Opening Hemichannels in Nonjunctional Membrane Stimulates Gap Junction Formation

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ABSTRACT We studied gap junction formation in pairs of *Xenopus laevis* oocytes expressing connexins that form functional hemichannels and found no correlation between junctional conductance (G_j) and whole-cell hemichannel conductances (G_{hemi}) within the first few hours of pairing. However, opening hemichannels to a threshold current stimulated a rapid G_j increase. Moreover, cx46 hemichannel current stimulated cx40 G_j even though cx40 and cx46 do not form heteromeric or heterotypic gap junctions. Initial growth rate and final steady-state level of stimulated G_j were proportional to the product of hemichannel conductances. External calcium affected the growth rate of stimulated G_j but not the final steady-state value. Time constants of formation were short in low $[Ca^{2+}]_{out}$ (3 min in 200 μM Ca^{2+}) and long in high $[Ca^{2+}]_{out}$ (15 min in 1 mM Ca^{2+}), but in oocyte pairs pretreated with lectins to reduce steric hindrance imposed by large membrane glycoproteins the time constant was short and Ca^{2+}-independent. We suggest that hemichannel activity stimulates G_j by collapsing the extracellular volume between membranes to allow the end-to-end binding between hemichannels. These studies suggest the possibility that functional hemichannels could trigger or enhance junctional formation in vivo in response to appropriate stimuli.

INTRODUCTION

Several members of the connexin protein family, including *Xenopus laevis* cx38, rat cx46, and mouse cx50, form functional hemichannels in nonjunctional plasma membrane. This paper shows that opening functional hemichannels dramatically stimulates the rate and extent of gap junction formation between oocytes and that functional hemichannels, like nonfunctional hemichannels composed of different connexins, assemble into gap junction channels. A variety of experiments in *X. laevis* oocytes suggests that gap junctions form from a pool of precursors already in the plasma membrane and not by coordinated insertion and assembly of precursors at sites of cell-cell contact (Blakytny and Harding, 1997; Ireland and Mrock, 1997).

The presence of hemichannels in nonjunctional membrane demonstrates that at least some fraction of the connexin protein residing in the plasma membrane is already in the form of hexameric assemblies (DeVries and Schwartz, 1991, 1992; Ebihara et al., 1995; Ebihara and Steiner, 1993; Ebihara, 1996). However, there is little evidence that gap junctions assemble from preformed functional hemichannels. There is disagreement over whether or not functional hemichannels are correctly processed in oocytes, and functional channels might be an aberrant fraction of the total hemichannel pool, nonfunctional hemichannels being the true precursors of gap junctions (Zampighi et al., 1989).

External pH, external Ca^{2+} concentration (Dahl et al., 1991, 1992; DeHaan, 1994; el-Sabban and Pauli, 1994; Meyer et al., 1992), cell adhesion molecules (DeHaan, 1994; el-Sabban and Pauli, 1994; Meyer et al., 1992), disulfide exchange rates (Dahl et al., 1991), and many other factors affect gap junction formation, but their modes of action are difficult to establish due in part to an inability to quantify the state and abundance of the gap junction precursors.

Here we show that gap junction channels assemble from functional hemichannels, further that increases in junctional conductance likely result from the accumulation of precursors at existing junctional sites and finally that the rate of growth of gap junction plaques can be limited by the availability of precursors or other endogenous factors. We find evidence for several distinct steps in gap junction formation. First, opposing cell membranes must come close enough together to allow formation of gap junctions; thus, promoting membrane apposition generates potential junctional sites. Second, functional hemichannels in opposing plasma membranes assemble across the narrow extracellular cleft region of the junction site to form functional gap junction channels. Third, the newly formed gap junction channels stabilize the junctional site, and subsequent plaque growth is determined by the availability of functional hemichannels and the physical forces opposing junctional expansion. Variations in the time constant of junctional formation suggest that these forces are mediated at least in part by lectin-specific endogenous carbohydrate interactions.

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METHODS

In vitro transcription

Cx40, cx46, and cx50, previously subcloned into the SP64T transcription vector, were a kind gift of David Paul (Harvard). Constructs were linearized with restriction endonucleases and capped mRNAs were transcribed in vitro with SP6 RNA polymerase using the Message Machine kit (Ambion, Austin, TX) according to the manufacturer's instructions. Purity and yield of transcribed mRNA were assessed by agarose gel electrophoresis. Ethidium

bromide staining intensity was compared to a known standard to assess yield. Connexin mRNAs were stored as 1 μg/μL master stocks at −80°C.

Oocyte preparation

Ovarian lobe tissue containing oocytes in all stages of development was surgically removed from adult female *X. laevis* (obtained from the following suppliers: *Xenopus* I, Ann Arbor, MI; NASCO, Fort Atkinson, WI; and Pacific Biological, Sherman Oaks, CA) anesthetized in 0.3% tricaine chilled to 4–6°C. The tissue was teased apart into smaller clumps containing 6–12 oocytes and incubated on a rotating platform at 17°C for 1 h in Ca^{2+}-free ND96 containing 1.5 mg/ml collagenase and trypsin inhibitor. After washing with Ca^{2+} free ND96, Stage V–VI oocytes were selected from the population, manually defolliculated if necessary, and incubated at 17°C for 24 h in ND96 supplemented with 25 mM NaPyruvate and either 0.05 mg/ml gentamicin or penicillin-streptomycin. Oocytes were injected with 3–5 ng of cx38 antisense RNA that suppresses endogenous gap junction expression (Barrio et al., 1991; Hennemann et al., 1992; Bruzzone et al., 1993). Oocytes were injected with 23–46 nL of cRNA (Ambion kit) coding for either cx46 or cx50 from stock concentrations ranging from 0.5 μg/μl to 0.0005 μg/μl and then incubated as above but in the presence of 1 mM $CoCl_2$ to reduce hemichannel steady-state conductance. Voltage clamp experiments began 18–48 h after cRNA injections.

ND96 contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, and 10 mM HEPES, pH 7.4. Ca^{2+}-free ND96 consisted of ND96 with no added calcium. Different calcium concentrations were achieved by adding calcium to this solution from a 1-M stock. The osmolarity of all solutions, measured using a Vapor Pressure Osmometer (Wescor, Logan, UT) was 200–210 mmol/kg. In experiments where pH was varied ND96 was prepared using buffers appropriate to the particular pH range (pH 6.0–6.7, PIPES; pH 7.3–8.2, HEPES; pH 8.7–9.7, CHES).

Pairing oocytes for gap junction formation experiments

Typically, 24–72 h after cRNA injections, oocytes were transferred to Petri dishes coated with 2% agaraose for removal of their vitelline layer before pairing. The vitelline layer was usually separated from the plasma membrane by incubation in hypertonic solution and then manually removed with No. 5 Dummont forceps. The stripping solution varied in calcium concentration and osmolarity without affecting experimental results. Devitellinized oocytes were incubating for 20–30 min in ND96 supplemented with 1 mM $CoCl_2$ to prevent hemichannel activity. Oocytes that resisted flattening were selected for pairing in teflon or tygon wells. After 30 min to 1 h incubation to ensure oocyte viability and structural integrity, oocytes were paired in Teflon wells by gently pressing together repeatedly over the course of several minutes. Tight-fitting barriers were avoided to allow for maximum exposed surface area during perfusions. Instead, tygon boats were prepared by splicing tygon tubing down its center, cutting small sections, and using a hot air gun to heat the inside surface. This caused the ends to curl up forming a curved well that maintained two oocytes in contact by gravitational forces. Adherence can be verified by gently displacing one oocyte away from the other and observing whether or not the unmanipulated oocyte plasma membrane tends to follow.

The overall visible contact area after 2 h of pairing was typically 1/4–1/3 the diameter of the oocyte and remained constant during most experiments, although some batches of oocytes tended to flatten out with time, leading to an increase in visible contact area. Oocytes from different preparations tended to flatten at different rates. The oocyte pairs were incubated in separate cylindrical chambers of 0.8-ml volume chambers in the above incubation media unless otherwise noted.

Electrophysiology

Voltage clamp recordings of macroscopic membrane currents were obtained using a two-electrode voltage clamp. AxoClamp-2B and 2A voltage clamps (Axon Instruments, Foster City, CA) using a 1× L headstage for voltage recording and a 10× MG headstage for passing current were used in one or two oocyte configurations. The bath potential was clamped to 0 mV using a 100× VG headstage. Voltage-recording and current-passing electrodes were pulled from borosilicate glass on a horizontal puller (Flaming-Brown P-87, Sutter Instruments, Novato, CA). The internal pipette solution consisted of 150 mM KCl, 10 mM EGTA, 10 mM Hepes, pH 7.2–7.4. Voltage-recording electrodes had resistances between 1 and 3 Megohms. Current-passing electrodes had resistances of 0.1–0.3 Megohms with a 1-mm agarose bridge at the tip to prevent leakage of KCl into the oocyte. Command pulses and current measurements were generated using Pclamp 6.0 data acquisition software (Clampex 6.0) to control the amplifiers interfaced to a PC through a Digidata 1200 A/D converter. Currents were filtered at 50–200 Hz and acquired directly to hard-drive.

After pairing for a minimum of 2 h, the oocytes were clamped to a potential equal to the average of the two resting potentials using dual two-electrode voltage clamps (Axoclamp 2A and 2B amplifiers, Axon Instruments). Hemichannel currents and gap junctional currents were recorded at various times and during perfusions with various solutions. Ten to fifteen times the bath volume was sufficient to fully exchange bath medium. At the end of each experiment, the membrane potentials of oocytes were recorded under the original bath conditions to assess viability of the oocytes.

RESULTS

Opening hemichannels greatly stimulates junctional conductance

Characteristics of unstimulated gap junction formation

The initial focus of these experiments was to simply determine if a quantitative relationship existed between the population of gap junction channels and the populations of functional hemichannels in an individual oocyte pair. In 15 different oocyte batches, paired oocytes expressing functional cx46 hemichannels developed little or no detectable coupling during the first few hours of physical contact. Initial junctional conductances ($G_{j,o}$) measured 2–4 h after pairing oocytes varied between different oocyte batches, but typically there was little early coupling unless the oocyte pairs expressed very large hemichannel currents or had large visible contact areas. In pairs with detectable early coupling, G_j increased linearly and slowly at rates independent of hemichannel expression level, which was determined by applying the same standard electrophysiological protocol to assay whole-cell hemichannel conductance in all oocytes. In 47 oocyte pairs obtained from 15 different batches (8 frogs) and injected with 0.005–0.2 ng of cx46 cRNA, $G_{j,o}$ assayed 2–4 h after pairing ranged between 0 and 4 μS, and initial rates of junctional conductance increase (as measured by the initial slope of the conductance time curve, dG_j/dt) ranged from 0–3 μS/hr. (Any delay between the time of physical pairing and the initial onset of observable gap junction formation was not determined.) Batch-to-batch variability and low initial junctional conductances observed after short pairing times probably reflect variation in endogenously expressed surface glycoproteins that sterically interfere with gap junction formation (Blakytny and Harding, 1997; Dahl et al., 1992). These results are consistent with the variability

in coupling levels and time course observed previously between different batches of oocytes or within the same batch of oocytes injected with the same amount of connexin mRNA (Dahl et al., 1992).

