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Formation of hybrid cell–cell channels

(gap junctions/oocyte expression/hybrid channels/voltage gating/rectification)

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ABSTRACT **The oocyte cell–cell channel assay was used to demonstrate that connexin-43 is a cell–cell channel-forming protein as previously shown for connexin-32. Expression of connexin-32 in one and connexin-43 in the other oocyte of a pair results in the formation of junctional conductances at rates similar to those observed when only one or the other connexin is expressed in both oocytes of a pair. This suggests that hybrid cell–cell channels form in the oocyte system. Hybrid channels also form when a connexin-43 mRNA-injected oocyte is paired with a noninjected oocyte expressing endogenous connexin. The latter hybrids have properties apparently contributed by both types of hemichannels. Pure connexin-43 channels are not voltage gated, whereas pure oocyte channels are voltage dependent; hybrids of these channels rectify.**

Cell–cell channels, contained in gap junctions of different tissues, were generally believed to consist of identical proteins. This belief was based on their uniform appearance in the electron microscope, on the observation that antibodies against a liver gap junction protein crossreact with junctional proteins from a variety of tissues, and the apparent formation of cell–cell channels between different types of cells (1–5). Recently, however, the existence of a family of different gap junction proteins has been demonstrated (6–12). The question, therefore, arises whether the channels that form between different cell types (heterologous junctions) can be hybrids composed of different hemichannels. The subunit composition of heterologous junctions cannot be assessed easily (13) because it has recently been shown that a single cell type can express more than one junctional protein (8), thus raising the possibility of heterologous junctions containing only one type of protein.

With the availability of cDNA clones for two different gap junction proteins (6, 9) and a functional expression system, we can readdress the question of hybrid channel formation (14, 15). The near identity of the presumed extracellular regions of the heart and liver connexins (6, 9, 16–18) leads one to expect the formation of hybrid channels. In this paper, we describe the formation of such hybrid channels as well as hybrid channels between heart and *Xenopus* oocyte connexins. Of particular interest are the gating properties of the latter hybrids because of the marked differences in gating of the parent channels. A preliminary report of this work has been given elsewhere (19).

MATERIALS AND METHODS

Source of mRNA. Connexin-43 mRNA was transcribed *in vitro* from a cDNA clone isolated by Beyer *et al.* (9). To increase translation efficiency of the mRNA, a 201-base-pair segment immediately preceding the initiation codon (AUG) was replaced by the sequence -GACC(AUG)-, and the altered cDNA was recloned in pGEM-3Z (Promega Biotec). After linearization of the plasmid with *Ssp* I, transcription was carried out with SP6 RNA polymerase as described (15). Connexin-32 mRNA was transcribed from a connexin-32 cDNA as described (15). For some experiments, total mRNA from rat heart was used (14).

Preparation of Oocytes. The preparation of *Xenopus laevis* oocytes and the microinjection of mRNA have been described (15).

Measurement of Junctional Conductance. Junctional conductance was determined by the dual voltage clamp technique (20) as described (15). Both oocytes of a pair were independently voltage clamped to the same holding potential, usually close to the membrane potential of ≅50 mV. (For determination of voltage dependence of junctional conductance, oocyte pairs that exhibited membrane potential differences of >5 mV were rejected.) Transjunctional voltage steps of 5 sec duration were established by alternatingly stepping one oocyte of a pair to depolarizing and hyperpolarizing potentials. This procedure was then repeated with the other oocyte. In the case of hybrid channels, to avoid any artifactual asymmetry, the same pulse protocols were applied to both oocytes. In addition, the pulse protocols were repeated after rotating the pairs by 180° with respect to the electrodes and reimpalement.

