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Sensitivity of gap junctional conductance to H ions in amphibian embryonic cells is independent of voltage sensitivity

(glutaraldehyde/retinoic acid/chemical modification/electrical coupling/intercellular communication)

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ABSTRACT **In vertebrate embryos gap junctional conductance (g_j) is reduced by transjunctional voltage (V_j) and by cytoplasmic acidification; in each case sensitivity is comparable to those of other channels gated by voltage and ligand–receptor binding. We show here that the mechanisms by which V_j and intracellular pH (pH_i) gate g_j are apparently independent. Partial reduction of g_j by lowering pH_i neither attenuates nor enhances further reduction by V_j. Certain drugs irreversibly (glutaraldehyde, 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) or reversibly (retinoic acid) abolish dependence of g_j on pH_i without appreciably affecting kinetic properties of voltage dependence or the shape of the steady-state V_j–g_j relation. These findings suggest that the mechanisms by which pH_i and V_j act on the gap junction are at least partially distinct and presumably involve separate regions of the junctional macromolecules.**

Gap junctions, which are found in many tissues, allow small molecules and ions to pass between cells. In excitable systems, gap junctions allow rapid impulse transmission and promotion of synchronous activity. In inexcitable and especially embryonic tissues, gap junctions probably transmit chemical signals or metabolites that may play a fundamental role in regulation of tissue growth and differentiation and in maintenance of tissue function.

Gap junction channels in certain tissues can be opened or closed by physiologically reasonable changes in intracellular concentration of H ions and by small potentials generated across the junctional membranes or between the cell interior and extracellular space (1). The H ion sensitivity of gap junctions of fish and amphibian blastulae (shown by acidification by membrane-permeant weak acids) is well fit by a Hill relation, with an apparent pK for channel closure of about 7.3; this gating process is moderately cooperative in that the Hill coefficient is between 4 and 5 (2). At the normal pH of 7.6 the channels are calculated to be 96% open. In several other tissues, including mammalian liver and heart and invertebrate neurons, pK values are 6.4–6.9 and Hill coefficients range from 1 to 8 (3). Coupling in the adult mammalian lens appears insensitive to H ions (4) and may not be mediated by gap junctions (5).

Gap junctional conductance (g_j) in fish and amphibian blastulae is also reduced by application of transjunctional voltage (V_j). In amphibia, the sensitivity is comparable to that of Na activation in axonal membranes (6), and g_j is reduced by about 50% by V_j values of ±15 mV; in the teleost *Fundulus*, voltage dependence is about half as steep (7).

We report here that in amphibian blastomeres, gating of junctional channels by H ions and V_j are independent processes. Over the effective range of gating, neither treatment modifies sensitivity to the other. Moreover, several compounds that react with proteins in markedly different ways reduce the dependence of junctional conductance on intracellular pH (pH_i) without affecting voltage dependence. One of these compounds (glutaraldehyde) acts irreversibly and also blocks conductance completely at higher concentrations (8, 9). One (retinoic acid) causes a transient reduction in g due to cytoplasmic acidification and then reversibly blocks pH dependence. The third [1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)] has no effect on g other than to reduce pH dependence, which it does irreversibly. EEDQ was previously shown to block pH dependence of g_j in crayfish septate axon (10), a system in which voltage-dependent gating is absent.

Further study of these and other reactive agents may lead to biochemical characterization of sites that confer pH and voltage sensitivity on the gap junction channel.

METHODS

Experiments were carried out on pairs of cleavage-stage blastomeres of amphibians (*Rana pipiens*). Frogs were obtained from Carolina Biological Supply (Burlington, NC) in late autumn to early spring. Ovulation was induced by injection of human chorionic gonadotrophin and mature eggs were stripped from the females; sperm were added as a suspension from minced testes. Cells from midblastulae were isolated mechanically as pairs in the presence of up to 0.05% (wt/vol) colchicine to inhibit mitosis. Single-strength Holtfreter's solution was used, containing 60 mM NaCl, 0.65 mM KCl, and 0.25 mM $CaCl_2$, buffered to pH 7.6 with 5 mM Hepes.

