Gap Junction Gating Sensitivity to Physiological Internal Calcium Regardless of pH in Novikoff Hepatoma Cells

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ABSTRACT Gap junction conductance (G_j) and channel gating sensitivity to voltage, Ca^{2+}, H^+, and heptanol were studied by double whole-cell clamp in Novikoff hepatoma cell pairs. Channel gating was observed at transjunctional voltages (V_j) > ±50 mV. The cells readily uncoupled with 1 mM 1-heptanol. With heptanol, single (gap junctional) channel events with unitary conductances (γ_j) of 46 and 97 pS were detected. Both Ca^{2+}-loading (EGTA·Ca) and acidifying (100% CO_2) solutions caused uncoupling. However, CO_2 was effective when $Ca^{2+}{}_i$ was buffered with EGTA (a H^+-sensitive Ca-buffer) but not with BAPTA (1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid) (a H^+-insensitive Ca-buffer), suggesting a Ca^{2+}-mediated H^+ effect on gap junctions. This was tested by monitoring the G_j decay at different pCa_i values (9, 6.9, 6.3, 6, and 5.5; 1 mM BAPTA) and pH_i values (7.2 or 6.1, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 2-(*N*-morpholino)ethansulphonic acid, respectively). With $pCa_i \geq 6.9$ (pH 7.2 or 6.1), G_j decreased to 10–70% of initial values in ~40 min, following single exponential decays (τ = ~28 min). With pCa_i 6–6.3 (pH 7.2 or 6.1), G_j decreased to 10–25% of initial values in 15 min (τ = ~5 min); the Student *t* gave a $P = 0.0178$. With pCa 5.5 the cells uncoupled in less than 1 min (τ = ~20 s). Low pH_i affected neither time course nor shape of G_j decay at any pCa_i tested. The data indicate that these gap junctions are sensitive to $[Ca^{2+}]_i$ in the physiological range (≤500 nM) and that low pH_i, without an increase in $[Ca^{2+}]_i$, neither decreases G_j nor increases channel sensitivity to Ca^{2+}.

INTRODUCTION

Direct cell-to-cell communication is mediated by membrane channels accessible to small cytosolic molecules (reviewed in Loewenstein, 1981; Peracchia, 1980, 1987; Délèze, 1987; Ramón and Rivera, 1987). Each channel is formed by the extracellular interaction of two oligomers (connexons), each composed of six identical proteins (connexins) radially arranged around the pore.

Several groups have succeeded in monitoring single channel activity in intact cells (Veenstra and DeHaan, 1986, 1988; Burt and Spray, 1988; Rook et al., 1988; Rüdisüli and Weingart, 1991; Lal and Arnsdrof, 1992; Anumonwo et al., 1992). Channel conductance (γ_j) varies depending on the preparation and the gap junctional protein (connexin, Cx) expressed. In cells expressing Cx43, γ_j usually ranges from 45 to 64 pS, but smaller (25–30 pS) and larger (75 and 160 pS) conductances have also been reported (Veenstra and DeHaan, 1988; Veenstra, 1991a).

The channels are equipped with gates that close in response to voltage and certain cytosolic changes. Channel gating and consequential functional uncoupling between neighboring cells is generally believed to be primarily a safety mechanism for protecting cells from injured neighbors (Délèze, 1965, 1970). However, evidence for a channel gating sensitivity ranging from low micromolar to high nanomolar $[Ca^{2+}]_i$ (Noma and Tsuboi, 1987; Veenstra and DeHaan, 1988; Peracchia, 1990a; Lazrak and Peracchia, 1992) suggests that gap junction permeability may change during normal cellular activities. This indicates that the gap junction channel is likely to be an active participant of physiological regulatory mechanisms.

Several uncoupling agents such as Ca^{2+}, H^+, voltage, cAMP, general anesthetics, unsaturated fatty acids, phosphorylation by kinase C, etc., have been identified (Spray et al., 1979, 1991; Loewenstein, 1981; Peracchia, 1980, 1987; Rose and Rick, 1978; Délèze and Hervé, 1983; Délèze, 1987; Obaid et al., 1983; Ramón and Rivera, 1987; Verselis and Bargiello, 1991), but the gating mechanisms are still unclear. Most cells are sensitive to transjunctional voltage (V_j), $Ca^{2+}{}_i$, $H^+{}_i$ and general anesthetics, while a few are sensitive to cAMP, fatty acids, kinase C and changes in membrane potential (V_{i-o}). Although H^+ and Ca^{2+} affect gating in all the cells tested, there is no proof that they act on the channel gates independently from each other.

The role of Ca^{2+} in coupling regulation, first proposed by Loewenstein (1966) for gland cells of insect embryos, following an earlier observation in cardiac myocytes (Délèze, 1965), was later confirmed with simultaneous monitoring of $[Ca^{2+}]_i$ and electrical coupling (Rose and Loewenstein, 1976), and through Ca^{2+}-injection experiments (De Mello, 1975; Délèze and Loewenstein, 1976). The role of H^+ was first proposed by Turin and Warner (1977, 1980) in amphibian embryonic cells simultaneously monitored for pH_i and electrical coupling. Spray et al. (1981a) supported the H^+ hypothesis for gap junction regulation by showing that channel conductance of amphibian embryonic cells is a sensitive function of pH_i; this lead them to propose that junctional conductance changes are a direct effect of protons on channel protein. However, a variety of conflicting data have been reported (Ramón and Rivera, 1987; Peracchia, 1987, 1991; Pressler, 1989). Although there is evidence that Ca^{2+}

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affects the channels independently from H^+, it is unclear whether H^+ acts independently from Ca^{2+} (Peracchia, 1990a).

To define in detail gap junction gating sensitivity to Ca^{2+} and H^+, the present study has tested the effects of internal solutions well buffered to various pH values and $[Ca^{2+}]$ values on the junctional conductance (G_j) of Novikoff hepatoma cell-pairs, and the effects of acidification on G_j of cells internally buffered for Ca^{2+} with either EGTA (a H^+-sensitive Ca-buffer) or BAPTA (a fast and H^+-insensitive Ca-buffer). Data on voltage dependence and single channel conductance were also obtained. A preliminary account of this study has been published (Lazrak and Peracchia, 1992).

METHODS

Cell culture and medium

The Novikoff hepatoma cell line N1-S1-67 (CRL 1604, American Type Culture Collection, Rockville, MD) was kindly provided by Dr. Ross G. Johnson (University of Minnesota, St. Paul, MN). These cells grow in suspension in a sealed Erlenmeyer flask at 37°C and do not need to be provided with a 5% CO_2 atmosphere, as monolayer cultures, because they generate the CO_2 needed for their development.

Cell suspensions were grown in S-210 medium (GIBCO BRL, Life Technol. Inc., Gaithersburg, MD), supplemented with (in millimolar): $NaHCO_3$ 50, Pluronic FC 68 (Sigma Chemical Co., St. Louis, MO; added to prevent cell/glass adhesion), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 10 (pH 7.2) and 1% penicillin-streptomycin. The medium was filtered, sterilized, and stored at 4°C. Just before use, newborn calf serum (GIBCO) and glutamine (GIBCO or Sigma; diluted from 200 mM stocks) were added to the medium to final concentrations of 10% and 1%, respectively.

Prior to the renewal of the cell culture, the cell clumps were dissociated into individual cells by a 5-min trypsinization at 37°C (2.5% trypsin; Sigma). The cells in suspension were counted and diluted to 3×10^5 to 4×10^4 (Johnson et al., 1974). For best results and yield stability the cells were diluted daily.