RESULTS

Connexin-43 Is a Cell–Cell Channel-Forming Protein. mRNA transcribed *in vitro* from connexin-43 cDNA, when injected into *Xenopus* oocytes, is capable of directing the formation of cell–cell channels between paired oocytes. The induction is observed over a nonmeasurable or low background of endogenous oocyte channels (Fig. 1, Table 1). If oocytes are first paired and then injected with mRNA, junctional conductance appears with a lag period of ≥4 hr. When pairing of the oocytes is delayed for 18 or 42 hr after the injection of mRNA, junctional conductance appears earlier and rises with a steeper slope. This suggests that the oocytes accumulate a pool of channel precursors that increases with time after mRNA injection. The minimum size of this pool can be estimated from the difference between conductances measured with or without delayed pairing. The actual pool size is probably much larger because not all precursors can be expected to become channels and not all channels are likely to be open at any particular time. The input resistance of mRNA-injected oocytes is not lower than that of uninjected oocytes. For example, the oocytes shown in Fig. 1 exhibited a membrane conductance of 1.21 ± 0.08 (18) μS 18 hr after mRNA injection, while the corresponding uninjected oocytes had a membrane conductance of 1.48 ± 0.11 (15) μS.

The gating properties of the connexin-43 channels are distinct from those of endogenous oocyte channels (Fig. 2). The conductance of endogenous oocyte channels remained constant following injection of buffered solutions containing 100 μM free Ca^{2+}. In contrast, connexin-43 channels exhib-

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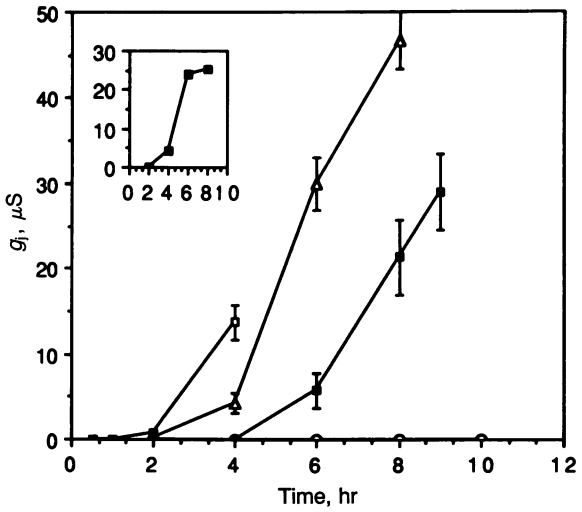

FIG. 1. Time course of formation of cell–cell channels in oocyte pairs in response to injection of connexin-43 mRNA. Each point represents the mean conductance (g_j) of 18 oocyte pairs (±SEM). The same pairs were reimpaled for conductance measurements at various time points after pairing at time 0. All oocytes (stage V) were obtained from one ovary. After mRNA injection the oocytes were paired immediately afterwards (■), 18 hr later (△), or 42 hr later (□). Uninjected oocytes were paired correspondingly. Since none exhibited detectable junctional conductance, only one set of time points (corresponding to mRNA-injected oocytes paired without delay) is shown (○). (*Inset*) Time course of cell–cell channel formation from precursors. Points represent Δg_j values (△ minus ■) with (18 hr) and without delayed pairing.

