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GATING OF GAP JUNCTION CHANNELS

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ABSTRACT Gap junctional conductance (g_j) in various species is gated by voltage and intracellular pH (pH_i). In amphibian embryos, g_j is reduced to half by a 14 mV transjunctional voltage (V_j), a change that in fish embryo requires ~28 mV. Crayfish septate axon and pairs of dissociated rat myocytes show no voltage dependence of g_j over a range of $V_j > \pm 50$ mV. In fish and amphibian blastomeres, g_j is steeply decreased by decrease in pH_i (n, Hill coefficient: 4.5) and the apparent pK_H (7.3) is in the physiological range. In crayfish septate axon the pK_H is lower (6.7) and the curve is less steep ($n = 2.7$). Rises in cytoplasmic Ca can also decrease g_j but much higher concentrations are required (>0.1 mM in fish blastomeres). Voltage and pH gates on gap junctions in amphibian embryos appear independent. In squid blastomeres pH gates exhibit some sensitivity to potential, both transjunctional and between inside and outside. A pharmacology of gap junctions is being developed: certain agents block g_j directly (aldehydes, alcohols, NEM in crayfish); others block by decreasing pH_i (esters that are hydrolyzed by intrinsic esterases, NEM in vertebrates, and, as in the experiments demonstrating the effect of pH_i, weak acids). Certain agents block pH sensitivity without affecting voltage dependence (retinoic acid, glutaraldehyde, EEDQ), further indicating separateness of pH and voltage gates. These studies demonstrate a dynamics of gap junctional conductance and variability in gating in a series of possibly homologous membrane channels.

INTRODUCTION

Intercellular communication is mediated in many tissues by gap junctions (cf. Bennett and Goodenough, 1978; Bennett, 1977). The gap junction in thin section after lanthanum infiltration shows the cells' membranes to be separated by a gap of 2–4 nm spanned at regular intervals by electron transparent structures. In freeze-fracture the gap junction is generally a macular array of 8–10 nm particles that cleave with the P face in all tissues of most nonarthropod phyla, and with the E face in arthropods. There is general agreement that the gap junction particles are proteinaceous oligomers and in several tissues a 25–27 kD protein has been isolated that presumably represents the monomer (cf., Nicholson et al., 1983). Gap junction channels connect the cytoplasms of coupled cells to provide a pathway for communication without access to extracellular space. They are relatively large channels, allowing the flux of molecules at least 12 Å in diameter and with molecular weights in excess of ~1,000 daltons. The functions that gap junctions serve in coupled tissues are presumed to be current flow in excitable tissues and exchange of nutrients and transfer of signaling molecules in inexcitable ones.

Study of biophysical properties of gap junction channels is progressing in several laboratories. Of particular interest are gating mechanisms that have recently been demonstrated. One of our strategies is to compare the gating properties of gap junctions in various preparations; our long-range goal is to understand more clearly how ions move through channels and how the channels are under cellular control. In this paper we review different forms of gating in different species and tissues, suggest how these differences might arise, and discuss some implications for junctional structure.

RESULTS

Gating of g_j by Voltage

Amphibian Embryo. Junctional conductance (g_j) can be altered by applied potentials in some systems but not in others. The voltage dependence of junctional conductance has been well-characterized in blastomeres of amphibia which are readily dissected to yield coupled cell pairs (Spray et al., 1981*a*; Harris et al., 1981). Initially it was observed that during application of long constant-current pulses to one cell of a pair, voltage in the directly polarized cell increased while voltage in the other cell decreased (Spray et al., 1979). The behavior was symmetrical for currents of either sign in either cell, and single cells were electrically linear, indicating that the active element was the junctional membrane.

A dual voltage-clamp technique was used to study steady-state and equilibrium properties of the junctions.

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Each cell of a coupled pair was clamped to a common holding potential with a separate voltage-clamp circuit. When one cell was stepped to a new voltage, the other cell's clamp supplied current to hold its potential constant; this current was equal in magnitude and opposite in sign to current through the junctional membrane. Junctional current divided by the transjunctional voltage gives g_j.

It was found that junctional conductance decreases to a new steady-state value for transjunctional voltages of either polarity (Fig. 1). For each polarity the steady-state conductance as a function of voltage is well-fit by a Boltzman relation of the form $g_j = \{(g_{max} - g_{min})/1 + \exp[A(V_j - V_0)]\} + g_{min}$.

In this equation g_{max} is the maximum conductance, g_{min} is the minimum conductance for large transjunctional voltages ($g_{min} < 0.05\ g_{max}$ in amphibia), V_j is the transjunctional voltage, V_0 is the voltage for which g_j lies halfway between g_{max} and g_{min}, and A is a parameter expressing voltage sensitivity. The Boltzman relation applies to an equilibrium distribution between open and closed states where the energy difference between them is a linear function of transjunctional voltage.

For junctions in amphibian embryos the g_j vs. V_j relation is steep, g_j having about the same voltage sensitivity as the m gate of the sodium channel in squid axon. The conductance g_j is dependent only on transjunctional potential and not on potential between cytoplasm and exterior. Thus the g_j–V_j relation was the same when determined at various potential differences between cytoplasm and external medium.

The time course of the decrease in g_j when V_j is stepped from zero is well-fit at each voltage level by a single exponential $g_j = g_\infty + (g_0 - g_\infty) \exp(-t/\tau)$ where g_0 and g_∞ are initial and steady-state values of conductance and τ is a time constant dependent on transjunctional voltage. This observation suggests that the conductance change is a reversible first-order process with forward and reverse rate constants solely a function of voltage. This inference is supported by the finding that the time constant (for voltages of a single polarity) is the same independent of the initial value of conductance and whether conductance is increasing or decreasing. Opening and closing rate constants, α and β, were calculated from the time constants and steady conductances over the range of clamping voltages used, and were well fit by exponential relations of the form $\alpha = \lambda \exp(-A_\alpha[V - V_0])$, and $\beta = \lambda \exp(A_\beta[V - V_0])$.