Experimental solutions

Before each experiment, 20- to 24-h-old cells were suspended in a standard external salt solution (SES, Table 1) at 22 ± 1.5°C. In an attempt to increase the intracellular Ca^{2+} by external treatments, cell pairs were superfused with an EGTA·Ca solution (Table 1), a modified SES containing 0.5 mM EGTA and 2.3 mM $CaCl_2$ ($[Ca^{2+}]_o$ = 1.8 mM). In these conditions the EGTA·Ca complex is electroneutral and diffuses into the cells, where it dissociates causing an increase in $[Ca^{2+}]_i$ (Mullins and Brinley, 1975). For CO_2-acidification experiments cell pairs were perfused with SES bubbled with 100% CO_2; the cells were whole-cell clamped with pipettes containing standard internal solution (SIS; Table 2) buffered for Ca^{2+} with 0.5 mM of either EGTA (SIS-1, Table 2) or BAPTA (1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; SIS-2, Table 2).

For testing the effects of different pH_i and pCa_i, the internal solution was buffered to pH 7.2 or 6.1 with HEPES (pKa = 7.5 at 25°C) or MES (2-[*N*-morpholino]ethansulphonic acid, pKa = 6.1 at 25°C), respectively, and $[Ca^{2+}]_i$ was buffered to pCa 9, 6.9, 6.3, 6, or 5.5 with 1 mM BAPTA (Table 2). Ca^{2+} buffers (solutions A–G, Table 2) were calculated using the following absolute dissociation constants: BAPTA-Ca K_D = 107.15 nM, BAPTA-H K_{D1} = 436.5 nM, BAPTA-H K_{D2} = 339 nM, and BAPTA-Mg K_D = 0.01 M, at 22°C and 100 mM ionic strength.

TABLE 1 External solutions

	SES	EGTA-Ca
	mM; pH = 7.2	
$CaCl_2$	1.8	2.3
EGTA		0.5
HEPES	10	10
$MgCl_2$	2	2
KCl	2.7	2.7
NaCl	145	145
Glucose	5.5	5.5

Electrophysiology

Cell suspensions were transferred onto a polystyrene dish (LUX 5221; Nunc Inc., Naperville, IL), and the cells were allowed to settle at the bottom of the dish for 30 min at 37°C before the dish was mounted on the stage of a Diaphot inverted microscope (Nikon Inc., Garden City, NY) for electrophysiology. The cells were continuously superfused at 0.5 ml/min (T = 22 ± 1.5°C).

All the experiments were performed using the standard double whole-cell patch-clamp procedure (White et al., 1985; Neyton and Trautmann, 1985; Weingart, 1986). Briefly, patch pipettes were made from capillary tubing (Kimax-51; Kimble Glass Inc., Langhorn, PA) with a vertical puller (700 B; David Kopf Instr., Tujunga, CA). For single channel recording the pipettes were coated with Silgard (Dow Corning Corp., Midland, MI) and fire-polished thereafter, while in all the other experiments the pipettes were pulled just before use. The resistance of pipettes filled with SIS-1 or SIS-2 (Table 2) ranged from 3 to 6 MΩ.

Each pipette was connected to a separate patch clamp amplifier (EPC-7; List Electronics, distributed by Medical Systems Corp., Greenvale, NY). The pipettes were lowered to the cells by two hydraulic micromanipulators (Narishige, distributed by Medical Systems Corp., Greenvale, NY) and the steps involved in the formation of the giga-seals and whole-cell configuration were followed on an oscilloscope screen (Model V-355; Hitachi Denshi America, Ltd., Woodbury, NY). The head stage of each amplifier was connected to the cell interior through a series resistance (R_{p1} and R_{p2}). The pipette resistance (R_{p1} and R_{p2}), increased two to four times following membrane rupture and establishment of whole-cell configuration (10–20 MΩ); 70–90% of this resistance was compensated by the amplifiers. The initial resistance was less than 10% of the parallel combination of plasma membrane ($R_m \approx 1$ GΩ) and seal ($R_s \geq 1$ GΩ) resistances.

Pulse generation and data acquisition were performed by means of an IBM-AT compatible computer equipped with Pclamp software (Axons instruments, Inc., Foster City, CA) and A/D–D/A interface (Labmaster TL-1; Axons instruments, Inc.). During acquisition the current traces were filtered at 3 kHz, and current-voltage (I-V) curves were plotted (Laserjet III; Hewlett-Packard, Rockville, MD). Current traces displaying single channel events were filtered at 3 kHz during acquisition, and some traces were digitized at 100–200 Hz during analysis. Amplitude of transitions in junctional current (I_j) were measured, counted, and plotted as current-amplitude histograms. The results from several records (as those shown in Fig. 3) were summed, appended to the same ASCII file, and plotted as a histogram using Fetchan and Pstat software (Pclamp, Axon Instruments).

For studying the electrical properties of the junctional membrane, the cells were initially voltage-clamped to the same holding potential (V_H = −40 mV), so that no junctional current would flow at rest (I_j = 0 pA). A V_j gradient was created by imposing a voltage step (V_1) to cell 1, while maintaining V_2 at −40 mV; thus, $V_j = V_1$. The negative feedback current (I_2), injected by the clamp amplifier in cell 2 for maintaining V_2 constant, was used for calculating G_j, as it is identical in magnitude to the junctional current (I_j), but opposite in sign ($I_j = -I_2$); using the Ohm's law: $G_j = I_j/V_j$.

RESULTS

Novikoff hepatoma cell culture

This cell line was established in 1959 by Swim (unpublished data) from the ascitic fluid of a Novikoff hepatoma, a chemi-

TABLE 2 Internal solutions

	SIS-1	SIS-2	A	B	C	D	E	F	G
					mM				
$CaCl_2$			0.5	0.3	0.81	0.6	0.9	0.75	0.98
BAPTA		0.5	1	1	1	1	1	1	1
EGTA	0.5								
HEPES	10	10	10		10		10		10
MES				10		10		10	
$MgCl_2$	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
KCl	145	145	145	145	145	145	145	145	145
NaCl	6	6	6	6	6	6	6	6	6
ATP	5	3	3	3	3	3	3	3	3
pCa	9	9	6.9	6.9	6.3	6.3	6	6	5.5
pH	7.2	7.2	7.2	6.1	7.2	6.1	7.2	6.1	7.2

cally induced rat liver tumor (Novikoff, 1957) and later adapted for culture by Plageman and Swim (1966). In spite of their name, these cells are not believed to originate from hepatocytes, but rather from Kupffer or endothelial cells (Meyer et al., 1991). When cultured at 37°C, the cell population doubles in about 12 h, following an initial exponential growth that reaches a logarithmic phase in 12–14 h. During growth, the cells form clumps of various size, but there is no need of protease treatment for obtaining cell pairs, as 10–20% of the clumps are formed of two cells of equal size. In SES (Table 1) the cells are 12–13 μm in diameter.

Basic electrical properties of Novikoff hepatoma cells

The Novikoff hepatoma cell line is suitable for studying the junctional membrane properties by double whole-cell clamp for a number of reasons: the small cell size (12–13 μm), for which fast exchange between pipette solution and cell interior takes place; the high resistance of the plasma membrane (1.1 ± 0.4 GΩ), for which series resistances cause only small errors in G_j calculations (less than 10%); the simple culturing procedure; and the high yield of cell pair formation. Membrane potential (V_m), measured in current-clamp mode in individual cells bathed in SES just after the establishment of whole-cell configuration, averaged -50 ± 13.5 mV ($n = 13$). Nonjunctional membrane resistance (R_m), determined in single cell in voltage clamp mode, ranged from 0.75 to 1.2 GΩ. Membrane capacitance, determined from the amplitude of the capacitive current, was 7.35 ± 2.55 pF ($n = 13$). G_j ranged from less than 1 to 200 nS, with a mean value of 53.55 ± 17.62 nS ($n = 84$), and was relatively insensitive to V_m (Fig. 1 *A*).

