Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 5494–5497, August 1986
Cell Biology

Isolated liver gap junctions: Gating of transjunctional currents is similar to that in intact pairs of rat hepatocytes

(single channels/electrical coupling/channel incorporation/intercellular communication)

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Contributed by M. V. L. Bennett, March 21, 1986

ABSTRACT **We have shown previously that conductance of rat liver gap junctions is blocked by an affinity-purified polyclonal antibody generated against rat liver junctional membranes, is not affected by moderate transjunctional or transmembrane potentials, and is reversibly decreased by cytoplasmic acidification and perfusion with octanol. We have now recorded currents from isolated liver gap junctions using patch electrodes dipped through a layer of mixed lipids whose concentrations match those of isolated liver appositional membranes. These currents are blocked by the same polyclonal antibody, are insensitive to moderate voltages imposed across the pipette tip, and are reversibly blocked by similar concentrations of H ions and octanol as are junctions *in situ*. The currents are likely to be gap junctional in origin; their block by low pH and other agents indicates that the gating mechanisms are intrinsic to the gap junctions themselves and presumably result from conformational change in the channel-forming protein.**

Gap junctions are aggregates of intercellular channels whose presumed roles in specific tissues are to mediate electrotonic coupling, metabolite exchange, and intercellular signalling (cf. ref. 1). It has recently become clear that, like other channels in biological membranes, gap junction channels are gated by specific stimuli (cf. refs. 2–4), thus broadening the potential regulatory role that these junctions may serve.

The gap junctions of liver are particularly well characterized ultrastructurally and biochemically (5–8), and the gene for this protein has been cloned (9). Because this tissue might provide a preparation in which the various properties of gap junctions could be correlated, we have recently characterized their gating properties in pairs of dissociated hepatocytes (10). One question that has remained unanswered, however, is whether the demonstrated gating properties are intrinsic to the gap junction channel protein or involve accessory proteins, perhaps cytoplasmic molecules, that interact with gap junctions to influence their opening or closing (11–13). We now have addressed this issue by recording from isolated gap junctional membranes incorporated into lipid membranes formed at the tips of patch electrodes.

MATERIALS AND METHODS

Several methods have been used in these studies, one of which is illustrated in Fig. 1*A*. Unpolished patch pipettes were pulled from fiber-containing capillary glass and filled with electrolyte (generally 150 mM KCl/5 mM EGTA/5 mM Hepes, pH adjusted to 7.2 or as indicated); their resistance was generally 5 MΩ or less and they were often dipped into squalene before use. They then were dipped into a grounded well containing electrolyte solution (the same as within the pipette or as indicated) atop which a layer of freshly prepared lipids had been added (Fig. 1*A*; well *i*).

Lipid mixtures generally contained (mol%): phosphatidylcholine (32), phosphatidylethanolamine (20), phosphatidylinositol (9), phosphatidylserine (8), and sphingomyelin (16), which are the molar ratios determined in appositional subfractions of rat liver plasma membranes (Table IV, p. 661, ref. 14). In a few experiments, phosphatidylcholine alone or with 10 mol% cholesterol was used, or the lipid mixture had the following composition: phosphatidylcholine (46), phosphatidylethanolamine (27), phosphatidylinositol (7), phosphatidylserine (14), and cholesterol (6). Lipids were obtained from Sigma and stored frozen under N_2; stock solutions were prepared in chloroform mixed in appropriate ratios, dried under a N_2 stream, and stored frozen. In each experiment the lipid mixture was freshly dissolved in *n*-hexane and applied to the surface of compartment *i* (Fig. 1) in this solvent. Generally, a single dip through the surface was adequate to obtain a seal of >10 GΩ (Fig. 1*B*); in cases in which the resistance was lower (to as low as 5 MΩ, the resistance of the uncompensated patch pipette), a second dip almost always achieved a high-resistance seal. (Although the number of stacked membranes at the pipette tip is presumably resolvable by capacity measurements, time constants of the membranes in these studies were not determined.) The pipette was then moved to the chamber (well *ii* in Fig. 1*A*) containing isolated liver gap junctions (0.01–1 μg of gap junction per ml of electrolyte solution) previously sonicated for 2 min in a microbath sonicator, where stepwise increases in conductance were often detected (Fig. 1*C*). After a conductance increase was observed, the pipette was placed in the third well (well *iii* in Fig. 1*A*), in which stable recordings were often obtained (Fig. 1*D*). All voltages indicated are those of the patch pipette.