Each cell was penetrated with independent electrodes (5–10 MΩ, filled with 3 M KCl) for measuring voltage and passing current; conductances of junctional and nonjunctional membranes were either calculated by applying the pi-tee transform to digitized input and transfer voltages (11) or measured directly with the dual voltage clamp technique (6). In the latter procedure, independent two-electrode voltage clamp circuits hold the cells at a common potential; then one cell is stepped to a command potential (V_j) while the other cell is held at the initial level. The current supplied by the clamp holding the potential of the latter cell constant is equal in magnitude to current flowing through the junctions (I_j). Dividing this current by the voltage step gives junctional conductance (g_j).

For determining pH_i a Thomas-type, recessed-tip pH microelectrode was often inserted into one of the cells (cf.

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Abbreviations: EEDQ, 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; g_j, gap junctional conductance; pH_i, intracellular pH; V_j, transjunctional voltage; I_j, junctional current; V_{i-o}, inside–outside voltage.

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ref. 2). The difference in voltage between the pH and recording electrodes measures pH_i.

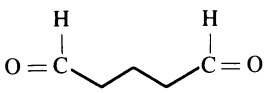
Drugs (Fig. 1) were freshly dissolved directly in perfusion solutions or added from stock solutions in dimethyl sulfoxide. Final dimethyl sulfoxide concentrations were always <1%. This concentration of dimethyl sulfoxide alone had no effect on g_j or its gating by H ions or V_j. Chambers used for EEDQ experiments were discarded after a single experiment.

RESULTS

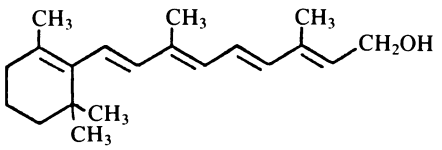
Independence of Gating by V_j and pH_i. To test for interaction between H and voltage gating, test pulses that decreased g_j were applied while g_j was also decreased by cytoplasmic acidification. Examples are shown in Fig. 2*A* for a cell pair before and during CO_2 exposure. Values of pH_i in the traces were about 7.6 for the top trace and 7.4, 7.3, and 7.2 for the second, third, and fourth traces, respectively, estimated from the change in g_j and the g_j–pH_i relation determined previously (2).

For each of the records shown and for pulses of opposite polarity, steady-state conductance is a nearly constant fraction of peak conductance, about 18%. Furthermore, the time courses of decline in g_j during the pulses are very similar (Fig. 2*B*), and each is well fit by a single exponential with a time constant of about 0.2 sec. The peak conductance shown for the smallest current is about 27% of the peak conductance in the control record. The constancy of the ratio of steady-state to initial conductance and the similar time course of the conductance decrease at normal and reduced pH_i suggest that acidification adequate to close >70% of the channels does not appreciably affect voltage gating of the remaining open channels.

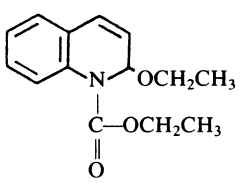
Effects of Glutaraldehyde, Retinoic Acid, and EEDQ on g_j. Glutaraldehyde and retinoic acid produced dose-dependent decreases in g_j, whereas EEDQ had no effect on g_j (not shown). The dose–response relation for a 2-min exposure to glutaraldehyde followed by a 5-min rinse showed a half-maximal effect between 1 and 10 μM. The decrease in g_j was irreversible, and longer exposure to even 1 μM glutaraldehyde (the lowest concentration tested) markedly reduced



Glutaraldehyde



Retinoic acid



EEDQ

FIG. 1. Structural formulae of the drugs used in this study.

