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Gap Junction Formation and Functional Interaction between Neonatal Rat Cardiocytes in Culture: A Correlative Physiological and Ultrastructural Study

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Summary. The time course of gap junction formation and growth, following contraction synchronization of cardiac myocytes in culture, has been studied in a combined (electro)physiological and ultrastructural study. In cultures of collagenase-dissociated neonatal rat cardiocytes, pairs of spontaneously beating myocytes synchronized their contractions within one beat interval within 2–20 min after they apparently had grown into contact. 45 sec after the first synchronized beat an appreciable junctional region containing several small gap junctions was already present. In the following 30 min, neither the area of individual gap junctions nor their total area increased. 75 min after synchronization both the area of individual gap junctions and their total area had increased by a factor of 10–15 with respect to what was found in the first half hour. In the period between 75 and 300 min again no further increase in gap junctional area was found. In double voltage-clamp experiments, gap junctions between well-coupled cells behaved like ohmic conductors. In poorly coupled cells, in which the number of functional gap-junctional channels was greatly reduced, the remaining channels showed voltage-dependent gating. Their single-channel conductance was 40–50 pS. The electrophysiologically measured junctional conductance agreed well with the conductance calculated from the morphometrically determined gap-junctional area. It is concluded that a rapid initial gap junction formation occurs during the 2–20 min period prior to synchronization by assembly of functional channels from existing channel precursors already present in the cell membranes. It then takes at least another 30 min before the gap-junctional area increases possibly by *de novo* synthesis or by recruitment from intracellular stores or from nonjunctional membranes, a process completed in the next 45 min.

Key Words beat-rate synchronization · morphometry · junctional conductance · voltage clamp · single-channel conductance

Introduction

Isolated myocytes from the heart show spontaneous activity as is demonstrated by their independent action potentials or contractions. Such cells may be regarded as pacemaker cells of their own. A well-known feature of cultured heart cells is that upon growing together they synchronize their previously independent contractions (De Haan & Hirakow, 1972, Jongsma, Masson-Pévet & Tjernina, 1987). Depending on plating density and culturing time, one can obtain either a population of synchronized cell pairs and small synchronous clusters or a confluent synchronously beating monolayer. From electrophysiological and morphological studies a considerable amount of evidence has been gathered showing that gap junctions mediate direct intercellular communication between cells—both electrically and chemically—in nearly every tissue, whether in vivo or in vitro (Loewenstein, 1981). In heart, gap junctions play a most important role since they are responsible for impulse propagation and thus for coordinated contractions.

Dissociated and cultured heart cells are a suitable model system to study pacemaking, conduction and the interrelation of the two (Veenstra & De Haan, 1986). Jongsma et al. (1987) published a study in which they determined the time course of beat-rate synchronization between pairs of neonatal rat heart cells in vitro, using a noninvasive opto-electronic technique to monitor the contractions of each cell. They found that in 50% of the pairs studied, the cells synchronized within one beating interval, while in the remaining cell pairs the first synchronized beats were preceded by a 4–65 sec period of partial synchronization. In all cases there was a delay of several msec between the synchronized contractions. In most experiments this delay remained at the value arrived at in the first synchronized beat, although in a few cases the delay decreased during a short period before reaching a constant value. It was concluded that physical coupling and therefore gap junction formation were complete before beating synchronization occurs. In a more recent electrophysiological study on gap junction channel formation between freshly isolated pairs of neonatal rat heart cells manipulated into contact, it appeared that

only 10 to 15 gap-junctional channels are sufficient for action potential propagation with a delay of 7–10 msec (Rook, Jongsma & Van Ginneken, 1988). From the results in both studies, however, it was not possible to determine whether, after an initial formation of a sufficient number of communicating channels to ensure beating synchrony of a cell pair, the gap-junctional area remained constant or increased any further.

In an attempt to answer this question about the kinetics of gap-junctional growth we investigated the relationship between gap junction size and the time course of synchronization in a combined physiological and ultrastructural study.

Materials and Methods

Experimental Set-Up

Cultures of neonatal rat heart cells were prepared as described previously (Masson-Pévet, Jongsma & DeBruijne, 1976; Jongsma et al., 1987) and plated in thin plastic culture dishes (Falcon 3006 Tissue Culture Film Liners; bottom ~0.15 mm thickness). The cultures were incubated in a pH and humidity controlled incubator at 37°C. The growth medium consisted of bicarbonate-buffered Ham's F10 (Flow laboratories) supplemented with 8–13% serum and 0.20–0.33% L-glutamine. The serum consisted of a 1 : 1 mixture of horse serum and fetal calf serum (Flow laboratories). No antibiotics or fungicides were added. The electrolyte composition of the growth medium was (in mM): NaCl 129.0, KCl 4.4, $CaCl_2$ 1.0, $MgSO_4$ 0.6, $NaHCO_3$ 14.0. The osmolarity of the medium, including serum, was 290 mOsm.

After 20–24 hr of culturing, the culture dishes were placed on the stage of an inverted microscope (Nikon Diaphot TMD) provided with an optically clear heating plate, held at a temperature of 36.5 ± 0.2°C. The cultures were observed with phase contrast optics at a magnification of 40 × 10. The preparation was superfused using a similar method as described previously (Jongsma et al., 1987). The superfusing salt solution had the same ionic composition as the growth medium except for $CaCl_2$, which was increased to 2.0 mM while 5.6 mM glucose was added as an additional substrate. During the superfusion, serum was added to the fluid to a final concentration of 6%. Two contracting cells were located which were so close to each other that growing together could be expected within the duration of an experiment. An opto-electronic set-up described by Jongsma et al. (1987) was used to monitor the contractions of each cell of a pair and to determine the exact moment of their synchronization. At different points of time after synchronization had occurred, the cells were instantaneously killed by pipetting glutaraldehyde fixative into the culture dish.

Electron Microscopy

Prior to fixation the superfusion was stopped and the heating current was switched off. 4 ml 4% glutaraldehyde in 0.15 M Na-cacodylate buffer, pH 7.4, was added to the content of the culture dish, resulting in a final aldehyde concentration of 1.5–2%. Care was taken not to displace the cell pair out of the viewing field of the microscope. During the fixation, which lasted 10–15 min, the position of the cell pair was marked on the bottom of the culture dish by carefully scratching a rectangular mark closely around the cells by means of a needle mounted on a micromanipulator. In addition, a deep and clearly visible mark was drawn by hand around the circumference of the illuminated area of the preparation. After completion of the fixation, the culture dish was removed from the microscope stage, the cells were rinsed with 0.15 M Na-cacodylate buffer and postfixed in 2% OsO_4 in water for 1 hr at room temperature. The culture was block stained in 2% magnesium-uranyl acetate in 50% ethanol. Dehydration was performed in graded ethanols, followed by infiltration with Araldite D. Under microscopic control a 500-μm paper diaphragm—presoaked in Araldite—was lowered over the cell pair, leaving an unobscured area about twice as large as the fine marks around the cells. The Araldite was cured for 24 hr at 60°C. Next, a capsule filled with Araldite was placed on top of the marked area, and this assembly was cured for another two days. The capsule, including the part of the culture dish underneath, was separated from the rest of the dish, and a pyramid was trimmed using the paper diaphragm and the fine markings as guide lines. Serial ultra-thin sections were cut on a Reichert Ultracut E microtome adjusted to a cutting thickness of 90 nm. Since cultured heart cells generally are very flattened, the sections were cut under an angle of 7° with respect to the bottom of the culturing dish. This slightly oblique plane of section through the cell pair and its substratum proved to facilitate the recognition of the various cell parts in each section. The sections were mounted on Formvar-coated slot grids (slot size: 2 × 0.6 mm) stained with Mg-Uranyl acetate and lead citrate, viewed in a Philips 420 electron microscope fitted with an eucentric tiltable preparation stage, allowing for the analysis of membrane areas having an oblique orientation to the plane of section. Thus we were able to analyze 50 to 70% of the generally tortuous junctional area between the cell pairs. The electron microscope was calibrated with a calibration grid with 2160 lines · mm^{-1}.