RESULTS

Currents as recorded in well *ii* (Fig. 1*C*) were often resoluble into discrete amplitudes (incrementing in ≈150-pS steps) at low-conductance levels. As the membrane achieved a higher conductance, the currents were noisier, and dwell times at apparent intermediate states may indicate rapid transit between discrete levels that are insufficiently resolved in these records. These increasing currents presumably represent addition of new channels to the patch. Membrane currents recorded in well *iii* (Fig. 1*D*) could display discrete transits between a low-conductance state (stable in the central part of the record) and an apparent open state corresponding to an additional conductance of about 150 pS. Throughout this record were rapid events, generally <10 pS conductance, that were probably not completely resolved at the frequency response of these recordings (*ca.* 80 Hz). Some other patches showed more states that were not always integral multiples of

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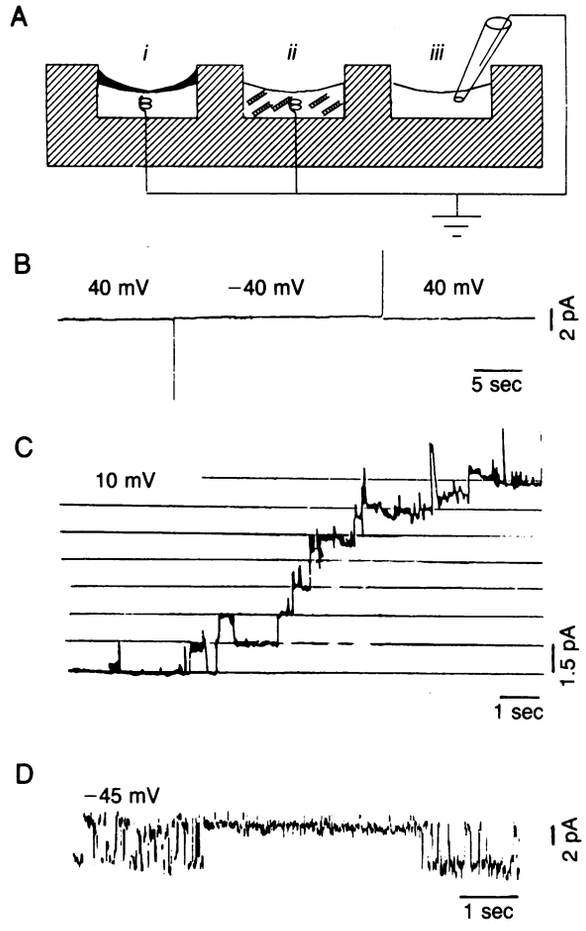

FIG. 1. Method of recording currents through isolated gap junctions. (*A*) A three-well slide (volume of each chamber: 0.3 ml) contained electrolyte solution in each compartment. Wells *i* and *ii* were grounded through Ag/AgCl wires; well *iii* was grounded via an agar bridge to allow solution changes without shifts in electrode potentials. Lipid mixtures dissolved in *n*-hexane were layered onto the surface of compartment *i* and sonicated gap junction fragments were added to compartment *ii*. Patch electrodes were dipped successively in the three compartments while recording currents through the pipette tip (records in *B–D*). After a seal was formed in compartment *i* and channel activity or macroscopic conductance was obtained in compartment *ii*, the electrode was moved to the third compartment (*iii*), which contained inflow and outflow tubes for the addition and removal of test solutions. (*B*) Conductance of the pipette after a single dip of the pipette through the surface of well *i* was generally quite low (10^{-10} S or less), as evidenced here by the very small current (*I*, transients are entirely capacitative) measured in response to ±40 mV command voltage steps. (*C*) Conductance of the pipette tip in chamber *ii* often increased incrementally (steps corresponding to conductance steps of about 145 pS in this record with a constant driving force of 10 mV). (*D*) Conductances acquired in well *ii* were often stably maintained in well *iii*.

a minimal conductance (Figs. 2*A* and 3 *A* and *D*). These different conductance steps may represent channels with different conductances or multiple states of a single channel.