Voltage dependence of the junctional membrane

For studying voltage dependence of junctional conductance, voltage steps of 10 mV ($\pm$100 mV maximum) and 0.4–1-s duration were applied to either cell of the pair, while maintaining the other at holding potential ($V_H = -40$ mV). For limiting the error induced by the remaining uncompensated series resistances, the V_j-I_j relationship was analyzed only in cell pairs coupled with $G_j \leq 8$ nS. In these cell pairs, at V_j values greater than $\pm$ 50 mV two symmetrical levels of I_j were observed (Fig. 1 *B*): an instantaneous ($I_{j\,inst}$) and a steady state ($I_{j\,ss}$). The voltage dependence of G_j was determined by measuring both I_j levels and plotting them with respect to V_j (Fig. 1 *C*). $I_{j\,inst}$, measured during the first 5–10 ms after the application of the voltage pulse, increased linearly as a function of V_j (Fig. 1, *B* and *C*). In contrast, $I_{j\,ss}$, measured at the end of each pulse, was linear only at V_j values of $\pm$ 50 mV, and started decreasing (deviating from linearity) at higher V_j values (Fig. 1, *B* and *C*). The slope of the curves (Fig. 1 *C*) is the junctional conductance G_j; in this example, $G_{j\,inst}$ was 5.77 ± 0.19 nS (mean $\pm$ SE, *open squares*) and $G_{j\,ss}$ 5.40 ± 0.22 nS (mean $\pm$ SE, *filled squares*) in the $\pm$ 50 mV range; the latter decreased to 2.8 ± 0.4 nS (mean $\pm$ SE) when V_j reached $\pm$ 100 mV.

To illustrate the relationship between $G_{j\,ss}$ and V_j, the normalized G_j ($n = 7$) was plotted with respect to V_j (Fig. 1 *D*). Normalized G_j values approached 1 at V_j values as high as $\pm$ 60 mV and decreased to a minimum of 0.44 at $\pm$ 100 mV. The curve was fitted to a two-state Boltzmann's distribution of the form:

$$(G_{j\,ss} - G_{j\,min})/(G_{j\,max} - G_{j\,ss}) = \exp[-A(V_j - V_0)],$$

where V_0 is the V_j value at which the voltage-sensitive conductance is half of the maximal value, $G_{j\,ss}$ is the experimentally derived normalized G_j value, $G_{j\,max}$ is the theoretical maximum normalized G_j, $G_{j\,min}$ is the theoretical minimum normalized G_j, and A is a constant expressing the strength of the interaction of the channel with V_j (Harris et al., 1981; Spray et al., 1981b). The constant A is equivalent to nq/kT, where n is the equivalent number of electron charges q that act as the gating mechanism by sensing changes in V_j, while k and T represent Boltzmann's constant and temperature (K). For this plot, the Boltzman's parameters (Fig. 1 *C*) were: $G_{j\,max} = 0.987$, $G_{j\,min} = 0.42$, $n = 1.7$ ($A = 0.067$), and $V_0 = -70$ mV, for negative values, and $V_0 = +72.5$ mV, for positive values. The cells used for the Boltzmann fit had a G_j of 5.344 ± 2.61 (mean $\pm$ SD, $n = 7$) at $V_j = 20$ mV.

Effects of heptanol on junctional conductance

Superfusions with 1 mM 1-heptanol in SES were used to reduce the junctional conductance to a sufficiently low level for detecting single channel activity in gap junctions. The uncoupling effect of heptanol is shown in Fig. 2. Junctional

