

Temperature Dependence of Embryonic Cardiac Gap Junction Conductance and Channel Kinetics

Yan-hua Chen, Robert L. DeHaan

Department of Anatomy and Cell Biology, Emory University Health Science Center, Atlanta, Georgia 30322

Received: 5 November 1992/Revised: 17 June 1993

Abstract. We have investigated the effects of temperature on the conductance and voltage-dependent kinetics of cardiac gap junction channels between pairs of seven-day embryonic chick ventricle myocytes over the range of 14–26°C. Records of junctional conductance (G_j) and steady-state unit junctional channel activity were made using the whole-cell double patch-clamp technique while the bath temperature was steadily changed at a rate of about 4°C/min. The decrease in G_j upon cooling was biphasic with a distinct break at 21°C. In 12 cell pairs, Q_{10} was 2.2 from 26 to 21°C, while between 21 and 14°C it was 6.5. The mean G_j at 22°C (G_{j22}) was 3.0 ± 2.1 nS, ranging in different preparations from 0.24 to 6.4 nS. At room temperature, embryonic cardiac gap junctions contain channels with conductance states near 240, 200, 160, 120, 80 and 40 pS. In the present study, we demonstrate that cooling decreases the frequency of channel openings at all conductance levels, and at temperatures below 20°C shifts the prevalence of openings from higher to lower conductance states: all 240 pS openings disappear below 20°C; 200 pS openings are suppressed at 17°C; below 16°C 160 and 120 pS events disappear and only 80 and 40 pS states are seen. Temperature also affected the voltage-dependent kinetics of the channels. Application of a 6 sec, 80 mV voltage step across the junction (V_{j80}) caused a biexponential decay in junctional conductance. Decay was faster at lower temperatures, whereas the rate of recovery of G_j after returning to V_{j0} was slowed. Cooling reduced the fast decay time constant, increased both recovery time constants, and decreased the magnitude of G_j decay, thus leaving a 10–16% larger residual conductance (G_{ss}/G_{init} , ± 80 mV V_j) at 18 than at

22°C. From these results we propose that embryonic chick cardiac gap junctions contain at least two classes of channels with different conductances and temperature sensitivities.

Key words: Gap junctions — Ion channels — Heart — Patch-clamp technique — Temperature

Introduction

Gap junction channels connect the cytoplasm of neighboring cells and allow ions and small molecules to pass from one cell to another, thereby coupling the cells both electrically and metabolically (for reviews, *see* Loewenstein, 1981; Bennett et al., 1991; Bennett & Verselis, 1992; Chen & DeHaan, 1993a). A striking characteristic of the gap junctions in embryonic chick heart cells is that, at low transjunctional voltages (V_j), the junctional channels open to six, evenly spaced unit conductance states (γ_j) near 40, 80, 120, 160, 200 and 240 pS. Voltage steps beyond ± 20 mV tend to suppress the larger γ_j states (Chen & DeHaan, 1992). The relation between channel activity and gap junction conductance (G_j) has been studied in a variety of vertebrate and invertebrate preparations (for reviews, *see* DeHaan, Chen & Penrod, 1989; Bennett et al., 1991; Veenstra, 1991), but the role of multiple channel conductance states remains poorly understood.

A change in temperature can affect biological processes in two ways. Processes that are dominated by diffusion of ions or molecules across membranes or through channels generally have a temperature coefficient (Q_{10}) on the order of 1.1–1.5, reflecting an activation energy (E_a) of 7–9 kcal/mol near room temperature. Activities that depend upon changes

in protein conformation such as enzyme catalysis or metabolically driven membrane transporters and pumps are more steeply temperature sensitive (Stein, 1986; Hille, 1992), with temperature coefficients on the order of 2–5 and an E_a of 10–40 kcal/mol (e.g., Kimmelberg, 1975; Matsuda & Iwata, 1985).

Substantial information exists concerning the effects of temperature change on the properties of excitable membranes. Cooling tends to depolarize membranes and reduce the magnitude of voltage-dependent currents (e.g., Hakoziaki, Matsumoto & Sasaki, 1989). In many preparations, the size of the unit channel conductance is reduced slightly ($Q_{10} = 1.1$ – 1.5), reflecting the slowing of ion movement through the open channel. In contrast, macroscopic current magnitude and kinetics are affected more dramatically ($Q_{10} = 2$ – 5) as a result of the slowing of gating processes. In most cell types, lowering temperature increases time constants for activation and inactivation and decreases the open-state probability (P_o) of channels despite a mean prolongation of channel open dwell times (Jonas, 1989; Kohlhardt, 1990). These results have generally been interpreted to mean that opening of ion-selective channels is associated with a substantial decrease in entropy (Jonas, 1989; Correa, Bezanilla & Latorre, 1992).

It has been known for many years that cooling reduces conduction velocity in mammalian Purkinje fibers, in part by increasing longitudinal resistance (Corabeouf & Weidmann, 1954). However, only recently have direct measurements been made of the response of mammalian cardiac gap junctions to temperature change (Sugiura et al., 1990; Bukauskas & Weingart, 1993). Mammalian cardiac gap junction preparations were reported to be insensitive (e.g., Noma & Tsuboi, 1987) or only weakly responsive (Fishman et al., 1991; Moreno, Fishman & Spray, 1992) to V_j at physiological temperatures, and to have unit channel conductance levels of 50–60 pS (Burt & Spray, 1988; Rudisuli & Weingart, 1990). It was therefore of considerable interest to investigate the temperature dependence of a cardiac gap junction preparation that is known to be more highly voltage sensitive (DeHaan et al., 1989; Veenstra, 1990) and to have multiple conductance states (Chen & DeHaan, 1992). In the present study, we show a sharp temperature response of G_j that results from an overall decrease in the frequency of channel opening with cooling and a shift from a prevalence of large channel conductance states to smaller states. Preliminary reports of portions of this study have been published previously (Chen & DeHaan, 1989, 1990).

Materials and Methods

CELL CULTURE

Ventricles of seven-day chick embryo hearts were dissociated into suspensions that included single cells, pairs, and cell clusters, using the four-cycle trypsinization procedure now routine in this laboratory (Nathan & DeHaan, 1979). The cells were suspended in culture medium 21212 and plated in glass chambers (0.5 ml capacity) which had been treated with concentrated sulphuric acid (18 M) to adjust the adhesiveness of the surface to allow cells to attach without flattening (Fujii, Ayer & DeHaan, 1988). The cultures were prepared at a density of 1×10^5 cells/chamber and incubated in an atmosphere of 85% N_2 , 10% O_2 , 5% CO_2 , at 37°C for 24 hr (pH 7.4). All measurements were made on cell pairs after 22–28 hr incubation in vitro. Pairs were selected for experiments in which the two members were of similar size (13–18 μ m diameter) and in which the diameter of the contact area between them was 2–10 μ m.

