Cell-to-cell coupling studied by diffusional methods in myocardial cells

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Summary. The diffusion of large molecular substances from cell to cell in multicellular and enzymatically isolated cell pairs is described. Permeability of the gap junctional membrane to these molecules and the critical diffusing diameter of the myocardial gap junctional channel are discussed.

Key words. Cell-to-cell coupling; diffusion of large molecules; gap junctional permeability; paired cardiac myocytes; gap junctional channel.

Introduction

Cardiac muscle cells are connected to each other at the intercalated disks[29], sites in part occupied by specialized structures called gap junctions[27]. Since the proposal by Weidmann[34] and Barr et al.[2] that the intercalated disks possess a low electrical resistance, numerous electrophysiological studies on cell-to-cell communication have been performed[6,8,13,14,19,30,31,36,39,40]. It has now been generally established that the gap junctions are the site of electrical coupling between adjacent cells[24]. Moreover, pores have been morphologically recognized in the gap junctional membrane by electron microscopic studies[17,18] and they are supposed to be responsible for the cytoplasmic continuity.

Another approach to the problem is the use of indicator probes to study the kinetics of cell-to-cell diffusion. Weidmann[35], in 1966 first showed that potassium ions diffuse through the gap junctions in both calf and sheep ventricular muscle. These experiments supported the idea that the gap junctions represent the low resistance pathway for electrical current.

Cell-to-cell diffusion: Experiments on multicellular preparations

In 1970, when I was working in Prof. Weidmann's laboratory, I extended the diffusion experiments by studying the movement of a larger molecule, the fluorescent dye Procion Yellow (mol. wt 697), in sheep and calf Purkinje fibers using the 'cut-end method'. In this method two Perspex chambers are separated by a dental rubber membrane through which a Purkinje strand was passed (see fig. 1, inset). One end of the strand was cut with fine scissors while it was being perfused with an isotonic sucrose solution containing 12.3 mM NaCl and 20 mM Procion Yellow, but no added Ca^{2+}. A time of 15 min was allowed for the dye to enter the cells and then the cut end was made to heal over by switching to Ca^{2+}-containing Tyrode solution. After several hours the fiber was removed from the chamber, frozen, cut into transverse sections of 60-µm thickness, and the distribution of the dye measured using an UV-microscope.

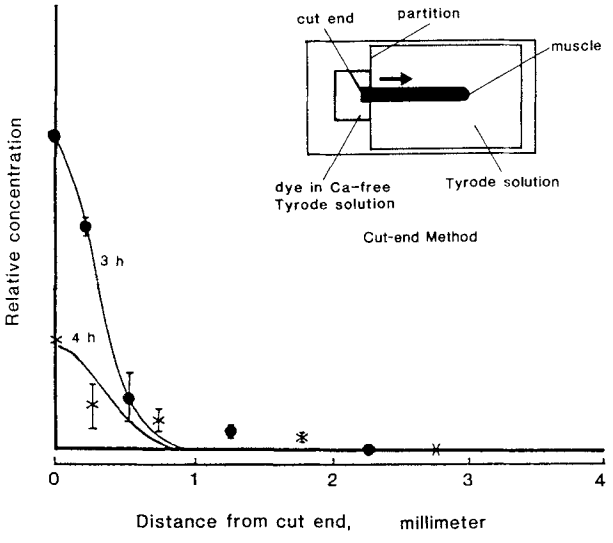

Figure 1. The inset shows a diagram of the 'cut-end method'. In experiments on sheep Purkinje fibers, the relative concentration of Procion Yellow against distance from the cut end was plotted at time intervals of 3 (●) and 4 (×) h. Solid lines represent the theoretical curves calculated from the equation

$$C = \frac{1}{2} C_o \left\{ \mathrm{erf} \frac{h - x}{2\sqrt{Dt}} + \mathrm{erf} \frac{h + x}{2\sqrt{Dt}} \right\} e^{-kt}$$

Vertical bars indicate ±SD. (from Imanaga[11]).

Figure 1 illustrates a plot of the relative concentration of the dye against distance from the cut end. The concentration of the dye decreased as the dye diffused intracellularly. Four hours after the experiment, traces of Procion Yellow were recognized at a distance of about 2400 µm from the cut end. With a cell length of the order of 100 µm for sheep Purkinje fibers[20], this result suggested that the dye must have crossed about 20 cells. This was the first demonstration of the passage of a large molecule through cardiac gap junctions. Since this work, numerous studies on diffusion of large molecular substances have been carried out using the 'cut-end method'[4,5,9,21,33,37], or by an electrophoretic injection method[25]. While these experiments demonstrated the diffusion of large molecular substances from cell to cell, they did not allow continuous measurements of the intracellular tracer distribution. Moreover, tracer profiles could only be determined after a time interval of several hours. In order to overcome these problems tracer distribution in cell pairs was studied.