The time course of the conductance changes is more complex when a transjunctional voltage is reversed in polarity. If, after a rectangular pulse of one polarity, voltage is stepped to a new value of opposite sign, current does not simply relax exponentially to its steady-state value. Instead it rises transiently above the value of decay for the second pulse alone. Calculations show that this behavior is consistent with two gates in series where all the transjunctional voltage drop occurs across the closed gate. Thus the closed gate must open before the transjunctional voltage can act on the open gate to cause it to close.

Using steady-state and kinetic parameters determining junctional conductance, we calculated the expected response to current injection for comparison with current clamp data (Harris et al., 1983). The agreement between calculation and experiment was generally good.

Although rate constants were reasonably well fit by exponential functions of voltage for the relatively small clamping pulses applied, these exponential relations appeared to be inaccurate for larger voltages. For calculating responses under current clamp where large voltages occur, to obtain satisfactory agreement it was necessary to assume closing rates that were slower than predicted by the exponential relations. Similarly, with reversal of transjunctional voltage the opening rates appeared to be slower than predicted. These deviations may represent a form of viscosity at the molecular level and have no great significance for structural models of the gap junctional molecule.

The voltage-current relation obtained under current-clamp conditions for an ascending then descending ramp of applied current shows a region of hysteresis. In this region the system is bistable and cells can be stably coupled or stably uncoupled depending on whether current has decreased from a large value or increased from a small value. Transitions from coupled to uncoupled states are regenerative; a decrease in g_j increases V_1 and decreases V_2, thus increasing V_j and further decreasing g_j. The bistability is also predicted by calculations from the voltage-clamp data.

The voltage dependence of g_j between amphibian blastomeres provides an intriguing mechanism that may gate intercellular communication during tissue differentiation. A gradient of nonjunctional membrane potentials due to permeability changes, electrogenic pumping, or other factors could shift the gap junctions past threshold for the regenerative change to an uncoupled state, thus forming a boundary between the regions.

From the foregoing data and morphological considerations we derived an explicit model of voltage gating in the gap junction macromolecule (see below). The apparent symmetry of the gap junction about the plane midway between the apposed membranes and the effects of reversal of transjunctional voltage suggest that there is a voltage gate in each of the apposed membranes. The voltage sensitivity can be accounted for by dipole moment changes between open, transition, and closed states of the channels. The exponential rates imply that the energy differences are linear functions of voltage, which would hold if dipole moment differences were constant, independent of voltage. The reasonableness of these properties for proteins in general has been argued in respect to the acetylcholine receptor molecule (Neher and Stevens, 1977).

Fundulus Embryo. Although voltage dependence of g_j between blastomeres of the killifish *Fundulus*

was not previously observed in current-clamp experiments, it is readily demonstrated with voltage clamp (cf. Fig. 1; White et al., 1982). The voltage sensitivity is less than in amphibian embryos (A is ~1/4 its value in amphibia), the transition is shifted towards larger voltages (V is 28 mV instead of 14 mV) and the minimum conductance is greater (~20% of its maximum value).

Preliminary studies show that kinetics of channel closure in *Fundulus* blastomeres are more complex than for amphibian junctions, two exponentials being required to fit the currents recorded at larger voltages. The additional time constant is slow and we have not yet ruled out the possibility that ionic accumulation or depletion is responsible. In this system, as in amphibia, g_j is not influenced by the potential difference between cytoplasm and exterior. The degree of sensitivity to V_j is so low as to be of doubtful physiological significance.

Other Tissues. A response similar to that in amphibian blastomeres is seen in the *Limulus* ommatidium, but complexities of structure make detailed comparisons unreasonable (Smith and Baumann, 1969). A number of coupled systems of neurons in vertebrates show no indication of voltage dependence, but because of lack of knowledge of the actual transjunctional potentials it is difficult to exclude some degree of voltage dependence (cf., Bennett, 1977). Two other systems that we have studied (crayfish septate axon [see also Johnston and Ramon, 1982] and pairs of ventricular myocytes [White et al., 1983]) show no dependence of g_j on potential (cf., Fig. 1). For large and long voltage pulses of either polarity, g_j is constant. Electrotonic synapses that rectify are also known (Furshpan and Potter, 1959; Auerbach and Bennett, 1969; Baylor and Nicholls, 1969). These junctions differ in being unidirectional and very much faster in reaching steady state (rates have not been determined). The mechanisms, if similar to those in blastomeres, must involve much smaller activation energies to allow the faster rates.

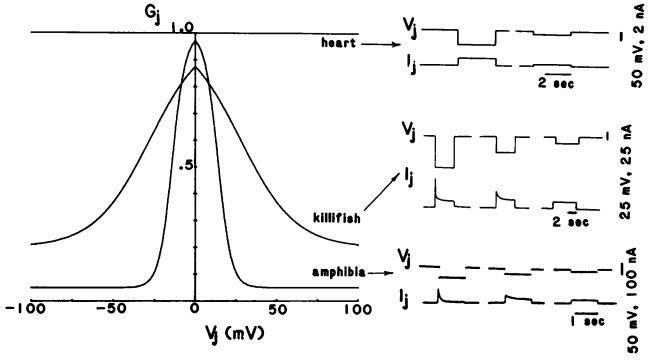

FIGURE 1 Effects of transjunctional voltage on normalized junctional conductance (G_{max} = 1) between pairs of dissociated ventricular myocytes from adult rat heart, blastomeres of killifish (*Fundulus*) and blastomeres of amphibia. Curves for blastomeres are pairs of Boltzmann relations symmetrical about the zero V_j axis. G_{max} is obtained by extrapolation of steady-state junctional currents for small voltages. Representative voltage-clamp records are shown to the right. In heart, I_j is not affected by V_j. In *Fundulus* and amphibian blastomeres, I_j declines during larger clamping pulses indicating decrease in junctional conductance.

As discussed below, the voltage-sensitive gate and pH gate in vertebrate gap junctions appear to be separate. A pH gate also has been shown in several invertebrate systems and it exhibits varying degrees of voltage sensitivity.