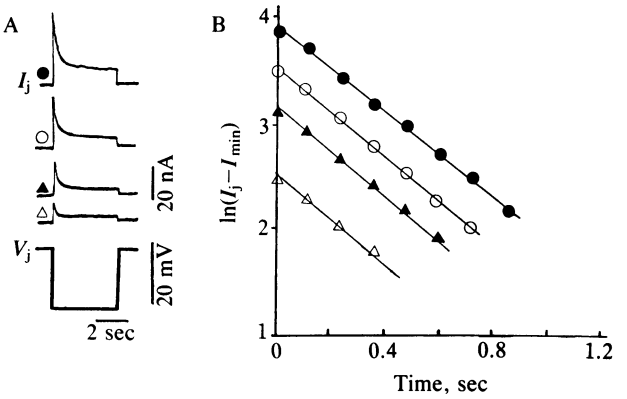


FIG. 2. Voltage dependence is not affected by cytoplasmic acidification that substantially reduces g_j. A pair of coupled cells was voltage-clamped and one cell was stepped to produce a V_j of 18 mV. CO_2-equilibrated saline was passed over the cells followed by normal saline. (*A*) I_j measured before and during CO_2 application at time indicated by symbols (●, control; ○, lumin; ▲, 1.3 min; △, 1.5 min). V_j applied during each current record. During each V_j step, I_j is at a maximum value, I_{max}, at the start of the pulse and decreases exponentially to a steady-state value, I_{min}, that, for this V_j, is about 18% of I_{max}. CO_2 application reduces I_{max} and I_{min} proportionately as the cells acidify. Recovery was complete upon rinsing. (*B*) In this semilogarithmic plot from records in *A*, the time courses of decay of I_j to I_{min} at normal and reduced pH_i values are linear and parallel, indicating that the decay is exponential with the same rate constant unaffected by acidification.

conductance. Exposure to fixative concentrations (10–100 mM) of this agent abruptly decreased g_j to zero and blocked coupling, even after prior partial reduction in g_j by exposure to lower concentrations. Uncoupling by low glutaraldehyde concentrations was never accompanied by appreciable intercellular acidification (pH_i stable to within 0.1 pH unit, not shown); presumably cytoplasmic buffers absorb the protons released in the aldehyde reaction with amino acid residues (12).

The dose-dependent decrease in g_j caused by retinoic acid was totally explicable in terms of measured cytoplasmic acidification. For short exposures g_j decreased in parallel with pH_i and recovered as pH_i increased during rinsing; the pH_i–g_j relation obtained was similar to that reported for other weak acids and esters (not illustrated; see refs. 2 and 13). For long exposures (>5 min), g_j recovered while the cell remained acidic (not illustrated).

Effects of These Agents on pH Dependence of g_j. Glutaraldehyde, EEDQ, and retinoic acid dramatically reduced the sensitivity of g_j to pH_i (Fig. 3*A*, solid line shows normal g_j–pH_i relation). In one experiment shown in Fig. 3*A*, brief treatment of a cell pair with 20 μM glutaraldehyde reduced g_j by 75%, a level that was stable after excess drug was washed away (5-min rinse in normal solution). The conductance remaining was little affected by CO_2 exposure that reduced pH_i from 7.5 to 6.8 (Fig. 3*A*, ○).

After a 15-min exposure to retinoic acid, exposure of cell pairs to CO_2-equilibrated saline solution had no effect on g_j, although pH_i was markedly reduced (to 6.9 in Fig. 3*A*, ×). The reduction in pH_i sensitivity accounts for the recovery of g_j during long exposures to retinoic acid.

EEDQ did not reduce g_j, but it did reduce pH sensitivity of g_j (Fig. 3*A*, ■, and Fig. 3*B*, in which the time course of acidification is shown). At the lowest pH value for which g_j was determined after EEDQ treatment, 6.75, g_j was decreased by 30%. This curve is consistent with the drug displacing the pH_i–g_j relation to the left by about 0.6 pH unit.

The pH_i insensitivity of g_j produced by EEDQ and glutaraldehyde was irreversible for up to 2 hr of rinsing the cells with normal solution (the longest time tested). Effects of