Morphometry

From electron micrographs the following morphometric data were derived: surface area and volume of each cell, surface area of the junctional zone between the cells and the area of the gap junctions found. Cell volume and surface area were obtained from low power micrographs (2500 ×) by measuring the cell perimeters in every tenth section—or more frequently if the cell shapes changed drastically within a few sections—using a digitizing tablet (Morphomat 10, Zeiss) connected to a PDP 11/73 computer. From the perimeter of the cell parts in the measured sections, the total number of sections and the section thickness, the computer calculated area and volume of each cell part in every section by interpolation and reconstructed the surface area and volume of each cell by adding up the measured and interpolated results of all sections.

The membrane area of the junctional zone was derived from micrographs of every section in which this zone could be found, at a final magnification of 15,000 ×. We defined the junctional zone as those regions in which the membranes of both cells were separated by an extracellular space of 50 nm or less. The total junctional area was calculated in a similar way as described above: the measured junctional length in each section was multiplied by the section thickness and the results were summed.

Gap junctions were analyzed from micrographs with a final

magnification of 80,000×. If the gap junctions were so small that they could only be observed in one section, we assumed them to have a disc-like shape (Masson-Pévet, 1979). Their area was calculated by taking their measured length as their diameter. Larger gap junctions, observed in several successive sections, were always oriented in such a way that a disc-like appearance was highly improbable. In those cases their area was calculated by adding up the area of their component part in each section. To obtain the real "height" of the gap junction components in tilted sections, corrections for the tilting angle α of the microscope preparation stage were made by dividing the section thickness by cos α.

Calculation of Junctional Conductance from Morphometric Data

From the literature (Manjunath & Page, 1985) and from freeze-fracture experiments on cultured cardiocytes carried out in our own laboratory (M. Masson-Pévet, *unpublished results*) it is known that the packing density of particles representing individual channels in rat cardiac gap junctions is about 12,000/μm^2 in fixed material. Thus, the number of communicating channels between cell pairs can be estimated from the morphometrically determined gap-junctional area. Recent studies have shown that the conductance of single rodent heart gap junction channels is about 50 pS (Burt & Spray, 1988; Rook et al., 1988; Rüdisüli & Weingart, 1989). The intercellular conductance was estimated by multiplying the total number of gap junction channels by their conductance.

Electrophysiology

In three experiments we tried to correlate the actual intercellular coupling between two cells with their gap-junctional area. To this end, the intercellular conductance at the end of a synchronization experiment as described above was electrophysiologically measured, prior to fixation for electron microscopy. Because single myocytes in culture are difficult to impale with conventional microelectrodes, we applied the whole-cell patch-clamp technique according to Hamill et al. (1981). Prior to the impalements the superfusion was stopped, the heating current was switched off and the growth medium in the culture dish was changed with serum-free HEPES-buffered Ham's F10 medium at room temperature (22°C). Suction microelectrodes were prepared and filled with a solution mimicking the intracellular fluid as described previously (Rook et al., 1988). This solution had the following composition (in mM): KCl 10; K-gluconate 130; Na_2ATP 10; $MgCl_2$ 5; HEPES 5; EGTA 10; $CaCl_2$ 1. The solution was brought to pH 7.1 with KOH and the combination $CaCl_2$/EGTA resulted in a free Ca^{2+} concentration of $\sim 5 \times 10^{-8}$ M. Two electrodes were each connected to the input stage of custom-built clamp amplifiers allowing both current or voltage-clamp recordings. The electrodes were lowered onto the cell surfaces and gigaohm seals (>10 GΩ) were formed by applying gentle suction. Next, access to the cell interiors was gained by disrupting the membrane under the patch by an extra pulse of suction. Access resistance from pipette interior to cell interior was estimated from the initial fast rising step in voltage displacements—recorded in current clamp mode—induced by application of 50-pA pulses to either cell. Access resistances ranged between 20–100 MΩ for each pipette. Action potentials of coupled cell pairs could be measured under current-clamp conditions. To determine the coupling resistance (R_j), both cells were voltage clamped near their resting potential, resulting in a net zero membrane current for each cell. Generally this was around −60 mV. Next, the holding potential of one of the two cells was changed stepwise to varying de- or hyperpolarized levels for different times, resulting in a constant voltage drop across the intercellular junction during the pulse. In principle, the current recorded in the cell held at a constant potential is a direct measure of the junctional current (I_j). Thus, the junctional resistance can easily be derived by dividing the applied transjunctional voltage difference by the measured I_j. In practice, however, one has to account for the access resistance of the electrodes to the cell interiors (R_{sA} and R_{sB}) which may cause an overestimation of the actual R_j, especially when cells are well coupled. To correct for the access resistances, we used an equation as derived previously (Rook et al., 1988).

$$R_j = \frac{I_A \cdot R_{sA} - I_B \cdot R_{sB} + (\Delta E_B - \Delta E_A)}{\Delta E_A / R_{mA} - I_A \cdot (1 + R_{sA}/R_{mA})}$$

in which I_A and I_B represent currents through electrodes A and B, R_{sA} and R_{sB} denote access resistances from electrodes to cells A and B, R_{mA} is membrane resistance of cell A and ΔE_A and ΔE_B denote the change in potential of electrodes A and B.

Results

We followed the process of beat-rate synchronization between pairs of spontaneously active cardiocytes and in 10 experiments we determined morphological data such as cell volume and area, the extent of the region of contact and of the gap-junctional area therein. The successive experiments cover a period of time from 45 sec to 5 hr after the first sychronized beat (Tables 1 to 3). It generally took 2–20 min before cells synchronized after they apparently had grown together. 45 sec after the first synchronized beat (expt. *1* in Table 1), a considerable junctional zone can already be present, as is illustrated by the electron micrograph in Fig. 1*A*. Although myocytes grown together in culture generally develop an appreciable zone of cell contact, a different type of cell contact may be encountered. In such a case the cells are interconnected by one or more thin threadlike protrusions. These threads sometimes are hardly visible under the light microscope and may even span a distance of several cell diameters. In Fig. 1*b* a low power electron micrograph of such a cell pair—fixed 12 min after synchronization (expt. *5* in Table 1)—is shown. Table 1 summarizes the morphometric results concerning synchronization time, surface area, cell volume and junctional area. There is no clear-cut relationship between synchronization time and junctional area, nor between cell surface area or cell volume and the latter.

The inserts in Fig. 1*A* and *B* give examples of gap junctions found between the respective cell pairs.

