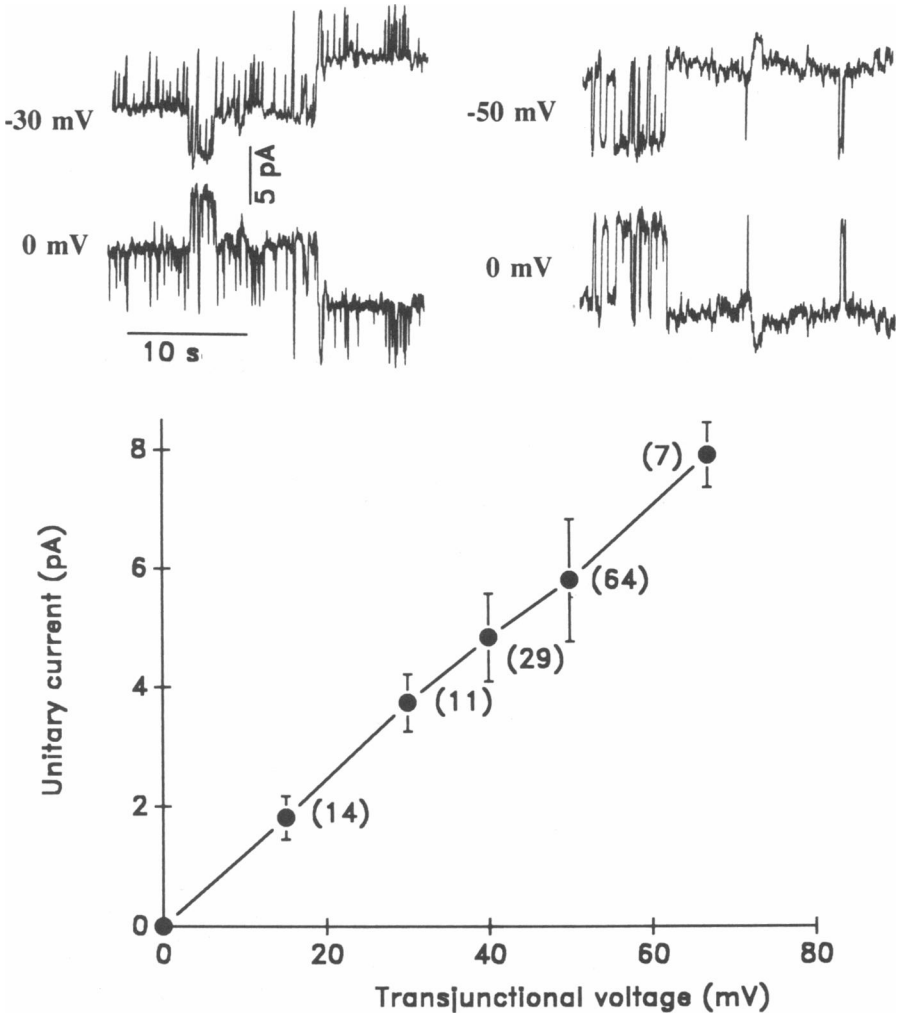

FIGURE 7 Unitary conductance of connexin32 channels is not voltage dependent. When the driving force applied across the junction (transjunctional voltage, V_j) was increased from 0 to 70 mV, the unitary current increased linearly. The slope of the plot corresponds to ~125 pS; representative events at two holding potentials are shown above plot. The number of events measured for each driving force is indicated in parentheses.

cence using antibodies directed against rat connexins 26, 32, or 43 and against a brain gap junction-associated antigen did not reveal discrete intercellular staining; and hybridization studies using cDNA probes for rat connexin32 and human connexin43 showed no homologous RNA (Eghbali et al., 1990; Fishman et al., 1990). It remains possible that the rat antibody probes did not recognize human connexin homologues due to differing amino acid sequences, or that the low abundance of connexins in SKHep1 cells prevented their detection with antibody or cDNA probes. However, these data are also consistent with the possibility that the 30 pS channel represents a novel connexin, which has not yet been identified biochemically. A gap junction channel of similar unitary conductance has been found between rat heart fibroblasts (Rook et al., 1989), a pancreatic tumor cell line (Somogyi and Kolb, 1988), a rat smooth muscle cell line (Moore et al., 1991), and in cell line derived from "oval" cells in rat liver (WB cells: Spray et al., 1991*a*), again without positive identification of corresponding connexin type.