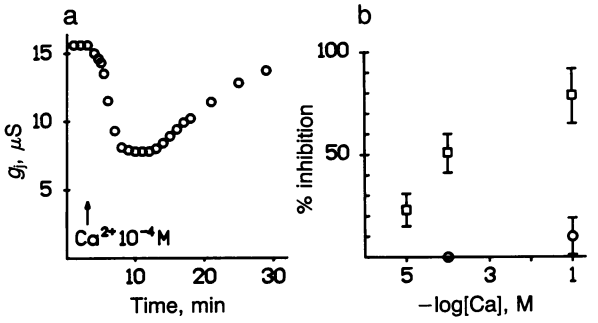

FIG. 2. Response of cell–cell channels to injection of Ca^{2+} buffers into oocyte pairs expressing connexin-43. (*a*) A transient reduction of junctional conductance, g_j, occurs in response to injection of Ca^{2+} buffers (100 μM) near the junction. The expression of connexin-43 cell–cell channels in the oocyte was the result of injection of mRNA transcribed *in vitro* from connexin-43 cDNA. (*b*) Concentration dependence of inhibition of junctional conductance by calcium. Channels expressed in oocytes in response to the injection of total rat heart mRNA (□) and in noninjected oocytes (○). Each point represents the mean ± SEM of conductance measurements on five oocyte pairs. For the determination of the percentage inhibition, conductance at the peak of the Ca response is compared to the conductance observed before the injection of calcium. The buffers were pressure injected as close to the membrane junction as possible. No quantification of $[Ca^{2+}]$ at the junction was attempted. However, the Ca^{2+}-activated Cl^- conductance that is present in oocytes (22) was used for control of Ca injections. Only oocyte pairs were analyzed in which the Ca-induced nonjunctional membrane currents increased by similar amounts after injection of the same buffers. Composition of Ca buffer solutions (23): 10 μM Ca, 9.5 mM CaCl/10 mM EGTA/200 mM Pipes, pH 7.5; 10 μM Ca, 60 mM $Ca(OH)_2$/100 mM potassium citrate, pH 7.5; 100 mM Ca, 100 mM $CaCl_2$/100 mM Pipes, pH 7.5.

ited reduced conductance following injection of buffered solutions containing as little as 10 μM free Ca^{2+}.

The most discerning feature that distinguishes endogenous channels from connexin-43 channels (and from connexin-32 channels, for that matter) (21) is their different response to transjunctional voltage. Endogenous channels are voltage sensitive (Fig. 3). A transjunctional voltage of either polarity reduces channel conductance. It is interesting to note that even at high transjunctional voltage a residual conductance remains. The mean residual conductance of 25 oocyte pairs at 50 mV was 29.7% ± 1.3% (SEM; range, 10–36%). In contrast, connexin-43 channels exhibit no voltage-dependent closure. The complete insensitivity to transjunctional voltage can only be observed in paired oocytes without detectable background of endogenous channels. Such a condition is easily obtained with delayed pairing (Table 1). If endogenous as well as connexin-43 channel proteins coexist in both oocytes of a pair, an intermediate response to transjunctional voltage is observed: the slope of the g_j versus transjunctional voltage plot is less steep, and a high residual conductance remains (Fig. 3). With conductance (g_j) ratios (injected/noninjected oocyte pairs) of 2, 3, and 24, residual conduc-

Table 1. Junctional conductances of oocyte pairs

	Control oocytes	Connexin-43 mRNA injected oocytes	Connexin-32 mRNA injected oocytes	Connexin-43/ connexin-32 mRNA injected oocytes	Connexin-43 mRNA injected/control oocyte pairs
Exp. 1	0.08 ± 0.01 (36)	2.24 ± 0.54 (34)			
Exp. 2					
3 hr	0 (56)	0.04 ± 0.01 (54)			
5 hr	0.12 ± 0.02 (56)	0.85 ± 0.19 (54)			
Exp. 3					
3 hr	0 (29)	0.02 ± 0.01 (30)	0.02 ± 0.01 (10)	ND	
5 hr	0.01 ± 0.01 (29)	0.19 ± 0.07 (30)	0.34 ± 0.12 (10)	0.16 ± 0.06 (20)	
Exp. 4					
3 hr	0 (18)	0.08 ± 0.03 (15)			<0.01* (15)
5 hr	0.20 ± 0.04 (18)	1.34 ± 0.32 (15)			0.45 ± 0.06 (15)

Conductances are expressed in μS, and the means ± SEM are given. Detection limit for measuring junctional conductance is <0.01 μS. The number of oocyte pairs analyzed is given in parentheses. Each experimental condition is applied to sibling oocytes. Oocytes were injected with mRNA and paired immediately afterwards, and junctional conductance was determined 16–18 hr later (Exp. 1). For Exps. 2–4, pairing was delayed by ≈16 hr, and junctional conductance was determined 3 and 5 hr after pairing. [Note: As described previously, the levels of endogenous oocyte channels are variable and are in part determined by the maturation state of the oocyte (21). Furthermore, activities of different mRNA preparations vary. Therefore, data should only be compared within the same line.] For each line, sibling oocytes of the same maturation stage were selected. From this pool, aliquots were used for the different experimental conditions. For Exps. 1 and 2, data from different oocyte preparations were pooled, while Exps. 3 and 4 used a single preparation each. 0 indicates that none of the pairs exhibited detectable junctional conductance (straight line of i_j at highest gain and 50-mV test pulses). Note that g_j values are lower than shown in Fig. 1. Less mRNA was injected for these experiments to be able to measure voltage dependence of g_j. ND, not determined.