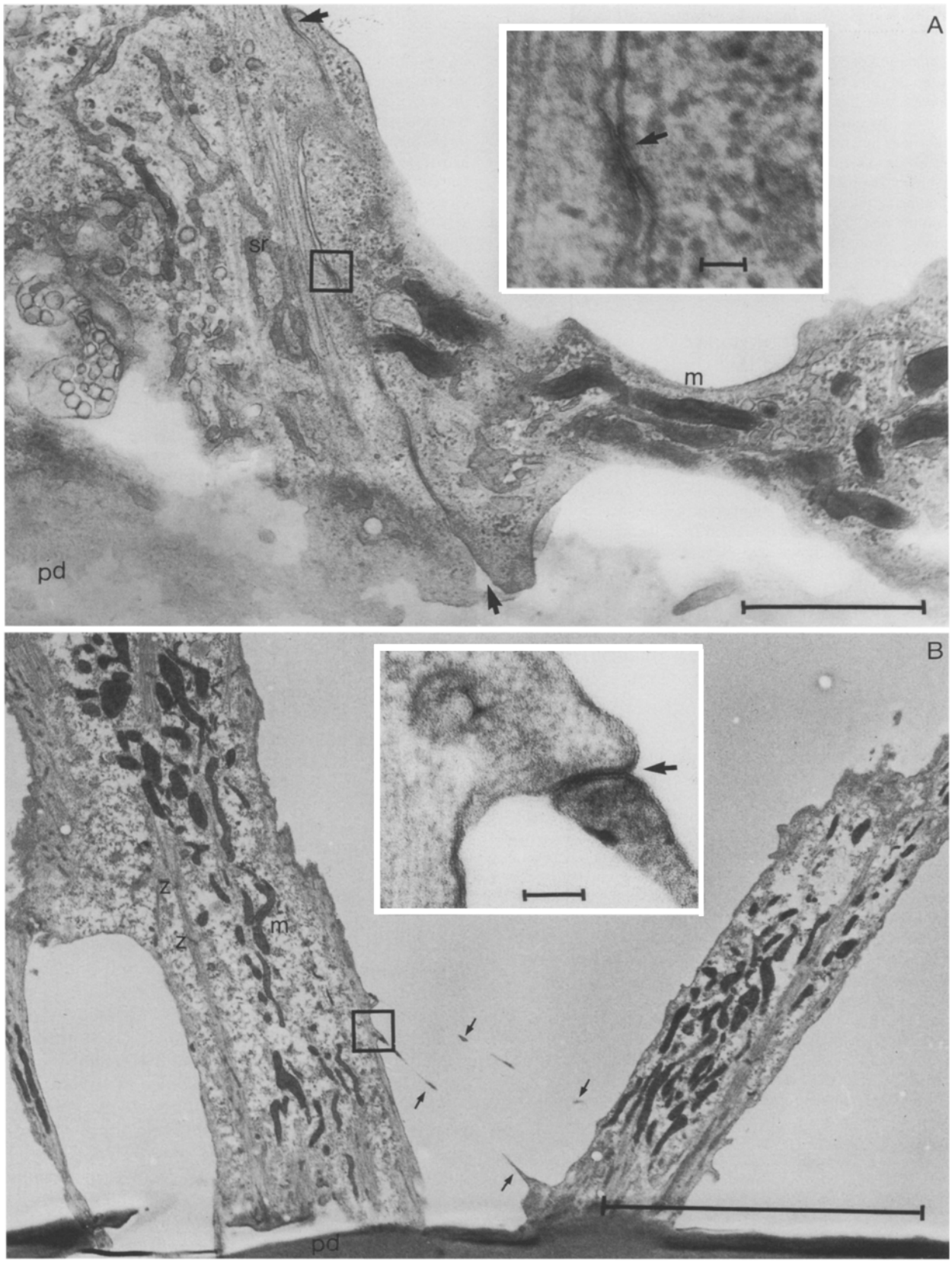

Fig. 1. General aspect of two types of cell connections and examples of gap junctions found between synchronized cells. (*A*) Low-power electron micrograph showing relatively large junctional region (arrows) between cell pair fixed only 45 sec after contraction synchronization. Scale bar: 1 μm. *Insert:* Detail of junctional zone between this cell pair (area indicated by rectangle) showing a small gap junction (arrow) with a diameter of 60 nm. Scale bar: 100 nm. (*B*) Low-power electron micrograph of cell pair fixed 12 min after synchronization. The cells are interconnected by two thread-like protrusions (arrows). Scale bar: 10 μm. *Insert:* Detail of cell contact. Thread arising from right cell makes contact with left cell via a well developed gap junction (arrow). Scale bar: 100 nm. *m*: mitochondria; *sr*: sarcoplasmic reticulum; *z*: z-lines in myofibrils: *pd*: bottom of petri dish

Table 1. Relationship between synchronization time, volume and surface area of each cell of a pair, and the junctional area between cell pairs[a]

Expt. no.	Synchronization time	Cell volume (μm^3)		Cell surface area (μm^2)		Area junctional zone (μm^2)
		Cell *A*	Cell *B*	Cell *A*	Cell *B*	
1	45 sec	1230	1080	358	343	2.3
2	1.5 min	1586	1253	511	422	1.8
3	3 min	1257	1152	408	342	3.2
4	6 min	2035	1041	597	412	0.6
5	12 min	2494	1597	754	539	0.4
6	30 min	1348	1026	415	343	9.8
7	75 min	1924	2162	557	520	0.8
8	120 min	1515	1292	434	417	10.4
9	210 min	1643	1422	538	412	74.0
10	300 min	1599	1637	483	478	>75.0

[a] Apart from the two largest junctional zones, which were found at longest synchronization times, there is no clear relationship between junctional zone area and the other parameters.

Insert in Fig. 1*A* is a detail of the junctional zone of the cell pair fixed 45 sec after synchronization. A small gap junction—one of the four found—with a diameter of 60 nm is discernible. The insert in Fig. 1*B* shows the junctional zone of the cell pair which had synchronized 12 min before fixation. This cell contact consisted of only two thin threads (arrows in Fig. 1*B*). Where these threads made contact with the neighboring cell, we found a gap junction only in one of the two contact regions (arrow in insert). Figure 2*A* to *E* shows details of the junctional zone of a cell pair fixed 210 min after synchronization (expt. *9*). In addition, the effect of tilting the preparation in the electron beam is demonstrated. In Fig. 2*A* the junctional membranes containing three regions of close membrane contact possibly containing a gap junction are shown (*1*, *2* and *3*). In the upper part of the figure the orientation of the cell membranes in the section is oblique and their course in the junctional region can only be guessed (between arrows). In Fig. 2*B* the preparation was tilted 20° clockwise with respect to the horizontal plane and the membrane areas in the upper part of the figure are now clearly visible. Moreover, in the regions *2* and *3* the presence of a gap junction seems evident. Figure 2*C* and *D* shows higher magnifications of these regions in which the angle of tilt was adjusted optimally to identify the gap junctions. Under these conditions, the junctional region in *1* is completely obscured (Fig. 2*B*). Tilting the preparation 20° counter-clockwise, however, clearly reveals that also in this region a gap junction is present (Fig. 2*E*).

In Table 2 the results concerning synchronization time, area of individual gap junctions found and the estimated total gap-junctional area actually present are summarized. From this table it can be concluded that during the first 30 min after synchronization the total gap-junctional area hardly increases, if at all. In expt. *4* only one small gap junction was found. Taken together, the mean area of the individual gap junctions in the first half hour after synchronization is 3200 ± 700 nm^2 (mean ± SE, n = 12, the large gap junction of expt. *5* excluded). If the relatively large gap junction of expt. *5* is included in the calculation of the mean individual gap junction area, the standard deviation turns out to be larger than the mean. Therefore, this gap junction is omitted in this particular calculation. The mean total gap-junctional area found in the first 30 min is 9300 ± 2000 nm^2 (mean ± SE, n = 6, including expt. *5* since the one gap junction making up the total gap-junctional area in this case is not anomalous when compared to total areas in the other experiments in this group).