Properties of cx46-hemichannel-stimulated gap junction formation

Surprisingly, while quantifying the hemichannel expression level, the junctional coupling increased rapidly in some oocyte pairs after opening cx46 hemichannels in both oocytes by lowering $[Ca^{2+}]_{out}$ and depolarizing the membrane potential. Within tens of minutes, the stimulated G_j often reached a steady-state magnitude that would have required 8–24 h to reach in unstimulated oocyte pairs.

A priori, one might expect this increase in junctional conductance to be mediated by a decrease in $[Ca^{2+}]_{out}$ or by an effect of the voltage rather than by a direct effect of the open hemichannels themselves. But for cx46, one can separate the individual effects of $[Ca^{2+}]_{out}$ and voltage because the voltage dependence of steady-state hemichannel activation is very calcium sensitive. The voltage producing half-maximal activation shifts from −27 mV in 0-added Ca^{2+}-media to +14 mV in 0.5 mM $[Ca^{2+}]_{out}$ (Ebihara and Steiner, 1993), so clamping oocytes to potentials more negative than −60 mV allows $[Ca^{2+}]_{out}$ to be varied between 2 mM and 10 μM without significantly increasing steady-state hemichannel conductance. Conversely, maintaining high $[Ca^{2+}]_{out}$ (>2 mM) allows significant depolarization without activating cx46 hemichannels.

Fig. 1 illustrates the two protocols used to separate the effects of opening cx46 hemichannels from the effects of voltage and calcium concentration. Fig. 1 *A* shows a typical G_j time course before and after briefly opening hemichannels in both oocytes by applying depolarizing voltage pulses. To control for effects of voltage, the pulse was first applied to oocytes bathed in solutions containing elevated $[Ca^{2+}]_{out}$ and 1 mM $CoCl_2$, which effectively closes all hemichannels. The bathing solution was then changed, reducing the divalent concentration and eliminating $CoCl_2$, thus promoting the opening of hemichannels with the same depolarizing voltage. The hemichannel currents activated by these voltage pulses (+20 mV for 10 s) are shown in panels *a* and *b* in Fig. 1 *A*. Note that junctional formation is stimulated only by open hemichannels and not by applied voltage.

Fig. 1 *B* introduces the convention followed throughout this paper that hemichannel conductances are shown by open symbols and junctional conductances by solid symbols. In this figure, hemichannels were activated continuously by a small elevation in the holding potential after lowering $[Ca^{2+}]_{out}$. Here, paired oocytes were initially clamped at −50 mV in 2.0 mM $[Ca^{2+}]_{out}$ closing all hemichannels and giving a baseline rate of G_j increase. The holding potential, V_h, was then stepped to −20 mV for 5–10 min and little change in G_{hemi} was seen due to the presence of high $[Ca^{2+}]_{out}$. V_h was then returned to −50 mV so that $[Ca^{2+}]_{out}$ could be reduced without increasing G_{hemi}. To insure complete solution exchange in the contact region, oocytes were maintained in low $[Ca^{2+}]_{out}$ for 5–10 min before depolarization. Note that gap junction formation does not take place in low $[Ca^{2+}]_{out}$ until hemichannels are opened as V_h is depolarized from −50 mV to −20 mV. (In some oocyte batches, or in oocyte pairs expressing low levels of hemichannels, there is a latency period between the onset of hemichannel activation and the onset of G_j increase.) The experiments described in Fig. 1 show that either membrane potential or $[Ca^{2+}]_{out}$ can be altered dramatically without stimulating an increase in G_j as long as cx46 hemichannels remain closed. Thus a rapid increase in G_j is stimulated only by conditions that specifically open hemichannels.

The kinetics of stimulated G_j

The initial rates of increase of stimulated G_j (dG_j/dt) ranged from 10 to 150 μS/h, more than a 100-fold increase over the baseline prestimulated rate. Within minutes to tens of minutes, the rate of stimulated G_j relaxed exponentially to zero or to a constant slow linear rate that was significantly greater than the initial prestimulated baseline rate. Time-dependent changes in G_j were usually well fit by a single exponential growth curve of the form

$$G_j(t) = G_{j,o} + G_{j,max}(1 - \exp(-t/\tau)),$$

where $G_{j,o}$ is the prestimulated junctional conductance, $G_{j,max}$ is the net increase in stimulated steady-state junctional conductance, and τ is the time constant of relaxation to the new steady-state $G_{j,ss}$. The solid lines in Fig. 1, *A* and *B*, are fits of the above form with parameters shown in the figure legend.

Poststimulation steady-state junctional conductance is proportional to the product of hemichannel conductances in the two oocytes

Assuming that gap junction formation proceeds as a second-order bimolecular reaction between two populations of hemichannels, as suggested by earlier studies on cx32, then the quantity and initial formation rate of gap junction channels should be proportional to the product of hemichannels contributed by each membrane. We used this relationship to demonstrate a positive correlation between functional hemichannel densities and stimulated gap junction channels, but as discussed later the range of data was limited by several factors. Fig. 2 shows that the change in steady-state junctional conductance induced by hemichannel activation is proportional to the product of normalized hemichannel conductances expressed by the two oocytes in contact, but the proportionality constant varies from batch to batch, and we cannot eliminate the possibility that the hemi- and junctional channel populations are related by other functional forms. Because pairing orientation affects junc-

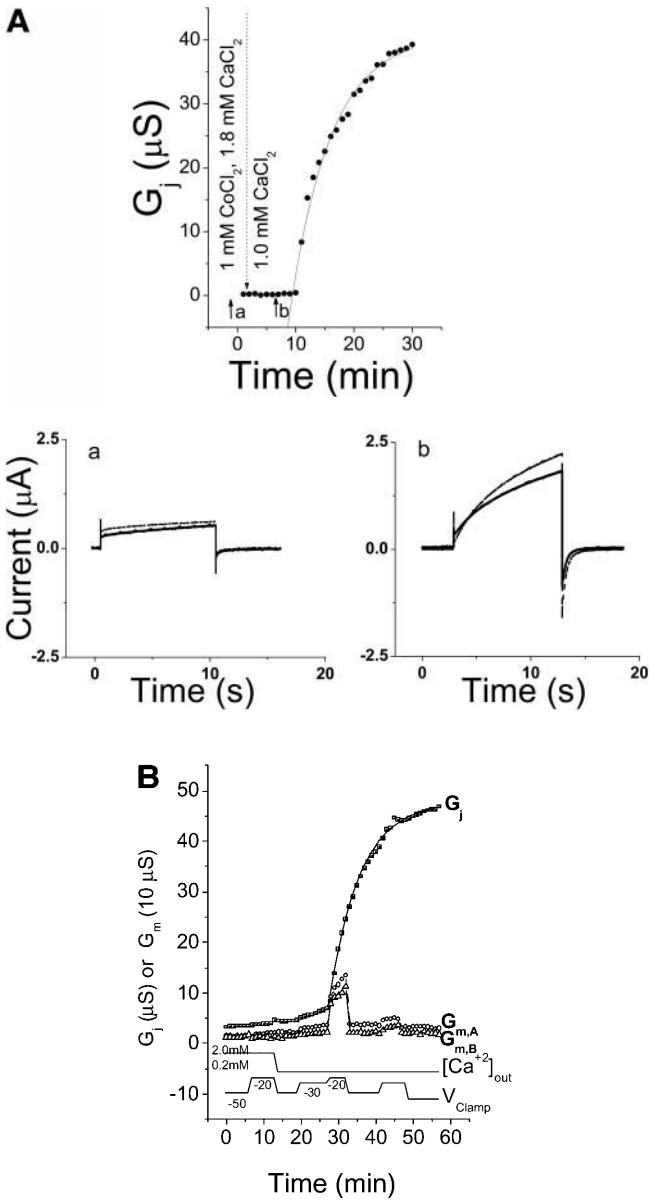

FIGURE 1 Opening cx46 hemichannels stimulates gap junction formation. (*A*) The time course of gap junctional conductance between cx46-expressing oocytes clamped to −30 mV. Each point represents the junctional conductance (G_j) determined by applying a 20-mV hyperpolarizing voltage step to one oocyte and dividing the junctional current recorded in the nonstepped oocyte by the transjunctional potential. The vertical dotted arrow indicates when the external medium was changed from ND96 supplemented with 1 mM $CoCl_2$ to 1 mM $CaCl_2$ ND96. The small arrows (*a* and *b*) show when the whole-cell current traces shown below in panels *a* and *b* were taken. The hemichannel currents were excited by a 10-s depolarizing pulse to +20 mV. (*B*) The effects of $[Ca^{2+}]_{out}$ and membrane potential on stimulating gap junction formation are mediated specifically through hemichannels, not through changes in voltage or calcium concentration alone. The time courses of junctional and hemichannel conductance were obtained by first stepping both oocytes simultaneously to −10 mV to measure individual hemichannel conductances and then by stepping one oocyte −20 mV relative to the other oocyte to determine junctional conductance. Junctional conductance is shown by solid symbols and hemichannel conductance by open symbols, a convention followed throughout this paper. Changes in holding potentials and external calcium concentrations are shown on the graph. Oocyte pairs were initially clamped to −60 mV to prevent hemichannel activation during decreases in $[Ca^{2+}]_{out}$. The holding potential was then elevated in 10- to 20-mV increments until a change in whole-cell conductance was elicited. After a change in junctional current was observed, the holding potential was returned to more negative potentials to close hemichannels during the course of junction development.

tional formation, these results reflect only vegetal-vegetal oocyte pairings in which the stimulated G_j growth rate was insensitive to subsequent changes in hemichannel current (but see Discussion for the effects of pairing orientation later). The variation in expression levels between oocytes injected with the same amount of cx46 cRNA is reflected in proportionally different amounts of G_j being stimulated by hemichannel activation. Simply put, the greater the hemichannel expression, the greater the stimulated G_j.

The scatter in Fig. 2 *A* indicates significant variability between different batches of oocytes. Many factors, including the total membrane area, number of cell-cell contacts, spatial distribution of hemichannels, temperature, and the general physiological condition of the oocytes probably contribute to variability in the relationship between the product of hemichannel levels and the final magnitude of the stimulated gap junctional coupling. But for any given batch of oocytes, plots of $G_{j,ss}$ versus $G_{hemi,A} \times G_{hemi,B}$ were well fit by straight lines constrained to pass through the origin. Thus, the multiple factors affecting the proportionality between stimulated G_j and the size of the hemichannel pools are relatively constant for oocytes obtained from the same batch even though they vary considerably from batch to batch.