*In 3 of the 15 pairs, conductance exceeded the detection threshold.

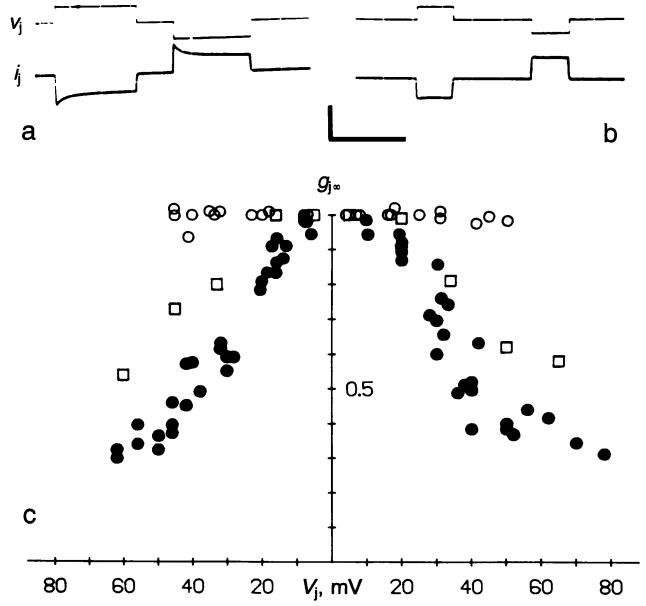

FIG. 3. Effect of transjunctional voltage on endogenous and connexin-43 mRNA-induced cell–cell channels expressed between oocyte pairs. Transjunctional current, i_j, in oocyte pairs expressing endogenous channels (*a*) declines with time and reaches a steady-state value when a transjunctional voltage (V_j) of 40 mV is applied. Constant transjunctional currents are seen in oocyte pairs expressing connexin-43 channels (*b*). Calibration: horizontal, 5 sec; vertical, 100 mV; 50 nA (*a*), 200 nA (*b*). (*c*) Steady-state chord conductances are plotted as a function of transjunctional voltage of endogenous channels (●; three pairs) and for connexin-43 channels (○; three pairs). An example of voltage dependence of an oocyte pair expressing both endogenous and connexin-43 channels (□) is included. $g_{j\infty}$ is normalized to conductance measured with transjunctional voltage pulses ≤10 mV.