At post-synchronization times of 75 min or more, both the area of individual gap junctions and the total gap-junctional area per time point is 10–15 times larger than found during the first 30 min. Here, the mean area of individual gap junctions found is 30,200 ± 5000 nm^2 (± SE, n = 19), while their mean total area is 159,500 ± 2100 nm^2 (± SE, n = 3, expt. *10* excluded, since morphometric data were not complete). In this period of time, ranging from 75 min to 5 hr after synchronization, again an increase in total gap-junctional area could not be detected. As mentioned in Materials and Methods, we generally were able to analyze 50–70% of the junctional membranes. It is very likely that, during the time periods studied, gap junctions are randomly distributed in the junctional region. For this reason we estimated the gap-junctional area actually present 1.4–2 times larger than the area which could be determined with certainty (Table 2). For the 12- and 300 min experiments (expt. *5* and expt. *10*) this reasoning did not

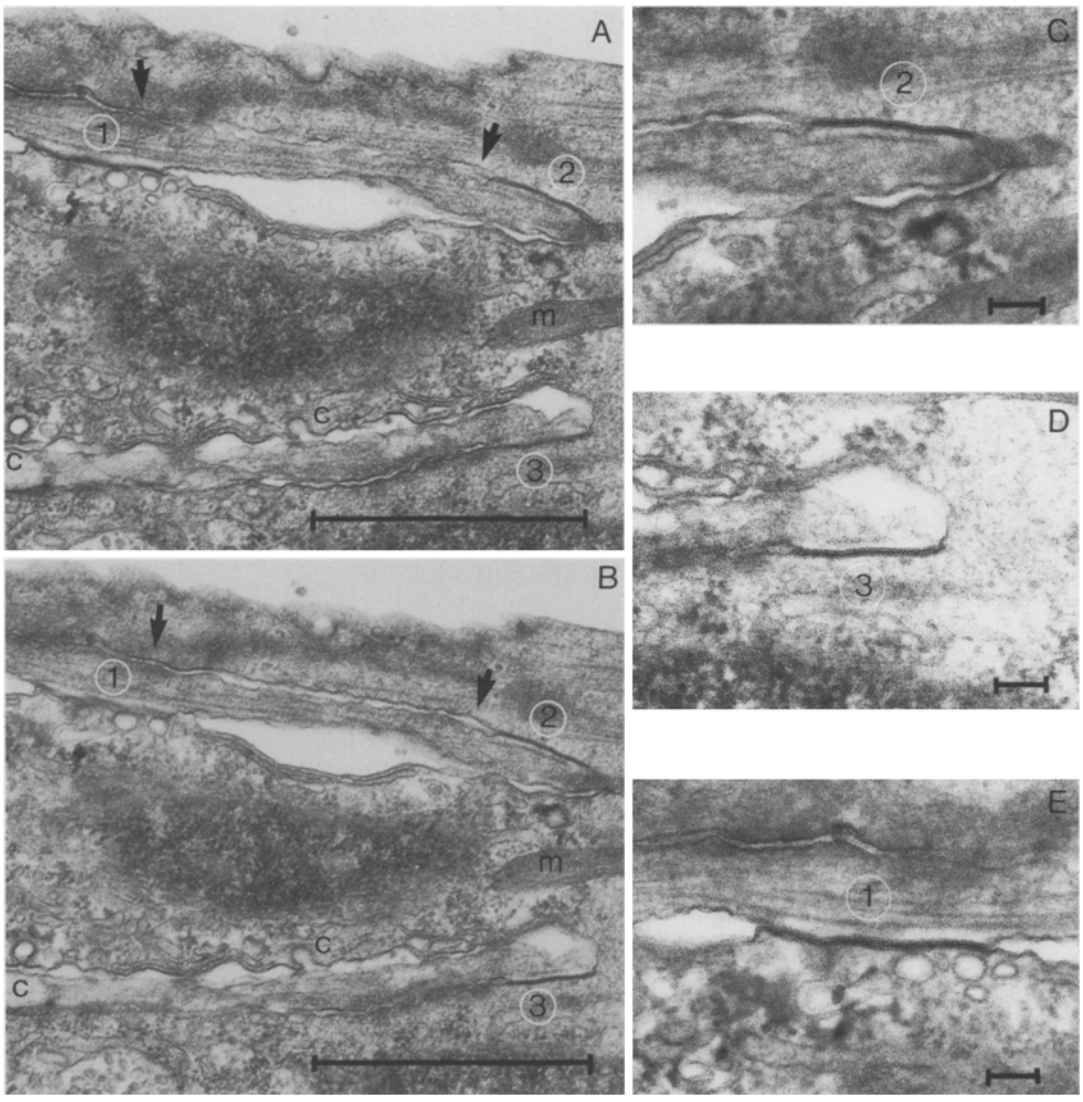

Fig. 2. Part of junctional region between cell pair fixed 210 min after synchronization. (*A*) Membranes in upper part of micrograph cannot be traced due to their oblique orientation in the section (between arrows). Three regions presumably containing a gap junction are indicated: *1*, *2* and *3*. (*B*) Same section as in *A* tilted 20° clockwise with respect to the electron beam, resulting in a good resolution in areas *2* and *3* and of junctional region between arrows. Areas *2* and *3* seem to contain a large gap junction. (*C* and *D*) Detail of regions *2* and *3* at optimal tilting angles (15° and 25°, respectively) showing a large gap junction in each region. (*E*) Detail of region *1* after tilting the section 20° counter-clockwise revealing another large gap junction. *m*: mitochondrion; *c*: caveolae; scale bars *A* and *B*: 1 μm, *C* to *E*: 100 nm

hold true, however: in the former case, because of the nature of the cell contact, we are quite certain that the gap junction found was the only one present, while in the latter case the junctional zone was too tortuous to be analyzed completely.

As described in Materials and Methods, one can estimate the intercellular conductance between cell pairs from the morphometrically determined number of gap junction channels and their single-channel conductance. In Table 3 the relationship between synchronization time, the estimated gap junctional area, the number of communicating channels therein and the calculated intercellular conductance, based on a single gap-junctional channel conductance of 50 pS, is given. In order to verify our assumptions leading to the estimation of intercellular conductance based on morphometric data, we tried to correlate the electrophysiologically measured junctional conductance with the morphometrically determined gap-junctional area in three experiments. Two experiments were performed in less than 30 min (expts. *11* and *12* in Table 4, and one at approximately 120

Table 2. Relationship between synchronization time, number and area of individual gap junctions and the total gap-junctional area found in each experiment[a]

Expt. no.	Synchronization time	Area of individual gap junctions found (10^3 nm^2)	Total gap junctional area found (10^3 nm^2)	Estimated gap junctional area actually present (10^3 nm^2)
1	45 sec	0.5 1.8 2.8 2.8	7.9	11.3–15.8
2	1.5 min	0.5 2.8 5.5	8.8	12.6–17.6
3	3 min	9.0	9.0	12.9–18.0
4	6 min	1.0	1.0	1.4–2.0
5	12 min	17.7	17.7	17.7
6	30 min	1.8 4.0 5.5	11.3	16.1–22.6
7	75 min	14.3 51.9 80.0	146.2	208.9–292.4
8	120 min*	5.5 5.6 5.6 11.7 14.7 16.5 34.6 37.7 54.2	180.5	257.9–361.0
9	210 min	43.7 57.2 59.8	160.7	229.6–321.4
10	300 min	12.4 15.6 78.4	106.4	>152

[a] Last column: estimated gap-junctional area actually present in each experiment. As explained in Materials and Methods, 50–70% of the junctional zone could be analyzed with respect to presence and area of gap junctions therein. Assuming random distribution of gap junctions in junctional zone, their total area was estimated to be 2–1.4 times larger than found.