Bimodality of unitary conductances and lack of conductance substates

Our demonstration that stable transfection of a cell line with connexin32 cDNA leads to the appearance of large gap junction channels raises questions about interaction of the exogenous protein with the occasionally observed endogenous one, and also allows us to comment on

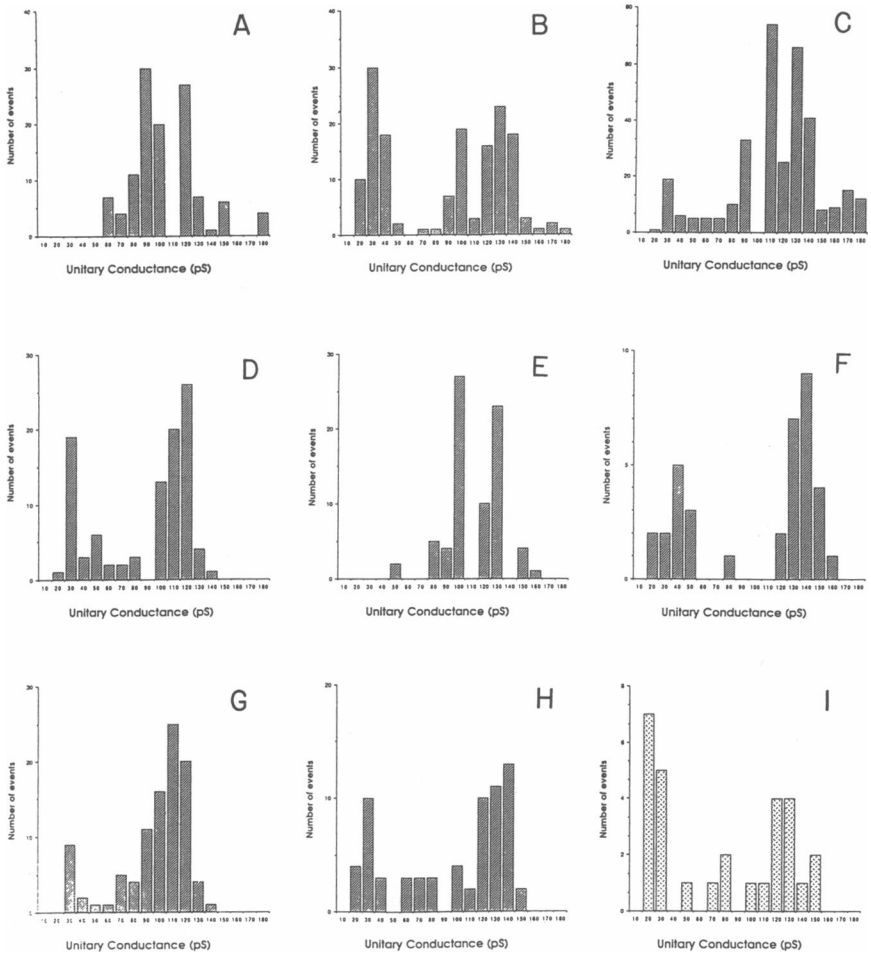

FIGURE 8 Transition histograms of unitary conductances recorded in nine experiments where at least 100 single channel events were clearly measureable after reducing γ_j by exposure to 2 mM halothane (*A–H*) or 0.5 mM heptanol (*I*). Individual histograms indicate variability in amplitude of the largest events and in relative frequency of events of the two sizes. Note that the endogenous channel is present in most, but not all histograms.