Gating of g_j by Intracellular pH (pH_i)

As has now been shown in many tissues, increase in cytoplasmic acidity decreases junctional conductance (Turin and Warner, 1980; Rose and Rick, 1978; Reber and Weingart, 1982; Giaume et al., 1980; Iwatsuki and Petersen, 1979). We have quantified the relation between g_j and pH_i in several preparations by simultaneously measuring pH_i in one cell of a pair and g_j while pH_i was changed using externally applied CO_2 and various weak acids. For fish and amphibian blastomere pairs, g_j is a sensitive function of pH_i, with an apparent pK_H of 7.3 and a Hill coefficient of ~4.5 (Spray et al., 1981 *b*). Normal pH_i in these tissues is ~7.6, so that slight cytoplasmic acidification can substantially decrease g_j (Fig. 2). The g_j–pH_i curve is similar to that recently published for pH_i dependence of longitudinal resistance in Purkinje strands from ungulate heart (Reber and Weingart, 1982), although in that case normal pH_i is ~7.2. Gap junctions of cardiac Purkinje fibers may thus be maximally sensitive to slight changes of pH_i in either direction.

The pH dependence of g_j in crayfish septate axon is less steep (Hill coefficient 2.4) and the apparent pK_H (6.75) is

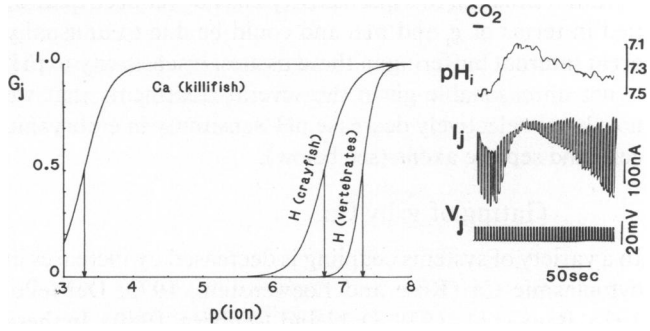

FIGURE 2 Effects of Ca and H ions on junctional conductance. Curves are Hill equations fit by eye to normalized data from many experiments. (cf., Spray et al., 1981 *b*, 1982 *a*). pH was measured in vertebrate blastomeres and crayfish septate axon with ion-selective electrodes. Ca concentrations at the junctional membrane of *Fundulus* blastomeres were fixed at known concentrations by an internal perfusion technique. Arrows are drawn vertically at the half-maximal conductance level for each curve to indicate apparent pK. An experiment demonstrating pH-dependence of g_j between *Rana* blastomeres is shown on the right. A pair of cells was subjected to dual voltage clamp during and after CO_2 exposure. I_j is the clamping current in one cell while voltage in the other cell, V_j, was pulsed to the indicated values. G_j is measured as the pulse amplitude in I_j when V_j was pulsed a constant amount; g_j declined and then recovered with nearly the same time course as the change in pH_i. The drift in the I_j record is a result of change in the nonjunctional membrane of that cell.

lower than that of the vertebrate cells examined (Fig. 2). Normal intracellular pH of crayfish axon is also lower, ~7.1. There is some dependence of input conductance on pH_i, and the coupling coefficient reaches maximum value near normal pH_i, decreasing on either side (Carvalho et al.).[1]

A category of potent compounds that block g_j reversibly through decreasing pH_i is a family of substituted benzyl esters (Spray et al., 1982 *b*; Nerbonne et al., 1982). These compounds, a typical one being *o*-nitrobenzyl acetate (oNBA), are membrane-permeant and act at low concentration (1–2 mM) in vertebrate and squid embryos, crayfish septate axon, and *Chironomus* salivary gland. The decrease in pH_i that occurs can be ascribed to cytoplasmic liberation of protons during hydrolysis by intracellular esterases. All the ester molecules are uncharged and can cross the cell membrane, whereas for a weak acid two pH units above its pK only 1% is in the permeant undissociated form. Thus the esters are effective at a 100-fold lower concentration, which may account for their lesser toxicity that we have generally observed. These compounds seem promising agents for cytoplasmic acidification and studies of effects of uncoupling cells. We also emphasize that caution should be observed when cells are loaded with membrane-permeant esterified Ca and pH indicators, for cytoplasmic acidification will be produced by hydrolysis. Furthermore, biologically active esters such as tumor promoters may acidify cells, as we have directly measured.

Relative insensitivity to exposure to CO_2 and other acidifying agents has been reported for lens and rods in the toad retina (Schuetze and Goodenough, 1982; Griff and Pinto).[2] Although this insensitivity has not yet been quantified in terms of g_j and pH_i and could be due to unusually great internal buffering in these tissues, insensitivity to pH_i is not unreasonable given the several treatments that we now know selectively decrease pH_i sensitivity in embryonic cells and septate axons (see below).

Gating of g_j by Ca

In a variety of systems coupling is decreased by increases in cytoplasmic Ca (Rose and Loewenstein, 1975; DeMello, 1975; Baux et al., 1978; Dahl and Isenberg, 1980). In these experiments, uncoupling may have been partially due to increased nonjunctional conductance of the cell. The question then arises whether cytoplasmic acidification acts on junctional conductance through increase in cytoplasmic-free Ca. In three studies little or no increase in cytoplasmic Ca was observed during acidification adequate to greatly decrease junctional conductance (Hess and Weingart, 1980; Rink et al., 1980; Bennett et al., 1978). The simplicity of the pH_i vs. g_j relation as well as the rapid reversibility are consistent with a direct action of protons on the junctional macromolecules in these cells. Ca also acts independent of pH as shown by combined pH electrode and aequorin studies in *Chironomus* salivary gland (Rose and Rick, 1978) and by an intracellular perfusion technique in *Fundulus* blastomeres. In the latter case, one cytoplasmic aspect of the junctions between a pair of cells was perfused with solutions well buffered to specific Ca and H activities (Spray et al., 1982 *a*). Values of g_j were inferred from changes in conductance of the perfused membrane, with control measurements indicating that g_j dominated any effect on nonjunctional membrane. Sensitivity to pH was comparable to that in intact preparations. Much higher Ca concentrations were required to reduce g_j (Fig. 2). When pH was held constant at 7.6, changing pCa from 6 to 4 had no effect; but between 0.1 and 1.0 mM free Ca, the junctional conductance decreased to a low level (and might have decreased more at higher Ca concentrations). The range of possible Hill plots for the effect of Ca on g_j showed a pK_{Ca} of ~3.3 (corresponding to 0.5 mM Ca) and a slope of ~2 (about half that determined previously for pH). This curve is similar to that shown for the low-affinity Ca-binding site in membranes isolated from cardiac ventricular cells (Nishiye et al., 1980).