* In expt. *8* (120 min) the addition of serum to the bath was stopped 10 min after synchronization to determine whether serum depletion had an effect on gap-junctional growth (*see* Discussion).

min (expt. *13* in Table 4) after contraction synchronization had occurred. In Fig. 3*A* synchronous action potentials recorded in current clamp from both cells of the pair in expt. *12*, impaled within 30 min after synchronization, are shown. There was no measurable delay between these action potentials (*not shown*). The rate of firing was rather variable, a common observation is isolated heart cells (Jongsma et al., 1987). After switching to voltage-clamp conditions, the voltage in either cell of the pair was changed stepwise with amplitudes varying between −50 mV (hyperpolarization) to +100 mV (depolarization) from holding potential while the junctional current was monitored in the other cell—held at a constant potential. In Fig. 3*B* a record of the junctional current resulting from a transjunctional voltage step of 100 mV for 1 sec is depicted. In Fig. 3*C* the amplitude of the junctional current is plotted as function of the applied transjunctional voltage. The linear relationship between current and voltage demonstrates the ohmic behavior of the gap-junctional conductance. A small deviation from this linearity can be observed where a ΔV_j of 40–50 mV was applied by hyperpolarizing either cell (ΔV_j applied in cell *A*: triangles, in cell *B*: squares; *see* legend Fig. 3*C* for further explanation). This is caused by a large drop in membrane resistance of myocardial cells upon strong hyperpolarization due to the activation of the large inward rectifying current I_{K1} (Clay & Shrier, 1981; Payet, Rousseau & Sauvé, 1985). This, in combination with the series resistance of the recording pipettes, attenuates the voltage control—and thus ΔV_j—in the cell which had been hyperpolarized, resulting in a smaller I_j than expected. After correction for the access resistances of the recording pipettes, we calculated the junctional conductance to be 8 nS. Morphometric analysis of the same cell pair resulted in a total gap-junctional area of 9700 nm^2. Taking in account that, like in most of the experiments described before, only 50–70% of the contact area could be analyzed completely, we estimate that the actual gap-junctional area was 13,860–19,400 nm^2, containing 166–233 junctional channels. For this cell pair, the junctional conductance calculated from the morphometric data is 8.3–11.7 nS, being well in the range of the electrophysiologically measured conductance.

The junctional conductance of the other cell pair impaled within 30 min after synchronization (expt. *11*, Table 4), appeared to drop from about 1 nS to a few pS, as measured under voltage-clamp conditions. Since in this case the junctional conductance became extremely low, we were able to record gating events of single gap-junctional channels. An example is shown in Fig. 4*B* in which gating of single gap junction channels appears as discrete transitions in the junctional current (I_A), recorded in the cell held at constant potential, during the application of a ΔV_j of 100 mV. The current recorded in the cell in which the potential was changed (I_B) is the sum of

Table 3. Synchronization time *vs.* estimated total gap-junctional area, estimated number of channels therein assuming a density of ~12,000 channels/μm^2 and the junctional conductance made up by this number of channels based on a single channel conductance of 50 pS.

Expt. no.	Synchronization time	Estimated gap-junctional area actually present (10^3 nm^2)	Estimated number of communicating channels	Estimated junctional conductance (nS)
1	45 sec	11.3–15.8	135–190	6.8–9.5
2	1.5 min	12.6–17.6	151–211	7.6–10.5
3	3 min	12.9–18.0	155–216	7.8–10.8
4	6 min	1.4–2.0	17–24	0.9–1.2
5	12 min	17.7	212	10.6
6	30 min	16.1–22.6	193–271	9.7–14.0
7	75 min	208.9–292.4	2507–3509	125.4–175.5
8	120 min	257.9–361.0	3095–4332	154.8–216.6
9	210 min	229.6–312.4	2755–3875	137.8–192.9
10	300 min	>152	>1824	>91.2

Table 4. Comparison of electrophysiologically measured gap-junctional conductance with morphometrically estimated gap junction area and conductance[a]

Expt. no.	Synchronization time	Area of individual gap junctions found (10^3 nm^2)	Estimated gap junctional area actually present (10^3 nm^2)	Estimated junctional conductance (nS)	Measured junctional conductance (nS)
11	<30 min	4.3	>4.3	ND	1 nS and less
12	<30 min	0.7 2.0 2.0 5.0	13.9–19.4	8.3–11.7	8
13	~120 min	1.8 1.8 2.8 3.8 26.3	52.0–73.0	31.0–44.0	42

[a] Note the rather good correlation between measured and estimated values in expts. *12* and *13*: apparently most of the gap junction channels are in the conducting state.

the resulting membrane current and the junctional current flowing into the other cell. Consequently the junctional gating events simultaneously appear in I_B as transitions of equal amplitude though of opposite sign. The current changes indicated a single-channel conductance of 40–50 pS. At values of ΔV_j larger than 50 mV the channels showed voltage-dependent gating as is illustrated in Fig. 4*A* in which the amplitude of I_j is plotted against ΔV_j at the beginning and at the end of 1-sec transcellular voltage steps of various amplitude. At values below 50 mV the relationship between ΔV_j and I_j has a linear appearance and—apart from small variations due to gating of the gap junction channels—I_j remains constant during the voltage steps. At values larger than 50 mV the I_j at the end of each voltage step is smaller than at the beginning of the step. Under these conditions the channels tend to close rather than to open. In this case we were not able to perform a complete morphometric analysis of the cell pair. Nevertheless, a small gap junction with an area of 4300 nm^2, the equivalent of 50 channels, was found.

The measured conductance between the cell pair impaled 2 hr after synchronization (Table 4, expt. *13*) was 42 nS. The morphologically determined gap-junctional area was 52,000–73,200 nm^2, which should account for 624–878 channels resulting in a junctional conductance of 31–44 nS. Compared with the morphometric data for cell pairs synchronized for this period of time, this value is rather low (*see* Table 3).

Discussion

Development of Regions of Contact and Synchronization between Cell Pairs

In this study we followed the synchronization process between pairs of spontaneously beating cardiocytes growing together in culture. At different points of time after the first synchronized beat, cell pairs were analyzed morphometrically. The plasma membranes of neonatal rat heart cells in culture show a high degree of motility: lamellopod and filo-