SOLUTIONS

The culture medium 21212 contained (by volume): 25% M199, 2% heat-inactivated horse serum, 4% fetal bovine serum, 67.5% K^+ -free Ham's F12 (all from GIBCO, Grand Island, NY), 1% L-glutamine, 0.5% pen-G (Sigma) and 1.3 mM KCl. The external bath solution (DBS) used for electrophysiological recordings was composed of (in mM): NaCl, 142; KCl, 1.3; $MgSO_4$, 0.8; NaH_2PO_4 , 0.9; $CaCl_2$, 1.8; dextrose, 5.5; and HEPES 10 (Baker Chemical, Phillipsburg, NJ). The pH was titrated to 7.4 by the addition of 1 N NaOH. Two intracellular pipette solutions were used in different experiments: IPS #57 and IPS #66. IPS #57 contained (in mM): K-Glutamate, 120; NaCl, 15; KH_2PO_4 , 1; $MgCl_2 \cdot 6H_2O$, 4; $CaCl_2 \cdot 2H_2O$, 4.7; EGTA, 5; HEPES, 10; Na_2 -ATP, 3; Na_2 -phosphocreatine, 3; pH = 7.1 (adjusted by 1 N KOH). We found that preparations survived longer after the patch was ruptured with K-glutamate in the IPS in place of KCl. IPS #66 contained (in mM): CsCl, 130; $MgCl_2 \cdot 6H_2O$, 4; $CaCl_2 \cdot 2H_2O$, 0.068; EGTA, 10; HEPES, 5; Na_2 -ATP, 3; Na_2 -phosphocreatine, 3; pH = 7.1 (adjusted by 1 N CsOH). CsCl was used because it gave excellent seals and blocked I_K ; the permeability ratio (P_{Cs}/P_K) is near unity (Brink & Fan, 1989). The two solutions were calculated (Godt & Lindley, 1982) to buffer internal free $[Ca^{2+}]_i$ either to high (4×10^{-6} M) (IPS #57) or low ($<1 \times 10^{-8}$ M) (IPS #66) level. Na_2 -ATP and Na_2 -phosphocreatine were added to the IPS just before use to block I_{KATP} and to improve the metabolic state of the cells.

ELECTROPHYSIOLOGICAL MEASUREMENTS

Cells were washed with DBS at room temperature and placed in a temperature-controlled microscope chamber for experiments. We used the whole-cell double voltage-clamp technique to measure both gap junctional current (I_j) and current through single junctional channels (i_j) as described previously (Veenstra & DeHaan, 1988; Chen & DeHaan, 1992). Patch electrodes had tip resistances of 2–6 M Ω . Each electrode was connected to a List model L/M EPC-7 or EPC-5 patch-clamp amplifier (List Medical Systems, NY). Current responses to voltage-clamp steps were continuously recorded on VCR tape (Panasonic Model AG-1250-

P VHS recorder) with a Neuro-corder Model DR-484 digitizer (Neuro Data Instruments, NY) sampling at 22 kHz. Sequences of voltage command steps were provided by a programmable step waveform generator designed and built by W.N. Goolsby in this laboratory. Before analysis, records were filtered with an Ithaco Model 4302 4-pole Bessel filter (Ithaca, NY) and analyzed with a Nicolet 4094 digital storage oscilloscope interfaced with an IBM computer. Sample rates at least twice the frequency of the filter bandwidth were used to reduce aliasing error. Filtering the data at 0.5 or 1.0 kHz yielded the best compromise between time resolution for single channel analysis and separation of events from noise. The transition time for the output of the filter from 10 to 90% of the response to an instantaneous input step was 530 μ sec at 1 kHz.

G_j was calculated from I_j/V_j , measured from a series of 500 msec voltage-clamp pulses designed to keep both members of the cell pair in the physiological voltage range: cell 1 was held at -10 mV and cell 2 was stepped from -10 to -50 mV to produce steps from 0 mV V_j (V_{j0}) to V_{j40} . The initial peak of I_j (I_{init}) was defined as the current in cell 1 (in which membrane potential, V_1 , was held constant) measured 200 μ sec after onset of the 40 mV step in V_2 . G_{init} (I_{init}/V_j) is a measure of the conductance at the end of the previous 6 sec period of V_{j0} , when G_j has recovered fully from the previous voltage pulse (Chen & DeHaan, 1992). Throughout this paper, $G_j = G_{\text{init}}$ unless otherwise defined. To pool results from the 12 experiments included in the present study, the measured change in G_j with temperature was normalized in bins of 1°C to its value at the reference temperature of 22°C (G_{j22}), and plotted against $1/T \cdot 10^3$ ($^\circ\text{K}^{-3}$). Each segment of the biphasic Arrhenius plots was fit separately with a least-squares regression line to optimize the correlation coefficients. A small sample T -test (Kleinbaum & Kupper, 1978) was used to verify that the slopes through the upper and lower temperature ranges were different. Values of Q_{10} and E_a were calculated from the linear fits.

Unit channel conductance ($\gamma_j = \Delta i_j/V_j$) was determined from the magnitude of the opening current transitions (Δi_j) recorded from the two electrodes during long periods of V_{j80} as symmetrical signals of equal size and opposite polarity (Veenstra & DeHaan, 1988). We measured unit channel events (where an event is defined as an opening, see Fig. 2B, inset) directly from the oscilloscope screen as previously described (Chen & DeHaan, 1992). In the text we refer to the sizes of such events by their nominal conductance states ($\gamma_{40}, \gamma_{80}, \dots, \gamma_{240}$). A transition from the closed to open state was defined as a monotonically changing sequence of points from one i_j level to the next, preceded and followed by dwell times of at least 1 msec during which current fluctuated no more than ± 1 pA around the mean. When the data were filtered at 1 kHz, summing of two channel openings could be distinguished from a single transition by a distinct shoulder if the openings were separated by at least 300 μ sec. We have shown previously (Chen & DeHaan, 1992) that the probability of two or more independent 40 pS channels opening within a 300 μ sec period was far too small to account for the frequency of 80–240 pS events observed (see Table 2).

TEMPERATURE CONTROL

In all experiments, measurements were begun with the preparation at room temperature, after which the bath was slowly warmed to 26 – 27°C or cooled to near 14°C . We controlled the temperature of the bath solution (DBS) with a Peltier device (Cambion) connected to a temperature controller designed and built by W.N.

Goolsby. The cell-containing chamber was gravity perfused with DBS (0.75 ml/min). The bath solution was cooled or warmed at a rate of about $1^\circ\text{C}/15$ sec. Temperature was monitored by a thermister probe accurate to $\pm 0.1^\circ\text{C}$ in the chamber during experiments. For this study, room temperature is defined as $22 \pm 0.5^\circ\text{C}$.