Oocytes were manipulated into physical contact until they began to adhere to each other, but the extent of visible contact that subsequently developed before voltage clamp experiments could not be controlled and greatly influenced the relationship between G_j versus $G_{hemi,A} \times G_{hemi,B}$. A rough estimate of contact area was derived from the diameter of the visible contact site measured under a dissecting scope fitted with an ocular micrometer. Oocytes from the same batch, paired on the same day after harvesting, developed very similar contact areas normally ranging between 0.25 and 0.4 mm (in visible linear dimension). However, in some batches of oocytes, the visible contact area between paired oocytes was significantly larger because the oocytes flattened out. These oocyte pairs developed much larger junctional conductances for a given hemichannel expression level than oocytes which retained their spherical shape. No attempts were made to normalize data for visible contact area because the geometry of the contact area, especially in flattened oocytes, could not be accurately deduced from a single linear measurement.

Fig. 2 *B* is a frequency histogram of the ratio of junctional conductance to the product of the hemichannel conductances of the two oocytes ($G_j/G_{hemi,A} \times G_{hemi,B}$) for oocyte pairs shown in Fig. 2 *A*. The distribution is bimodal and divides

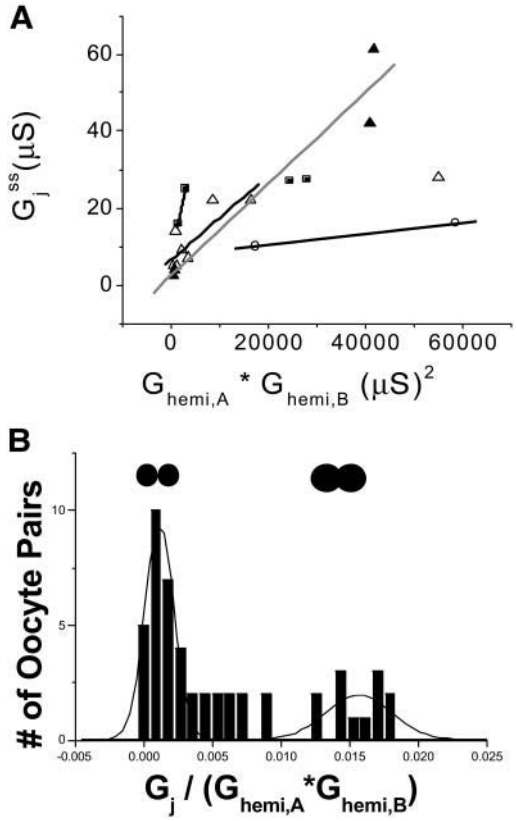

FIGURE 2 The magnitude of stimulated G_j is proportional to the product of hemichannel expression levels. (*A*) The change in steady-state junctional conductance stimulated by hemichannel activation was plotted against the product of normalized whole-cell hemichannel conductances for each oocyte pair. To obtain a quantitative measure of expression levels, hemichannel currents elicited in 0.5 mM $[Ca^{2+}]_{out}$ by a 12- to 20-s voltage pulse to +30 mV were fit to a single or double exponential and the extrapolated steady-state current was divided by the driving force for hemichannels using −10 mV as the reversal potential. Data was collected from 15 different batches of oocytes. Gap junction formation was stimulated by a variety of protocols described in the text. Pairing conditions and $[Ca^{2+}]_{out}$ varied between experiments. All data is from vegetal-vegetal pairs where the stimulated growth in junctional conductance reached a stable steady-state level. (*B*) A frequency histogram of the relationship between stimulated G_j and the product of normalized whole-cell hemichannel conductances. The low conductance peak is from oocytes with low contact area and the high conductance peak is from oocytes with large contact areas, as shown schematically by drawings of oocyte pairs shown above the peaks. A Gaussian fit of the data (*solid line*) was used to determine the centers of the peaks.

one population of oocyte pairs whose contact areas were much larger than normal (high proportionality constant) from a second population with much smaller contact areas (low proportionality constant). In general, larger $G_j/(G_{hemi,A} \times G_{hemi,B})$ values were associated with greater visible contact areas, suggesting that the number of junctional sites formed by hemichannel activation is proportional to the area of contact. The silhouettes of oocyte pairs over the two peaks are intended to convey an approximation to the difference in the shapes and areas of contact that produced the different distributions.

Initial rate of stimulated G_j increase is proportional to hemichannel densities

Fig. 3 *A* shows the initial rate of stimulated G_j increase plotted against $G_{hemi,A} \times G_{hemi,B}$. As discussed in the next section, $[Ca^{2+}]_{out}$ affects the rate of stimulated G_j increase. Hence, the initial rates of gap junction formation were compared only for those oocyte pairs in Fig. 2 for which G_j was stimulated in <0.2 mM $[Ca^{2+}]_{out}$. The frequency histogram of the $(dG_j/dt)/(G_{hemi,A} \times G_{hemi,B})$ proportionality constants is shown in Fig. 3 *B*. These data show that the initial rate of gap junction formation is again proportional to the product of hemichannels expressed by the contacting oocytes. Also, from the frequency histogram, we see the same bimodal distribution for $(dG_j/dt)/(G_{hemi,A} \times G_{hemi,B})$ that we saw for $G_j/G_{hemi,A} \times G_{hemi,B}$. If the bimodal distribution results from a difference in cell-cell contact area, as suggested above, then the dis-

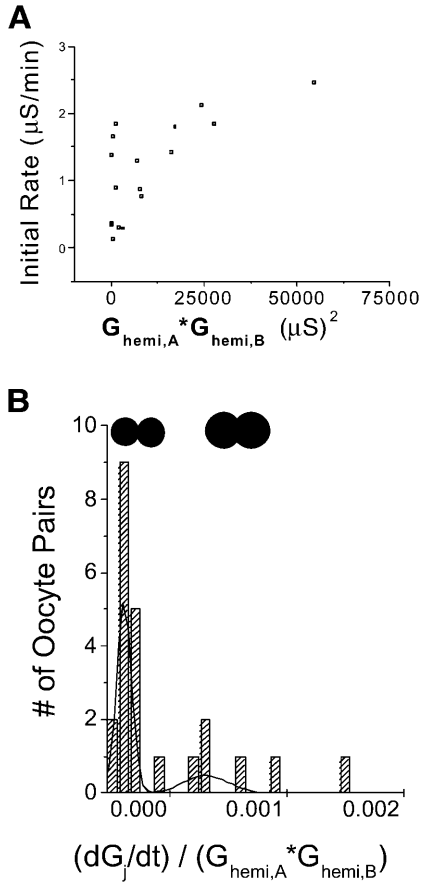

FIGURE 3 Initial rates of G_j increase are proportional to the G_{hemi} product. (*A*) The initial rate of stimulated G_j was plotted against the product of normalized whole-cell hemichannel conductances for the oocyte pairs shown in Fig. 2. Here, only oocyte pairs where G_j was stimulated in 0–0.2 mM $[Ca^{2+}]_{out}$ were used. (*B*) A frequency histogram of the relationship between the initial rate of stimulated G_j and the product of normalized whole-cell hemichannel conductances. The low conductance peak is from oocytes with low contact area and the high conductance peak is from oocytes with large contact areas. Gaussian fits (*solid line*) were used to determine the centers of the peaks.

tributions of both $G_j/G_{hemi,A} \times G_{hemi,B}$ and $(dG_j/dt)/(G_{hemi,A} \times G_{hemi,B})$ should be influenced in a similar manner, and this turns out to be the case. In a Gaussian fit of the distributions (shown as a *solid line*), the two peaks were separated by a factor of 11 in the $G_j/G_{hemi,A} \times G_{hemi,B}$ histogram and 9.8 in the $(dG_j/dt)/(G_{hemi,A} \times G_{hemi,B})$ histogram. This is precisely what would be expected if the visible contact area between contacting oocytes influenced both the extent and initial rate of stimulated G_j by the same mechanism.

The steady-state level of junctional conductance is not determined by a balance between formation and degradation

The poststimulated steady-state level of coupling could represent either the depletion of available precursors combined with the absence of turnover, or alternatively an active balance between the rates of gap junction formation and degradation. In order to gain better insight into the nature of the steady-state G_j reached after stimulation, we needed some way to eliminate one of the reaction pathways. For this purpose, we chose to work with cx50 hemichannels, which we have previously shown could be reversibly blocked by treatment with the histidine modifying reagent diethyl pyrocarbonate (DEPC) (Beahm and Hall, 2002). Cx50 expressing oocytes pretreated with DEPC before pairing developed no detectable coupling within 4–6 h of contact compared to nontreated oocytes (Fig. 4). This finding does not rule out the possibility that DEPC-modified hemichannels assemble into nonfunctional gap junction channels but does suggest that functional cx50 hemichannels are the precursors to functional cx50 gap junction channels.

In a separate set of experiments, gap junction formation was specifically initiated by opening cx50 hemichannels. After G_j reached a stable level, DEPC was applied for 30 s to render cx50 hemichannels nonfunctional while periodically assaying G_j. As shown in Fig. 4, G_j remained stable during the first 20–30 min after DEPC treatment and when assayed many hours later. Nonspecific effects of DEPC on gap junction formation were ruled out on the basis that the reagent had no effect on cx46 hemichannels or stimulated cx46 gap junction formation (data not shown). These data suggest that cx50 homotypic gap junctions are very stable structures that show little to no turnover for up to 6 h. Hence, the steady-state G_j is not the consequence of a dynamic equilibrium between formation and degradation rates, but can only be explained by a depletion of available hemichannels or restricted plaque growth.