To test whether the conductances recorded in our experiments were attributable to incorporation of gap junctions, polyclonal antibodies raised against isolated gap junctions were added to the bath. These antibodies were shown previously to react with the 27-kDa protein that is the principal junctional constituent (15) and to block gap junctional conductance and dye permeability when injected into hepatocyte pairs (16). Preimmune serum had no marked effect on conductance of the membranes (six experiments) at final concentrations in the bath as high as 15 μg/ml (Fig. 2*A*). The gap junction antibody, however, abruptly decreased patch conductance in each of the nine experiments in which it was added at concentrations as low as 0.1 μg/ml (response to 0.5 μg/ml is shown in Fig. 2*B*). No reversal of the effect was seen in two experiments in which the compartment was rinsed continuously for 20 min. The apparent channel blockade was not affected by repeatedly switching the command voltage back and forth between +40 and −40 mV.

The physiological characteristics of gap junctional conductance between dissociated pairs of hepatocytes include absence of voltage dependence at least in the range ±40 mV, pH dependence over the narrow pH range 6.6–6.2, and reversible block by octanol (10). To test whether sensitivity of conductance of isolated gap junctions to pH was qualitatively similar to that obtained *in vivo*, the pH of the bathing solution was changed by perfusion (in well *iii* as shown in Fig. 1*A*) from pH 7.2 to 6.6 to 6.0 and back to 6.6 in a preparation in which conductance was initially high (Fig. 3*A*). Though steps in currents are poorly resolved in these records (from a membrane with minimal conductance at pH 6.6 of about 0.2 nS), those step-like currents that do occur have amplitudes of 150–500 pS. Conductance was not changed in changing bath pH from 7.2 to 6.6 (not shown) but was profoundly reduced upon exposure to pH 6.0 solution, a pH range over which the junctional conductance *in situ* is reduced substantially (10). The reduction in conductance was reversed by rinsing in saline at pH 6.6 (Fig. 3*A*, third trace). Similar reduction in conductance was obtained in the four other preparations in which this pH range was tested and also in five experiments in which pH was reduced to 5.8 by bubbling the bathing solution with 100% CO_2, even when the buffering power of the electrolyte within the pipette was elevated to 10 mM with Hepes at pH 7.4.

To establish whether the conductances recorded in these studies were voltage dependent, low-conductance patches with apparent single-channel activity were held at various command potentials (±40 mV) for 0.5–2 min (Fig. 3*B*) and conductance of the pipette tip (current divided by voltage) was averaged over that time period (open circles, Fig. 3*C*). Additionally, we examined the effects of imposed voltage on conductance of >20 low-resistance (presumably multichannel) membranes by applying 2-sec command steps (filled circles, Fig. 3*C*) or a staircase of voltage steps (Fig. 3*C Inset*). Current was constant and proportional to voltage throughout the pulses and increased proportionately to voltage in the staircase; as in pairs of hepatocytes, junctional conductance was not affected by voltage over the range ±40 mV.

Octanol reduces gap junctional conductance when applied to hepatocyte pairs (10) and other tissues (17, 18) at concentrations of 0.1 mM or less, and we tested its effects on the currents associated with isolated gap junctions. Upon addition of even very low concentrations (0.01–0.1 mM) of octanol, patch conductance was markedly reduced and recovered rapidly when rinsed with normal solution (Fig. 3*D*; this record is representative of conductance changes seen in 12 applications performed on five preparations).

DISCUSSION

In summary, the recorded currents are like gap junctional currents *in situ* in that they are blocked by a gap junction antibody (16), show pH dependence within the appropriate range, are not appreciably voltage dependent, and are reversibly blocked by octanol (10). Moreover, the amplitudes of unitary currents were reduced by <10% in the presence of 150 mM NaCl, suggesting little selectivity for Na vs. K ions, and by <50% in the presence of 150 mM $CsSO_4$ (not illustrated), indicating that channel size is large; large size and absence of charge selectivity are other characteristics of gap junction channels (19, 20) that should be explored in future experiments on this preparation.