Results

THE EFFECT OF TEMPERATURE ON G_j

To determine the influence of temperature, G_j was measured periodically starting at room temperature. In a typical experiment (Fig. 1A, B), the bath solution was warmed to 26°C and then cooled slowly to 14°C over a period of 20 min, before warming again to 26°C . Thus, G_j was measured during two or three passes through the reference temperature, 22°C . Immediately after rupture of the membrane patch in the experiment illustrated, G_j is 0.93 nS. For this cell pair, the average conductance at 22°C (G_{j22}) was 0.80 nS. Among the twelve preparations included in the present study, G_{j22} , i.e., the conductance at constant temperature, varied by more than 30-fold, from 0.2 to 6.4 nS, and averaged 3.0 ± 2.1 nS (mean \pm SD).

As temperature changed, the conductance rose and fell in close correlation with warming and cooling of the bath. When the conductance measurements were plotted against temperature in $^\circ\text{C}$ the relationship of $G_j(T)$ was curvilinear. When the measurements of G_j from this experiment were replotted as an Arrhenius plot, a regression line through the entire data set had a correlation coefficient (r) of 0.9992. We found that r could be optimized by fitting regression lines to the data as two separate segments: 26 – 21°C ($r = 0.9997$) and 20 – 14°C ($r = 0.9998$). A small-sample T -test demonstrated that the two slopes were significantly different at the 99% confidence level ($p = 0.008$). The lines crossed at 21.6°C . Q_{10} for the biphasic temperature dependence was 2.9 for temperatures above the break and 9.0 for the lower temperature range. To compare temperature effects in different cell pairs, the change in G_j with temperature for each of the twelve preparations was normalized to G_{j22} . Figure 1D shows the mean $G_j(T)$ at each temperature from the twelve experiments, displayed in an Arrhenius plot. Although the entire 14 – 26°C range was not sampled in all of the experiments, we again found that the linearized slope of $G_j(T)$ between 14 and 21°C was significantly steeper than that in the higher temperature range ($p = 0.002$). The two lines in the fits of the pooled data met to form a break at 21.0°C . Q_{10} for the slopes from 26 to 21°C was 2.2, while between 21 and 14°C , Q_{10} was 6.5.

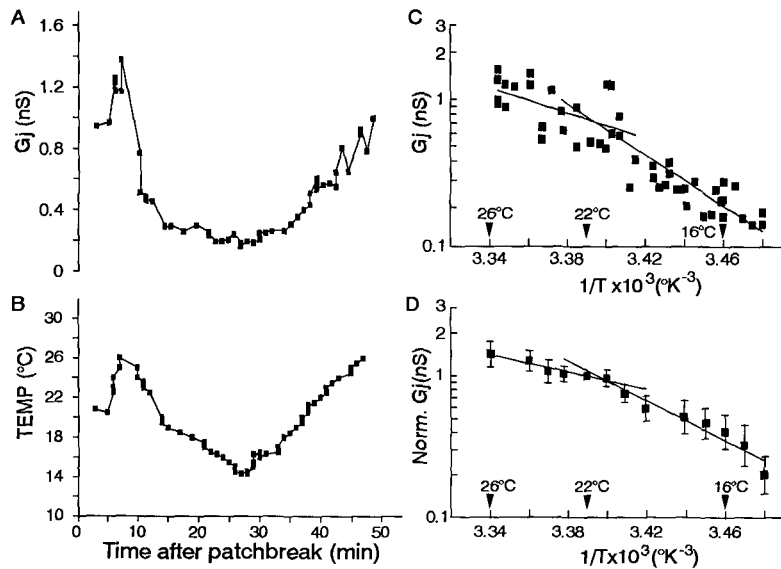


Fig. 1. The effect of temperature on junctional conductance (G_j). (A) Periodic measurements of G_j were begun with the bath at room temperature, shown in (B). In this experiment, measurements were begun at 21°C, after which the bath solution was warmed to 26°C, and then cooled slowly to 14°C over a period of 20 min before warming again to 26°C. Each point in A represents an average of 2–3 measurements at the indicated temperature. G_j at the beginning of the experiment for this cell pair was 0.93 nS. G_j rises to a maximum of 1.4 nS and falls to a minimum of 0.2 nS, in close correlation with bath temperature. (C) Arrhenius plot of the data in A and B as G_j against $1/T \cdot 10^3$ ($^{\circ}\text{K}$). For this cell pair (#040187, IPS #57) $G_{j22} = 0.8$ nS; break in linear slopes at 21.6°C; $Q_{10} = 2.9, 9.0$; $E_a = 17.9, 37.5$ kcal/mol. (D) Arrhenius plot of mean G_j . Measurements of G_j in each of 12 experiments were normalized to G_{j22} in bins of 1°C. Mean $G_{j22} = 3.0 \pm 2.1$ nS; break at 21.0°C; $Q_{10} = 2.2, 6.5$; $E_a = 13.7, 31.9$ kcal/mol.

EFFECT OF TEMPERATURE ON UNITARY CONDUCTANCE LEVELS OF CHANNEL OPENINGS

Examples of unit channel openings recorded at different temperatures in a weakly coupled cell pair are shown in Fig. 2A. Two such events are shown at greater amplification and higher sweep speed in Fig. 2B (inset) to illustrate the shoulderless, monotonic current transitions that we classified as unit channel openings. It can be seen from inspection of the main part of Fig. 2B, where 1,670 such events are pooled from four cell pairs and plotted against temperature, that cooling has three effects. (i) It causes a small reduction in the size of each conductance state; i.e., the average size of each unit conductance state (except for the γ_{160} state) increases slightly with temperature, producing a slope greater than 1.0 (Table 1). The anomalous behavior of the 160 pS state may result from missorting of states at the upper or lower end of the temperature range. Calculating a mean Q_{10} for temperature effect on γ_j from the slopes of each of the six states gives a value of 1.08 ± 0.11 . (ii) Cooling decreases the frequency of opening (F_o) to each state (except the γ_{40} state; Table 2); and (iii) below 20°C cooling progressively suppresses the activity of the larger states.

Table 1. Total openings and temperature coefficient of changes in unit channel conductance states between 14 and 27°C

Mean γ_j (pS)	SD	N	Q_{10}
44.1	9.0	278	1.325
79.8	8.8	526	1.094
120.6	10.0	217	1.036
155.7	9.7	410	0.970
199.2	9.1	73	1.032
244.5	13.6	166	1.033

The unit channel conductances (γ_j) of 1,670 events pooled from four different cell pairs were measured at V_{j80} over the temperature range from 14 to 27°C, sorted by conductance level as described previously (Chen & DeHaan, 1992), and averaged. Q_{10} values were calculated from linear fits of each conductance level and had a mean of 1.08 ± 0.11 .