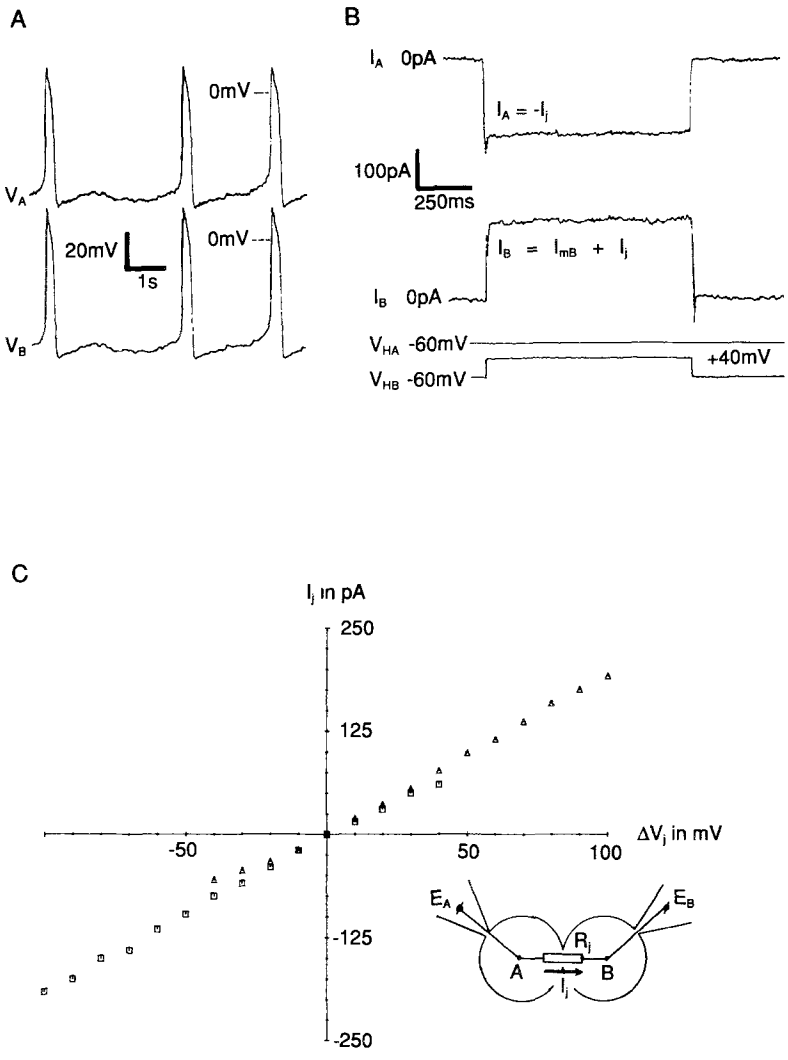

Fig. 3. (*A*) Action potentials of two spontaneously active rat heart myocytes recorded in current clamp about 30 min after they had synchronized their contractions. The cells showed a diastolic depolarization together with a rather noisy membrane potential; consequently, their rate of firing was irregular. (*B*) Same cell pair under double voltage-clamp conditions. Cell *B* was stepped 100 mV from holding potential for 1 sec; I_A: equivalent of the junctional current (measured as $-I_j$) due to the applied transcellular voltage difference, recorded in cell *A* held at a constant potential. I_B: current recorded in depolarized cell is sum of membrane currents (I_{mB}) and I_j. Note the absence of voltage dependency in the junctional current. (*C*) The junctional current (I_j) as function of the applied transcellular voltage (ΔV_j). The graph is a combination of two recordings: first, cell *A* was stepped (ΔE_A) −50, −40, --- +100 mV from holding potential (−60 mV) while I_j was measured in cell *B* (triangles). Next, the same protocol (ΔE_B) was applied to cell *B* while I_j was measured in cell *A* (squares). In each case the transcellular voltage difference applied may be expressed as: $\Delta V_j = \Delta E_A - \Delta E_B$. Thus, when cell *A* was stepped +100 mV from holding potential, ΔV_j was +100 mV, which resulted in a I_j of +190 pA flowing via the gap junctions (R_j) from *A* into *B* (recorded as −190 pA in cell *B*). When cell *B* was stepped 100 mV, ΔV_j was −100 mV with respect to the gap junctions and a I_j of −190 pA flowed from *B* to *A* (recorded as −190 pA in cell *A*; *see* recording depicted in *B*). The $\Delta V_j/I_j$-relationship clearly demonstrates the ohmic behavior of the gap junctions between this cell pair

pod structures are rapidly formed and retracted. Once one or more of such membrane processes from one cell "touches" the membrane of another cell, these regions of cell contact frequently remain intact and eventually may grow considerably in size. From the first moment that two cells of a pair have come into contact—as far as can be observed at the light-microscopical level—it takes 2—20 min before the cells synchronize their contractions. Part of the observed variability may be of biological origin; part of it arises from the difficulty in determining when cells actually are in contact while observing them with a light microscope. It appeared that the area of the region of contact between two cells growing together in culture was not strictly dependent on the time elapsed after their synchronization (Table 1), as demonstrated by the size of these regions at 6 and 75 min (expt. *4* and *7*) and particularly at 12 min (expt. *5*), in which the cells were only connected by two thread-like protrusions. Neither was there any relationship between cell volume or cell surface area and junctional area. Apparently the degree of coupling is only dependent on the number and size of the gap junctions.

Identification of Gap Junctions

In conventional ultra-thin sections gap junctions can be recognized as regions where the cell membranes come into close apposition, leaving only a small 2–4 nm intercellular "gap." An important condition in identifying these structures is that both membranes, and preferably the gap in between, can be observed. In principle, this condition is only met when the membranes are orientated perpendicular to the plane of observation. Therefore, identification of gap junctions which are obliquely orientated in the sections

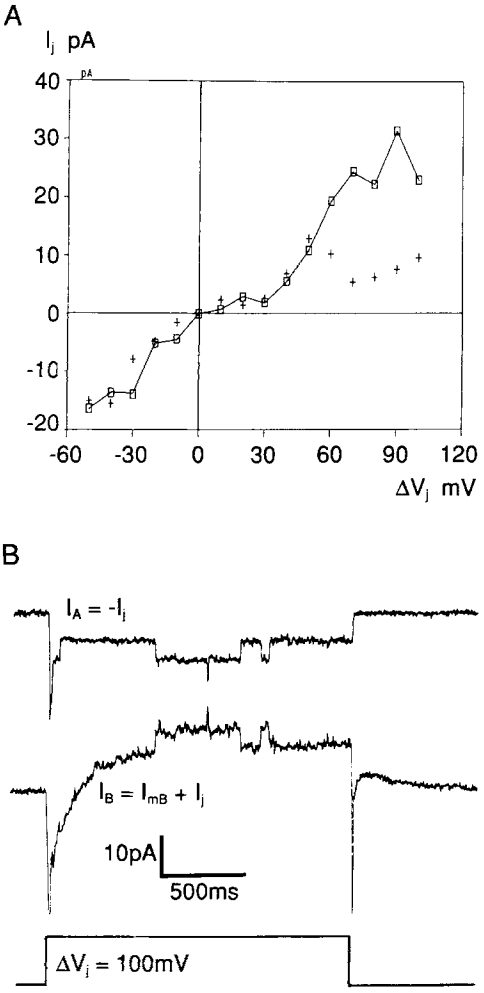

Fig. 4. (*A*) $\Delta V_j/I_j$ relationship of a poorly coupled cell pair, demonstrating voltage sensitivity of gap junction channels. □: amplitude of I_j recorded at the beginning, and +: at the end (quasi-steady state) of 1-sec ΔV_j voltage steps. Voltage sensitivity is prominent at values of $\Delta V_j > 50$ mV (*see* text for further explanation). (*B*) Double voltage-clamp recording from the poorly coupled cell pair demonstrating gating of single gap junction channels. Holding potential (bottom trace) of cell *B* was changed from −60 to +40 mV for 2 sec while the potential in cell *A* was kept constant (*not shown*), resulting in a ΔV_j of 100 mV. Current recorded in cell *A* is equivalent of junctional current ($-I_j$); current recorded in cell *B* is sum of membrane current (I_{mB}) caused by the voltage step and junctional current I_j flowing into cell *A*. Current transitions resulting from gating of gap-junctional channels occur simultaneously in both current recordings and are of equal amplitude and of opposite sign. The amplitude of current changes indicates a single-channel conductance of 40–50 pS

is hampered. The eucentric tiltable specimen stage used in the electron microscope permitted both clockwise and counter-clockwise rotations up to 45° out of the horizontal plane. This means that in a sector of 90° all junctional membrane areas could be orientated optimally with respect to the electron beam. Assuming that the junctional membrane areas were orientated randomly in the sections (i.e., in a sector of 180°), this means that at least 50% of the junctional regions could be analyzed and the gap junctions therein resolved. In practice, we frequently could resolve gap junctions with a slightly oblique orientation (up to about 15°) in the sections, thus the upper limit of resolution should be about 70%.