Cell-to-cell diffusion: Experiments on isolated cell pairs

A method of single cell isolation from myocardium has been developed recently by Powell et al.[26]. By this enzymatic dissociation technique[26,32], not only single cells but also cell pairs can be obtained. It has been demonstrated that the junctional membrane is electrically intact in these cell pairs, despite the treatment with collagenase[14,19,31,39]. Such cell pairs represent a suitable preparation for studying cell-to-cell diffusion by fluorescent dyes, for not only can the dye concentration be measured in a single cell, but its distribution in both cells can be continuously monitored[12].

In this study, patch pipettes with a tip diameter of 2–5 µm were fabricated as described by Hamill et al.[7]. They were filled with pipette solution (in mM: potassium aspartate, 130; KCl, 10; EGTA, 0.01; HEPES, 5; sodium ATP, 5; $MgCl_2$, 1; creatine phosphate, 5; pH was adjusted to 7.4 with KOH), containing 0.1% of a fluorescent dye. To perform an experiment, a pipette was attached to one cell of a cell pair. After a giga ohm seal was established, the cell membrane at the tip of the pipette was ruptured by brief suction, allowing the dye to diffuse from the pipette into the cell. Fluorescence of the dye was observed by means of an inverted microscope,

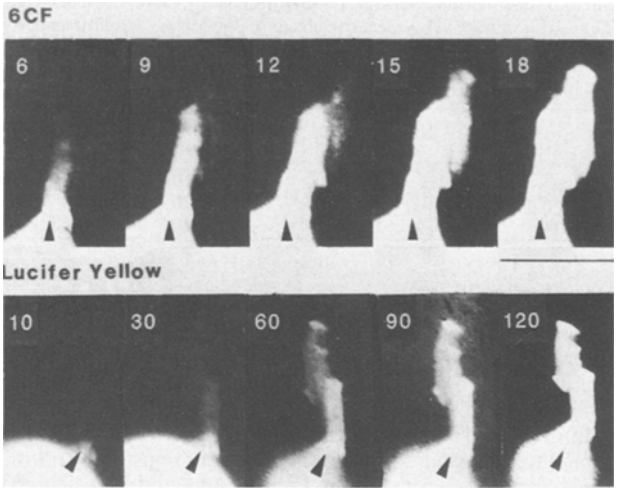

Figure 2. Diffusion of fluorescent dyes studied in ventricular cell pairs. The photomicrographs demonstrate intracellular and intercellular movement of 6-carboxyfluorescein (6CF; upper panels) and Lucifer Yellow (lower panels). The numbers indicate the time in seconds after the beginning of diffusion. The arrows point to the location of the pipette tip. The horizontal bar corresponds to 100 μm.

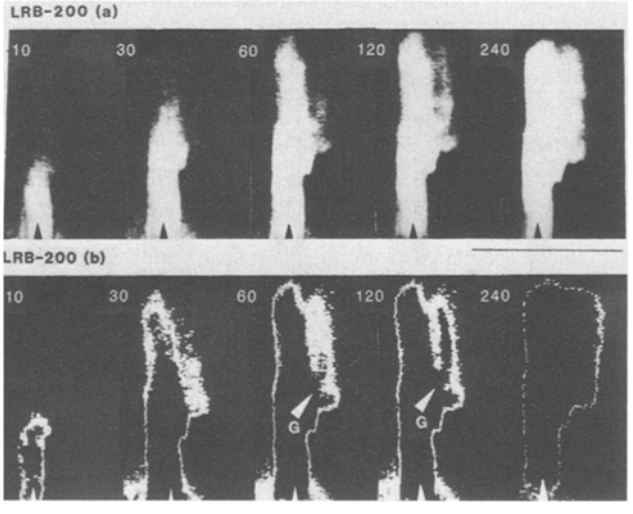

Figure 3. Photomicrographs illustrating the diffusion of Lissamine Rhodamine B-200 (LRB-200) in a cell pair. Panel *a*: Regularly processed images. White areas indicate the dye. Panel *b*: Reversed images obtained by means of an edge processing image analyzer. Black areas indicate the dye. The numbers indicate the time in seconds after the beginning of diffusion. The arrows (G) mark the location of the gap junction. The horizontal bar corresponds to 100 μm.

and monitored with a television camera. To estimate the concentration of the dye within the cell, the fluorescent images on the TV screen were analyzed with a photodiode.

This method, however, was subject to a number of limitations. For example, the TV camera had an automatic intensity control system and the calibration curve was found to be non-linear when a wide range of dye concentrations was tested. Moreover, the fluorescent signal depends on the thickness of the cells and bleaching of the dye has also to be taken into account. For these reasons, careful calibration of the optical signal was crucial and the following method was used. The camera system was controlled manually and the relationship between light intensity and dye concentration was tested. To do this, tissue culture dishes were filled to a depth of 5 mm with various dye concentrations in 0.2% agarose solution. A linear relationship between fluorescent intensity and dye concentrations between 1 and 6 μM was found[12]. On the assumption that the thickness of a ventricular cell is 10 μm, dye fluorescence would be almost linearly related to dye concentration in a cell up to about 3 mM. Therefore, measurement of fluorescent intensity by the photodiode quantitatively represents changes in the intracellular dye concentration[12].