EFFECT OF TEMPERATURE ON PREVALENCE OF CHANNEL OPENINGS TO EACH STATE

Under the conditions used, where channel activity was reduced by the steep transjunctional potential ($V_j = 80$ mV), the frequency of opening to any level

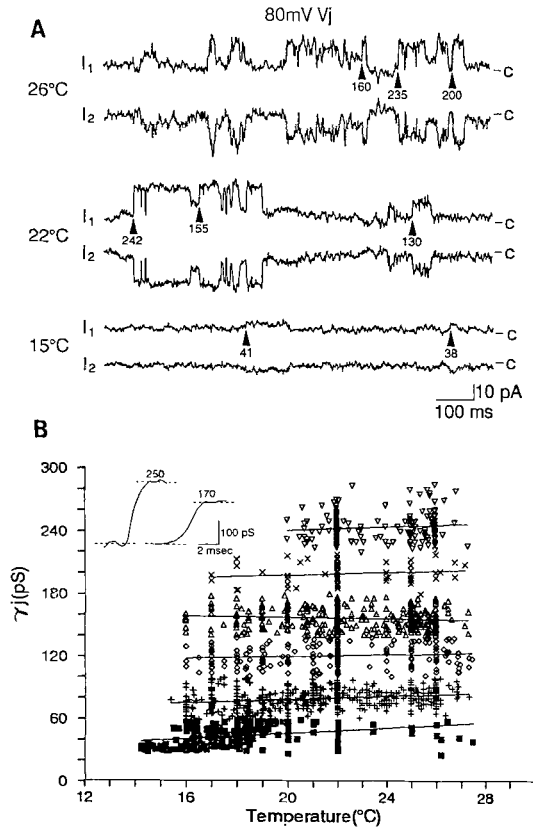


Fig. 2. The effects of cooling on single channel currents. (A) I_j from a single preparation at 15, 22, and 26°C ($V_j = 80$ mV; records filtered at 1,000 Hz; $G_{j22} = 2.7$ nS). Examples of unitary channel openings to different conductance levels are evident at 22 and 26°C (arrowheads), whereas only γ_{40} openings are seen in this record at 15°C. Note that background noise decreases with cooling. (B) Two records are shown in the inset at high sweep speed to illustrate monotonic current transitions that met the criteria for unit channel openings. Each point in the main figure represents a channel opening at the temperature indicated. The large number of events at 22°C reflects the long recording periods at that temperature during which control data were accumulated. A total of 1,670 channel openings are pooled from four different cell pairs. Changes in temperature had a small effect on the conductance of each state (mean $Q_{10} = 1.08$), but had a dramatic effect in shifting the prevalence of openings to smaller states. Between 26 and 20°C openings to all six conductance states are evident; at lower temperatures larger openings are progressively suppressed. The parameters of each conductance state (mean and SD of γ_j , number of openings, and Q_{10}) are listed in Table 1. (cell pair 040187, IPS #57; 081387, IPS #57; 111491, IPS #66; 022092, IPS #66).

was low. This is seen in Table 2, where the number of opening events (N) and F_o are shown during recording epochs of 300 sec at 22 and at 18°C. These low values result mainly from the openings tending to be clustered in poorly defined bursts of activity, separated by periods of many seconds of quiescence. Nonetheless, the greatest effect of temperature change was on prevalence, i.e., F_o of each con-

ductance state. At temperatures above 20°C, all six conductance states were observable. Below 20°C, 240 pS events ceased. Openings to the 200 pS level disappeared at temperatures below 17°C. At 16°C γ_{40} , γ_{80} , γ_{120} , and γ_{160} openings were found, while at 14–15°C only γ_{40} and γ_{80} events could be seen, and these were rare. The total number of openings at all temperatures to each conductance level is listed in Table 1.

In association with the shift in prevalence of openings to lower conductance states, overall F_o is also substantially reduced (Table 2). For example, the total channel openings (to all levels) fell from 693 to 275, comparing a 300 sec recording epoch at $22 \pm 1^\circ\text{C}$ with a similar period at $18 \pm 1^\circ\text{C}$ (Table 2, column N). At 22°C more than one-fourth (27.7%) of the openings were to the 160 pS state, yielding a frequency of 38.4 events/min. At 18°C, 240 pS events disappeared and the F_o of γ_{160} openings declined to only 10.8/min. Only the F_o of 40 pS openings appeared to be unaffected. That the frequencies of both the γ_{200} and γ_{240} openings fell to very low values before the smaller states were much affected, reveals the greater temperature sensitivity of the larger states.

THE KINETICS OF VOLTAGE-DEPENDENT CONDUCTANCE DECAY AND RECOVERY

At room temperature, both the magnitude and rate of voltage-dependent decay in I_j increased in response to increasing steps in V_j . Moreover, both the voltage-dependent decay and recovery of junctional conductance were best fit by biexponential functions with a fast time constant (τ_1) of 20–150 msec and τ_2 of a few seconds (Chen & DeHaan, 1992). The effects of cooling from 22 to 18°C on the amplitude and kinetics of current decay and recovery are shown in Table 3. In examples of the current responses illustrated in Fig. 3, I_{init} at 18°C is smaller than at 22°C, reflecting the lower resting conductance at the cooler temperature (I_{init} at 18°C averaged 49% of that at 22°C). Furthermore, the early component of the current decay is steeper than at 22°C, that is, τ_1 becomes smaller with cooling (Table 3). In some preparations, decreasing the temperature caused τ_1 to disappear altogether. The decay thus became monoexponential with the remaining time constant resembling τ_2 .

After the onset of an applied V_j pulse, conductance reached a new steady-state value (G_{ss}) in 6–8 sec. It is apparent from Fig. 3 that cooling had a greater relative effect on G_{init} than on G_{ss} . This is reflected in an increase in the ratio $G_{\text{ss}}/G_{\text{init}}$ with cooling, for example from 0.24 to 0.34 between 22 and 18°C at V_{j80} (Table 3). These effects on decay

Table 2. Frequency of opening (F_o) to each conductance state at 18 and 22°C ($V_j = 80$ mV)

18 ± 1°C					22 ± 1°C				
Nom. γ_j (pS)	γ_j^a (pS)	N^b	% Total	F_o^c (N/min)	γ_j^a (pS)	N^b	% Total	F_o^c (N/min)	
40	41.7 ± 7.4	72	26.2	14.4	44.6 ± 6.9	62	8.9	12.4	
80	79.9 ± 7.9	87	31.6	17.4	81.1 ± 7.8	167	24.1	33.4	
120	120.4 ± 8.8	59	21.5	11.8	118.8 ± 8.0	102	14.7	20.4	
160	159.5 ± 8.6	54	19.6	10.8	159.9 ± 10.0	192	27.7	38.4	
200	200.6 ± 12.5	3	1.1	0.6	199.3 ± 7.7	83	12.0	16.6	
240		0	0	0	242.1 ± 11.3	87	12.6	17.4	
Totals		275	100.0	55.0		693	100.0	138.6	

^a Mean ± SD, ^b Number of openings to each γ_j during a 300 sec recording epoch. ^c Frequency of openings to each γ_j (events/min).