Calculation of Gap-Junctional Area

As described in Materials and Methods, we used two methods to calculate the area of individual gap junctions, depending on the appearance of their profiles in the sections. If the profile length was less than 100 nm and in each adjacent section the same gap junction was not present, we assumed a plaque with a disc-like appearance having a diameter equal to the profile length. The area of all gap junctions found in the first 30 min after synchronization could be calculated in this way. These results are independent of section thickness. The possibility that the plaques are not completely macular is discussed below. Some of the gap junctions found after longer synchronization times were considerably larger in diameter than 100 nm and/or appeared in more than one section. In these cases we used the section thickness as a measure for the "height" of the components in each section. In these cases the validity of the calculation of gap junction areas from the length of individual gap junction profiles as they appeared in successive sections is dependent on section thickness. During sectioning, care was taken to obtain ribbons of serial sections with a constant silvery/gold appearance, indicating that the pre-set 90-nm section thickness on the microtome indeed resulted in reproducible sections. In the microscope section thickness was once more determined according to a method proposed by Small (1968) by measuring the minimal width of folds which were present in some sections. The smallest folds should closely approximate twice the section thickness. Section thickness measured this way varied between 80 and 110 nm (94.1 ± 7.4, mean ± SD, $n = 7$).

On photo micrographs having a final magnification of 80,000×, the smallest areas of close membrane apposition had a diameter of about 1.6 mm, i.e., a true diameter of 20 nm. Together with some longer gap-junctional profiles, which could easily be identified as such, these were the only membrane specializations that could be found in the experiments during the first 30 min. Therefore, these focal contacts were interpreted as very small gap junctions. It is obvious that with the TEM techniques we

used, one cannot be sure whether such contacts actually contain the number of gap-junctional particles which theoretically could be present. In freeze-cleave studies on gap junctions in developing mammalian embryonic heart, it has been reported that gap junction formation started with the formation of small linear arrays of 9-nm particles (Gros et al., 1978) or that macular gap junctions showed particle-free areas (Shibata, Nakata & Page, 1980). This implies that our assumption that small gap junctions in our preparation are of macular shape has to be considered with some caution. It could mean that the gap-junctional area at the shorter synchronization times is overestimated and that a slight increase during the first 30 min could not be detected. On the other hand, the electrophysiological measurements of the junctional conductance between cell pairs agreed rather well with the calculated junctional area and the number of channels therein between the very same cells (Table 4, expts. *12* and *13*). We therefore conclude that our estimations on gap-junctional area are not too far off.

Electrical Properties of Gap Junctions

We demonstrate that the single gap junction channel conductance between neonatal rat heart cells in culture is 40–50 pS (Fig. 4*B*). This value is in agreement with recent reports on the single gap junction channel conductance in neonatal rat heart, both in freshly isolated cells (Rook et al., 1988) and in cultured cells (Burt & Spray, 1988), as well as in isolated cells from adult guinea-pig heart (Rüdisüli & Weingart, 1989). In well-coupled cell pairs the gap junctions showed no voltage dependency (Fig. 3), as is commonly found in heart tissue, including rat heart (Weingart, 1986). However, when the number of functional channels was low, such as in the poorly coupled cell pair (Table 4, expt. *11*), marked voltage-dependent gating of the channels was present (Fig. 4*A*), an observation in agreement with recent findings on gap junction channel gating properties in rat heart cell pairs in the process of coupling (Rook et al., 1988), in embryonic chick heart cells (Veenstra, 1990) and in rat lacrimal cell pairs in the process of uncoupling (Neyton & Trautmann, 1985).

We have previously demonstrated that when only a few channels are present, the probability of the channels to be open (P_o) at a ΔV_j of 100 mV can be estimated to be about 0.25. However, at low values of ΔV_j, P_o is much higher (possibly 0.85 at a ΔV_j of 50 mV). In the same publication (Rook et al., 1988) we hypothesize—based on some experimental proof—that the cytosols act as an access resistance to the channel pores (Hille, 1984): the current density near the channel mouth results in a cytosolic voltage drop. When the number of gap junction channels is low, only a small junctional current is flowing; thus the cytosolic voltage drop is also small. In the case of large gap junctions, the current density near the gap junction channels is high, resulting in a large cytosolic voltage drop. Consequently, these channels sense a considerably smaller transjunctional voltage drop than applied between the electrodes. As a result their P_o is large, even at large intercellular voltage differences. This means that large gap junctions show no voltage sensitivity (*see*, e.g., Fig. 3) and, additionally, that on the average most channels will be in the open state. This also explains why the morphometrically calculated number of gap junction channels correlates well with the electrical measurements. To test this hypothesis we constructed a simple mathematical model based on the calculation of the electrical field at any point in a system of two cells connected by idealized gap junction particles arranged in a hexagonal array. The resulting partial differential equation of Laplace was solved using a finite difference representation of this equation in polar coordinates. The results show that a junction consisting of one channel senses 98% of the applied transjunctional voltage. With increasing numbers of channels this figure decreases and a junction consisting of 100 channels only senses 60% of the applied voltage (Jongsma et al., 1990).

Gap-Junctional Formation and Growth

In a study on beat-rate synchronization of neonatal rat heart cell pairs in culture (Jongsma et al., 1987), it appeared that synchronization occurred within one beat interval or sometimes after a very short period of partial synchronization. It was concluded that gap junction formation sufficient for cell coupling should be completed in the 2–20 min period prior to synchronization in which the cells apparently had grown into contact. In the present study this notion was confirmed, since 45 sec after synchronization a number of small gap junctions was already found. Moreover, in an electrophysiological study on gap junction channel formation between freshly isolated neonatal rat heart cells manipulated into contact, it was found that the formation of only 15 channels and possibly even less, was quite sufficient to ensure action potential propagation, be it with a delay of ~10 msec (Rook et al., 1988). Like freshly isolated cardiocytes, neonatal rat heart cells in culture have a relatively small membrane area and consequently have a high membrane resistance: typically 2–5 GΩ at resting potential. The degree of coupling between cells is not only dependent on junctional resistance

but also on their membrane resistance. Current injected in one cell of a pair may flow through both the junctional and membrane pathways (Watanabe & Grundfest, 1961; Bennett, 1966; Weingart, 1986). Thus, the higher the membrane resistance of the current-injected cell, the more current may pass through the gap junctions to the adjacent cell. In addition, the higher the membrane resistance of the latter cell, the larger the voltage deflection caused by the junctional current will be. These features explain why cultured cell pairs growing together usually synchronize their contractions within one beat interval. It also explains the absence of a measureable delay between action potentials in the cell pair in expt. *12* (Fig. 3, Table 4), despite the fact that the gap-junctional area still was rather small (14,000–19,000 nm^2, i.e., 160–230 channels).

In addition, we show that the process of gap junction formation between pairs of cultured neonatal rat cardiocytes occurs in two distinct steps: first, a rapid formation of relatively small gap junctions (nevertheless quite sufficient for action potential propagation) just after the cells have grown into contact, followed by a 10 to 15-fold increase in size and total area of the gap junctions between 30 and 75 min after synchronization (Tables 2 and 3).