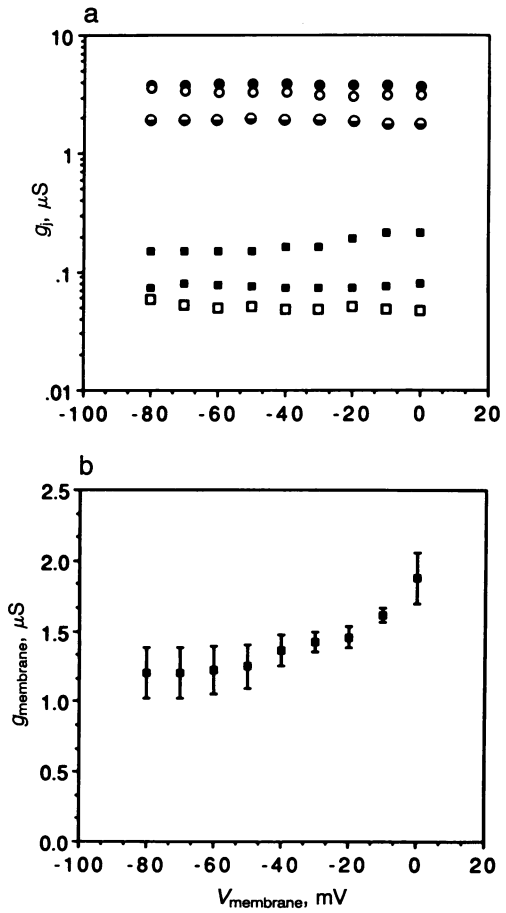

FIG. 4. Effect of membrane potential on junctional conductance (*a*) and nonjunctional membrane conductance (*b*). Junctional conductance (g_j) was determined by stepping both oocytes of a pair to the indicated membrane potentials and applying small (≅5 mV) test pulses to one oocyte. Data from three connexin-43 mRNA injected pairs (circles) and from three uninjected pairs (squares) are shown. Nonjunctional membrane conductance (g_m) was determined the same way by adding i_1 and i_2 (i_2 has a negative sign); the same values were obtained by a bath current monitor, recording the sum of the currents. Means ± SEM of three uninjected oocyte pairs are shown.

tances of 41%, 66%, and 89%, respectively, were observed at 50 mV transjunctional voltage. (Each value represents the mean of three oocyte pairs.)

Junctional conductances of neither endogenous nor mRNA-induced channels are dependent on the oocytes' membrane potential (Fig. 4*a*). Only if oocytes are held for longer periods of time (several minutes) at potentials of −10 to 0 mV, a gradual decline of junctional conductance of connexin-43 channels, but not of endogenous channels, can be observed. This coincides with an increase in conductance of the nonjunctional membrane for ions (possibly including Ca^{2+}) (Fig. 4*b*).

Formation of Hybrid Channels Between Different Connexins. The question of whether hybrid channels can form was addressed by pairing oocytes in which one had been injected with connexin-32 mRNA and the other with connexin-43 mRNA. Such pairs exhibited conductances similar to those observed with pairs expressing identical connexins (Table 1). While this result suggests that hybrid channels can form, a more rigorous test for the existence of hybrid channels would be the formation of channels with hybrid gating properties. When connexin-43 mRNA-injected oocytes were paired with noninjected oocytes, the junctional conductance was higher than that observed in uninjected pairs (Table 1). Testing such pairs for voltage dependence of their junctions revealed a strong asymmetry. No voltage-dependent closure was observed when the test pulses for determining junctional conductance were hyperpolarizing uninjected cells or depolarizing connexin-43 mRNA-injected cells. In contrast, a voltage-dependent closure, indistinguishable from that seen with endogenous channels, was observed with test pulses depolarizing the uninjected cells or hyperpolarizing the mRNA-injected cells (Fig. 5). Depending on the level of expression of endogenous channels, this asymmetry varies. If the ratio of mRNA-induced g_j to endogenous g_j is high, the asymmetry is pronounced. With lower ratios (induction against a high background of endogenous g_j) partial voltage dependence can be observed with hyperpolarization of uninjected cells or depolarization of mRNA-injected cells.

DISCUSSION

Connexin-43 is a genuine cell–cell channel-forming protein. Paired oocytes injected with mRNA synthesized *in vitro* from a connexin-43 cDNA clone exhibit cell–cell conductances similar to those obtained with expression of connexin-32 (15). The rapid appearance of cell–cell conductance in oocytes paired with a delay after mRNA injection is comparable to that observed with connexin-32 mRNA (21). It has been shown that under these conditions a pool of channel precursors accumulates that is readily available for junction formation after pairing, and that it is the size of this pool that is rate-limiting in junction formation (21).

It should be noted that no change in input resistance of the oocyte membrane was observed as a result of connexin-43 expression in unpaired oocytes. Therefore, if the pool of precursors is located in the membrane and if it is in the form of hemichannels, such hemichannels must be closed.