Table 3. Voltage-dependent parameters of G_j decay and recovery

	Decay		G_{ss}/G_{init}	Recovery	
	τ_1 (ms)	τ_2 (sec)		τ_1 (ms)	τ_2 (sec)
18 ± 1°C					
V_{j80}	33.9 ± 3.1	3.7 ± 5.5	0.34 ± 0.11	189.6 ± 26.2	5.6 ± 3.1
V_{j-80}	31.9 ± 2.0	2.2 ± 3.3	0.46 ± 0.11	285.5 ± 100.3	30.9 ± 21.5
n^*	4	4	5	2	2
22 ± 1°C					
V_{j80}	66.6 ± 26.1	1.9 ± 1.5	0.24 ± 0.07	90.7 ± 90.1	2.4 ± 0.8
V_{j-80}	43.0 ± 7.4	1.1 ± 1.1	0.30 ± 0.04	83.5 ± 37.4	1.7 ± 0.7
n^*	4	4	5	2	6

* n = number of cell pairs.

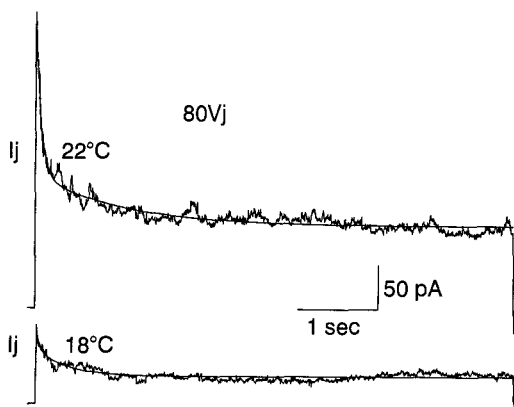


Fig. 3. G_j decay at 22 and 18°C. In these responses, I_{init} is 341 pA at 22°C and 90 pA at 18°C. The decay currents were best fit by biexponential functions at both temperatures with $\tau_1 = 63.2$ msec at 22°C decreasing to 37.2 msec at 18°C; $\tau_2 = 1.1$ sec at 22°C and 0.37 sec at 18°C. The ratio G_{ss}/G_{init} at 22°C is 0.27, and increases to 0.36 at 18°C, indicating that there is less current decay at the lower temperature. (cell pair 111491, IPS #66).

cause large shifts in the $I_j - V_j$ curves (Fig. 4A). At 22°C, I_{init} deviates only slightly from linearity with V_j , whereas I_{ss} shows a pronounced voltage depen-

dence at values of V_j beyond ± 20 mV. At 18°C, both I_{init} and I_{ss} are distinctly nonlinear but the difference between them, which represents the decay current, is small. The difference in response at the two temperatures can be seen more clearly when initial and steady-state conductances are plotted in G_j/V_j curves (Fig. 4B).

Recovery of conductance after returning from V_{j80} or V_{j-80} to V_{j0} (Fig. 5) remained biexponential in all cases. In contrast to the decay process, both recovery time constants increased with cooling (Table 3), i.e., the recovery process was slowed by lower temperatures.

Discussion

GAP JUNCTION CONDUCTANCE VARIES WITH TEMPERATURE

In principle, cooling could affect G_j in three ways. (i) Reducing the rate of ion diffusion through the open channel pores would decrease γ_j of any size channel or conductance state with a temperature coefficient on the order of 1.1–1.3. (ii) Decreasing

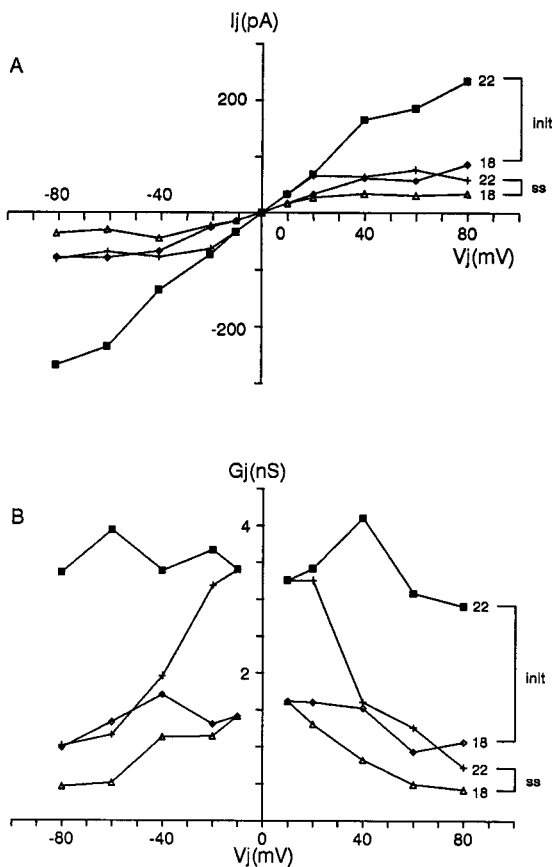


Fig. 4. $I_j - V_j$ and $G_j - V_j$ curves at 22 and 18°C. (A) Values of I_{init} were pooled from repeated 6-sec steps in V_j to ± 10 , ± 20 , ± 40 , ± 60 , and ± 80 mV. Each voltage pulse was separated from the last by a 6 sec period of V_{j0} . At 22°C, I_{init} (current measured 200 μ sec after the onset of the V_j pulse) increases in nearly linear fashion with the steps in V_j over the range examined, but steady state current (I_{ss}) is voltage dependent at V_j above ± 20 mV. At 18°C, I_{init} is smaller and not as linear as at room temperature, reflecting the slower recovery at low temperature. The magnitude of current decay is also smaller at 18°C (57% at V_{j80}) than at 22°C (75% at V_{j80}). (B) Data from (A) converted to $G_j (= I_j/V_j)$. (cell pair 100891, IPS #66).

P_o of channels would be expected to have a stronger effect on G_j . (iii) In a junction with channels having multiple conductance levels of widely different sizes, altering the prevalence of opening of channels to their different size configurations from large conductance states to small could dramatically alter G_j . We found a sharp dependence of G_j on temperature, with a distinct break in slope at 21°C (Fig. 1D). From 26 to 21°C, mean Q_{10} was 2.2, while from 21 to 14°C it was 6.5. These slopes are equivalent to activation energies of about 14 and 32 kcal/mol, respectively. From measurements of unit channel activity, we found channel openings to all six conductance states down to 20°C, but a progressive suppression of large conductance states below 20°C (Fig. 2). That is, in addition to the expected slowing of ionic diffusion