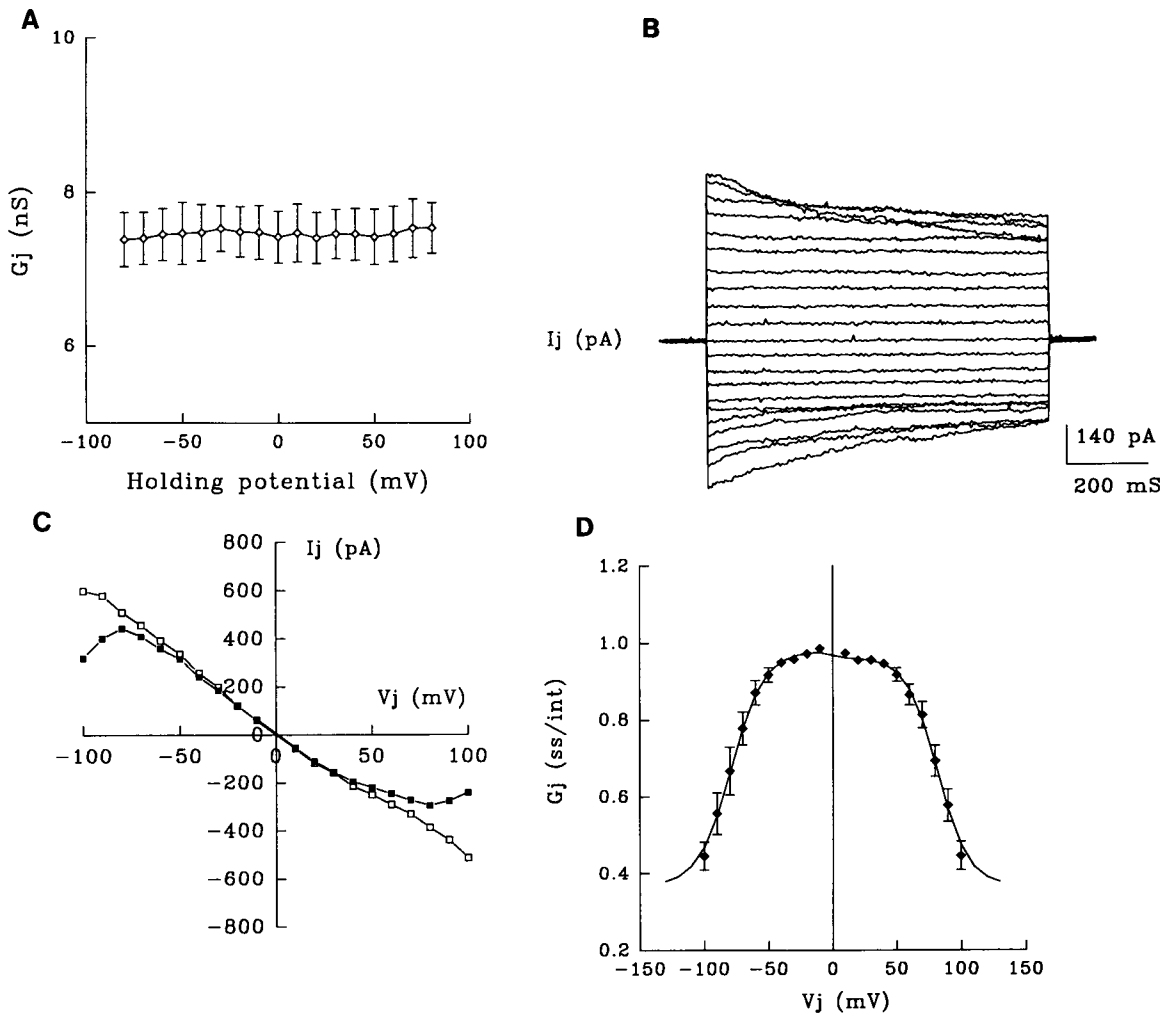

FIGURE 1 (*A*) Relationship between junctional conductance ($G_j \approx 7.382 \pm 0.790$ nS; mean ± SD, $n = 5$) and holding potential (V_H). Note that G_j is insensitive to membrane potential, at least in the ± 80 mV range. The junctional current was determined by imposing to either cell a voltage step of 10 mV and 650-ms duration. (*B*) Illustration of a family of I_j traces generated by pulsing one cell of the pair every 10 s to different V_j values (±100 mV, 820 ms, 10 mV increments), from a holding potential (V_H) of −40 mV. With V_j values ≤ ± 50 mV, I_j is relatively constant throughout the duration of the pulse. In contrast, with V_j values ≥ ± 50 mV, I_j undergoes a time- and voltage-dependent relaxation from its largest instantaneous value ($I_{j\,inst}$) toward a lower steady state value ($I_{j\,ss}$). (*C*) Current-voltage relationship for both instantaneous (*hollow squares*) and steady state (*filled squares*) junctional currents. The I_j/V_j relationship is linear and has a slope of 5.77 ± 0.19 nS (mean ± SE) for $I_{j\,inst}$ and 5.40 ± 0.22 (mean ± SE) for $I_{j\,ss}$ over the ± 50 mV V_j range, while it deviates from linearity and starts decreasing at greater V_j values. Similar results were obtained in six other cell pairs in which G_j ranged from less than 1 to ~8 nS. (*D*) Boltzmann fit of normalized G_j averaged from experiments similar to that shown in *B*. The cells had a G_j of 5.344 ± 2.61 (mean ± SD, $n = 7$) at $V_j = 20$ mV. G_j decreases as a function of V_j, reaching its half maximum value at transjunctional voltages ($V_j = V_0$). The parameters of the Boltzmann fit are $G_{max} = 0.987$, $G_{min} = 0.42$, $n = 1.70$, $V_0 = +72.5$ mV for positive voltages, and $V_0 = -70$ mV for negative voltages.

currents were visualized by applying a voltage pulse of +50 mV (820-ms duration) every 10 s. Heptanol caused a drastic decrease in I_j (Fig. 2 *A*), as G_j decreased from 26.6 nS to less than 0.1 nS (Fig. 2 *B*) within 2.5 min. The uncoupling was almost completely reversible upon return to standard saline, such that I_j and G_j recovered to ~90% of their initial values in ~10 min.

Single channel activity

Single channel events were clearly observed when transjunctional voltage steps of 100 mV (2–4-s duration) were applied to cell pairs uncoupled with 1 mM 1-heptanol (G_j values ≤ 0.5 nS), but single channel events were also seen with transjunctional voltage lower than 100 mV. Channel activity is manifested by steplike current transitions that occur simultaneously in both traces and have the same amplitude but opposite polarity (Fig. 3, *A* and *B*). Different records showed either one (Fig. 3 *A*) or more (Fig. 3 *B*) gap junction channels. During acquisition the data were sampled at 3 kHz. Records exhibiting discrete single current steps, as in Fig. 3, were selected for examining the time domain of the signals. Channel open time ranged from 10 to 550 ms (mean = 168 ms); possible voltage dependence of channel open time could not be determined. The speed of channel opening and closing ranged from <2 to 20 ms, varying among cell pairs.

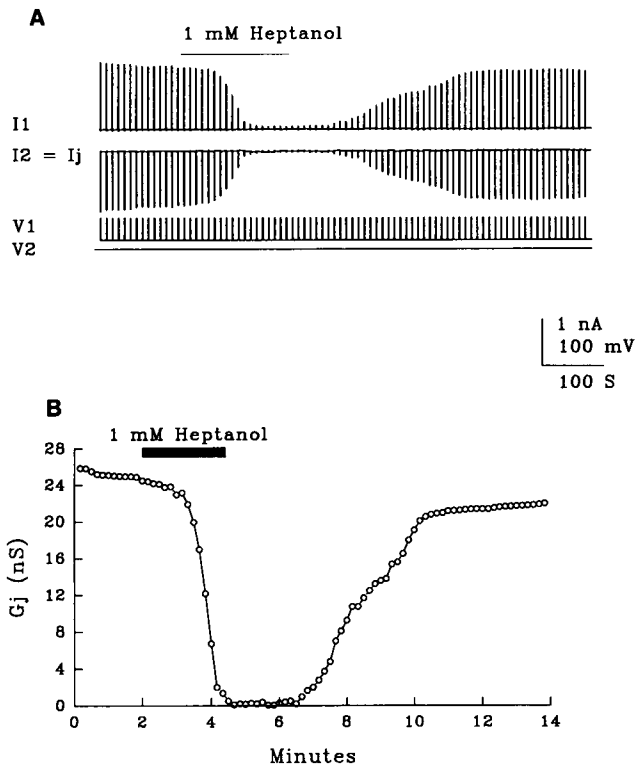

FIGURE 2 (*A*) Whole-cell currents and voltages monitored from a cell pair before, during and after superfusion with 1 mM 1-heptanol. Voltage pulses of 50 mV and 820-ms duration, were applied to cell 1 every 10 s. Heptanol caused rapid and reversible uncoupling, reflected by a dramatic decrease in I_j and I_1. (*B*) Time course of G_j in the experiments shown in *A*. With heptanol, G_j decreased from 26.6 nS to less than 0.1 nS in ~3 min, and recovered to 95% of its initial value within 9 min of washout.

Although most channels had similar opening and closing kinetics, some channels opened fast and closed slowly, others opened slowly and closed fast.

The range of single channel conductances is shown in the event histogram (Fig. 3 *C*). Two Gaussian distributions are seen peaking at unitary conductances of 46.31 ± 7.88 pS (mean ± SD; 1173 events) and 97.1 ± 11.6 pS (mean ± SD; 233 events). The 97 pS conductance does not appear to result from the simultaneous opening of 46-pS channels because individual channels with this conductance could be identified in many records (Fig. 3 *B*). However, whether the 46-pS conductance is a stable substate of the 97-pS channel or a different channel could not be determined.