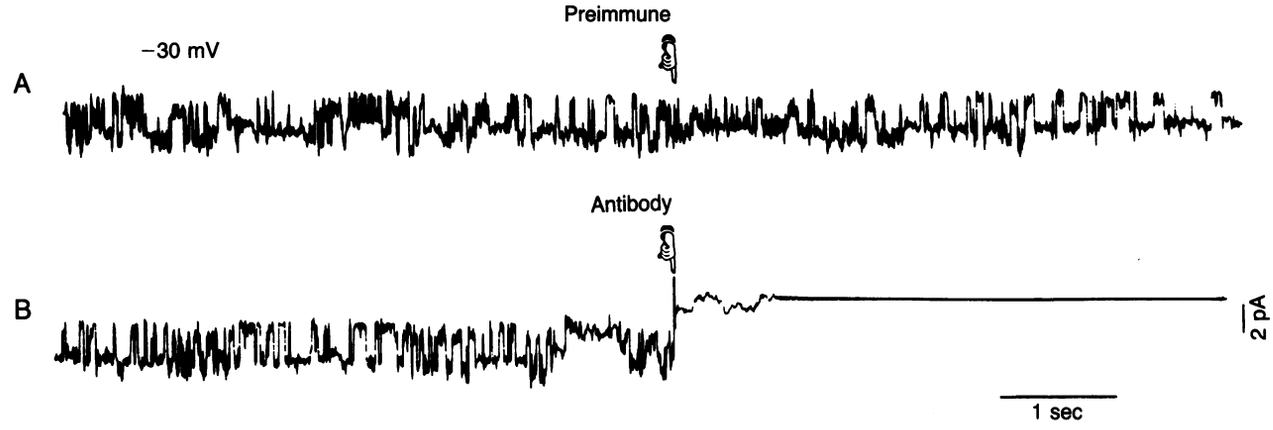

FIG. 2. Currents recorded under the conditions illustrated in Fig. 1 were blocked by an antibody to the gap junction protein (0.15 μg, added to the 0.3-ml bath at the arrow shown in *B*) but not by preimmune serum (5 μg, added at the arrow shown in *A*). Records in *A* and *B* are separated by 70 sec; a constant driving force of −30 mV was maintained throughout this record. Conductance at the start of the record is about 0.3 nS; at the end of the record, conductance is <1 pS.

The question may be raised whether these currents could be through hemichannels that are hypothesized to exist in single membranes as channel precursors or dissociation products. Though we cannot rule out this possibility, it seems more likely that these recordings are from complete gap junction units because antibody blockade of macroscopic