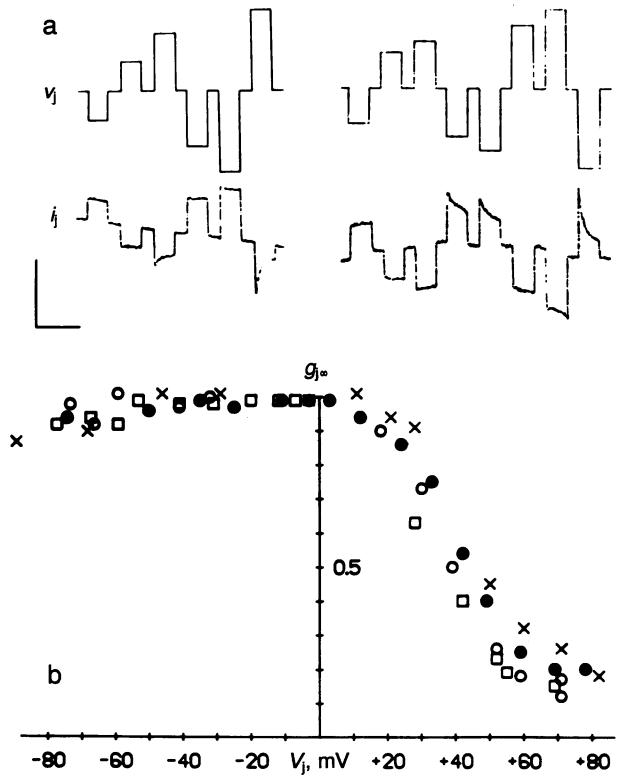

FIG. 5. Voltage dependence of cell–cell channels between uninjected and connexin-43 mRNA-injected oocytes. (*a*) Voltage steps (redrawn to scale) of increasing amplitude and alternating polarity (hyperpolarization of the stepped oocyte is shown as an upward deflection) are imposed on the mRNA-injected oocyte (V_j) (left tracing). Resulting currents required to maintain the membrane potential of the uninjected oocyte constant are shown in the lower trace (i_j). The holding potential for both oocytes was −50 mV. Calibration: horizontal, 10 sec; vertical, 40 mV; 10 nA. Stepping the voltage of the noninjected cell of the same pair produces mirror image tracings (right tracing). (*b*) Normalized steady-state junctional conductances in two hybrid oocyte pairs (pair 1, × and ●; pair 2, ○ and □) are plotted as a function of transjunctional voltage. The polarity of the transjunctional voltage is given in relation to the uninjected oocyte—i.e., depolarization of the uninjected oocyte or hyperpolarization of the mRNA-injected oocyte are shown as positive voltages. The voltage dependence of these channels is asymmetric: they close in response to depolarization of the uninjected (● and □) or hyperpolarization of the mRNA-injected (× and ○) oocyte and are unaffected by opposite polarization of the two respective cells. For all measurements, both cells were held at the same potential ±2 mV.

The cell–cell channels formed in response to connexin-43 mRNA injection into oocytes exhibit gating properties as seen in the parent tissue (refs. 24 and 25; see, however, ref. 26 for dissenting view of Ca effects). Even though these channels face the same cytoplasmic milieu as endogenous oocyte cell–cell channels, the two types of channel are distinct. Connexin-43 channels are sensitive to injection of Ca^{2+} buffers, while endogenous oocyte channels are not. Furthermore, connexin-43 mRNA-directed channels are insensitive to transjunctional voltage. Endogenous channels, on the other hand, exhibit a voltage dependence resembling that observed in channels in early embryos (20, 27). The fact that the different gating properties of different channels are seen in the same cytoplasmic environment indicates that the information for their specific gating resides in the individual connexin sequences.