In previous reports it has already been shown that gap junction channel formation can be a rapid process, taking only a few minutes or even seconds while it occurs between apparently nonspecific membrane regions. It has therefore been proposed that a pool of precursors of gap junction hemichannels or of complete connexons is present in the membranes of individual cells (Loewenstein et al., 1981). This notion is corroborated by the evidence that gap junction formation even occurs in the presence of metabolic inhibitors and inhibitors of protein synthesis (Tadvalkar & Pinto da Silva, 1983). Although the mechanism of channel formation and of assembly into gap junctional plaques is unknown, several models have been put forward. According to a model proposed by Loewenstein (1981), these channel precursors can move randomly in the fluid cell membrane matrix. The moieties in one membrane could interlock with their counterparts in an adjacent cell membrane by short-range attractive forces—for instance, by van der Waals forces—at their extracellular ends, thus overcoming the electro-repulsive forces between the cell membranes. This type of channel-assembly is only possible when adjacent cell membranes come into close apposition of about 2 nm, a condition which is met in the so called "formation plaques" (Johnson et al., 1974). The size of gap junctions formed by this mechanism would be limited by the number of proto-channels present and the size of the formation plaques in the junctional membrane area. The diffusion away from the gap-junctional area is prevented by the bending forces of the cell membranes at the edge of the formation plaque. Abney, Braun and Owicki (1987) proposed a model derived from statistical mechanics of lateral interactions among membrane proteins, in which, apart from the cell membranes, also the channel-forming structures were repulsive at their intercellular faces. In this model gap-junctional channels are kept together despite repulsive forces between each hemichannel in the junctional plaque by compressing forces which arise from the extra-junctional glycocalyx, which has been excluded from the formation plaque during its formation.

The likely explanation of the time course of gap-junctional development in our preparation is as follows: as the cells grow into close contact, which takes place in the 2–20 min period prior to synchronization, the stock of channel forming structures is responsible for the rapid formation of functional communicating channels as soon as adjacent cell membranes are near enough to serve as formation plaques (Johnson et al., 1974; Gros et al., 1978). During this process probably most of the channel forming moieties available in the developing junctional region are involved, since in the next 30 min after the first synchronized beats no appreciable increase in total gap junctional area seems to occur. The rate of channel formation and the number of channels achieved, is limited by the availability of precursors in the junctional membrane of either cell. Obviously there is a difference in potency to form gap junction channels between cells, since the gap junction area found at several time points after synchronization can be variable, which is especially clear in expt. *4* (Tables 2 and 3) in which only 17–24 gap junction channels had been formed. In their study on beat-rate synchronization between cultured heart cells, Jongsma et al. (1987) reported that occasionally cell pairs uncoupled again after some period of synchronization. It is likely that in these cases the number of gap junction channels formed was so low that accidental closure or removal of only a few channels resulted in an instantaneous desynchronization. A variable and limited availability of channel precursors could also explain why there is no clear-cut relationship between the junctional area between cell pairs and the gap-junctional area therein, as is clear when Tables 1 and 2 are compared.

After the initial process of channel formation described above, it takes at least 30 min before the gap junctions increase in size, since a notable increased gap-junctional area could only be found after periods of synchronization of 75 min or more. Probably the existing small gap junctions serve as focal

points for further accretion of channels into larger gap-junctional plaques, since the number of gap junctions found per experiment does not increase in time (Table 2), with exception of expt. *8* in which the effect of serum deprivation on gap-junctional growth was studied (*see below*). Apparently the increase of gap-junctional area in this second phase of formation also varies between one cell pair and another, as is demonstrated by the 120-min electrophysiological experiment (expt. *13*, Table 4). In this case, gap-junctional area and intercellular conductance are rather low when compared with the morphometric data for the other cell pairs synchronized for the longer periods of time.

A possible explanation of the large increase in gap-junctional area between 30 and 75 min after synchronization is *de novo* synthesis of gap-junctional protein, followed by insertion of new channels in the junctional membrane. However, it has been shown that cytoskeleton disruptors induce the assembly of gap junctions in epithelial cells (Tadvalkar & Pinto da Silva, 1983), which may point to a cytoskeletal control of the distribution of channel precursors in the cell membrane. Thus the possibility of recruitment of connexons from nonjunctional membranes instead of, or in addition to, *de novo* synthesis cannot be ruled out. When cultures of neonatal rat cardiocytes are incubated with a site-directed antibody against connexin 43—a cardiac gap-junctional protein (Beyer et al., 1989, El Aoumari et al., 1990)—numerous cells do not only show immunofluorescent labeling of the junctional regions but also in the cytoplasm and in perinuclear regions which might represent Golgi structures (M.B. Rook, B. de Jonge, D. Gros and H.J. Jongsma, *unpublished*). Although as yet little is known about the pathways of junctional proteins from the site of transcription to the plasma membrane, it may be possible that there are intracellular pools of gap junction channel precursors. Recruitment of channels from such stores can also be involved in the second phase of gap-junctional growth.

Addition of serum to the medium is essential for proliferation and synchronization of cardiac cells in culture. To investigate whether serum depletion impairs the second phase of gap-junctional growth encountered after 30 min of synchronization, we stopped the addition of serum to the superfusion fluid 10 min after synchronization had occurred in expt. *8* (indicated with an asterisk in Table 2). Assuming that the initial serum concentration of 6% declines exponentially, one can calculate the serum concentration to be only 0.1% after another 20 min, while still declining. Two hours after synchronization had occurred, the cell pair was prepared for electron microscopy as described before. Surprisingly, the total gap-junctional area found in this experiment fitted nicely in the range of areas found at the synchronization times larger than 30 min. An unusual large number of 9 gap junctions was found, however, of which three were as large as generally found at these time points while the other six were more or less comparable in size with gap junctions found during the first 30 min. It has previously been shown that a rise in intracellular cAMP concentration, which can be brought about by extracellular administration of cAMP, by phosphodiesterase inhibitors, hormones and, interestingly, also by serum deprivation, has a stimulatory effect on gap junction formation (Flagg-Newton, Dahl & Loewenstein, 1981; Radu, Dahl & Loewenstein, 1982). As determined by freeze-fracture electron microscopy, a cAMP-induced gap junctional growth is associated with an increase in frequency of gap-junctional clusters, particularly of smaller and presumably newer clusters (Flagg-Newton et al., 1981). We speculate, although this experiment has not been repeated, that in expt. *8* serum depletion caused a similar effect.

The question remains whether the gap-junctional area would increase any further over periods longer than investigated in this study. If a junctional region of ~75 μm^2 (Table 1, expts. *9* and *10*), containing a 0.23–0.32 μm^2 gap-junctional area (Tables 2 or 3, expt. *7* to *9*) is arbitrarily taken as a value for synchronized cell pairs which have grown together to a maximal extent, the gap junctions represent 0.3–0.4% of the total junctional membrane area. This value agrees well with the ratio of gap junction area to plasma membrane area in 18 dpc mouse embryonic hearts while it is only about half the value found in adult mouse heart (Gros et al., 1979). Moreover, the morphometric data indicate that the junctional resistance can be as low as 7–5 MΩ (in terms of conductance, 140–200 nS, Table 3, expts. *7–9*). These values approach the junctional resistances of about 1.7 MΩ found between cell pairs isolated from adult rat heart (Weingart, 1986). It therefore seems likely that after the second phase gap junction formation between cell pairs is completed or nearly completed. Parameters like size and number of gap junctions could change, however, as the cultures grow into confluent synchronized monolayers and each individual cell has to be coupled to numerous others.

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