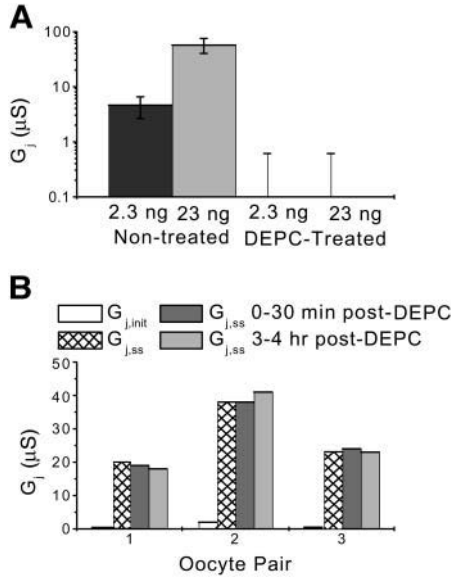

FIGURE 4 Steady-state G_j is not due to balance of formation and degradation rates. Depleting available gap junction precursors does not alter the steady-state junctional conductance for 3–4 h. Two groups of oocytes were injected with either 2.3 or 23.0 ng of cx50 cRNA and incubated in ND96-S + 1 mM $CoCl_2$ at 17°C for 2 days before voltage-clamp experiments. Single oocytes from each cRNA group were treated with the histidine-modifying reagent DEPC. DEPC treatment consisted of washing oocytes for 3–5 min in pH 6.0 ND96 followed by a 1-min wash in a freshly prepared solution consisting of 3.3 μL DEPC dissolved in 50 mL of pH 6.0 ND96, and then washed again in pH 6.0 ND96 for 3–5 min before being returned to ND96 + 1 mM $CoCl_2$. DEPC-treated and nontreated oocytes from each cRNA group were assayed for hemichannel expression by exposure to 0.1 mM $CaCl_2$ ND96, pH 7.6. Nine oocyte pairs were prepared from either DEPC-treated or nontreated oocytes for each cRNA group and incubated in ND96. Oocytes, 4–6 h after pairing, were voltage clamped to −40 mV and G_j was assayed by imposing 10-s transjunctional potentials ranging from 10 to 80 mV in 10-mV increments. Data is presented as the mean ± SE from n number of experiments. Pretreating cx50-expressing oocytes with DEPC completely eliminated hemichannel current and prevented the formation of functional gap junction channels, suggesting that functional hemichannels are the gap junction channel precursors. In experiments from a different batch of oocytes, G_j was stimulated in three different cx50-expressing oocyte pairs by perfusing the oocyte pair with 0-added $CaCl_2$ ND96, pH 8.0, to open cx50 hemichannels. The prestimulated and poststimulated G_j was assayed at 1-min intervals by imposing a 1-s, 20-mV transjunctional potential. After the stimulated G_j reached a stable level, DEPC was applied as described above and G_j was followed for an additional 20–30 min before removing the oocyte pair from the recording setup. G_j was reexamined in the same oocyte pairs 3-4 h after the initial time of DEPC treatment. The initial and steady-state G_j, and the G_j recorded at various times after DEPC treatment are shown for each of the three oocyte pairs. DEPC treatment did not alter G_j during the first 20- to 30-min recording period. The same DEPC solution was effective in eliminating hemichannel currents. G_j remained stable for up to 4 h even after rapidly depleting the pool of functional precursors (hemichannels), suggesting that the stable coupling level is not the result of an equilibrium reaction between the formation and degradation rates of gap junction channels.

Mechanism of stimulation

Stimulation of junction formation is a general property of hemichannel-forming connexins

We tested if the stimulation of gap junction formation was a general property of all functional hemichannels or simply a unique property of cx46 hemichannels. *Xenopus* cx38 and mouse cx50 also form functional hemichannels that can be opened in the plasma membrane of single oocytes, and we assayed the ability of these connexins to stimulate the growth rate of G_j between paired oocytes.

Cx38 is endogenously expressed by *Xenopus* oocytes and forms functional hemichannels that are similar to cx46 hemichannels in voltage and calcium dependence of macroscopic currents (Ebihara et al., 1989). This connexin is normally eliminated by cx38 antisense oligonucleotides to prevent contamination during experiments involving the functional expression of other connexins. In batches expressing relatively high levels of cx38 hemichannels, paired oocytes were subjected to the same protocol used in stimulating cx46 gap junction formation. Fig. 5 *A* shows that gap junction formation is stimulated only when $[Ca^{2+}]_{out}$ and holding potential are adjusted to activate cx38 hemichannels. These experiments were repeated with similar results in six oocyte pairs from two different oocyte batches. Hemichannel currents are shown as open symbols and junctional conductances as solid symbols

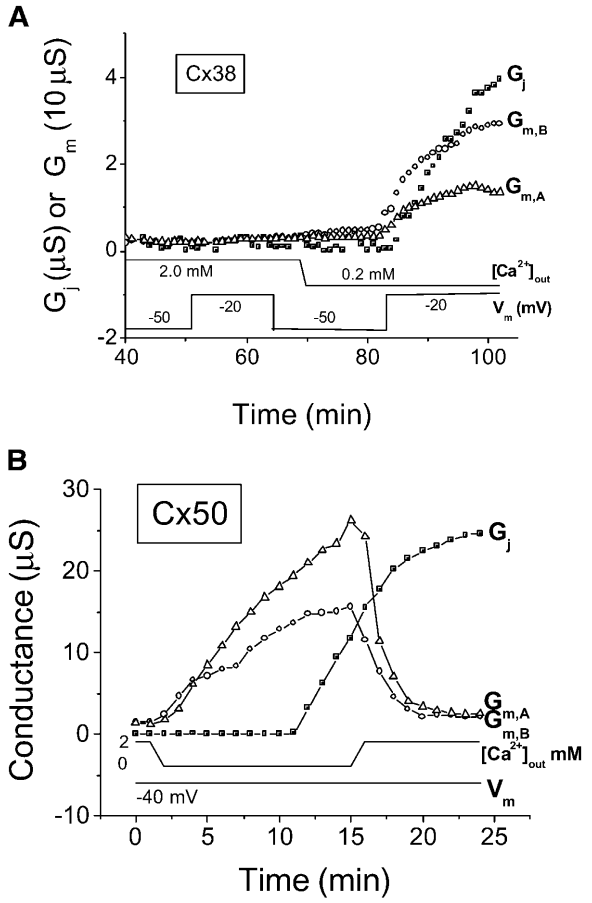

FIGURE 5 Gap junction formation is stimulated by opening cx38 or cx50 hemichannels. The responses of junctional and nonjunctional conductances to changes in $[Ca^{2+}]_{out}$ and/or holding potential are shown for noninjected oocyte pairs (*A*) and cx38 antisense-treated oocytes injected with cx50 cRNA (*B*). Two to three oocyte pairs were examined in four different oocyte batches. Cx38 gap junction formation could be stimulated in only two of the batches. The ability to stimulate cx38 coupling was correlated with the presence of an increased whole-cell conductance generated in 0.2 mM $[Ca^{2+}]_{out}$ after elevating the holding potential to −20 mV. The average stimulated cx38 G_j in two different batches was 4.2 ± 1.1 μS and 8.4 ± 3.1 μS. In four different oocyte batches, gap junction formation was successfully stimulated in all oocyte pairs expressing cx50 hemichannels.

Cx50 also forms functional hemichannels, but with properties quite different from those of cx38 and cx46 hemichannels (Zampighi et al., 1999; Beahm and Hall, 2002). Cx50 hemichannels are voltage-independent over the typical range of resting potentials, whereas those of cx38 and cx46 are not. Hence, in oocyte pairs expressing cx50, whole-cell hemichannel currents can only be increased by lowering $[Ca^{2+}]_{out}$. But although calcium gating rather than voltage gating opens cx50 hemichannels, opening the channels still stimulates gap junction formation just as opening cx46 and cx38 hemichannels by voltage does (Fig. 5 *B*). Note that when calcium is reduced in order to open hemichannels, there is often a delay before G_j is stimulated. This delay simply reflects the slow activation kinetics of the hemichannel pool and the fact that a threshold level of hemichannel current must be reached to stimulate G_j. These results clearly demonstrate that open hemichannels, not voltage or calcium concentration, are responsible for the stimulation of junctional formation. These experiments were successfully repeated in three different oocyte batches with oocytes that were pretreated with cx38-antisense oligonucleotides before cx50 cRNA injections.

Hemichannel current of cx46 can stimulate junctional channel formation of a connexin, which does not form hemichannels

If stimulation of gap junction formation depends solely on current flux and not on structural properties specific to cx46 hemichannels, then activating sufficient hemichannel current in only one of the paired oocytes should stimulate gap junction formation between any compatible connexins expressed in both oocytes. We tested if cx46 hemichannel current restricted to one oocyte could stimulate the assembly of cx40 gap junction channels. Cx40 does not form functional hemichannels and does not form functional heterotypic gap junction channels with cx46 (Bruzzone et al., 1993). Also, cx46 hemichannel currents recorded in oocytes coinjected with cx40 and cx46 cRNA were indistinguishable from those in oocytes injected with only cx46 cRNA, suggesting that cx40 and cx46 do not interact to form functional heteromeric hemichannels (data not shown).

The time courses of junctional and nonjunctional conductances were measured in (cx40 + cx46)/cx40, cx40/cx46, and cx40/cx40 oocyte pairs subjected to conditions that open cx46 hemichannels. Results are presented in Fig. 6. Again hemichannel currents are shown as open symbols and junctional conductances as solid symbols. In (cx40 + cx46)/cx40 pairs, opening the cx46 hemichannels in the one oocyte stimulated cx40 gap junction formation (Fig. 6 *A*). The voltage dependence of the stimulated G_j was indistinguishable from the voltage dependence of cx40 homotypic gap