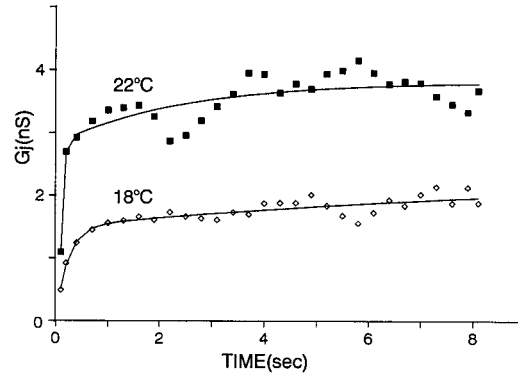


Fig. 5. Recovery of conductance after a step return from V_{j80} to V_{j0} at 22 and 18°C. Each data point during the recovery period is measured from a test pulse (100 msec, 10 mV) applied every 300 msec during the 8-sec period of $V_j = 0$ mV. Recovery was best fit by biexponential functions (Table 3) over a broad temperature range; both τ_1 and τ_2 increased progressively upon cooling. In the cell illustrated, τ_1 increased from 47 to 227 msec and τ_2 lengthened from 2.4 to 10 sec between 22 and 18°C (cell pair 100891, IPS #66).

through the open channels, cooling caused both a decrease in total F_o (to all but the 40 pS states), and below 20°C, a shift to smaller states. At room temperature, about one-fourth of all channel openings were to the γ_{200} and γ_{240} states while one-third were to 40 and 80 pS (Table 2). These figures match well the values reported earlier from a different data set (Chen & DeHaan, 1992). A decrease in temperature caused total F_o to decline sharply but unequally; openings to large channel states were more strongly suppressed than smaller openings. Upon cooling to 18°C, there were no further γ_{240} events and openings to 200 pS were rare, while more than half of the events were to the γ_{40} and γ_{80} states. Channels continued to open to 40 pS levels even at 14°C, though opening frequency at that temperature was very low. Since the effect of temperature on the diffusion of ions through the open channels had a Q_{10} of only 1.08, the larger effect on overall junctional conductance presumably reflects the differential reduction in F_o of the different conductance states. We interpret the average Q_{10} of 2.2 in the higher temperature range to reflect effects on γ_j and P_o alone, while the sharper temperature dependence below 21°C presumably results from the added effects of the progressive inhibition of larger openings.

Displaying the data in Arrhenius plots as in Fig. 1C and D requires treating steady-state G_j as a reaction rate, despite the obvious fact that it is in reality the net result of numerous rates. At the level of unit channel activity, junctional conductance may be described as the product of three parameters: the number (N) of channels of a given size that are active, the open-state probability (P_o) of those channels, and their unit channel conductance (γ_j), i.e.

$G_j = \gamma_j NP_o$. Each of these parameters is likely to be affected by temperature differently. Moreover, in a junction with multiple independent channels (or conductance states), the situation is even more complicated because G_j presumably depends on the sum of the activities of each conductance, i.e., $G_j = (\gamma_j NP_o)_1 + (\gamma_j NP_o)_2 + \dots + (\gamma_j NP_o)_i$, where the summed terms represent the activities of the first to the i th channel size (or conductance state). Hence, the values of E_a and Q_{10} that result from an Arrhenius fit are at best only apparent values because one does not know the number of reaction steps involved or energy barriers crossed to achieve a given change in G_j . Nonetheless, it is of some interest to compare the values found here with those derived from other gap junction preparations. For example, the Q_{10} for G_j in the median giant axon of the earthworm (Verselis & Brink, 1986), and in adult (Sugiura et al., 1990) and neonatal (Bukauskas & Weingart, 1993) mammalian heart cells was 1.2–1.5 ($E_a = 7$ –9 kcal/mol), consistent with an effect primarily on ionic diffusion through the open channels rather than on channel gating. In contrast, Q_{10} in the septate junction of the crayfish lateral giant axon was about 3 over the range from 5 to 20°C, equivalent to an E_a of about 18 kcal/mol (Payton, Bennett & Pappas, 1969).

At 14°C openings were extremely rare and were limited to 40 pS, although G_j did not fall to zero. This could result if some channels were locked in an open position, or if the closed state had a nonzero conductance. The prominent decrease in F_o at 14°C is in contrast to the behavior of channels in neonatal rat heart cells which, according to Bukauskas and Weingart (1993), continued to open and close with countable frequency down to –2°C. These authors have reported a Q_{10} for γ_j of 1.43 between 14 and 37°C and 1.14 between –2 and 13°C. In these preparations, γ_j was found to vary from 19.6 to 75.6 pS with a value at 22°C of 45 pS which is close to the γ_{40} conductance state found here. These results suggest that the γ_{40} state in both rat and chick heart is relatively resistant to cooling, and that temperature sensitivity increases with size of the unit conductance state.

PHYSIOLOGICAL MECHANISMS OF THE TEMPERATURE DEPENDENCE OF G_j

What are the mechanisms by which temperature might alter G_j and the kinetics of its voltage-dependent conductance decay and recovery as seen in the present work? The temperature sensitivity of gap junctions in *Chironomus* salivary gland cells was initially interpreted as secondary to a reduction in metabolic pumping which resulted in alterations in

intracellular ionic concentrations (Politoff, Socolar & Loewenstein, 1969). In support of this contention, Beers, Bridge and Spitzer (1989) found that cooling guinea pig myocytes could cause a release of Ca^{2+} into the cytosol which others have demonstrated would be of sufficient magnitude to reduce G_j (Noma & Tsuboi, 1987; Veenstra & DeHaan, 1988; Bennett et al., 1991). There is also evidence that phosphorylation plays a role in regulating G_j (reviewed in Bennett et al., 1991; Saez et al., 1993). Thus, the temperature dependence of G_j might reflect differential temperature effects on phosphorylation-dependent regulatory systems.

In contrast to the idea of an indirect effect, Payton et al. (1969) interpreted the rapid reversibility of temperature-dependent changes in junctional resistance in the crayfish lateral giant axon as evidence for a direct effect on the junctions themselves. The experiments presented in the present work also support the idea of a direct effect of temperature on channel gating. We used two different patch electrode solutions (IPS #57 and #66) that infused the cells with high concentrations of ATP and phosphocreatine and were strongly buffered to maintain constant pH_i and $[\text{Ca}]_i$. Moreover, we saw similar effects with $[\text{Ca}]_i$ buffered to very different levels. These conditions make it unlikely (but do not disprove) that the three most widely studied regulators of G_j : pH_i , $[\text{Ca}]_i$, or access to phosphorylated intermediates varied sufficiently with temperature to account for our results.

The observation that gap junction channels respond to cooling with small decreases in unit channel conductance and larger effects on gating-dependent parameters is consistent with findings on other membrane channels. For example, the Q_{10} of the unit conductance of nicotinic endplate channels (Nelson & Sachs, 1979) and cardiac and neural sodium channels (Jonas, 1989; Kohlhardt, 1990; Correa et al., 1992) was 1.1–1.4. In these preparations, however, the temperature dependence of current magnitude or kinetic parameters was 2–5, indicating that the response of channel gating to temperature was steeper.