The Hill coefficient for the divalent Ca is about half that for the monovalent H, which may reflect action on the same site. Competition between these ions for a single site at normal cytoplasmic ionic concentrations would be expected to be minimal, because the Ca affinity is so low (cf. Fig. 2).

The low apparent affinity of the gap junction to cytoplasmic Ca, ~10,000 times less than for pH_i, suggests that the relative importance of Ca ion in ordinary cellular controls of the gap junction channel is minimal. Still, cytoplasmic Ca approaches extracellular concentrations after membrane disruption and Ca may be important in pathological situations for isolating damaged cells.

Independence of Gating by pH and Voltage in Amphibian Embryos

In pairs of amphibian blastomeres, dependence of g_j on transjunctional voltage is not affected by pH_i changes over the range 7.7 to 7.0. At any given voltage equilibrium, conductances are a constant proportion of the maximal conductance, and the time courses of conductance decreases during long voltage steps are exponential with similar time constants (Fig. 3). These data suggest that pH_i over this range of values does not affect the voltage sensor in the channel and that forward and backward rate constants are unchanged. It is unlikely that rate constants are changed in opposite directions by the same amount so that the time constants are unaffected but steady state values are reduced. The simplest hypothesis is that voltage and pH act on separate gating mechanisms. Not only is the

[1]Carvalho, A. C., D. C. Spray, and M. V. L. Bennett. Manuscript in preparation.

[2]Griff and Pinto. Personal communication.

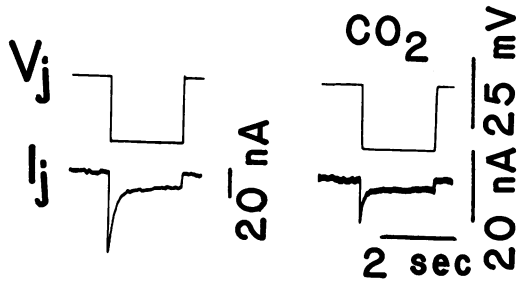

FIGURE 3 Independence of pH and voltage gates in pairs of amphibian blastomeres. Junctional currents recorded from a pair of coupled cells during a rectangular pulse in V_j had an initial peak with an exponential decline to a steady-state value. During exposure to CO_2 (*right*), peak and steady state values were reduced to ~25% of their initial (*left*) and recovery values. The ratio of steady-state to peak conductance and the time course of decrease in conductance were similar in the two conditions, suggesting that the two gates are independent.

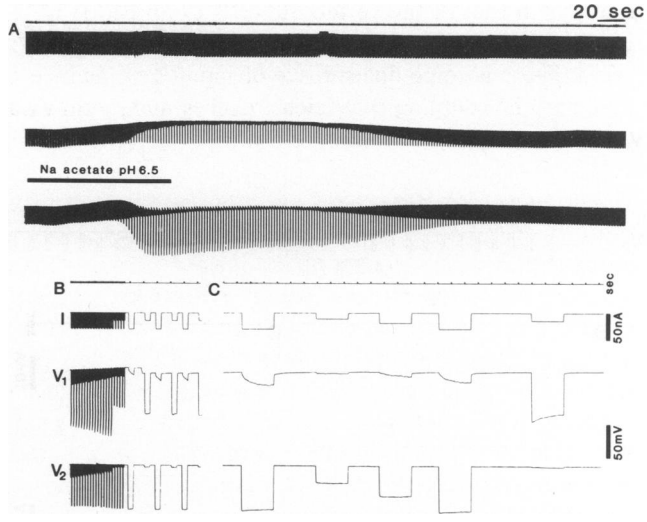

FIGURE 4 Reducing pH_i decreases g_j in pairs of squid blastomeres and reveals voltage dependence of g_j. *A*, transient exposure to sea water at pH 6.5 in which Na acetate was substituted for NaCl reversibly reduced g_j between a pair of squid blastomeres. Current pulses were delivered alternately in each cell. The larger vertical deflections in each cell measure the input resistance of that cell and the smaller deflections measure the transfer resistance from the other cell. *B*, when conductance was decreased a hyperpolarizing pulse in cell 2 produced a voltage in cell 1 (V_1) that increased during the pulse. Hyperpolarizing pulses in cell 1 produced only rectangular voltage responses in cell 2. The chart recorder speed was increased 10-fold midway during the record. *C*, the increase in V_1 for currents applied in cell 2 was greater for larger currents (first four pulses). Little increase in g_j occurred during current pulses applied in cell 1 (5th pulse). 1-s time marks occur above each record.

voltage-sensitive region unaffected by pH_i, but the pH_i-sensitive region is unaffected by voltage. Additional evidence of separateness of pH and voltage sensitive regions is provided by pharmacological agents that affect these gating properties independently (see below).