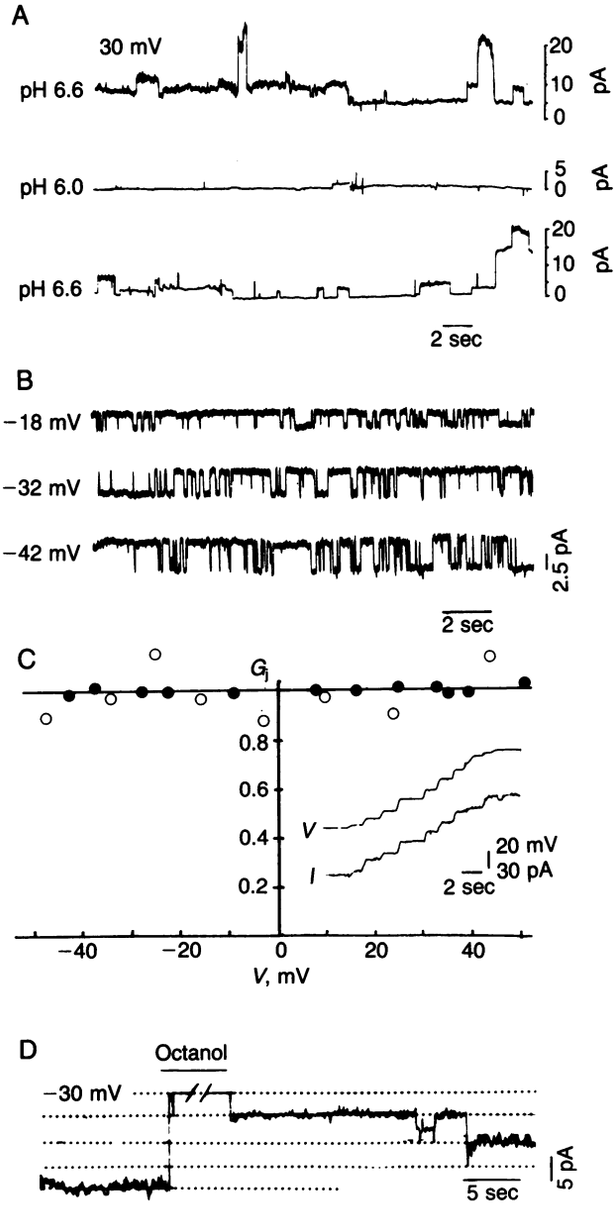

FIG. 3. Gating properties of incorporated gap junctional channels recorded under the conditions described in Fig. 1 and text. (*A*) Effects of bath pH on patch conductance at a constant driving force of 30 mV. At pH 6.6 (150 mM KCl/5 mM Hepes/5 mM EGTA) average conductance (mean current divided by voltage) during the uppermost record was about 0.4 nS. Two minutes after bath pH was changed with the same electrolyte titrated to pH 6.0 with HCl, average conductance was <1 pS (second record). Two minutes after reperfusing with pH 6.6 solution, average conductance recovered to about 0.2 nS (last record in *A*). The solution in the patch pipette was 150 mM KCl/5 mM Hepes/5 mM EGTA, pH 7.0. (*B* and *C*) Lack of appreciable voltage dependence of patch conductance in low- (*B*) and high-conductance (*C, Inset*) membranes. (*B*) The frequency of transitions between open (downward) and closed states is not markedly affected by command potentials over the range −18 to −42 mV; in each trace, step conductance changes average about 150 pS. (*C*) Conductance of membrane patches obtained over a range of command voltages normalized to their average values. Solid symbols represent conductance values obtained from a high-conductance membrane (1.2 nS at 10 mV) measured at the end of a 2-sec command step to each potential. Open symbols represent mean conductances (averaged for 2-min episodes) of apparent single-channel records similar to those displayed in Fig. 3*B*. (*Inset*) Current recorded in another high-conductance membrane to a series of voltage steps (progressively more pipette positive from zero transmembrane voltage at the beginning of the record); the current increased linearly with voltage up to the maximum applied—in this case, 65 mV. (*D*) Conductance of a membrane patch was reversibly decreased by perfusion with 0.01 mM octanol (duration of perfusion indicated by the solid line above the records). Patch conductance was initially about 0.7 nS and was abruptly decreased (to <1 pS). Within 1 sec of rinsing with normal bath solution (150 mM KCl/5 mM EGTA/5 mM Hepes, pH 7.2) a step in conductance of about 150 pS appeared, followed by a further increase 30 sec later of similar magnitude. (The apparently stable transition to an intermediate level may represent flicker between states that is unresolved at the frequency response of the system: limited to about 80 Hz by the chart recorder.) Dotted lines represent current increments corresponding to 150-pS conductance changes.

currents is due to action on the cytoplasmic aspect of the channel (16); its action on patches was always to reduce conductance by at least 90%, whereas 50% reduction would be expected if the cytoplasmic aspect of conducting hemichannels were randomly oriented facing in or out of the pipette.

Our data show that isolated gap junction membranes contain channels with unitary conductance on the order of 150 pS in 150 mM electrolyte solution, a size consistent with that predicted on geometric considerations (21) and recorded between pairs of cells with low-conductance nonjunctional membranes (22, 23). Apparent single-channel conductances between cells also are somewhat variable in amplitude, and transitions between states may be slow (tens of milliseconds). Further work will be required to understand these properties, whether they represent unresolved flickering between states, different states of single channels, or variability among channels. These channels are considerably smaller and not so obviously multistate as those recorded from lens junctional protein reconstituted into lipid bilayers (24).

The channels that we recorded from presumably are responsible for mediating electrotonic and dye coupling between liver and other coupled cells *in vivo*, for which considerable correlative and direct evidence already exists (4, 16, 25). Gating of currents through isolated gap junctions is similar to that of macroscopic junctional currents recorded from pairs of hepatocytes (10), and we therefore conclude that opening and closing of the gap junction channel by pH and octanol are attributable to conformational changes in the protein comprising it and do not, in these circumstances, require other molecules.

Our search over the past 5 years for identifiable single gap junction channel currents has been aided by the contributions of several other colleagues, including R. L. White (to whom we are especially indebted for the design and implementation of the electronics), D. Paul (supported in part by a Grass Foundation Fellowship to the Marine Biological Laboratory, Woods Hole, MA), and A. L. Harris. This work was supported by National Institutes of Health Grants NS 16524 and NS 19830 to D.C.S. and NS 07015 to M.V.L.B.

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