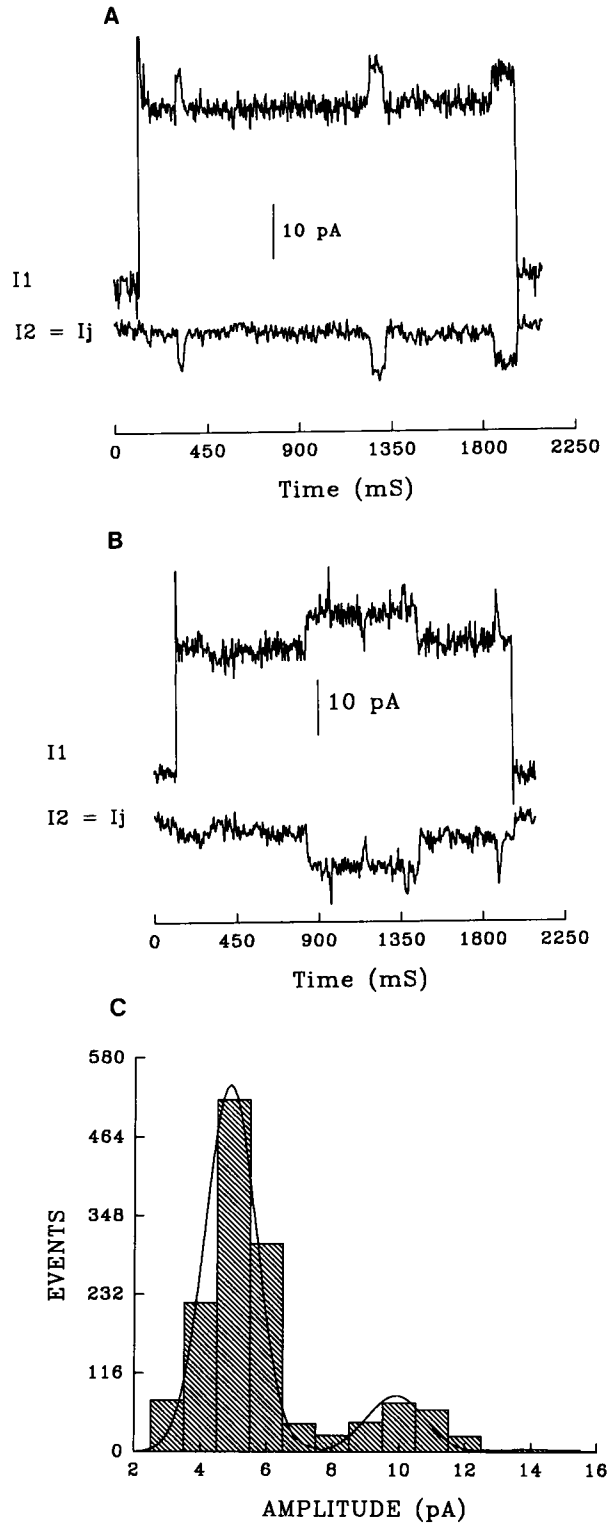

FIGURE 3 (*A*) Single-channel current events recorded from gap junctions of a cell pair uncoupled with 1 mM heptanol. In these records the channel activity was induced by the application of a +100 mV voltage step of 2-s duration to cell 1, single channel activity was seen with smaller and larger pulses (result not shown). Only rapid and symmetrical current deflections observable in both I_1 and I_2 (I_j) are representative of transitions between open and closed states. Three channel events of ~46 pS are visible in this record. V_H = −40 mV. (*B*) Similar record of single-channel current events showing the activity of two junctional channels of different conductance, ~46 and ~97 pS. (*C*) Event amplitude histogram and multiple Gaussian distribution of single channels. Event amplitudes are distributed around two mean values: 4.631 ± 0.778 pA (mean ± SD; 1173 events) and 9.71 ± 1.16 pA (mean ± SD; 233 events). With V_j gradients between ± 50 and ± 100 mV, we observed two mean conductances of 46.31 ± 7.78 pS and 97.1 ± 11.6 pS. The 97-pS conductance seems not to be the consequence of simultaneous opening of two 46-pS channels. The records were acquired at 3 kHz, and some of the data were filtered during analysis at 100–200 Hz (digital filter).

Effect of EGTA·Ca solutions at normal $[Ca^{2+}]_o$

Junctional conductance was sensitive to external procedures that increase $[Ca^{2+}]_i$. In an attempt to increase the intracellular Ca^{2+}, the cell pair under voltage clamp conditions was perfused with EGTA·Ca solutions ($[Ca^{2+}]_o$ = 1.8 mM; Table 1) and changes in I_j were measured before, during and after EGTA·Ca superfusion (Fig. 4 *A*). A constant voltage step of 10-mV and 650-ms duration was applied on either cell of the pair every 5 s, while V_H was held at −40 mV (Fig. 4 *A*).

G_j decreased by 72% within 2.5 min of EGTA·Ca superfusion (2 ml/min) (Fig. 4 *B*). When the cells were reperfused with normal solution, G_j recovered to ~90 of control values in ~7 min (Fig. 4 *B*). Normal recovery of G_j required 5 mM ATP in the pipette solution (Table 2, SIS-1). With 1 mM ATP, both time-course and magnitude of uncoupling were the same, but recovery did not take place even after extensive superfusion with SES normal solution.

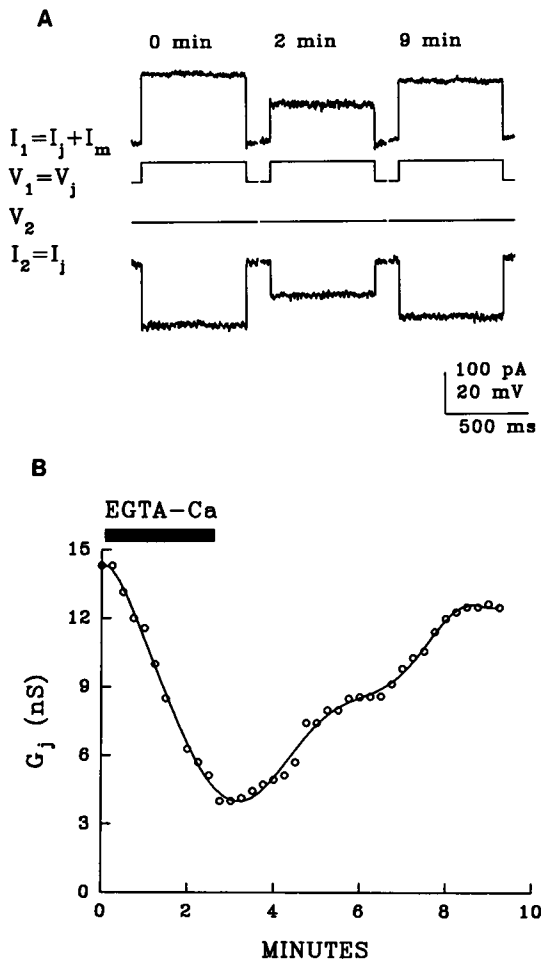

FIGURE 4 Changes in junctional conductance (G_j) in a Novikoff hepatoma cell pair superfused with an EGTA·Ca solution ($[Ca^{2+}]_o$ = 1.8 mM; Table 1). (*A*) Currents and voltages recorded during the experiment shown in *B* before (0 min) during (2 min) and after (9 min) uncoupling. Note the reversible decrease in I_j induced by exposure to EGTA·Ca. I_1 and I_2 are whole cell currents recorded from cell 1 and cell 2 under voltage clamp conditions. V_1 and V_2 are the holding potentials of each cell. A transjunctional voltage (V_j) of 10 mV and 650-ms duration is applied to cell 1 by stepping V_1 from a holding potential of −40 mV. V_2 is held constant at −40 mV; thus, $V_j = V_1$. Junctional current is seen in both current records with opposite polarity and similar amplitude. However, only I_2 is equal to I_j, as I_1 also contains a small nonjunctional membrane component (I_m) associated with the voltage step, such that $G_j = I_j/V_j$ (Ohm's law). Initial G_j = 16 nS. (*B*) Time course of junctional conductance (G_j); note that EGTA·Ca causes a rapid and reversible decrease in G_j.

Superfusion with solutions bubbled with 100% CO_2

Cytoplasmic acidification caused by exposure to 100% CO_2 had drastically different effects on G_j depending on the intracellular Ca-buffer used. With 0.5 mM EGTA in the patch pipettes (Table 2, SIS-1) CO_2 superfusions as short as 2 min had a very large effect on G_j (Fig. 5 *A*). In contrast, with 0.5 mM BAPTA (Table 2, SIS-2) even 4-min superfusions had minimal or no effect on G_j (Fig. 5 *B*). Furthermore, with EGTA the CO_2 effects were poorly reversible (Fig. 5 *A*) even after 20 min of washout.

This observation suggested that much, if not all, of the CO_2 effect on G_j (Fig. 5 *A*) might have resulted from a low pH_i-induced increase in $[Ca^{2+}]_i$, because the Ca-buffering capacity of EGTA (but not that of BAPTA) is strongly reduced by a decrease in pH. This was also suggested by previous evidence for a significant increase in $[Ca^{2+}]_i$ with acidification, and a closer correlation of G_j with $[Ca^{2+}]_i$ than with pH_i, in other cells (Peracchia, 1990a). Therefore, various pCa_i values and pH_i values were tested (see below).

Effect of internal Ca^{2+} and H^+ on junctional conductance

Immediately following the establishment of double whole-cell configuration, cell pairs display G_j values ranging from less than 1 to 200 nS, G_j spontaneously decays with time, even in cells exposed to internal solutions well buffered for Ca^{2+} (1 mM BAPTA) and containing 3 mM ATP, following a single exponential function (Figs. 6 and 7 *A*) with time constants (τ) greater than 20 min. To test the effect of different pCa_i values and pH_i values on G_j, we have compared G_j decay curves among cell pairs exposed to intracellular solutions well buffered for Ca^{2+} (with 1 mM BAPTA) to different pCa values (9, 6.9, 6.3, and 6) and for H^+ (with 10 mM HEPES or MES) to either pH 7.2 (Table 2, solutions A, C, and E) or 6.1 (Table 2, solutions B, D, and F), respectively.