Voltage Dependence of pH Gating in Squid Embryos

Pairs of blastomeres from cleavage stages of squid embryos are closely coupled electrically, and the coupling is markedly reduced by cytoplasmic acidification (Fig.4 *A*), although the relation between g_j and pH_i has not yet been determined. Although the large magnitude of the "resting" junctional conductance makes measurement difficult, it is apparently voltage independent. However, as junctional conductance is reduced by decrease in pH_i, a form of voltage dependence appears in which junctional conductance is increased by appropriate applied potentials. In Fig. 4*C*, hyperpolarization of cell 2 causes an electrotonic potential in cell 1 that increases to a new steady-state value with a time constant of ~1 s (much slower than the input time constant). The steady-state conductances are larger for larger voltages and the g_j vs. V_j relation does not saturate for V_j up to at least 60 mV (not illustrated). The dependence on voltage is complex and varies from preparation to preparation. The conductance may be increased by hyperpolarization of one of the cells, but not the other (Fig. 4 *C*). In other cases the effects are more symmetrical (Fig. 5 *A*). Hyperpolarization of one cell is generally more effective than equal depolarization of the other cell (compare the first long pulse in Fig. 5 *A* with Fig. 5 *C* and the third long pulse in Fig. 5 *A* with Fig. 5 *B*). Thus the conductance increase is not simply dependent on transjunctional voltage. Equal hyperpolarization of both cells can cause small increases in g_j, which thus occur in the absence of a transjunctional voltage (Fig. 5 *A*, second long pulse).

The interpretation of these results is still somewhat tentative because of limited data and difficulty in holding the preparations constant. It is likely that there are two gates per channel, one in each membrane, as postulated for voltage sensitivity in vertebrate embryos. A physiological reason for proposing two gates is that in preparations with asymmetrical properties, a reversed transjunctional voltage can accelerate the decrease in g_j that occurs in the absence of a voltage gradient. The cause of the asymmetrical electrical properties is unknown but may result from different H (or Ca) concentrations in the two cells. Measurement of voltage-dependent opening of channels would not be possible if two closed gates of opposite sensitivity to transjunctional voltage were in series. The appearance of voltage sensitivity in squid blastomeres is transitory as pH_i is lowered, and seems to occur in a narrow range where a substantial fraction of the channels may be closed on only one side.

The dissimilar effects on g_j of depolarization and hyperpolarization suggest that the gating macromolecules are sensitive to both transjunctional potential and potential between cytoplasm and exterior. The action of the inside-outside potential is seen directly when both cells are equally hyperpolarized (second long pulse in Fig. 5 *A*). A tendency for conductance to increase or decrease as a

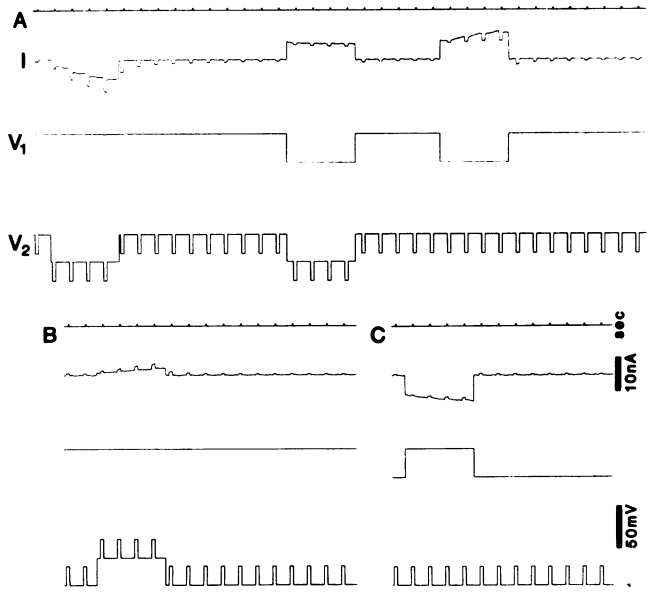

FIGURE 5 Dual voltage-clamp experiments with a pair of squid blastomeres during cytoplasmic acidification. Brief test-voltage pulses in cell 2 were superimposed on long pulses in either cell 1 or 2. The current trace shows currents in cell 1. *A*, when cell 2 was hyperpolarized (first long pulse) the junctional current associated with the long pulse and the brief currents associated with test pulses increased during the pulse, indicating increase in g_j. When cell 1 was stepped (third long pulse), the brief pulses show that g_j increased. The current during the long pulse is the sum of junctional and nonjunctional currents; the gradual rise represents change in g_j as indicated by the brief pulses. After the long pulses, conductance declined to its initial value over several seconds. When both cells were hyperpolarized, a smaller conductance change, about twofold, was seen (second long pulse). *B*, *C*, depolarizing either cell caused a smaller increase in g_j than equal hyperpolarization of the other cell, indicating that g_j is not simply dependent on transjunctional voltage.

result of hyperpolarized or depolarized inside-outside potential would add to the effect of a transjunctional potential when one cell was hyperpolarized and subtract when the other cell was depolarized. The summation of effects might also depend on locations of the voltage-sensitive components and voltage drops within the channels.

The suggestion that both transjunctional potential and inside-outside potential act on junctional channels seems reasonable on several grounds. The gap between apposed membranes should be accessible to small ions, and a large conformational change could well produce dipole moment changes with vector components both along the channel axis and perpendicular to it.

Junctions in *Chironomus* salivary gland have recently been shown to have pronounced dependence on inside-outside potential (Obaid et al., 1983). Some dependence on transjunctional potential is not yet excluded. The g_j-vs.-potential relation is simply shifted along the voltage axis by decrease in pH_i (and probably also by decrease in pCa_i). Voltage-sensitive and pH gating are interdependent and may well be mediated by the same structure (in contrast to the situation in amphibian blastomeres). We conclude, therefore, that pH-gating mechanisms exhibit a range of voltage sensitivities, quite sensitive to inside-outside potential in *Chironomus*; somewhat sensitive to transjunctional potential and slightly sensitive to inside-outside potential in squid blastomeres; and quite insensitive to either kind of potential in vertebrate embryos.

Pharmacological Properties of the Gap Junction Channel

We have examined the effects of many different types of drugs on properties of gap-junction channels, finding several that reduce g_j or its voltage or pH dependence. The substituted benzyl esters discussed above may be considered pharmacological agents because of their relatively great potency, although they act through pH_i.