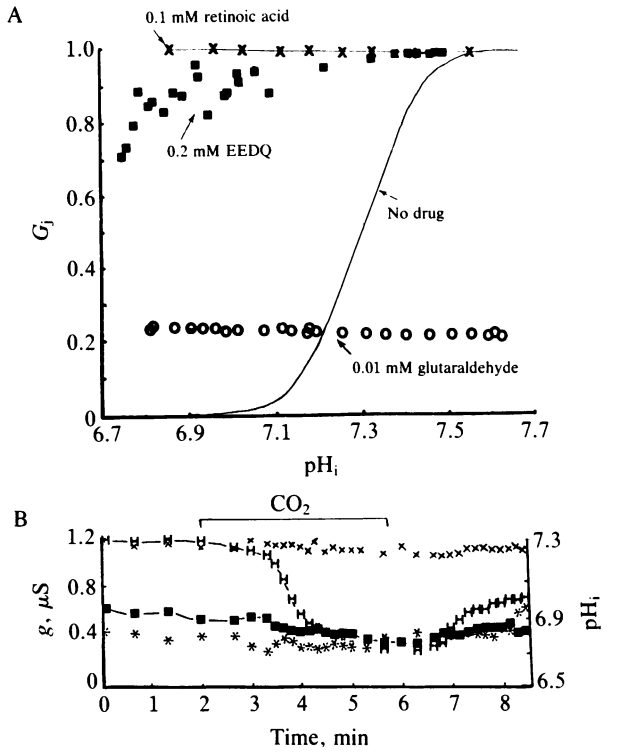


FIG. 3. Glutaraldehyde, EEDQ, and retinoic acid reduce sensitivity of g_j to acidification. (*A*) Graphs of relations between normalized conductance G_j and pH_i. Solid line, the normal relation for vertebrate embryonic cells from ref. 2. ○, Glutaraldehyde (10 μM for 2 min) reduced G_j to about 0.2. CO_2 exposure that decreased pH_i to 6.8 then had no effect on the remaining G_j. ×, After a 15-min treatment with retinoic acid, G_j was not affected by CO_2 exposure that decreased in pH_i to 6.9. ■, After EEDQ treatment (0.2 mM) G_j was only moderately reduced by CO_2 application. For the most acid condition attained, G_j was decreased to about 0.7. The data are consistent with a shift to the left of the pH_i–G_j relation by about 0.6 pH unit. (*B*) Graph of conductances and pH_i during an experiment in which CO_2-equilibrated saline was applied after a 10-min treatment with 0.2 mM EEDQ. pH_i (H symbols) was reduced (starting at 3 min), but g_j (■) and conductances of nonjunctional membranes (×, *) showed only a 30% decrease associated with decrease in pH_i from 7.3 to 6.7 and recovery to 7.0.

retinoic acid on pH dependence were completely reversible by 30 min of rinsing with normal Holtfreter's solution.

Effects of These Agents on Voltage Dependence. None of these compounds appreciably affected either the steady-state voltage dependence of g_j or its kinetics, as is illustrated in Fig. 4 for EEDQ at two concentrations (0.2 and 0.4 mM). For the control and both concentrations, the time course of the decline in I_j at V_j values of ±15 and ±30 mV was similar and the relation of steady-state g_j to V_j was the same.

DISCUSSION

The data presented here show independence of gating of g_j by pH_i and V_j in amphibian blastomeres; during acidification that markedly reduces g_j, voltage sensitivity of the remaining conductance is unchanged. In addition, three chemically unrelated compounds reduce sensitivity of g_j to pH_i without affecting voltage dependence. One implication is that H ions and voltage act at different sites on the gap junction channel. Our previous findings also suggest separability of response mechanisms for these two sorts of gating stimuli: in fish and amphibian embryonic cell pairs, sufficient intracellular acidification reduces g_j by >99%, yet a substantial voltage-insensitive component (up to 20%) remains at even very large transjunctional potentials (cf. ref. 14). In several other vertebrate systems, gap junctions are voltage insensitive, although pH sensitivity is readily demonstrated (mammalian heart, ref. 15; liver, ref. 23; superior cervical ganglion cells, unpublished data).

With respect to independence of pH and V_j gating of g_j, amphibian blastomeres contrast strikingly with two invertebrate systems in which the two gating mechanisms interact. At normal pH_i, g_j between squid embryonic cells displays little voltage dependence, but as pH_i is reduced (by bathing in membrane-permeant weak acids or acetate esters), g_j becomes dependent on V_j and, to a lesser extent, potential between cytoplasm and exterior (V_{i-o}) (13, 14, 16). In *Chironomus* salivary gland, g_j is quite sensitive to V_{i-o} and less sensitive to V_j. The effect of pH is to shift the V_{i-o}–g_j relation along the V_{i-o} axis (17).