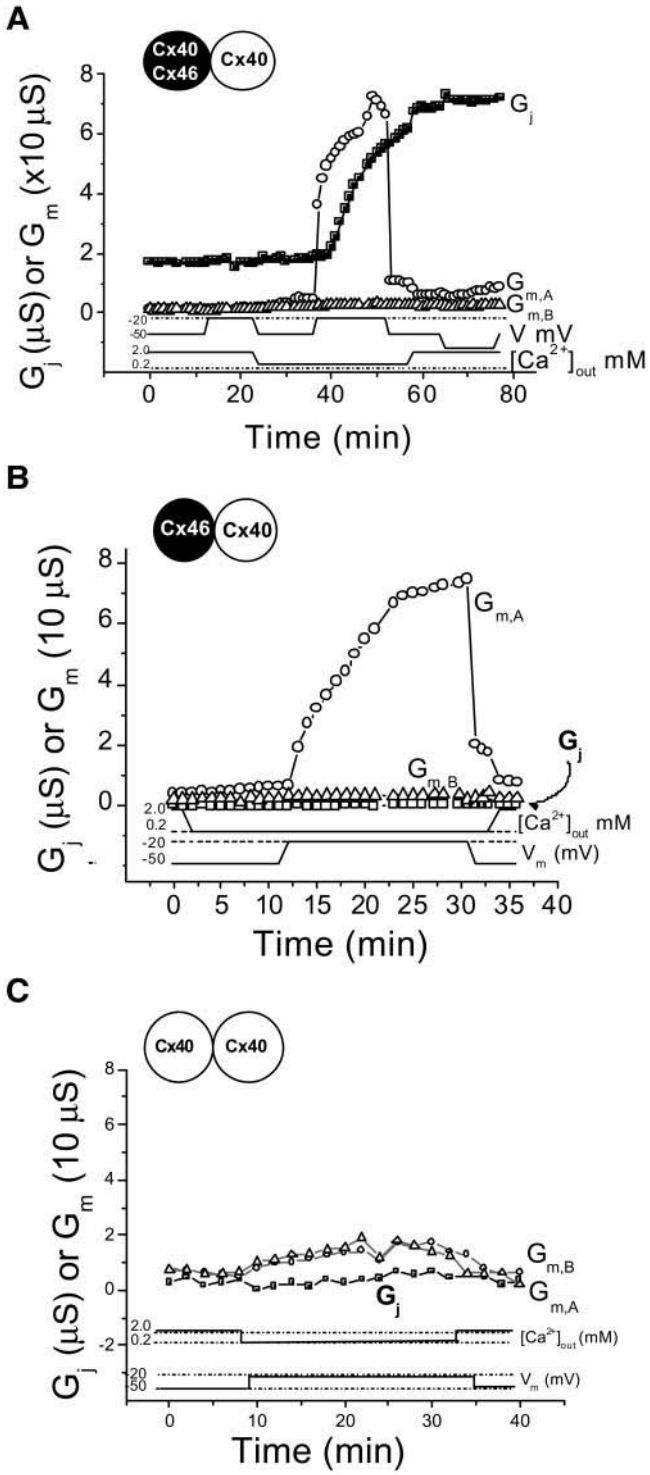

FIGURE 6 Opening cx46 hemichannels stimulates cx40 homotypic gap junction formation (*A–C*). Changes in junctional and nonjunctional conductances were recorded in different types of oocyte pairs subjected to conditions that open cx46 hemichannels, as described in Fig. 4 *A*. Cx38-antisense treated oocytes were injected with 0.1 ng cx46 cRNA, 0.4 ng cx40 cRNA, or a mix containing 0.1 ng cx46 and 0.4 ng cx40 cRNA. Oocytes, 1–2 days after cRNA injections, were paired in the following configurations: cx40/cx40, cx40/cx46, (cx40 + cx46)/cx40, (cx40 + cx46)/cx46. G_j was stimulated in (cx40 + cx46)/cx40 pairs (*A*) and (cx40 + cx46)/cx46 pairs (not shown) after activating cx46 hemichannel current in one or both oocytes. G_j was not stimulated in cx40/cx46 (*B*) or cx40/cx40 (*C*) pairs subjected to the same voltage and calcium conditions. However, cx40/cx40 pairs showed large coupling levels 18–24 h after pairing, whereas cx40/cx46 pairs developed no detectable coupling within 30 h of pairing. (*D*) Steady-state junctional conductances normalized to their values at ±10 mV and plotted against the transjunctional potential. The averaged G_j-V_j data for oocyte pairs of the same configuration were fit to a Boltzmann equation (*smooth lines*). The Boltzmann fit parameters were $V_o = 33.4$ mV, $A = 0.28$, $G_{min} = 0.25$ for five cx40/cx40 pairs, $V_o = 28.4$ mV, $A = 0.30$, $G_{min} = 0.25$ for four (cx40 + cx46)/cx40 pairs, and $V_o = 67$ mV, $A = 0.09$, $G_{min} = 0.01$ for two (cx40 + cx46)/cx46 pairs. A Boltzmann fit of cx46/cx46 G_j-V_j data obtained from other experiments is included for comparison.

junctions. Likewise, the G_j-V_j relationship for G_j stimulated in (cx40 + cx46)/cx46 oocyte pairs was indistinguishable from that of cx46 homotypic gap junctions, suggesting that cx40 and cx46 do not assemble into functional heterotypic gap junction channels (data not shown). Opening cx46 hemichannels in cx40/cx46 pairs did not result in any increase in junctional conductance. And indeed cx40/cx46 pairs never exhibited any junctional conductance at all (Fig. 6 *B*). Finally, the conditions previously used to activate large cx46 hemichannel currents ($[Ca^{2+}]_{out} = 0.2$ mM, $V_h = -20$ mV) did not stimulate G_j in cx40/cx40 oocyte pairs, supporting previous findings that current through functional hemichannels is required to stimulate gap junction formation (Fig. 6 *C*). In cx40/cx40 pairs, G_j developed slowly over the course of 10–28 h, indicating that active precursors were available for gap junction formation. The Boltzmann fit parameters for the G_j-V_j relationship in (cx40 + cx46)/cx40 oocyte pairs ($V_o = 28.4$ mV, $A = 0.30$, $G_{min} = 0.25$) were nearly identical to those in cx40/cx40 oocyte pairs ($V_o = 33.4$ mV, $A = 0.28$, $G_{min} = 0.25$). The fit parameters of G_j stimulated in (cx40 + cx46)/cx46 oocyte pairs ($V_o = 67$ mV, $A = 0.09$, $G_{min} = 0.01$) were similar to those previously determined for homotypic cx46 gap junction channels.

These results argue against the possibility that G_j arises from a change in the structural properties of cx46 hemichannels and suggest a mechanism where current through open hemichannels initiates gap junction formation. Opened cx46 hemichannels could conceivably form nonfunctional heterotypic gap junctions with cx40, and stimulate cx40 homotypic gap junction channels by a nucleation process, but this ad hoc explanation seems rather too contorted to be viable, especially when viewed in light of data presented in the next section.

A threshold level of hemichannel current is required to stimulate gap junction formation

To test for a possible threshold effect, incrementally larger hemichannel currents were generated until a rapid change in the G_j growth rate was observed. Fig. 7 shows the hemichannel currents required to initiate gap junction formation in two oocyte pairs obtained from the same batch but expressing

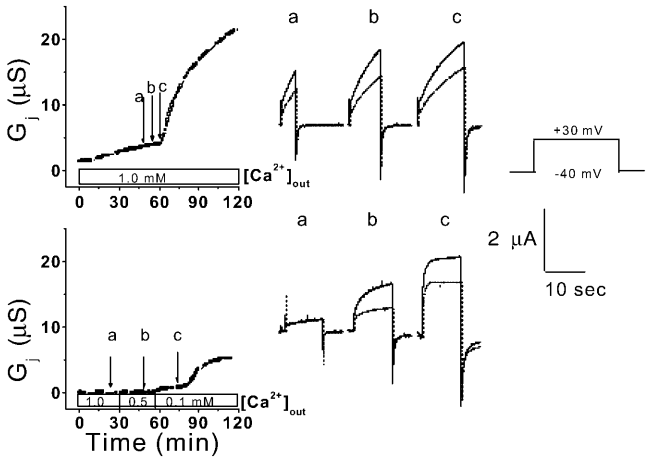

FIGURE 7 Gap junction formation is stimulated by a threshold level of hemichannel current that is independent of hemichannel expression levels. The left-hand panels show the time courses of gap junctional conductance for two oocyte pairs prepared from the same oocyte batch. Each time point represents G_j measured as described in Fig. 1. $[Ca^{2+}]_{out}$ is shown in the bar just above the time scale. Lettered arrows indicate when cx46 hemichannels were simultaneously activated in both oocytes. The right-hand panels (labeled *a*, *b*, and *c*) show whole-cell nonjunctional current traces with labels corresponding to the lettered arrows in the G_j time course shown in the left-hand panel. Hemichannel currents were elicited by simultaneously depolarizing both oocytes to +20 mV from a holding potential of −40 mV. Incrementally larger hemichannel currents were achieved by increasing the duration of the depolarizing pulse (*A*) or by applying the same voltage pulse after decreasing $[Ca^{2+}]_{out}$ (*B*). Despite the large difference in expression levels (assessed by comparing hemichannel currents in 1 mM $[Ca^{2+}]_{out}$), similar currents were required to initiate gap junction formation in each oocyte pair. The extent of G_j stimulated by the threshold currents was roughly proportional to expression level.

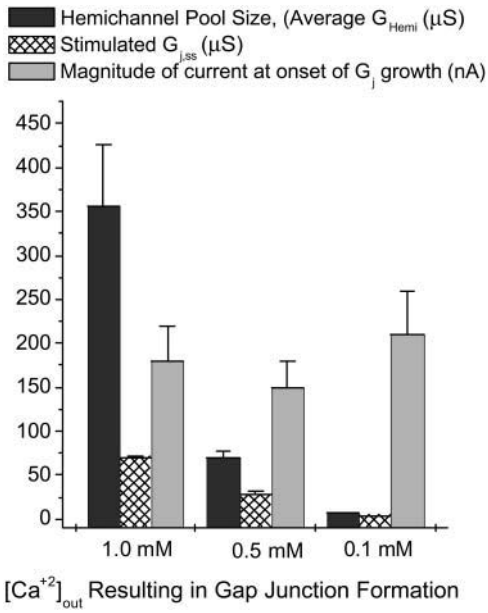

FIGURE 8 Lowering $[Ca^{2+}]_{out}$ stimulates gap junction formation only if the steady-state hemichannel current increases beyond a threshold level. Lowering $[Ca^{2+}]_{out}$ stimulates gap junction formation only when whole-cell hemichannel current increases beyond a threshold level. Paired oocytes were voltage clamped to −30 mV in ND96 supplemented with 1 mM $CoCl_2$. G_j was assayed periodically as described in Fig. 1. $[Ca^{2+}]_{out}$ was lowered every 15–20 min by perfusion with ND96 containing 1 mM, 0.5 mM, and 0.1 mM until G_j increased. The data from each oocyte pair were grouped by calcium level resulting in gap junction formation. Data in each group were averaged and are shown as mean and standard deviation for two to three pairs. Cx46 hemichannel expression level is expressed as maximum whole-cell hemichannel conductance measured in 0.5 mM external calcium (*solid columns*). The extent of stimulated increase in G_j is shown in the thatched columns. The cx46 hemichannel current that coincided with the initial increase in junctional conductance was measured as the difference between measured holding current and the holding current in the presence of 1 mM $CoCl_2$ (*shaded columns*).

different numbers of hemichannels. In the top set of panels, paired oocytes incubated in 1 mM $[Ca^{2+}]_{out}$ were depolarized to +30 mV for an increasing period of time to activate an increasing number of hemichannels. In this particular case, a 12-s pulse, but not 4- or 8-s pulses, stimulated gap junction formation. Hemichannel activation after the initial stimulation of G_j rarely produced any changes in the growth rate or extent of G_j. In oocyte pairs expressing fewer hemichannels, shown in the lower set of panels, incrementally larger hemichannel currents were generated by repeating the same voltage pulse protocol after stepwise reductions in $[Ca^{2+}]_{out}$. These experiments show that a minimum level of hemichannel current is required to stimulate gap junction formation and that the threshold current is independent of $[Ca^{2+}]_{out}$ and total hemichannel expression level.

Another indication that a calcium-independent threshold current is required to stimulate gap junction formation is presented below and in Fig. 8. The steady-state activation curve of cx46 hemichannels shifts toward more negative potentials with decreases in $[Ca^{2+}]_{out}$ *t*. Hence, at a fixed holding potential, lowering $[Ca^{2+}]_{out}$ opens an increasing fraction of the total hemichannel pool. In one experiment, oocyte pairs clamped between −20 and −30 mV were subjected to stepwise decreases in $[Ca^{2+}]_{out}$ until a rapid increase in G_j was observed. The whole-cell current coinciding with the onset of G_j growth was recorded. After the stimulated G_j reached a steady-state value the level of hemichannel expression was assayed by measuring the whole-cell conductance of each oocyte in 0.5 mM $[Ca^{2+}]_{out}$. In Fig. 8, oocyte pairs are binned into groups according to which calcium concentration led to G_j stimulation. Plotted for each group is the average hemichannel expression level (*solid bars*), the average hemichannel current at the time of G_j stimulation (*shaded bars*), and the extent of stimulated G_j (*hatched bars*). The data show that as the size of the available hemichannel pool decreases, an increasing fraction of the pool must be opened (by larger decreases in $[Ca^{2+}]_{out}$) to achieve a threshold current that stimulates gap junction formation. The average hemichannel current at the time of G_j stimulation was ~−200 nA regardless of $[Ca^{2+}]_{out}$ or total hemichannel expression levels. If reducing calcium did not result in a change in gap junctional conductance within 5–10

min, the time required for the holding current to reach steady-state, longer incubations of up to 30–45 min also failed to stimulate gap junction formation. These results show that at a fixed holding potential, lowering $[Ca^{2+}]_{out}$ does not stimulate gap junction formation unless a threshold level of hemichannel current is induced. Also note that the extent of stimulated G_j was proportional to the hemichannel expression level and not the calcium concentration.