speculations regarding substates of gap junction channels. In other systems heterologous channels can be formed after cells expressing homogeneous hemichannels (or connexons) are paired. *Xenopus* oocytes (which express an endogenous voltage dependent connexin) have been paired with oocytes injected with mRNA encoding less voltage dependent connexins (Swenson et al., 1989; Werner et al., 1989). The heterlogous pairs displayed asymmetrical voltage dependence, in which the steady-state junctional conductance in response to transjunctional voltages was similar to that predicted to arise from combination of connexons with and without voltage dependent gates. Cardiac fibroblasts, which display voltage dependent g_j and γ_j in the range of 20 pS, have been paired with cardiac myocytes, which are less voltage dependent and where γ_j is ~50 pS (Rook et al., 1989). Resulting pairs displayed asymmetric voltage dependence, and unitary conductances of the heterologous pairs were as expected from the series alignment of hemichannels with the distinct unitary conductances, so that combining a connexon from a 20 pS channel with one from a 50 pS channel gave a channel with unitary conductance of ~30 pS.

Our data indicate that the functional pairing of the endogenous and introduced hemichannels does not occur to an appreciable extent. The size and variance of γ_j for the small channel in transfectants (Fig. 9). is quite similar to that obtained in the parental cell line (Fig. 2). Heterologous pairings of these hemichannels would be expected to yield channels with γ_j of ~50 pS, whereas in this conductance range there were actually the fewest events recorded (Fig. 9). Exogenous expression studies in *Xenopus* oocytes have also suggested that certain connexins will mate with one another (rat connexin43-

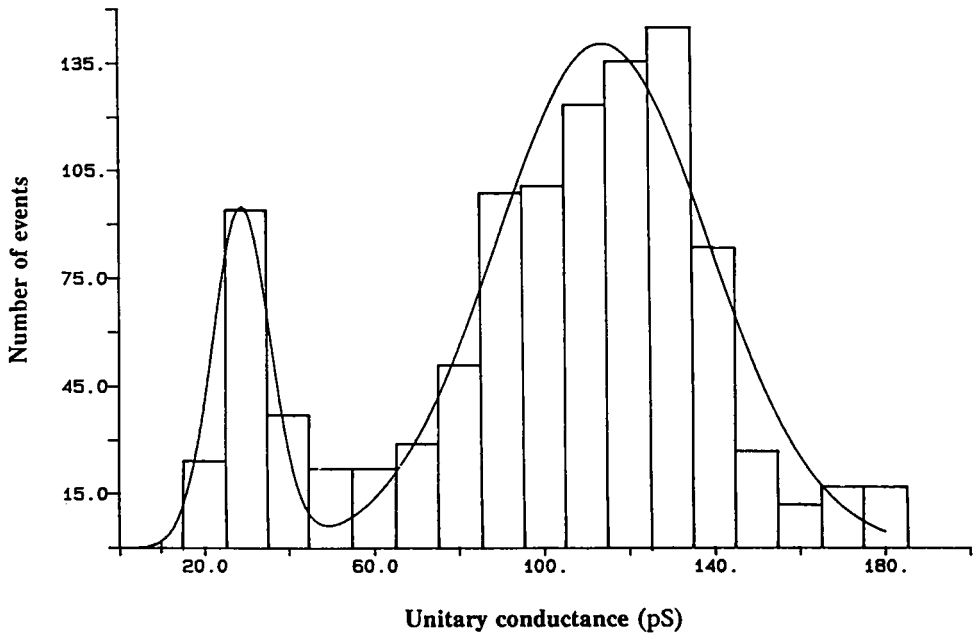

FIGURE 9 Histogram summarizing the total γ_j values of the individual experiments shown in Fig. 8. The resulting histogram shows two dominant γ_j values of 30 ± 9 pS, which corresponds to 15% of the total, and is similar to that seen in the parental line (cf Fig. 2), and 123 ± 22 pS, which was much more frequently observed. This larger channel is presumably formed of connexin32.