Voltage dependence of g_j presumably arises because there is a dipole moment change associated with the conformational change between open and closed states. Thus, the energies of open and closed configurations and the equilibrium distribution between them are affected by potential. H ions may cause conformational changes primarily by changing the local electric field. Interaction between applied potentials and H ions would arise where H ion binding sites

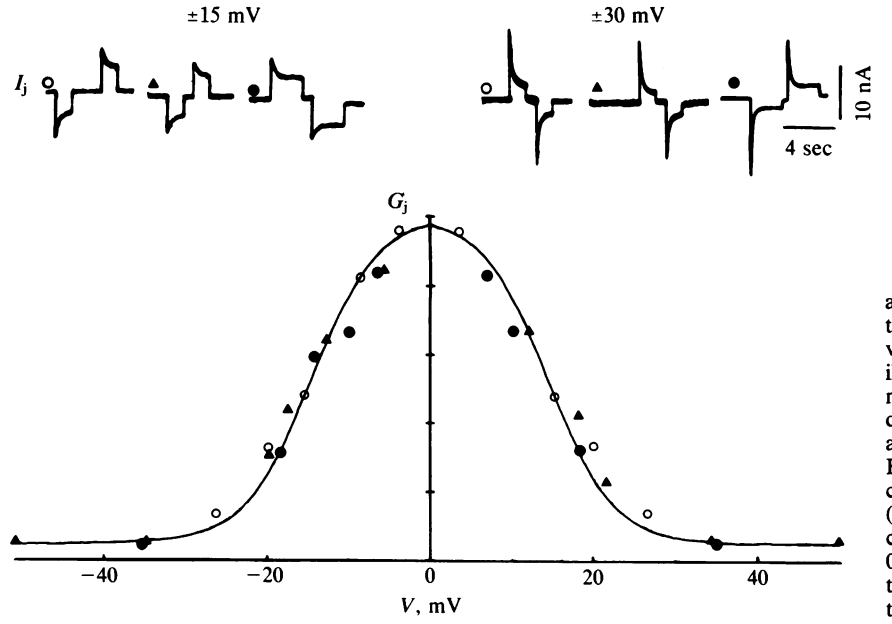


FIG. 4. Voltage dependence of g_j is not appreciably affected by EEDQ at concentrations that greatly reduce pH dependence. I_j values in a pair of voltage-clamped cells are illustrated for V_j values of ±15 mV and ±30 mV. The time course of relaxation of I_j during the pulses was similar in control (○) and after treatment with 0.2 (▲) or 0.4 mM (●) EEDQ for >10 min. The graph below indicates that the steady-stage G_j–V_j relation (where G_j is normalized g_j) was similar in control (○) and after treatment with 0.2 (▲) or 0.4 mM (●) EEDQ. The solid line is a plot of the Boltzmann relation shown to describe the effect of V_j on G_j (6).

were accessible to the potentials. The simple shift in the V_{i-o}–g_j relation in *Chironomus* is consistent with voltage and H ions acting at the same site, unlike the separation inferred for amphibian blastomeres. We have postulated that there are two voltage gates in amphibian junctions, one at either cytoplasmic end of the channel where they are sensitive to voltage gradient along the channel but not to the potential between channel interior and extracellular space. The H binding sites could be located on the cytoplasmic aspect of the channel far enough away from the channel mouth to be insensitive to V_j. The conformational change responsible for channel closure could be the same for pH_i and V_j, and V_j might simply act at a later stage of the reaction initiated by H ions.

As the primary structure of the gap junction protein becomes clearer, modeling of tertiary structure will become a reasonable enterprise, as is already true for the junctional protein of lens (cf. ref. 5) and the acetylcholine receptor (18). High-resolution structural studies of isolated liver gap junctions reveal a cytoplasmic domain of the molecule that could have a gating function (19) and change in tilt of channel monomers has also been proposed to be involved in gating (20). Gating mechanisms and the degree of interaction between them may provide important constraints in formulating and testing structural hypotheses.

Two of the agents found here to selectively decrease pH sensitivity of g_j, retinoic acid and glutaraldehyde, have been shown to uncouple cells. Retinoic acid reduces metabolic cooperativity in tissue culture (21) and this block of g_j is correlated with developmental abnormalities induced by vitamin A administration (22). The initial uncoupling by retinoic acid as a consequence of intracellular acidification is transitory; cells eventually recouple at low pH_i because of loss of pH sensitivity. Metabolic cooperativity is measured over a still longer time course, and there may be a later reduction in g_j that we did not evaluate.

Although the dynamic nature of g_j is only recently being appreciated, the opportunities for physiological action of such a sensitive channel include regulation of growth and differentiation, where the tissue boundaries may be established or eliminated based on physiological controls over junctional conductance. The presence of two (and perhaps more) gating mechanisms provides greater opportunities for cellular control.

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