If a population of channels can exist in only two states, closed and open, and the energy difference between the two states is a linear function of voltage, then the equilibrium distribution between the two states will be given by a classic Boltzmann relation, and the time course of the change in conductance of the population after an applied voltage step will follow a monoexponential curve. The voltage dependence of channels with multiple conductance states should not be fit by a Boltzmann relation, and should not give rise to monoexponential decay curves. However, with noisy signals, different conductance states may be resolvable into separate exponentials

only if they differ widely in magnitude. Thus, in our records above 20°C, the G_j -decay curves reflected the voltage sensitivity of all six channel states, presumably poorly resolved into two gating classes that yielded biexponential fits (Table 3). At 18°C, in contrast, where the most prevalent openings were to the γ_{40} and γ_{80} states, τ_1 became shorter or disappeared altogether leaving the decay curve to approximate a monoexponential with a slow time constant characteristic of the smaller states. Our data show that the magnitude of G_j decay also decreased as temperature was reduced, presumably again as a result of the decrease in F_o of the large conductance states.

MOLECULAR IMPLICATIONS

The connexon hemichannels that constitute gap junctions are formed from a multi-gene family of closely related proteins, the connexins (Beyer, Paul & Goodenough, 1990; Bennett et al., 1991; Willecke et al., 1991). Recent results from experiments combining the techniques of molecular biology and patch clamping suggest that junctional channels with multiple conductance states can be composed of a single class of connexins. Veenstra et al. (1992) obtained functional expression of each of the three connexins found in chick heart (Cx42, Cx43, and Cx45) by stable transfection of communication-deficient N2A cells. In cell pairs containing connexons composed only of Cx42, these workers found multiple-state channels with a maximal unit conductance of 236 ± 14 pS and substates at approximately 40 pS intervals of 201, 158, 121, and 86 pS. In contrast, other N2A cells expressing exclusively Cx43 showed a dominant γ_j of 44 ± 6 pS with rarer openings to 28 and 67 pS (Veenstra et al., 1992). These values of γ_j are in excellent agreement with the data reported here and previously (Chen & DeHaan, 1992), and they suggest that the five largest conductance levels that we recorded in embryonic heart cells reflect the activity of a single class of multi-conductance gap junction channels composed of Cx42. The γ_{40} state apparently results from the activity of coexistent Cx43 channels.

There is mounting evidence to support the contention that more than a single connexin can be expressed in a cell (Bennett et al., 1991). At least five connexins or connexin mRNAs have been identified in gap junctions of cardiac tissue: Cx40, Cx42, Cx43, Cx45, and Cx46 (Beyer et al., 1990; Willecke et al., 1991; Henneman et al., 1992; Kanter, Saffitz & Beyer, 1992; Paul, Bruzzone & Haefliger, 1993), some of which, e.g., Cx42 and Cx43, as noted, have different unit channel conductances and voltage sensitivities (Veenstra et al., 1992). Further, in hepatocytes, different connexins have been found

in a single gap junction (Traub et al., 1989; Spray et al., 1991). To be consistent with the present results, we need only assume that the two postulated populations of connexons in embryonic chick heart cells also have different temperature sensitivities. This hypothesis would not only be consistent with the present data, it could also account for our recent observation of rectifying junctions in occasional cell pairs (Chen & DeHaan, 1993b), since rectification has been reported to result from heterotypic junctions in which the connexon hemichannels in the two cells are formed from different connexins (Swenson et al., 1989; Rubin et al., 1992). A fuller discussion of various models of multi-conductance gap junctions is presented elsewhere (Chen & DeHaan, 1993a).

Our thanks to B.J. Duke, W.N. Goolsby, A. Daniel, and G. Rousseaux for assistance with aspects of this project, to F. Schmidt for help with the statistical analysis, and to L. J. DeFelice for valuable discussions of the data. The work was supported by NIH grant PO1-HL27385 to R.L.D. The manuscript was written in part while R.L.D. was a Fulbright Scholar and Visiting Professor in the laboratory of Dr. Anne Warner at University College London, UK.

References

- Beers, D.M., Bridge, J.H.B., Spitzer, K.W. 1989. Intracellular Ca^{2+} transients during rapid cooling contractures in guinea-pig ventricular myocytes. *J. Physiol.*, **417**:537–553
- Bennett, M.V.L., Barrio, L.C., Bargiello, T.A., Spray, D.C., Hertzberg, E., Saez, J.C. 1991. Gap junctions: New tools, new answers, new questions. *Neuron* **6**:305–320
- Bennett, M.V.L., Verselis, V. 1992. Biophysics of gap junctions. *Sem. Cell Biol.* **3**:29–47
- Beyer, E.C., Paul, D.L., Goodenough, D.A. 1990. Connexin family of gap junction proteins. *J. Membrane Biol.* **116**:187–194
- Brink, P.R., Fan, S.F. 1989. Patch clamp recordings from membranes which contain gap junction channels. *Biophys. J.* **56**:579–593
- Bukauskas, F.F., Weingart, R. 1993. Temperature dependence of gap junction properties in neonatal rat heart cells. *Pfluegers Arch.* **423**:133–139
- Burt, J.M., Spray, D.C. 1988. Single channel events and gating behavior of the cardiac gap junction channel. *Proc. Natl. Acad. Sci. USA* **85**:3431–3434
- Chen, Y.-H., DeHaan, R.L. 1989. Cardiac gap junction channels shift to lower conductance states when temperature is reduced. *Biophys. J.* **55**:152a
- Chen, Y.-H., DeHaan, R.L. 1990. The effects of varying pH and temperature on dye coupling through gap junctions in pairs of embryonic chick heart cells. *Int. Biophys. Congr.* **10**:316
- Chen, Y.-H., DeHaan, R.L. 1992. Multiple-channel conductance states and voltage regulation of embryonic chick cardiac gap junctions. *J. Membrane Biol.* **127**:95–111
- Chen, Y.-H., DeHaan, R.L. 1993a. Multiple channel conductance states in gap junctions. In: Gap Junctions. J.S. Hall, G.A.