While the voltage dependence of endogenous oocyte channels and those in the early embryos are qualitatively similar, there are notable quantitative differences including slope and residual conductance. A larger residual conductance remains at high transjunctional voltages in oocytes than in early embryos (20, 25). This residual conductance can vary from 10% to 35%. Oocytes expressing connexin-43 channels against a high g_j provided by endogenous channels form cell–cell channels with a reduced voltage dependence. The slope of the g_j versus V_j plot is inversely related and the residual conductance is directly related to the ratio of mRNA induced over endogenous g_j between paired oocytes. This raises the possibility that endogenous channels do not represent a homogeneous population. Alternatively, cell–cell channels in early embryos might be composed of proteins different from those expressed in oocytes or of the same proteins that were modified posttranslationally. It should be emphasized that in cases in which two types of connexin coexist within the same cell membrane, we cannot, at this time, distinguish between heteromeric hemichannels and different homomeric channels in parallel.

Paired oocytes expressing different connexins appear to form hybrid cell–cell channels that are made from two homomeric hemichannels. The hybrid channels can form between hemichannels consisting of connexin-32 and of connexin-43 subunits, respectively, as well as between hemichannels made from connexin-43 and endogenous oocyte connexins, respectively. The evidence for the existence of hybrid channels is provided by the increased junctional conductance that is observed between oocytes expressing different connexins as compared to oocyte pairs not injected with mRNA. The junctional conductances provided by hybrid channels made from connexin-32 and connexin-43 hemichannels increase at rates similar to those provided by pure connexin-32 or connexin-43 channels.

Oocyte pairs in which only one oocyte has been injected with connexin mRNA exhibit junctional conductances that are considerably larger than those observed between uninjected pairs, confirming an observation made earlier with injection of total mRNA (14). In some experimental conditions, junctional conductance could even be observed in hybrid pairs (only one oocyte injected), while the detection threshold was not exceeded in corresponding uninjected control pairs. The simplest explanation for this observation is that the presence of large numbers of channel precursors in the mRNA-injected oocyte increases the chance of docking with a rare hemichannel in the uninjected oocyte.

Alternatively, complete junctions could be provided by the mRNA-injected oocyte. Such a mechanism has been postulated for the transfer of the epidermal growth factor receptor between membranes (28). However, the properties of the channels formed between mRNA-injected and uninjected oocytes strongly argue for the formation of true hybrid cell–cell channels with each cell contributing one hemichannel. Such channels were found to respond to transjunctional voltage in an asymmetric fashion, whereas symmetric oocyte pairs (injected or noninjected) exhibit overall symmetric voltage gating properties.

Based on the kinetics of the voltage dependence of cell–cell channels in early embryos, Harris *et al.* (27) devised a model for channel gating by voltage that comprised two gates in series located within each hemichannel. The data presented here give experimental support to this model. Like embryonic channels, pure endogenous oocyte channels close symmetrically in response to applied transjunctional voltage of either polarity. The hybrid heart–oocyte channels are still voltage dependent but in a one-sided fashion. Both closure kinetics and voltage relationship of their steady-state junctional conductance, $g_{j\infty}$, are indistinguishable from those observed with pure oocyte channels. It seems likely, therefore, that the same gate that is found on both sides of pure oocyte channels is operative on only one side of the hybrid channels. In the hybrid channels, the gate is provided by the endogenous oocyte hemichannel, and it closes when this side

of the cell–cell channel faces the positive pole of the transjunctional voltage field.

The asymmetric response of the hybrid channels to transjunctional voltage results in rectification of transjunctional current. It is interesting to note that the first cell–cell channels discovered had rectifying properties (29). They were called rectifying electrical synapses and were found to have the typical appearance of gap junctions in the electron microscope (30). Based on the existing literature, they appear to be rare, but this does not exclude a wider distribution. Examples of rectifying channels have been found in the giant fiber synapse of the hatchet fish (31) and in various synapses of the leech (32).

Voltage clamp analysis of the crayfish electrical synapses (33) has shown that their voltage dependence is qualitatively similar to that exhibited by the heart–oocyte gap junctional hybrid channels. Therefore, the same building principles may apply. It should be noted, however, that despite the similarities, the gating structures of neuronal electrical synapses are likely to be different because of their different gating kinetics. Electrical rectifying synapses close orders of magnitude faster than the hybrid channels described here, and their residual conductance following voltage-dependent closure is substantially lower.

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