Crayfish axons are uncoupled by several sulfhydryl reagents at low concentration, including *N*-ethylmaleimide (NEM) and diamide, with no change in pH_i or sensitivity of g_j to pH_i (Carvalho and Ramon, 1982). Vertebrate embryos are uncoupled by 10 mM NEM but that uncoupling is accompanied by and explicable in terms of a drop in pH_i.[3] Diamide uncouples without changing pH_i, but other suhydryl reagents are ineffective.

Octanol decreases g_j in crayfish septate axon (Johnston et al., 1980), cardiac myocytes,[3] and vertebrate embryos (Fig. 6) without changing (in crayfish or embryos) pH_i or pH dependence of g_j.

Aldehydes (formaldehyde, valeraldehyde, glutaralde-

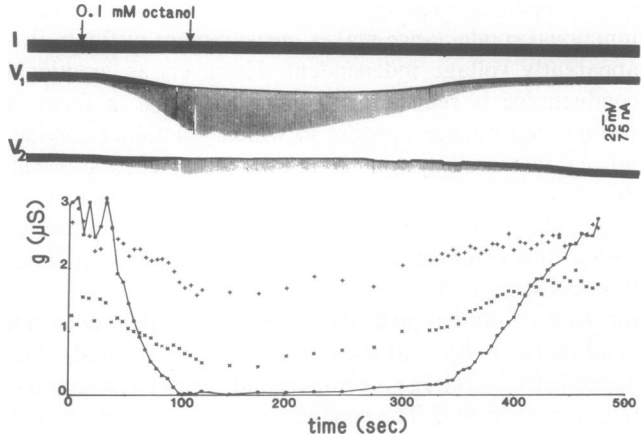

FIGURE 6 Octanol (0.1 mM) reversibly uncouples killifish (*Fundulus*) blastomeres. The upper three traces are similar to Fig. 4. The graph in the lower part of the figure shows conductances of junctional (filled symbols) and nonjunctional (pluses and crosses) membranes calculated from the input and transfer resistances. The conductances fall sharply when octanol is applied and then recover when it is rinsed away.

[3]Spray, D. C., R. L. White, A. C. Carvalho, A. L. Harris, and M. V. L. Bennett. Unpublished results.

hyde, and dihydroxybenzaldehyde) reduce g_j irreversibly (Spray et al., 1980). This channel blockade implies that the structural characterization of open channels is not possible using fixed material (cf., Bennett et al., 1972). Furthermore, the fixative glutaraldehyde reduces channel gating by pH_i (see below).

We have found three compounds that block pH dependence of gap junctions: retinoic acid, glutaraldehyde at 10 μM concentrations, and ethoxy-*N*-ethoxydihydroquinoline (EEDQ), a carboxyl reagent (Carvalho et al., 1982). The effect of retinoic acid is reversible, and retinoic acid and EEDQ can affect pH dependence without reducing g_j. If applied while pH is low, these compounds actually elevate g_j. Application of 0.2 mM EEDQ for a few minutes does not affect g_j but renders the conductance of the junctions largely insensitive to pH_i over the normal range (Fig. 7). Similar results were obtained for retinoic acid and glutaraldehyde. None of these compounds affects either steady state or kinetic properties of gap-junction voltage dependence (illustrated for EEDQ in Fig. 7).

SUMMARY

What We Know or Suspect About Gating of Gap-Junction Conductance

The data discussed above provide evidence of functional and pharmacological separability of voltage and pH gating mechanisms in amphibian and fish embryos. Because the gating mechanisms are separable, it is plausible that they involve conformational changes of different regions of the channel macromolecules.

For the sake of concreteness we suggest that a voltage-sensitive gate is located near each cytoplasmic end of the gap junction channel (Fig. 8 *B*). The open-closed transition of this gate could correspond to the structural change reported by Unwin and Zampighi (1980), although their

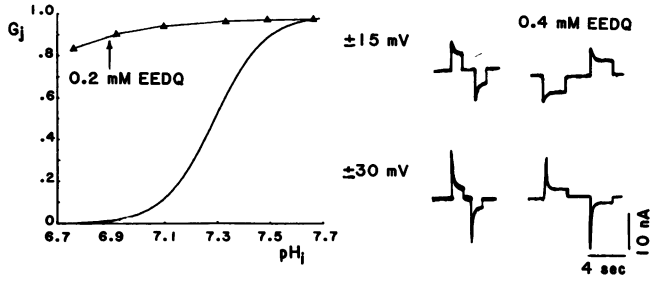

FIGURE 7 EEDQ reduces pH dependence of g_j in *Fundulus* blastomeres but does not affect g_j magnitude or voltage dependence. On the left the normal g_j-pH_i relation (*lower line*) is compared with the pH sensitivity after 0.2 mM EEDQ exposure (▲). On the right, currents due to applying rectangular transjunctional voltage pulses of +15 mV and +30 mV before and after 0.4 mM EEDQ have similar and steady-state values and time constants of decay, showing that voltage dependence is little changed.

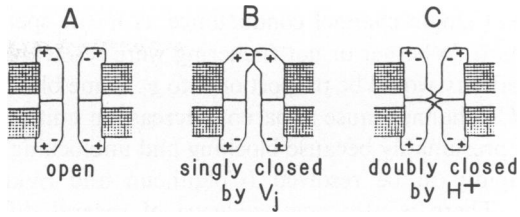

FIGURE 8 Schematic diagram of a vertebrate gap junction in each of three states. *A*, all gates open. *B*, one gate closed by transjunctional voltage. *C*, both pH gates in the closed configuration.

method of specimen preparation cannot be considered an uncoupling treatment. One reason, albeit a weak one, for choosing this location is the lack of dependence of this gate on inside-outside potential. Also, the experiments in which transjunctional polarity is reversed are consistent with the presence of two gates in series.

We suggest that the pH-dependent gate is near the center of the junctional structure (Fig.8 *C*), and that gating could result from a structural difference of the sort seen by Makowski et al. (1977), who, however, had no experimental control over whether the structure assumed the supposed open or closed configuration. At this location near the intercellular gap the gate could be more readily acted on by the voltage difference between channel interior and extracellular space, as in *Chironomus* and squid. It is reasonable that the pH gate is also the gate dependent on intracellular Ca.