Fig. 7 *A* shows single exponential decays of G_j in cell pairs buffered to pCa_i 6.9 (Table 2, solutions A and B) or 9 (Table 2). G_j decreased to 10–70% of initial values in ~40 min (Fig. 7 *A*) with a τ of 27.8 ± 6.32 min (mean ± SE; n = 13). Figs. 6 and 7 *B* show single exponential decays of G_j in cell pairs buffered to pCa_i 6.3 (Table 2, solutions C and D) or 6 (Table 2, solutions E and F). At these pCa_i values, G_j decreased to 10–25% of initial values in ~15 min (Figs. 6 and 7 *B*) with a τ of 4.87 ± 1.24 min (mean ± SE; n = 7). The difference between the two groups was statistically significant (P = 0.0178).

Experiments with pCa 5.5 (Table 2, solution G) resulted in extremely fast uncoupling with τ values of ~20 s (Fig. 8).

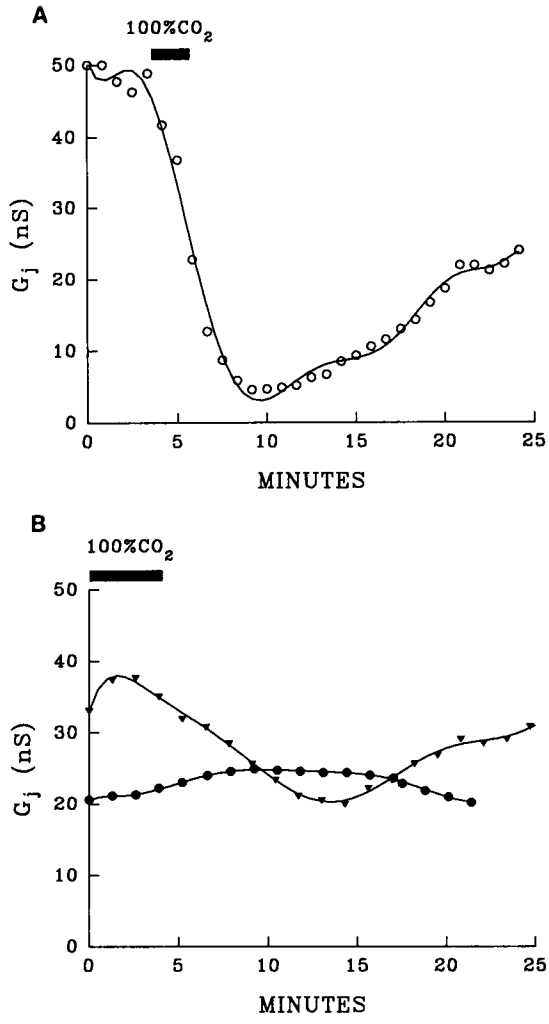

FIGURE 5 Time course of uncoupling in cell pairs perfused with a solution bubbled with 100% CO_2. (*A*) With intracellular solutions buffered for calcium with 0.5 mM EGTA (a Ca^{2+} chelator sensitive to pH; Table 2, SIS-1) CO_2 causes a fast and strong uncoupling, such that G_j decreases from 50 to ~5 nS in less than 2 min. (*B*) With solutions buffered with 0.5 mM BAPTA, (a Ca2+ chelator insensitive to pH; Table 2, SIS-2), CO_2 does not have a significant effect on G_j. In fact, in one cell pair (*filled triangles*) G_j paradoxically increases with CO_2 and start decreasing slowly and moderately only after the end of the CO_2 exposure, while in the other cell pair (*filled circles*) there is only a slow increase in G_j that plateaus and eventually start decreasing. Since in the former the slow decrease in G_j does not correspond in time with the CO_2 treatment, it is unlikely that it is a direct consequence of acidification. The results suggest an indirect action of H^+_i on channel gates, Ca^{2+} ions being the most obvious mediator.

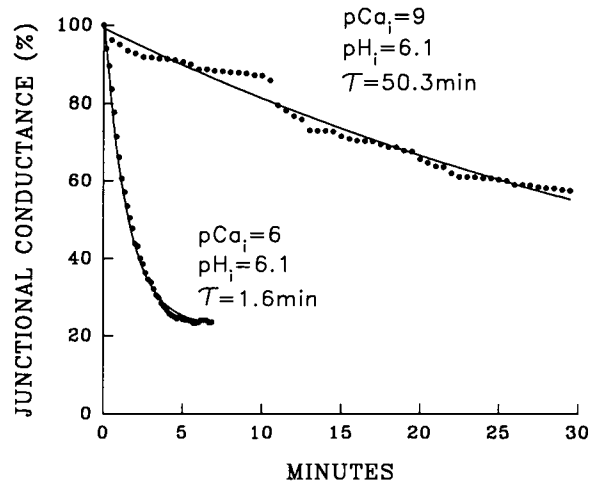

FIGURE 6 Time course of normalized G_j decay in cell pairs intracellularly buffered for Ca^{2+} with 1 mM BAPTA to either pCa_i 9 or pCa_i 6 and for H^+ with 10 mM MES to pH_i 6.1 (Table 2). With pCa_i 9 G_j decreases to ~60% of the initial value in 30 min (initial G_j = 48.80 nS) following a single exponential decay with a τ of 50.3 min, while with pCa_i 6 G_j decreases to ~27% of the initial value (G_j = 41.13 nS) in 5 min following a single exponential decay with a τ of 1.7 min.

Note in Fig. 8 the ~30-s delay between the establishment of whole-cell configuration (time zero) and the precipitous drop in G_j, likely to signal the time at which Ca^{2+} diffusing from the pipette has reached the gap junctions. This indicates that the exchange between pipette solution and cell interior takes place within a few seconds, as previously reported by Fenwick et al. (1982), Marty and Neher (1985), and Pusch and Neher (1988).

The pH of the pipette solutions affected neither time course nor shape of G_j decay at any pCa tested (Figs. 6, 7 *A*, and 7 *B*). At pCa_i 6.9 or greater τ was 31.7 ± 8.9 (mean ± SE; n = 9) with pH_i 6.1 and 19 ± 3.25 (mean ± SE; n = 4) with pH_i 7.2 (not a statistically significant difference).

DISCUSSION

The data indicate that gap junctions of Novikoff hepatoma cells are voltage-sensitive, contain channels with unitary conductances of 46 and 97 pS, are gated by nanomolar $[Ca^{2+}]_i$ regardless of pH_i, and are insensitive to H^+_i. The cells uncouple with CO_2 when they are internally buffered with EGTA, a Ca^{2+} buffer weakened by low pH, but do not with BAPTA, a Ca^{2+} buffer relatively insensitive to pH. Thus, in these cells the effect of low pH_i on G_j seems to be Ca^{2+}-mediated.

Novikoff hepatoma cells belong to a cell line derived from a chemically induced rat liver tumor (Novikoff, 1957), and are believed to originate from Kupffer or endothelial cells. Although these cells have been shown long ago to be electrically and dye coupled (Johnson et al., 1974), and have been used extensively for studying gap junction formation, prior to the present work the properties of their gap junctions have never been studied by double whole-cell clamp electrophysiology.