- Zampighi and R.M. Davis, editors. pp. 91–103. Elsevier, Amsterdam
- Chen, Y.-H., DeHaan, R.L. 1993b. Asymmetric gap junction current between embryonic chick ventricular cells. *IUPS Abstracts* p. 178
- Coraboeuf, E., Weidmann, S. 1954. Temperature effects on the electrical activity of Purkinje fibers. *Helv. Physiol. Pharm. Acta* **12**:32–41
- Correa, A.M., Bezanilla, F., Latorre, R. 1992. Gating kinetics of batrachotoxin-modified Na^+ channels in the squid giant axon. Voltage and temperature effects. *Biophys. J.* **61**:1332–1352
- DeHaan, R.L., Chen, Y.-H., Penrod, R.L. 1989. Voltage dependence of junctional conductance in the embryonic heart. In: *Molecular and Cellular Mechanisms of Antiarrhythmic Agents*. Luc Hondeghem, editor. pp. 19–43. Futura, Mount Kisco, NY
- Fishman, G. I., Moreno, A.P., Spray, D. C., Leinwand, L.A. 1991. Functional analysis of human cardiac gap junction channel mutants. *Proc. Natl. Acad. Sci. USA* **88**:3525–3529
- Fujii, S., Ayer, R.K., Jr., DeHaan, R.L. 1988. Development of the fast sodium current in early embryonic chick heart cells. *J. Membrane Biol.* **101**:209–223
- Godt, R.E., Lindley, B.D. 1982. Influence of temperature upon contractile activation and isometric force production in mechanically skinned muscle fibers of the frog. *J. Gen. Physiol.* **80**:279–297
- Hakozaki, S., Matsumoto, M., Sasaki, K. 1989. Temperature-sensitive activation of G-protein regulating the resting membrane conductance of *Aplysia* neurons. *Jap. J. Physiol.* **39**:115–130
- Hennemann, H., Suchyna, T., Lichtenberg-Frate, H., Jungbluth, S., Dahl, E., Schwarz, J., Nicholson, B.J., Willecke, K. 1992. Molecular cloning and functional expression of mouse connexin40, a second gap junction gene preferentially expressed in lung. *J. Cell Biol.* **117**:1299–1310
- Hille, B. 1992. *Ionic Channels of Excitable Membranes*. Second Edition. Sinauer Assoc., Sunderland, MA
- Jonas, P. 1989. Temperature dependence of gating current in myelinated nerve fibers. *J. Membrane Biol.* **112**:277–289
- Kanter, H.L., Saffitz, J.E., Beyer, E.C. 1992. Cardiac myocytes express multiple gap junction proteins. *Circ. Res.* **70**:438–444
- Kimmelberg, H.K. 1975. Alterations in phospholipid-dependent ($\text{Na}^+ + \text{K}^+$)-ATPase activity due to lipid fluidity: Effects of cholesterol and Mg^{2+} . *Biochim. Biophys. Acta* **413**:143–156
- Kleinbaum, D.G., Kupper L.L. 1978. *Applied Regression Analysis and other Multivariable Methods*. Duxbury, Belmont, CA
- Kohlhardt, M. 1990. Different temperature sensitivity of cardiac Na^+ channels in cell-attached and cell-free conditions. *Am. J. Physiol.* **259**:C599–604
- Loewenstein, W.R. 1981. Junctional intercellular communication: The cell-to-cell membrane channel. *Physiol. Rev.* **61**:829–913
- Matsuda, T., Iwata, H. 1985. Species difference in temperature dependence of cardiac ($\text{Na}^+ + \text{K}^+$)-ATPase activity. *Biochem. Pharmacol.* **34**:2343–2346
- Moreno, A.P., Fishman, G.I., Spray, D.C. 1992. Phosphorylation shifts unitary conductance and modifies voltage dependent kinetics of human connexin43 gap junction channels. *Biophys. J.* **62**:51–53
- Nathan R.D., DeHaan R.L. 1979. Voltage clamp analysis of embryonic heart cell aggregates. *J. Gen. Physiol.* **73**:175–198
- Nelson, D.J., Sachs, F. 1979. Single ionic channels observed in tissue-cultured muscle. *Nature* **282**:861–863
- Noma, A., Tsuboi N. 1987. Dependence of junctional conductance on proton, calcium, and magnesium ions in cardiac paired cells of guinea pig. *J. Physiol.* **382**:193–211
- Paul, D.L., Bruzzone, R., Haefliger, J.-A. 1993. Identification of novel connexins by reduced-stringency hybridization and PCR amplification using degenerate primers. In: *Gap Junctions*. J.S. Hall, G.A. Zampighi and R.M. Davis, editors. pp. 15–20. Elsevier, Amsterdam
- Payton, B.W., Bennett, M.V.L., Pappas, G.D. 1969. Temperature-dependence of resistance at an electrotonic synapse. *Science* **165**:594–597
- Polito, A.L., Socolar, S.J., Loewenstein, W.R. 1969. Permeability of a cell membrane junction. Dependence on energy metabolism. *J. Gen. Physiol.* **53**:498–515
- Rubin, J.B., Verselis, V.K., Bennett, M.V.L., Bargiello, T.A. 1992. Molecular analysis of voltage dependence of heterotypic gap junctions formed by connexins 26 and 32. *Biophys. J.* **62**:183–195
- Rudisuli, A., Weingart, R. 1990. Gap junctions in adult ventricular muscle. In: *Biophysics of Gap Junction Channels*. C. Peracchia, editor. pp. 43–56. CRC, Boca Raton, FL
- Saez, C.J., Nairn, A.C., Czernik A.J., Spray, D.C., Hertzberg, E.L. 1993. Rat connexin43: regulation by phosphorylation in heart. In: *Gap Junctions*. J.S. Hall, G.A. Zampighi and R.M. Davis, editors. pp. 275–281. Elsevier, Amsterdam
- Spray, D.C., Chanson, M., Moreno, A.P., Dermietzel, R., Meda, P. 1991. Distinctive gap junction channel types connect WB cells, a clonal cell line derived from rat liver. *Am. J. Physiol.* **260**:C513–C527
- Stein, W.D. 1986. *Transport and Diffusion across Cell Membranes*. Academic, NY
- Sugiura, H., Toyama, J., Tsuboi, N., Kamiya, K., Kodama, I. 1990. Adenosine-triphosphate (ATP) directly affects junctional conductance between paired ventricular myocytes isolated from guinea pig heart. *Circ. Res.* **66**:93–153
- Swenson, K.I., Jordan, J.R., Beyer, E.C., Paul, D. 1989. Formation of gap junctions by expression of connexins in *Xenopus* oocyte pairs. *Cell* **57**:145–155
- Traub, O., Look, J., Dermietzel, R., Brummer, F., Hulser, D., Willecke, K. 1989. Comparative characterization of the 21-kD and 26-kD gap junction proteins in murine liver and cultured hepatocytes. *J. Cell Biol.* **108**:1039–11051
- Veenstra, R.D. 1990. Voltage-dependent gating of gap junction channels in embryonic chick ventricular cell pairs. *Am. J. Physiol.* **258**:C662–C672
- Veenstra, R.D. 1991. Comparative physiology of cardiac gap junction channels. In: *Biophysics of Gap Junction Channels*. C. Peracchia, editor. pp. 131–144. CRC, Boca Raton, FL
- Veenstra, R.D., DeHaan, R.L. 1988. Cardiac gap junction channel activity in embryonic chick ventricle cells. *Am. J. Physiol.* **254**:H170–H180
- Veenstra, R.D., Wang, H.-Z., Westphale, E. M., Beyer, E.C. 1992. Multiple connexins confer distinct regulatory and conductance properties of gap junctions in developing heart. *Circ. Res.* **71**:1277–1283
- Verselis, V., Brink, P.R. 1986. The gap junction channel: Its aqueous nature as indicated by deuterium oxide effects. *Biophys. J.* **50**:1003–1007
- Willecke, K., Hennemann, H., Dahl, E., Jungbluth, S., Heynkes, R. 1991. The diversity of connexin genes encoding gap junction proteins. *Eur. J. Cell Biol.* **56**:1–7