Xenopus connexin38), whereas other do not (rat connexin32-*X.* connexin38) (Swenson et al., 1988).

Contribution of more than one connexin protein to the connexon, as has been postulated for connexins 26 and 32 in liver (Traub et al., 1989) is as yet without physiological demonstration. A model in which endogenous subunits could combine randomly would be expected to result in multiple conductance states, which is not supported by the distinct bimodality of the event histogram from transfected cells (Fig. 9). Although the large variance in the conductance of the larger channel might be interpreted as indicating heterogeneity in channel composition, it more likely represents interexperimental variability caused by instrumentation noise or small tip potentials arising from seal formation in the current record so that driving force was over- or underestimated.

As is mentioned above, numerous γ_j conductance values have been measured for gap junctions between various cell types, and in several preparations, channels of more than one size have been recorded. In some reports, it has been speculated that these distinct conductances represent conductance substates of individual channels (Veenstra and DeHaan, 1986; Somogyi and Kolb, 1989), although no study has demonstrated that exposure to any gating treatment changes the relative distribution of substate events. In the experiments presented here, we have recorded large and small junctional currents in cases where g_j was normally low or was reduced to low values by halothane or heptanol exposure. Our finding was that in response to these agents the macroscopic conductance could be increased or decreased without converting the channels between large or small unitary conductances. Furthermore, γ_j was not altered by voltage. Thus, for connexin32 gap junction channels expressed in these cells and for the gating treatments thus far explored, there appear to be no long-lived intermediary conductance states. Similar conclusions were derived from experiments on cardiac myocytes and WB cells after exposure to various uncoupling agents (see Spray and Burt, 1990; Spray et al., 1991*a*.

Why are there gap junction channels with different unitary conductance?

One answer might be in permitting cells of different origin or at different developmental stages to restrict or allow passage of larger molecular species. For example, the channel with the smallest γ_j (30 pS) is expressed between all transformed hepatoma lines that we have examined (unpublished observations, Moreno, A. P., M. Waltzman, and D. C. Spray). There are suggestions that gap junctions between some invertebrate species might

display higher permeability to larger molecules than those of vertebrates (Spray et al., 1990*b*; Michalke and Loewenstein, 1971), although there may be exceptions (e.g., Bodmer et al., 1988). However, connexins 43 and 32, for which γ_j differs by a factor of 2–3 in the same cell type (cf Fishman et al., 1990 and present paper), both allow passage of Lucifer Yellow (dye passage was used in both studies as a selection criterion and hundreds of intracellular injections on cell pairs with comparable ranges of g_j values have revealed no striking difference in rate or extent of dye transfer). Moreover, fibroblasts from heart rapidly exchange Lucifer Yellow between themselves and between cardiac myocytes (Burt et al., 1981) indicating that even the 20–30 pS channel permits flux of this large molecule. Presumably, the application of more quantitative techniques will be necessary to resolve this issue.

Another possibility is that differences in unitary conductance have little to do with diverse gap junction channel functions in different tissues. In excitable tissues, where intercellular flow of current is the function that the gap junction channel serves, the safety factor for impulse propagation is so large that functional conductance alterations are unlikely to play important roles except under grossly pathological conditions (see Spray and Burt, 1990; Spray et al., 1990*a*). Thus, unitary conductance may simply be an epiphenomenon, which nonetheless signifies the specific connexins forming the junctional channel.

More relevant to the issue of why different channel types are expressed in different cell types may be gating properties. It is now clear that sensitivity of these junctional channels to acidification and voltage varies according to connexin type, as does the capacity for phosphorylation and consequences for junctional conductance (see Spray and Bennett, 1985; Spray and Burt, 1990). Thus changes from expression of one connexin to another occurring during development or differentiation (see Dermietzel et al., 1989; Gimlich et al., 1990; Beyer, 1990) could confer upon the tissue differential sensitivity to second messengers and other factors and thereby endow junctions formed of different connexins distinct sets of regulatory mechanisms.

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