Our interpretations of our data generally assume that channel gating is an all-or-none phenomenon: a channel is either open or closed. The strongest evidence for this hypothesis is still the voltage dependence in which first-order changes in conductance are most readily explained in terms of probabilities or rates of transitions between open and closed states. Ultimately, single channel recordings will be required to establish the validity of this view. Subsidiary hypotheses are necessary to account for the residual conductance, g_{min}. There may be a population of voltage-insensitive channels (like those in some other tissues); channels may close partially; or the fraction of time that channels are open may not go to zero even with large voltages. The second possibility might be tested by dye flux measurements. If a channel were partially closed, it presumably would exhibit a lower permeability to molecules near the critical size for permeation than to small ions. The ratio of dye permeability to conductance would decrease instead of remaining constant.

If the gap junction had a gating mechanism in series with a selectivity filter, as is postulated for the Na channel (Strichartz, 1973), conductance might decrease in proportion to the change in permeability. It is difficult to conceive of nonselective partial gating, but flickering between open and closed states with a variable mean open time appears reasonable. If flickering of the channel gate were too rapid to be recorded, one would observe a gradual decline in

apparent single channel conductance as mean open time decreased. Whether or not flickering were resolvable, dye permeability would be proportional to g_j. Some blockers of the ACh channel cause apparent decrease in unit conductance, presumably because blocking and unblocking occur too rapidly to be resolved (Colquhoun and Sakmann, 1981). There is also now evidence of several different conductance states in ACh channels, but no change in selectivity has been reported.

The titration curve of g_j as a function of pH_i in vertebrate embryos is consistent with all-or-none closure produced by the pH gate as well. All-or-none gating is supported by preliminary experiments[3] in which Lucifer Yellow flux remains roughly proportional to g_j when g_j is changed severalfold by changes in pH_i. Lucifer Yellow is close to the limit of junctional permeation and should be sensitive to small constrictions in channel diameter. Evidence for all-or-none closure based on proportional restriction of flux of large and small molecules has recently been reported for *Chironomus* salivary gland (Zimmerman et al., 1983) in contrast to the earlier suggestions from the same laboratory of preferential exclusion of large molecules (Loewenstein, 1981). Single-channel data would be expected to reveal the behavior of the pH gating mechanism, as closure produced by a conformational change rather than by occupation of binding sites should be slow. Evidence of stepwise conductance changes were reported for response to increased intracellular Ca (Loewenstein et al., 1978), quite possibly operating on the pH gate. These transitions were not of constant amplitude, but resolution may have been a problem. As pointed out above, however, apparent gradations in conductance can result from unresolved variable flickering between constant open and closed states.

Comparison of junctions in different species provides a basis for conjecture on the structural necessities. In the two kinds of vertebrate embryos examined, voltage dependence is pronounced in one and somewhat weak in the other. If a dipole moment change between open and closed conformations is responsible for both instances of voltage dependence, a large change is not essential for the transition. With respect to evolution, more data may tell us whether the strong voltage dependence seen in amphibian embryos is more primitive or more advanced than the weak voltage dependence of the teleost. Voltage dependence of junctional conductance, like that in amphibian embryos, appears to occur in *Limulus* ommatidium (Smith and Baumann, 1969); if this be true, a wider homology between gap junctions seems likely. The lack of voltage dependence in junctions of cardiac myocytes of rat (White et al., 1983) and crayfish axon (Johnston and Ramon, 1982; confirmed by us) may mean that some gap-junction molecules have evolved to the point where they can no longer make the required conformational change. Alternatively, analogous gating may be possible but with only a negligible change in dipole moment. Rectifying synapses can be accounted for in terms of a single gate. As noted above, the rapidity of gating would require a lower energy barrier between open and closed states.

The pH gate (or pH-induced conformational change) appears frequently among vertebrates and invertebrates, and comparisons similar to those for voltage dependence can be made. The few cases of pH independence could involve an amino acid change that removed a necessary H-ion–binding site or lowered its affinity; other molecular changes might lead to a rigid molecule. The ability to block pH sensitivity with simple chemical treatments implies that mutational processes might be equally effective. The only invertebrate examined adequately has a pH sensitivity (and normal pH) shifted towards greater acidity. The junctions in squid embryos (from preliminary data with intracellular phenol red) also have their pH sensitivity shifted toward lower pH_i. The voltage sensitivity of the squid junctions, apparently of the pH gate, is relatively complex in that it depends on both transmembrane and inside-outside potentials. The suggestion that changes underlying this gating involve dipole moment change in both directions of field appears to provide no great theoretical obstacles. Similarly, the data from *Chironomus* cells can be fit into a general scheme where voltage dependence results from dipole moment changes associated with conformational changes. Conversely the lack of voltage effects on the pH gate in vertebrates means either lack of dipole moment change or a higher energy barrier between states.

It has recently become possible to speak of a pharmacology of gap junctions. One aim is to infer the junctional structure. A second is to find a specific blocker of junctional communication for analysis of the roles of gap junctions, which are still obscure in most inexcitable tissues. While a magic bullet plugging the channels is not yet in hand, a number of different treatments exist (e.g., substituted benzyl esters and octanol) that would be expected to have differing side effects. Thus a common outcome following multiple drug treatments may represent a common action on gap junctions.

The controls of junctional conductance discussed in this paper are undoubtedly basic to the role of the junctions in the organism's physiology. Cells can also control when junctions form and when they disappear, a topic not addressed here (but see Bennett et al., 1981; Spray et al., 1982 *c*). The rapid responses of decreasing conductance may in part be precursors of the longer-term changes of junction removal.

The study of gap junctions will go forward at both cell and tissue levels. The biophysical analysis should reveal aspects of junctional structure and also provide experimental controls over junctional communication that will lead to increased understanding of its functional significance.