Voltage sensitivity

The data clearly indicate that gap junctions of Novikoff hepatoma cells are voltage-sensitive, but voltage sensitivity is very moderate by comparison to other preparations (Spray et al., 1991). Junctions with low voltage sensitivity have been reported in systems expressing connexin 43 (Cx43) such as cardiac tissues (Veenstra, 1990, 1991a, 1991b; Wang et al.,

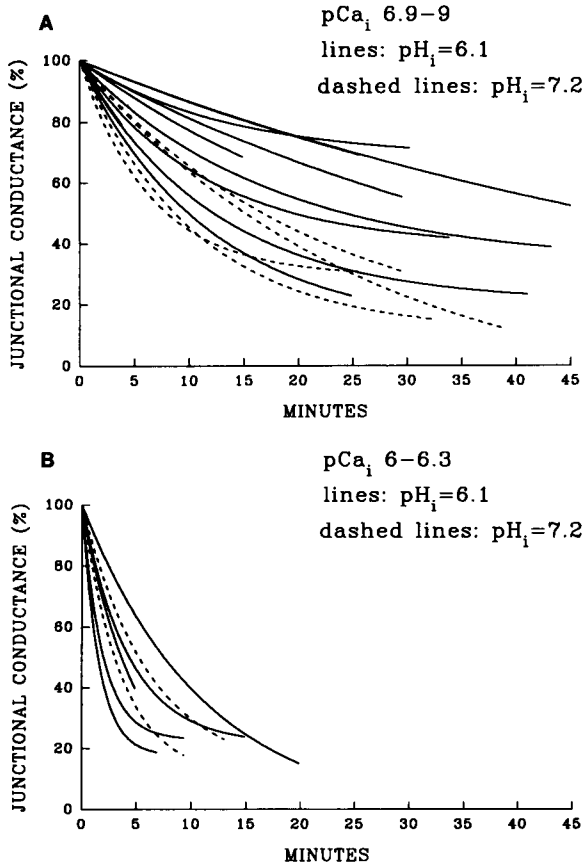

FIGURE 7 Single exponential decays of normalized junctional conductance in cell pairs intracellularly buffered to pCa$_i$ 6.9-9 (*A*; Table 2, solution SIS-2, A and B) and to 6.3-6 (*B*; Table 2, solutions C–F), at either pH$_i$ 7.2 or 6.1. Note that the acidic pipette solution affects neither time course nor shape of G_j decay, while the higher the $[Ca^{2+}]_i$ the faster the G_j decay. The mean initial G_j was 50.06 ± 20.5 nS (mean ± SD) for the plots shown in *A*, and 68.63 ± 35.9 nS (mean ± SD) for those shown in *B*. This indicates that the time constant (τ) of the exponential decay of G_j is independent of initial junctional conductance. Paradoxically, in most experiments the residual G_j was higher at pH$_i$ 6.1 than at pH 7.2.

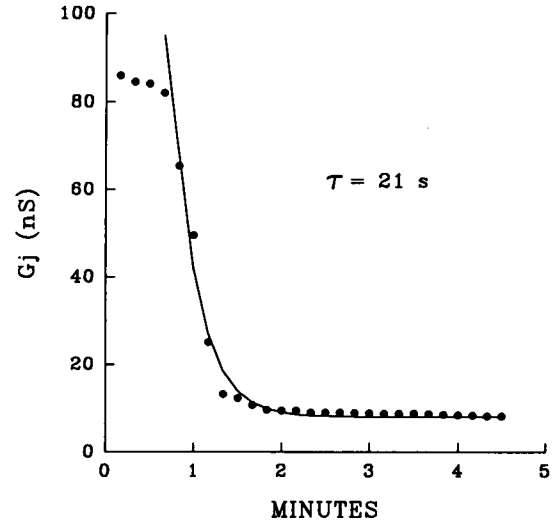

FIGURE 8 Time course of uncoupling of a cell pair intracellularly buffered to pCa$_i$ 5.5 and pH$_i$ 7.2 (Table 2, solution G). Initial G_j = 85.9 nS. The uncoupling followed a single exponential decay (*solid line*) with a τ of 21 s. Note the abrupt decrease in junctional conductance (*filled circles*) that occurs in less than 1 min after the rupture of the membrane patches. This demonstrates the fast exchange between pipette solution and cytosol.

1992; Lal and Arnsdorf, 1992; Anumonwo et al., 1992) lens epithelial cells (Cooper et al., 1989) and cells transfected with Cx43, such as *Xenopus* oocytes (Swenson et al., 1989) and SKHep1 cells (Moreno et al., 1992). Since also Novikoff hepatoma cells are believed to express Cx43 (Meyer et al., 1992; Starich and Johnson, personal communication), it is reasonable to believe that the low voltage sensitivity is related to the nature of the connexin expressed by this cell line.

Voltage sensitivity was greater in poorly coupled cells and increased in well coupled cell as coupling spontaneously deteriorated with time. This could be due to a change in series resistance. Because of uncompensated resistances in series with the junctional resistance, V_j could be reduced below the level at which voltage-dependent gating becomes apparent (Rook et al., 1988; Wilders and Jongsma, 1992). Therefore, in our study the analysis of the voltage sensitivity of the gap junction was limited to the cell pairs poorly coupled (G_j ranging from <1 to ~8 nS).

Single channel conductance

In order to observe single channel events the heptanol treatment was selected among other uncoupling methods, such as exposure to CO_2 or addition of calcium to the pipettes, because with heptanol the channel activity of the nonjunctional membrane is considerably reduced and nonjunctional membrane resistance is high. This reduces the noise level and minimizes the error in measuring current from single junctional channels.

Single channel activity was observed when nearly all of the channels were closed by heptanol in response to a voltage gradient across the junction. The two major unitary conductances observed (46 and 97 pS) are similar to those reported for most cells expressing Cx43 (Burt and Spray, 1988; Rook et al., 1988; Rüdisüli and Weingart, 1991; Lal and Arnsdorf, 1992; Anumonwo et al., 1992), the only possible exception being the embryonic chick heart cells, where two conductance levels (165 and 75 pS) were reported (Veenstra and DeHaan, 1986, 1988). Our results, showing low voltage dependence and a similar range of the single channel conductances, together with the immunoblot data of Meyer et al. (1992), suggest that Novikoff hepatoma cells express the same connexin (Cx43) of the adult rat heart (Beyer et al., 1987). However, whether small amounts of other connexins are coexpressed with Cx43 cannot be entirely excluded. Present evidence does not indicate that the 97-pS conductance represents the simultaneous opening of two 46-pS channels, because current steps of this size were often observed rising individually from baseline, suggesting that they result from the opening of individual channels. More work will be needed to determine whether the 46-pS conductance is a stable substate of the 97-pS channel or another channel.

Sensitivity of the junction to Ca^{2+} and H^+

The sensitivity of cell coupling to acidification has been tested for years in many systems using different approaches. In cell pairs, G_j was found to decrease in cells exposed to 100% CO_2, when the pipette solutions were buffered for calcium with EGTA (Burt and Spray, 1988). However, EGTA is not as reliable as BAPTA as intracellular Ca-buffer for several reasons. First, the Ca-EGTA affinity constant drops by two orders of magnitude with a decrease in pH from 7 to 6 ($K_{Ca\text{-}aff} = 1.8 \times 10^6$ and 1.8×10^4, at pH 7 and 6, respectively), while that of Ca-BAPTA decreases only slightly with the same pH drop ($K_{Ca\text{-}aff} = 7 \times 10^6$ and 1.8×10^6, at pH 7 and 6, respectively). Second, calculations of the gradient of $[Ca^{2+}]$ in the vicinity of a channel pore show that EGTA solutions are completely ineffective in buffering $[Ca^{2+}]$ within macromolecular distances from the pore, while faster buffers such as BAPTA are quite effective (Marty and Neher, 1985; Adler et al., 1991; Stern, 1992). This is consistent with our data showing a dependence of CO_2 uncoupling efficiency on the Ca-buffer used (EGTA versus BAPTA), and indicates that acidification might uncouple cell pairs via an increase in cytosolic Ca^{2+} which is not adequately buffered by EGTA. Indeed, one would expect the opposite result if H^+, rather than Ca^{2+}, were acting on the channel's gating mechanism, because, under the conditions of cytoplasmic acidification by 100% CO_2, EGTA would bind H^+ and release Ca^{2+} (for each Ca^{2+} released two protons would bind); thus, $[Ca^{2+}]_i$ would increase more (and $[H^+]_i$ would increase less) than with BAPTA. In fact, with lowered pH_i EGTA turns out to be a source of Ca^{2+} rather than a Ca^{2+} chelator.