The threshold current appears to be determined by the sum of currents in both oocytes. When paired oocytes expressing different hemichannel levels are compared to pairs expressing similar hemichannels levels, G_j was stimulated when the sum of whole-cell hemichannel currents exceeded the threshold level. To test if the combined currents in both oocytes define the threshold current, hemichannels were activated in one oocyte at a time or in both oocytes simultaneously, as shown in Fig. 9. In three out of four oocyte pairs, G_j increased only when hemichannels in both oocytes were opened at the same time. The magnitude of activated current in each oocyte did not change between pulse protocols, and a cumulative effect was ruled out because the activation pulse could be repeatedly applied to one oocyte or the other without altering the growth rate of G_j.

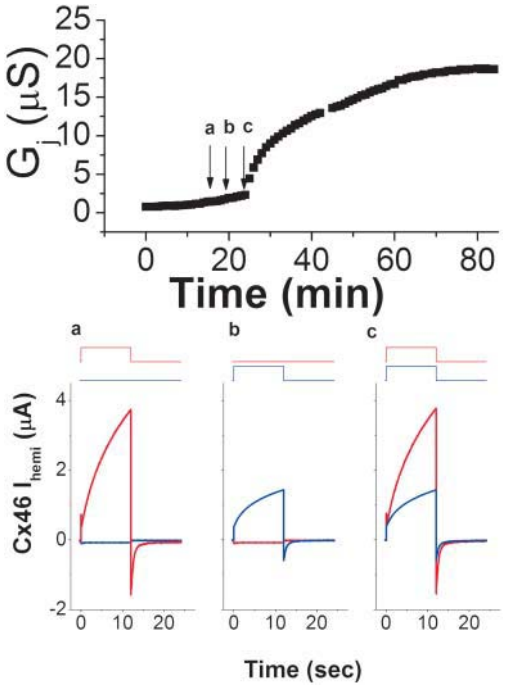

FIGURE 9 Synchronous stimulation is more effective than sequential stimulation Activating hemichannels simultaneously in both oocytes is more effective in stimulating gap junction formation than activating hemichannels sequentially in each oocyte. Cx46-expressing oocyte pairs were voltage clamped to −30 mV in a dual two-electrode voltage clamp configuration. Junctional and nonjunctional conductances were assayed periodically as described in Fig. 1. Hemichannels were activated by applying a 9-s depolarizing pulse to +30 mV to one of the oocytes, then to the other oocyte, and then to both oocytes simultaneously, allowing 2–5 min between pulse protocols to determine if the growth rate of G_j was affected (*bottom panel*). Note that if hemichannels are activated in only one oocyte, a 70-mV transjunctional potential occurs and the presence of junctional coupling can be seen as current recorded in the nonstepped oocyte. In five out of six oocyte pairs from two different batches of oocytes, gap junction formation was stimulated only when hemichannels were activated in both oocytes simultaneously. Even repeated activation of hemichannels in a single oocyte sequentially failed to stimulate gap junction formation.

Calcium entry is not the likely mechanism of stimulation

Two lines of evidence argue strongly against the possibility that calcium entry through open hemichannels results in an increase in the rate of gap junction formation. First, the threshold current required to stimulate gap junction formation is completely independent of $[Ca^{2+}]_{out}$. Given the same threshold current, much less calcium would enter oocytes bathed in low calcium media than oocytes bathed in high calcium media. Also external calcium can be completely removed (0 added calcium with 2 mM EGTA present) with no effect on the ability of hemichannel activation to stimulate gap junction formation (data not shown).

Second, increasing cytosolic calcium levels by the calcium ionophore A23187 fails to stimulate G_j whereas opening hemichannels in the same ionophore-treated oocyte pair does. Fig. 10 shows a representative ionophore experiment. Similar results were obtained in two other oocyte pairs. Perfusion with 1 or 5 μM A23187 in the presence of 2 mM $[Ca^{2+}]_{out}$ evoked a transient or sustained increase in whole-cell conductance, presumably through calcium-activated Cl^- channels. The whole-cell conductance produced by treatment with A23187 responded to changes in holding potential and $[Ca^{2+}]_{out}$ oppositely from cx46 hemichannels. The A23187-induced whole-cell conductance increased when $[Ca^{2+}]_{out}$ was increased or the holding potential was made more negative. In three oocyte pairs tested, perfusion with calcium ionophores never resulted in gap junction formation whereas activating hemichannels in the same oocyte pairs did stimulate gap junction formation. Increasing $[Ca^{2+}]_{out}$ from 0.2 mM to 2.0 mM in the presence of 5 μM A23187 caused a rapid decrease in the junctional conductance, a further indication that intracellular calcium was actually elevated by the ionophore.

Factors modulating junctional stimulation

Pairing orientation dramatically affects threshold current and final junctional conductance

Because some connexins are asymmetrically distributed on the surface of oocytes (Levine et al., 1993), we tested if pairing orientation affected the amount of G_j that developed between paired oocytes. The pairing orientation of oocytes did affect the poststimulated steady-state junctional conductance, but also the threshold current required for stimulating gap junction formation. Fig. 11 shows that in oocyte pairs expressing similar levels of cx46 hemichannels, vegetal-

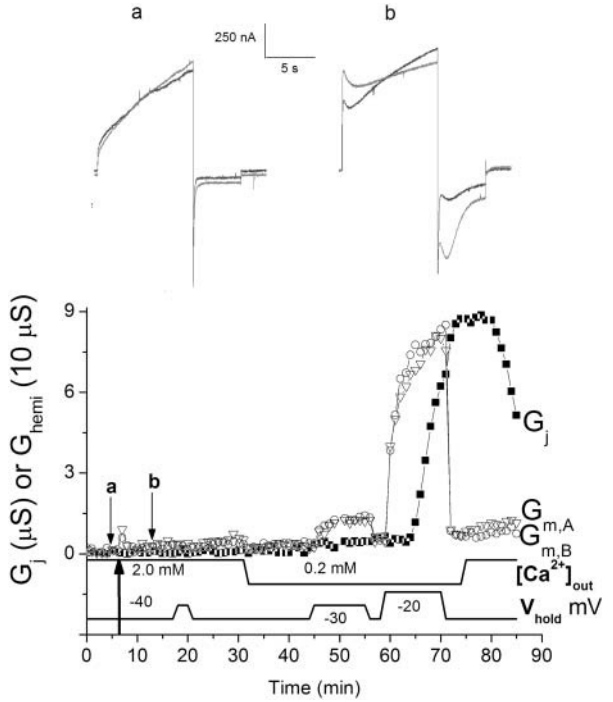

FIGURE 10 Increasing cytosolic $[Ca^{2+}]$ does not stimulate G_j growth. (*Top*) The time courses of junctional and nonjunctional conductances were recorded in oocyte pairs as described in Fig. 1. Oocyte pairs were perfused with 2 mM $[Ca^{2+}]_{out}$ ND96 containing 2 mM A23187 calcium ionophore (*green arrow*). A positive indicator that cytosolic calcium increased is the difference between the hemichannel currents activated before (*bottom*, *a*) and after (*bottom*, *b*) the addition of ionophore (*green arrow*). After ionophore addition, there is an increase in whole-cell conductance and a second transient component to whole-cell current traces elicited by depolarizing pulses (most evident in tail currents). This new current is very likely due to the well-known Ca^{2+}-activated chloride channel, an endogenous channel expressed abundantly in most batches of oocytes (Carpenter, 1987). The activation of this current confirms the entry of a significant amount of calcium. Note that activation of hemichannels does not produce sufficient calcium entry to activate the Ca^{2+}-activated chloride current in the absence of ionophore. But even when calcium entry mediated by ionophores could not stimulate G_j, opening cx46 hemichannels by lowering $[Ca^{2+}]_{out}$ and elevating the holding potential could.

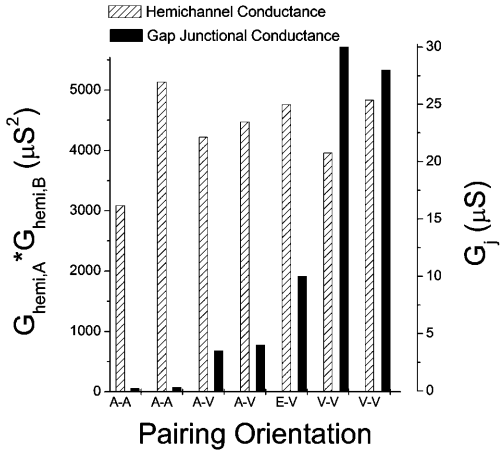

FIGURE 11 Pairing orientation affects both threshold current and the magnitude of stimulated cx46 G_j. Oocytes were paired in four orientations to produce different contact types: vegetal-vegetal poles (V-V), vegetal pole-equatorial region (V-E), vegetal-animal poles (V-A), and animal-animal poles (A-A). An increasing fraction of total hemichannels was opened by depolarizing pulses applied in decreasing $[Ca^{2+}]_{out}$ until a change in the growth rate of G_j was detected. G_j was then followed over time until a new steady-state condition was reached. The whole-cell hemichannel conductance, extrapolated if necessary to 0.5 mM Ca^{2+}, was used to quantify expression levels. The hemichannel expression levels and stimulated change in $G_{j,ss}$ were grouped according to pairing orientation. The $[Ca^{2+}]_{out}$ in which gap junction formation was stimulated is presented as a measure of I_{Thresh}. Not only did the pairing orientation affect the stimulated increase in G_j, but it also determined the fraction of hemichannels that must be opened to initiate gap junction formation.

vegetal pairings (V-V) resulted in the highest stimulated junctional conductance levels followed by equatorial-vegetal pairings (E-V) and then animal-vegetal (A-V) pairings. Little to no coupling could be stimulated in animal-animal (A-A) pairings, despite the presence of functional hemichannels. The threshold level of hemichannel current required to stimulate gap junction formation was also dependent on pairing orientation, but in the opposite manner. Stimulating gap junction formation in A-V pairs required much larger and longer hemichannel currents than it did in V-V pairs. We interpret these findings to imply that the density of hemichannels is greater in the vegetal poles of oocytes, and that a greater fraction of the total hemichannel pool had to be opened in nonvegetal pole pairings in order to generate the same threshold current level in the contact region.