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DISCUSSION

Session Chairman: Peter Rand *Scribes*: Lyanne Schlichter, Gary A. Gintant, and Juli Lai Weiss

BRINK: In your records, you show that V_j and pH_i affect g_j. There is a residual g_j especially in the voltage dependence. In the earthworm septate axon, we see a residual g_j in the pH dependence but don't see any V_j sensitivity (Verselis and Brink, this volume). Do you have any evidence that residual g_j is due to two populations of channels, or to incomplete closure of a homogeneous population, or a statistical mechanism, or something else?

SPRAY: When pH_i is reduced to 6.9–7.0 in fish and amphibian blastomeres, g_j is essentially zero, even at large V_j. There is residual g_j of 20% in fish and 5% in amphibians. This difference is one that distinguishes these two gates in the vertebrate embryonic gap junctions. Residual g_j might be due to a small percentage of voltage-independent channels, to open time not going to zero even at high V_j, or to partial closing of channels. One way to distinguish among these possibilities would be to determine whether permeability to a large molecule is abolished when only residual g_j remains. In our original experiments using Lucifer yellow in amphibian cells we saw no transfer, but the resolution might have been too low.

BRINK: When you voltage clamp amphibian junctions at various pH_i, you see a constant voltage dependence of the remaining g_j. Is this the best evidence for two separate gates?

SPRAY: The pharmacological block of the pH dependence without blocking the voltage sensitivity seems even stronger evidence for separate gates.

BRINK: The kinetics of voltage dependence of g_j are slow (τ is <100–200 ms) in amphibian cells. Are they similar for *Chironomus*?

ROSE: Yes, but there are at least two exponentials, in contrast to the single exponential Harris et al. reported for amphibia.

LECAR: If each connexon (hemichannel) has a gate dependent on transjunctional voltage, then the conductance vs. voltage curve should be symmetrical at about $V_j = 0$ mV. Your data do not show this, so you must have a different picture of the channel gating.

ROSE: I agree, we do have a different picture of channel gating in this tissue. The lack of symmetry at about $V_j = 0$ mV is one reason why we argue that V_j does not control g_j. Rather, we think that the gate in each hemichannel is controlled by the potential of the cell in whose membrane it is embedded; that is, one gate of the cell-to-cell channel by E_1, the other by E_2.

BENNETT: It is reasonable for gap junction channels to be sensitive to either V_j or $V_{i\text{-}o}$ or both. A large, charged macromolecule that undergoes a conformational change is likely to produce a dipole moment shift or a charge movement in the transjunctional (V_j) direction and/or orthogonal to it; that is, in the inside to outside ($V_{i\text{-}o}$) direction. In the squid embryo at normal pH_i, G_j is insensitive to voltage. However, at low pH_i, G_j becomes voltage-dependent. Then, rather than closing the channels as in the amphibian blastomeres, nonzero V_j opens the channels. G_j also depends slightly on $V_{i\text{-}o}$. Hyperpolarizing either cell increases G_j; hyperpolarizing both cells equally so that V_j is zero increases G_j but much less so (as illustrated in Fig. 5). Depolarizing either cell (such that $V_{i\text{-}o}$ is positive) also increases G_j but slightly less than do equal hyperpolarizations, although the V_j's are identical. Depolarizing both cells equally slightly decreases G_j. It is clear from these data that G_j is primarily dependent on V_j but that there is some sensitivity to $V_{i\text{-}o}$.

Injecting acid into one cell of a coupled pair produces an asymmetrical situation in which V_j positive on the side of the injected cell increases G_j, while V_j negative decreases it. (A small effect of $V_{i\text{-}o}$ is also present as in the symmetrical case.) This result can be most easily explained by picturing two mirror symmetric gates, one in each half of the channel, such that only one is affected when acid is injected on one side. (The asymmetry is not stable; and it is likely that a significant amount of acid crosses patent junctions in a few minutes.)

The effect of unilateral acidification helps to explain the symmetrical case as due to the effects on two gates in series. At moderate symmetric concentrations of H ions, many of the gates on each side are closed. Granted that the gates operate independently, some channels will have both gates open and be responsible for the "resting" G_j (see Diagram A). Some gates will be closed on only one side (Diagrams B and C) and some will be closed on both sides (Diagram D). Thus, single channels exist in four states. An applied V_j of a given polarity will affect these states differently, because V_j tends to open gates on one side and close gates on the other side. Take as a specific example channels with V_j on the right side positive, the polarity that tends to open the right gate (gate 2) and close the left gate (gate 1). Double open channels (A) will tend to close because of action on gate 1, thus decreasing G_j. Channels with only gate 2 closed (B) will tend to open, thus increasing G_j. Channels with only gate 1 closed (C) will tend to stay closed because the potential will keep gate 1 closed. Gate 2 in these channels will have little potential drop across it because of the closed gate 1 in series and will be little affected. The double closed channels (D) will have half the potential across each gate tending to keep gate 1 closed and gate 2 open. However, the effects on these gates will be less than where either gate is in series with an open gate. Moreover, the doubly closed channels can't be opened by V_j and the observed loss of voltage sensitivity at lower pH_i may be due to all channels being closed on both sides. Because the net effect of V_j in the symmetrical case is always to increase G_j, we infer that more channels are open than are closed, and that voltage sensitivity of opening is greater than that of closing. The greater effect on opening would be magnified at low conductance levels where more channels are singly closed on one side than are double open.

There is an interesting prediction of this model, namely that an applied V_j will produce cycling of single channels through the four states. With reference to the left-hand side of the reaction scheme in Diagram E, application of V_j positive on side 2 tends to move channels from gate 1 open, gate 2 closed (bottom) to doubly open (left) to gate 1 closed, gate 2 open (top). The transitions on the right side of the reaction scheme will be "pushed" in the same direction following V_j application. However, the transition from gate 1 open, gate 2 closed (bottom) to doubly closed (right) will be less sensitive to V_j because the open gate 1 that the potential tends to close will be in series with the closed gate 2. Similarly the transition from doubly closed (right) to gate 1 closed, gate 2 open (top) will be less sensitive to V_j because the affected gate 2 is in series with the closed gate 1. An applied V_j will tend to move channels from the equilibrium situation without applied V_j toward the gate-1-closed, gate-