The uncoupling effect of superfusions with Ca-EGTA solutions at normal $[Ca^{2+}]_o$ confirms that $Ca^{2+}{}_i$ independently affects coupling in Novikoff cells. EGTA·Ca^{2+} solutions were previously found to simultaneously increase $[Ca^{2+}]_i$ to >1 μM and junctional electrical resistance in crayfish axons studied with current clamp and Ca^{2+}-sensitive microelectrodes (Peracchia, unpublished data). EGTA·Ca^{2+} solutions cause an increase in $[Ca^{2+}]_i$ because the electroneutral EGTA·Ca^{2+} diffuses passively through membranes (Mullins and Brinley, 1975) and once in the cell readily dissociates, increasing $[Ca^{2+}]_i$. Evidence for the need of 5 mM ATP in the patch pipette for G_j recovery indicates that recoupling requires energy-dependent mechanisms likely to include Ca^{2+} extrusion and sequestration.

The interpretation of a $Ca^{2+}{}_i$-mediated low pH_i effect on coupling is supported by data on cell pairs exposed to different pH_i values and pCa_i values. In these experiments we have assumed that the ionic composition of the junctional environment reaches a rapid equilibrium with the pipette solution. This is suggested by the very short delay (~30 s) between the establishment of whole-cell configuration (time zero) and the precipitous drop in G_j occurring with pipette solution of pCa 5.5 (Fig. 8). The delay is likely to represent the time required for the diffusion of Ca^{2+} from the pipette interior to the junctional channels. Previous studies have clearly demonstrated that the whole cell patch-clamp procedure enables one to control the ionic composition of the cytosol. The diffusional equilibrium between patch pipette solution and cytosol is reached within seconds for ions and other small molecules, especially in cells as small as the Novikoff hepatoma cells (12–15 μm of diameter), as previously reported for Na^+ (15 s) by Fenwick et al. (1982) and Marty and Neher (1983), and for K^+ (15 s) by Marty and Neher (1985), in bovine chromaffin cells. The kinetics of the diffusional exchange between small cells and patch pipettes has been studied extensively by Push and Neher (1988) using compounds ranging in molecular weight from 23 to 150,000 daltons. Their data demonstrated a fast exchange of small compounds between pipettes and cytosol (especially for small ions such as Na^+ and K^+), and the existence of a relationship between the diffusional rate of a given molecule and various parameters such as molecular weight, pipette-cell assembly and pipette electrical resistivity. For insuring fast exchange between cytosol and pipette solution and low access resistance, in our study we always used pipettes with relatively large opening, ranging from 0.5 to ~2 μm (3–6 MΩ resistance when filled with SIS). In any event, no one can be absolutely sure about the exact ionic composition of the region near the junctional channels, and even $Ca^{2+}{}_i$ measurements with fura-2 or indo-1 are inadequate in providing the necessary high resolution data on $[Ca^{2+}]_i$ at the channel environment.

The dramatic decrease in time constant of uncoupling with a change in pCa_i from 6.9 to 6.3, regardless of pH_i, indicates that the channel gates are sensitive to nanomolar changes into $[Ca^{2+}]$ and are insensitive to pH_i, at least in the range between 7.2 and 6.1. Lowered pH_i does not seem to change channel sensitivity to Ca^{2+} as well, but paradoxically may in fact prevent some channels from closing. In the absence of an increase in $[Ca^{2+}]_i$, acidification may inhibit the mechanism causing spontaneous uncoupling, as with internal solutions well buffered for Ca^{2+} (BAPTA) and H^+ (HEPES or MES) both the time constant of the spontaneous G_j decay and the residual junctional conductance were greater at pH_i 6.1 than at pH_i 7.2. Our data on lack of synergism between Ca^{2+} and H^+ in reducing G_j contrast with previous evidence on cardiac cells (Burt, 1987; White et al., 1990), but are consistent with the recent data on crayfish axons subjected to cytoplasmic acidification while monitoring junctional resistance (R_j) and either $[Ca^{2+}]_i$ or $[H^+]_i$ (Peracchia, 1990*a*). In this study the curves describing the time course of changes in $[H^+]_i$ and R_j were found to differ significantly from each other in shape and peak time, and fast and slow changes in $[H^+]_i$ of similar magnitude had markedly different effects on R_j; in contrast, changes in $[Ca^{2+}]_i$ and R_j matched well with each other (Peracchia, 1990a). The increase in $[Ca^{2+}]_i$ with acidification resulted from Ca^{2+} release from caffeine and ryanodine-sensitive stores (Peracchia, 1990b). As in the crayfish study, in Novikoff cells Ca^{2+} appears to affect the channel gates at nanomolar concentrations.

Early data seemed to support a direct effect of H^+ on junctional conductance (Turin and Warner 1977, 1980;

Spray et al., 1981a; Spray and Bennett, 1985), but some inconsistencies were apparent (Peracchia, 1987, 1991; Ramón and Rivera, 1987). In the heart, a pH_i of 6.6 had only a small effect on junctional conductance (Reber and Weingart, 1982), H^+ affected healing-over only at pH <5 (De Mello, 1983) and internal longitudinal resistance correlated poorly with $[H^+]_i$ (Pressler, 1989). In crayfish, low pH solutions did not change junctional conductance in internally perfused axons (Johnston and Ramón, 1981; Arellano et al., 1986), and several inconsistencies between pH_i and electrical coupling were reported in cells of insect salivary glands (Rose and Rick, 1978).

In the past, the $[Ca^{2+}]_i$ effective on coupling has been a matter of controversy. While only $[Ca^{2+}]_i$ as high as 40–400 μM seemed to be effective in some systems (Oliveira-Castro and Loewenstein, 1971; Spray et al., 1982), low micromolar (Délèze and Loewenstein, 1976; Rose and Loewenstein, 1976; Weingart, 1977; Dahl and Isenberg, 1980; Neyton and Trautmann, 1986; Maurer and Weingart, 1987; Veenstra and DeHaan, 1988) to nanomolar (Noma and Tsuboi, 1987; Peracchia, 1990a) concentrations were found effective in others. In our study, pCa 6.3 (500 nM) affected G_j, while pCa 6.9 (126 nM) did not, suggesting that $[Ca^{2+}]_i$ at which most Ca^{2+}-driven physiological functions are activated may also affect cell coupling. This indicates that Ca^{2+} is a physiological regulator of cell coupling and that gap junctions may play a role in a variety of Ca^{2+}-mediated cellular activities. Indeed, preliminary data show a simultaneous increase in $[Ca^{2+}]_i$ to ~500 nM, decrease in G_j by ~30% and activation of $K^+(Ca^{2+})$ in Novikoff hepatoma cells in which a release of IP_3 was induced by ATP activation of purinergic receptors (Lazrak et al., 1993).

In conclusion, Novikoff hepatoma cells are well coupled by moderately voltage-sensitive gap junctions with single channel conductances of ~46 and ~97 pS. The gap junctions are sensitive to $[Ca^{2+}]_i$ in the nanomolar range, and $H^+{}_i$ seems neither to decrease G_j nor to increase channel sensitivity to $Ca^{2+}{}_i$.

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