The time constant of stimulated gap junction formation is sensitive to $[Ca^{2+}]_{out}$

$[Ca^{2+}]_{out}$ dramatically affected the time constant of stimulated gap junction formation without significantly altering the final steady-state magnitude of junctional coupling (Fig. 12). Fig. 12 *A* shows three representative examples of the time course of stimulated junctional conductance increase. All three curves, low Ca^{2+}, high Ca^{2+}, and high Ca^{2+} with lectin, are well fit with a single exponential (and sometimes a persistent linear component as for the particular example of high Ca^{2+}, 1.0 mM shown). These data show that the proportionality between $G_{j,ss}$ and $G_{hemi,A} \times G_{hemi,B}$ remains constant over the range of $[Ca^{2+}]_{out}$ from 0.1 to 1.0 mM. Although the magnitude of stimulated G_j did not depend on $[Ca^{2+}]_{out}$, the time constant is a strong function of Ca^{2+} concentration in the absence of lectin. Time constants derived from fitting stimulated G_j growth curves to a single exponential process are plotted as a function of $[Ca^{2+}]_{out}$ in Fig. 12 *B*. The time constant for reaching steady-state G_j increased from 4 min to 25 min as $[Ca^{2+}]_{out}$ increased from 0.2 mM to 1 mM. But the

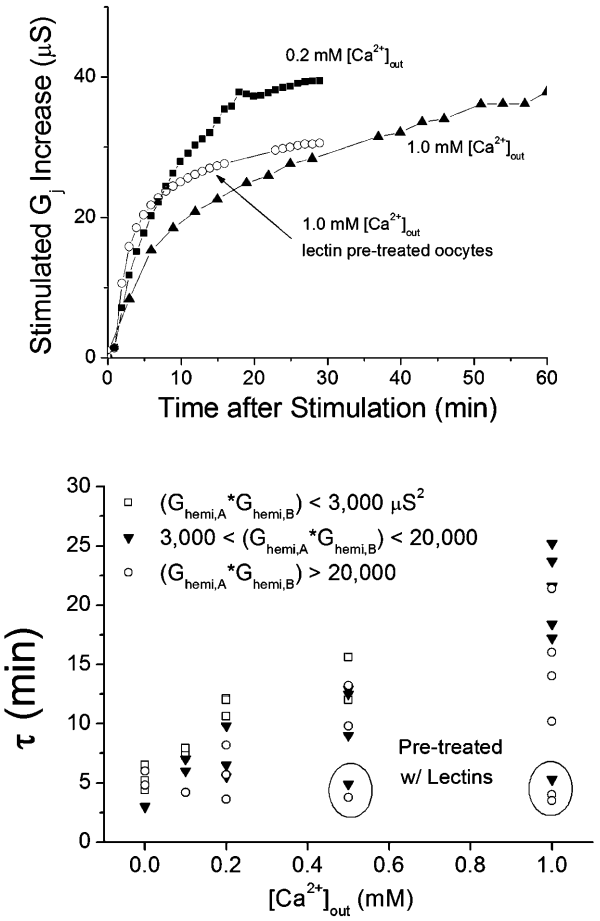

FIGURE 12 The time constant of stimulated gap junction formation is dependent on $[Ca^{2+}]_{out}$. (*Top panel*) Examples of G_j growth curves stimulated under different conditions. Oocyte pairs expressing similar hemichannel levels are shown to demonstrate that the extent (as opposed to the rate) of gap junction formation was not significantly altered by $[Ca^{2+}]_{out}$. Gap junction formation was stimulated in 0.2 mM $[Ca^{2+}]_{out}$, 1.0 mM $[Ca^{2+}]_{out}$, and 1.0 mM $[Ca^{2+}]_{out}$ for oocyte pairs pretreated with 20 μg/ml soybean agglutin before pairing. (*Bottom panel*) The time constants obtained after fitting G_j growth curves to a single exponential process. If the stimulated change in junctional conductance could be modeled by a single exponential process, data were fit to the equation $G_j(t) = G_{j,o} + G_{j,max} \times (1 - \exp(t/\tau))$. Time constants were binned according to the $[Ca^{2+}]_{out}$ present during gap junction formation. Different symbols are used to distinguish between different expression levels to show the inverse relationship between the time constant and the total expression level seen at higher $[Ca^{2+}]_{out}$.

dependence of τ on $[Ca^{2+}]_{out}$ was eliminated when stripped oocytes were pretreated with lectins (20 μg/ml soybean agglutinin for 20–30 min) before pairing. We reasoned that lectins might increase the diffusion rate of proteins in the membrane by cross-linking and condensing surface glycoproteins that might impede diffusion. In these pairs, the threshold current for stimulating gap junction formation was significantly lower than in untreated pairs and the time course of junctional coupling was very rapid even in high $[Ca^{2+}]_{out}$.

The time constants obtained in experiments using lectin-treated oocytes at two different Ca^{2+} concentrations are shown as circled data points in Fig. 12 *B*. In untreated oocyte pairs, the time constant of stimulated G_j growth was a roughly linear function of calcium concentration. But the time constant of stimulated G_j growth in lectin-treated oocyte pairs was fast and independent of Ca^{2+} concentration. This result suggests that the rate-limiting effect of $[Ca^{2+}]_{out}$ on the time course of gap junction formation might be mediated through a calcium-dependent modulation of cell-surface carbohydrate interactions that prevent hemichannel docking. This view is consistent with the observations of Dahl and colleagues (Dahl et al., 1992; Carpenter and Cohen, 1976) that both glycosidase and lectin treatment enhance gap junction formation. This hypothesis is further strengthened by the finding that when initial unstimulated coupling levels were large, suggesting low levels of endogenous factors hindering gap junction formation, time constants were short and independent of external calcium concentration.

DISCUSSION

The experimental data developed above provide a basis for understanding both gap junction formation and how opening hemichannels can stimulate it. The late stages of gap junction formation include the initiation of intimate cell-cell contact sites, the transport of precursors from both cells to junctional sites, assembly of precursors into a functional gap junction channel, and the accumulation and organization of individual channels into gap junction plaques. For large contact areas, such as those found in the paired-oocyte expression system, the growth rate of macroscopic G_j depends predominantly on two separate time-dependent processes: 1), the rate of establishing junctional sites where the distance separating plasma membranes is short enough to allow for interactions between opposing hemichannels; and 2), the rate of gap junction channel assembly occurring within these junctional sites. We demonstrate here that opening functional hemichannels, such as those formed from cx46, cx50, and cx38, can stimulate rapid increases in voltage-dependent G_j that most likely result from an increase in the area available for junctional formation rather than stimulation of the actual channel assembly reaction.

The kinetics of gap junction formation in paired *Xenopus* oocytes

The nature of the gap junction precursor

It is generally accepted that gap junction channels assemble from precursors residing in the plasma membrane. Neither protein synthesis nor trafficking to the plasma membrane is required for gap junction channels to assemble after cell contact is made (Dahl et al., 1992; Levine et al., 1993).

However, the state of oligomerization of the precursor is not usually identified directly and in principle could differ for different connexins. Also, it is still unclear whether or not functional hemichannels represent an aberrant fraction of the total hemichannel pool, the majority being nonfunctional. In over 20 different batches of oocytes, transient hemichannel activation resulted in an immediate increase in the growth rate of G_j that was unaffected by subsequent changes in hemichannel activation levels. When assayed under constant $[Ca^{2+}]_{out}$ conditions, the initial growth rate of stimulated G_j was proportional to the product of hemichannel levels and followed the second-order rate law $dG_j/dt = N \times k \times G_{hemi\ A} \times G_{hemi\ B}$, where k is the rate constant and N is a measure of the number of sites where junctional formation can occur. The time course of junctional conductance increase followed first-order exponential kinetics where the final steady-state value of junctional coupling was proportional to the product of hemichannel levels as given by $G_{j,ss} = N \times k_s \times G_{hemi,A} \times G_{hemi,B}$, where k_s is a constant. Together, these results suggest that gap junction channel assembly proceeds with the kinetics of a bimolecular reaction between the two populations of functional hemichannels in the paired oocytes. However, we cannot totally dismiss the possibility that G_j and standardized G_{hemi} are related by a different or more complicated equilibrium and rate equation. In certain individual batches of oocytes, the data fit the product rule better than in other batches. Also, the combined data showed that stimulated G_j was proportional to the average G_{hemi} in the oocyte pair, and that this correlation was only slightly less significant than the correlation between G_j and the G_{hemi} product. Unfortunately, the range of data is restricted by the fact that if hemichannel densities were too low we could not generate the threshold currents required to stimulate gap junction formation and if the hemichannel densities were too high then either oocyte viability was compromised or the stimulated G_j was too large to measure accurately. Although the exact form of the quantitative relationship between the numbers of functional hemichannels and gap junction channels remains difficult to determine, there is a definite positive correlation between these populations. We offer this positive correlation to suggest that hemichannels that function in nonjunctional membrane, such as those formed by cx46, cx50, and cx38, can assemble into functional gap junction channels just as readily as nonfunctional hemichannels.

The nature of steady-state G_j

Our results argue that the steady-state level of stimulated G_j results from either a depletion of available precursors or limited plaque expansion and that the newly formed gap junctions have a relatively long half-life in the paired-oocyte system. Modifying cx50 hemichannels with DEPC before pairing oocytes prevents formation of functional gap junction channels, but modifying the hemichannels after a steady-state G_j is reached has no immediate or long-term effect on the magnitude of G_j. If the stimulated $G_{j,ss}$ resulted from equilibrium between formation and degradation pathways, eliminating the forward reaction should have drastically altered the equilibrium value of $G_{j,ss}$ and a rapid loss of coupling should have been detected. On the contrary, gap junctions between oocytes appear to be very stable, with G_j remaining nearly constant for up to 24 h even after eliminating the population of functional precursors. Although this finding may contrast with what is known of connexin half-lives in mammalian cells, it is supported by other studies in *Xenopus* oocytes. Unlike mammalian cultured cells, the half-life of connexins and gap junctions in *Xenopus* oocytes appears to be much longer, up to 24 h (Zampighi et al., 1999; Zhou et al., 1999). Also, electron microscopic analysis of gap junctions between paired oocytes rarely detects the double-bilayer plaque structures that represent retrieved gap junctions (Guido Zampighi, personal communication).

A comparative analysis of gap junction formation experiments performed in different batches of oocytes provided qualitative evidence that the stimulated $G_{j,ss}$ can represent either the depletion of available precursors or limited plaque growth, depending strongly on the presence or absence of batch-specific endogenous factors. In some oocyte batches, the forces opposing gap junction formation are so great that channel assembly only occurs while hemichannel conductance remains high. Any condition that closes hemichannels, including hyperpolarized voltages or elevated $[Ca^{2+}]_{out}$, reversibly prevents further G_j increases until the hemichannels are opened again. As discussed later, hemichannel current is proposed to facilitate the establishment of junctional area, partly by removing or dispersing the volume of substance between two opposing membranes. Hence, it appears that the density of endogenous components that interfere with the formation and expansion of junctional areas is so great in some oocyte batches that it effectively limits plaque growth and de novo establishment of new junctional sites and thereby is most likely responsible for maintaining the observed steady-state level of coupling.

In some batches of oocytes or in oocytes pretreated with lectins to reduce the extent of steric hindrance interfering with gap junction formation, stimulated G_j continued to increase long after the stimulus (open hemichannels) was removed. In these oocyte pairs, we propose that the density of endogenous cell-surface components was not great enough to limit gap junction plaque expansion. Here, initial channel formation stabilizes junctional areas that then serve as traps where precursors diffusing into the perimeter of the trap have a high probability of docking with opposing precursors. Because a good ''trap'' depletes its surroundings, the exponential decay of G_j growth represents the depletion of available precursors.

Rate-limiting steps of gap junction formation

External calcium has been shown to have an inhibitory effect on the extent of cx32 gap junction formation between oocytes when measured a fixed number of hours after pairing (Dahl et al., 1992). However, it was not clear whether calcium acted on the channel precursors or on other aspects of the channel formation process. Also, because G_j was sampled at only one time point it was unclear if gap junction formation had proceeded to completion at the time G_j was assayed. In experiments presented here, $[Ca^{2+}]_{out}$ did not affect the extent of stimulated gap junction formation. It did, however, dramatically affect the rate of formation. The time constant of G_j growth can be as short as several minutes in $[Ca^{2+}]_{out}$ below 0.2 mM or as long as 30 min in 1 mM $[Ca^{2+}]_{out}$. Hence, we found that a major rate-limiting step in G_j development was very calcium sensitive.

There are several possible explanations for why calcium slows the rate of gap junction formation without affecting the extent of final G_j. 1), Calcium may decrease the affinity of interaction between opposing hemichannels or increase the time between hemichannel docking and the first opening of the gap junction channel. 2), Calcium affects nonconnexin factors involved in gap junction formation. For example, Ca^{2+} could stabilize interactions between cell-surface factors that hinder the expansion of gap junction plaques by preventing close membrane appositions. Alternatively, Ca^{2+} may increase the local concentrations of nonconnexin membrane components and thereby reduce the effective diffusion rate of hemichannels to junctional sites. 3), Ca^{2+} may determine the number of individual junctional sites that form in any given area of cell-cell contact after hemichannels are opened. Here, lower $[Ca^{2+}]_{out}$ may result in many junctional sites established within a single cell-cell contact site, thereby increasing the ''trapping'' efficiency of diffusing precursors. If this is the case, then for oocyte pairs expressing similar hemichannel densities, the size of the average gap junction plaque should be smaller when junction formation is stimulated in low $[Ca^{2+}]_{out}$. Many of these possibilities could potentially be distinguished by combining electron microscopic analysis and electrophysiology on the same oocyte pairs.

When oocytes were pretreated with lectins to reduce the extent of steric hindrance that opposes gap junction formation, the growth rate of stimulated G_j was independent of $[Ca^{2+}]_{out}$. Hence, calcium appears to reduce the rate of gap junction formation by affecting endogenous membrane components rather than hemichannel binding affinities. Increasing $[Ca^{2+}]_{out}$ after stimulating gap junction formation can rapidly eliminate G_j growth depending on the batch of oocytes, again suggesting that the sensitivity to $[Ca^{2+}]_{out}$ depends on batch-specific endogenous oocyte properties rather than the structural properties of hemichannels. Together, these findings suggest that although calcium undoubtedly affects hemichannel gating, its rate-limiting effect on gap junction formation is primarily mediated through nonhemichannel factors.

Possible mode of action by which hemichannel current stimulates gap junction formation

Initiation of new junctional sites versus increased docking affinity

That hemichannels are the precursors of gap junctions is not particularly surprising, but it is quite surprising and hitherto unobserved that opening hemichannels dramatically stimulates junctional conductance. The question is by what mechanism. The low rate of coupling before hemichannel current activation may result from reduced interactions between hemichannels owing to a paucity of junctional sites or from a low affinity of interaction between hemichannels that have never opened (the implication of this conjecture being that opening hemichannels might somehow increase the strength of their interaction and enhance the probability of docking). Our data rule out the altered affinity hypothesis.

Gap junction channels assemble from the end-to-end binding of two opposing hemichannels, a process referred to as ''hemichannel docking.'' Factors altering disulfide exchange rates affect the rate of gap junction formation, suggesting that the external loops of hemichannels, which contain three conserved cysteine residues per connexin polypepetide loop, undergo conformational changes during the docking process (Dahl et al., 1991). Also, conformational changes are associated with the voltage-dependent gating of cx46 hemichannels, and the gate that closes at negative voltages has been physically located close to the extracellular mouth of the channel (Campbell and Bode, 1983; Ali et al., 1989). Hence, changes in hemichannel conformation consequent to opening might increase the affinity of interaction between opposing hemichannels and thereby increase the rate of channel assembly occurring in preexisting junctional areas. However, several lines of evidence clearly rule out changes in hemichannel conformation as the initiating event in stimulating junctional conductance. 1), If a gating-induced conformational change increases hemichannel affinities, then we would expect to see increases in G_j that are proportional to the magnitude of activated hemichannel current rather than the threshold effect which is actually seen. Stimulation of gap junction formation requires a threshold level of hemichannel current that is independent of voltage, $[Ca^{2+}]_{out}$, and the total number of hemichannels in the membrane. 2), Conformational changes are not immediately followed by G_j increases as indicated by a latency period between the beginning of hemichannel activation and the onset of changes in G_j observed in some oocyte pairs. 3), Once stimulated, G_j increases rapidly even after the hemichannels are returned to their closed state. 4), If conformational changes consequent to cx46 hemichannel opening increase the affinity of interaction, then such changes should not

affect the formation of other types of gap junction channels. However, activating cx46 hemichannels that are coexpressed in only one of the paired oocytes can stimulate cx40 homotypic gap junction formation. Because cx46 does not form gap junctions with cx40, changes in cx46 affinity cannot promote formation of cx40 gap junctions.

Pretreatment of oocytes with lectins reduces the variability of junctional conductance measured between different batches of oocytes and results in up to 10-fold higher junctional conductances than in nontreated oocyte pairs. The mechanism appears to be a clearance of steric hindrance by aggregation and retrieval of lectin-bound proteins and can be mimicked by treatment with glycosidases (Levine et al., 1991). Likewise, we propose that the lack of junctional conductance measured within hours after pairing is due to a lack of sufficient membrane contact area resulting from the presence of steric hindrance imposed by membrane glycoproteins and/or proteoglycans. Hemichannel activation induces a rapid increase in the growth rate of G_j, and the magnitude of the threshold current is determined by batch-specific factors associated with contact area and steric hindrance opposing gap junction formation. Hence, it appears that hemichannel activation mimics the effects of reducing steric hindrance.

How functional hemichannels orchestrate the formation of junction sites

Opposing membranes must come together within a distance of 3–4 nm in order for hemichannels to dock. This is an extremely short distance relative to the average 20–40 nm that uniformly separates cell membranes at points of contact. To establish such potential junctional sites, all macromolecules that physically or electrostatically prevent short-range distances between two opposing membranes must be cleared from the contact site and the volume of solution separating the two membranes must be reduced. How cells establish this type of contact is unknown. It is believed that gap junctions form randomly at sites of cell-cell contact, and there is no evidence that close membrane associations are regulated.

Our data are consistent with the hypothesis that hemichannel current promotes local depletion of osmoles (probably mostly Na^+ and Cl^-) in the microenvironment. This loss of osmoles results in a collapse of extracellular space that increases membrane contact and establishes new sites where junctions can form or expands the area of existing sites. The possibility that osmotic forces induced membrane fusions between the two oocytes is ruled out on the basis that the stimulated G_j was still voltage dependent, strongly so in the case of cx50 experiments. Furthermore, although we could induce gap junction formation by swelling the oocytes in 30% ND96, the latency period between a change in osmolarity and a change in G_j was considerable (5–10 min) and G_j rapidly disappeared after removing the osmotic gradient. This finding, combined with the data showing that the threshold current is determined by the sum of currents in the contact zone, lead us to favor an osmotic effect mediated in the immediate vicinity of the contact area rather than a global swelling phenomenon.

Hemichannel current could potentially increase the number of contact sites by creating an osmotic gradient favoring transfer of water from the extracellular microenvironment into the oocyte or by reducing electrostatic screening by depleting the Ca^{2+} concentration. Production of an osmotic gradient would reduce extracellular volume and thus increase membrane contact, and the increase in electrostatic repulsion would tend to sweep charged glycoproteins from the Ca^{2+}-depleted regions, increasing the likelihood of membrane contact. It is possible that both effects occur.

Physiological and technical implications

The ability of hemichannel activity to stimulate gap junction formation provides a potential physiological role for functional hemichannels and forces us to reexamine the accepted dogma that gap junction formation proceeds in a random fashion where cell membranes come into contact. And because hemichannel activity can be regulated by both cellular and extracellular factors, there may be greater regulatory control over the establishment of gap junctions than previously expected.

Naturally occurring forces opposing gap junction formation must be overcome to bring membranes close enough to allow for the physical interaction between hemichannels. This may explain the intimate relationship between gap junction formation and cell adhesion molecules. Under cell culture conditions, adhesion proteins are required to establish a stable cell-cell contact region. Although cell-cell adhesion separates plasma membranes by a distance greater than that required for hemichannel docking, it provides a stable environment and may thereby increase the likelihood that membranes come even closer together to allow for hemichannel docking. Other types of channels and/or transporters may be used to collapse the interstitial volume to form junction sites between cells. These are probably transient sites that will disappear if there are no gap junction precursors available for channel assembly to stabilize the site. Using functional hemichannels to establish junction sites is the most efficient way of generating stable gap junctions because the gap junction precursors are guaranteed to reside in the junctional site when it is formed. An accelerated time course of gap junction formation may have physiological significance in situations involving transient cell-cell contacts, such as cell migration, leukocyte extravasation, and innervation of muscle during development.

Functional hemichannels may serve a physiological role in lens tissue, where the channels responsible for the ''leak'' currents of fiber cells remain unknown and may in fact be functional cx46 and/or cx50 hemichannels. Maintaining

a minimal external volume is of great importance in maintaining the clarity of the lens, and hemichannels may serve a role in regulating the external volume, aside from their role as a component of gap junction channels. Also, a spatial difference in the density of fiber cell gap junctions is thought to be critical for directing current flow, and consequent fluid flow, through lens tissue. The lens fiber cell hemichannels may preferentially stimulate gap junction formation depending on localized environmental cues, which may play a role in establishing and maintaining the observed high density of gap junction channels at the equator of the lens, where current flux through the lens is maximal.

Although physiological roles for functional hemichannels are only speculative at this stage, the technical implications of these findings are of some immediate experimental utility. The threshold current required to stimulate gap junction formation provides a new analytical tool for measuring the probability of cell-cell interactions that lead to gap junction formation. And the ability to control hemichannel activity provides unprecedented control over the initiation of gap junction formation. In most batches of oocytes all available contact sites are converted into junction sites within a very short time (seconds), so subsequent growth of junctional conductance represents the reaction kinetics of channel assembly. The ability to quantify the hemichannel pool allows for data from different experimental manipulations that aim to affect gap junction formation to be normalized to precursor availability. And finally, the ability to stimulate an accelerated time course of gap junction formation allows assessment of how different factors can affect the rate, extent, and growth form of junctional conductance in real time.

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