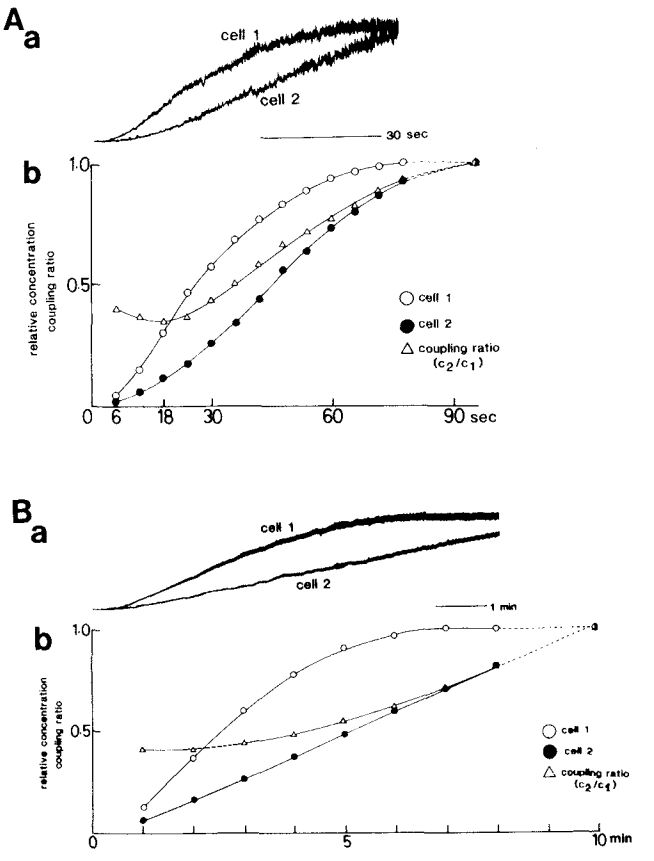

Figure 4. Diffusion of large molecules in ventricular cell pairs. Panel *Aa*: Plot of fluorescence intensity versus time in the case of 6CF. The signals were obtained from two photodiodes placed on the images displayed on a TV screen. One sensor was located 50 μm away from the pipette tip (cell 1), the other one across the gap junction (cell 2), 20 μm away from the first sensor. Panel *Ab*: Normalized plot of the dye concentration against diffusion time. Open circles indicate cell 1 and closed circles cell 2. Triangles correspond to the coupling ratio $[dye]_{cell\ 1}/[dye]_{cell\ 2}$. Panels *Ba* and *Bb*: Diffusion of LRB-200. Format identical to panel *Aa*.

When a cell underwent a contracture and thereby increased its thickness, the light intensity curve shifted. However, this could usually be recognized as a sudden shift in the contour of the fluorescence image. Since bleaching has been found in many fluorescent dyes[1,3,5,41], tests were also performed to determine the decrease in fluorescence as a consequence of the exposure to light. A low intensity light source was used to minimize bleaching. Under these conditions, bleaching was found to be rather small[12] and thus would not disturb the intracellular measurements.

To study the diffusion in ventricular cell pairs, three fluorescent dyes of different molecular weight were chosen: 6-carboxyfluorescein (6CF; mol. wt 376 Da), Lucifer Yellow (mol. wt 457 Da), and Lissamine Rhodamine B-200 (LRB-200; mol. wt 559 Da). Figures 2 and 3 show selected photomicrographs illustrating an experiment for each case. In these figures, the sequences of photographs emphasize the two processes involved; diffusion within the cytoplasm, and diffusion through the nexal membrane.

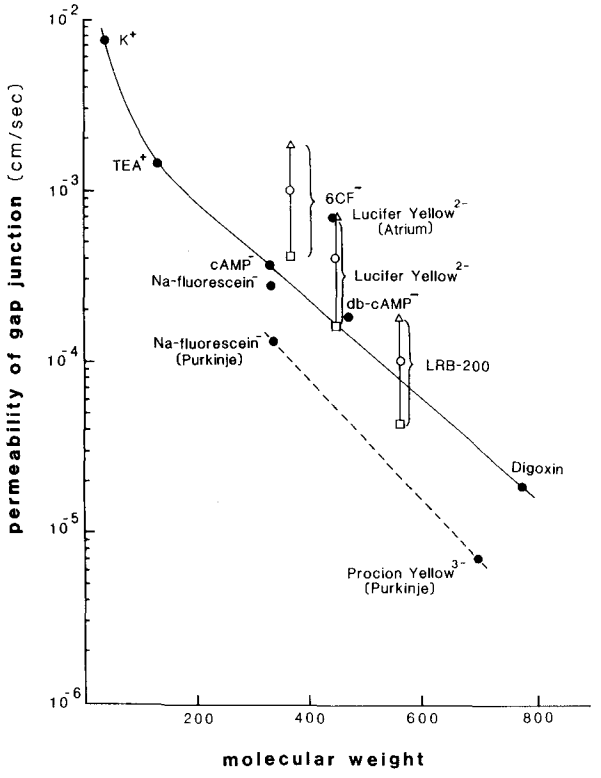

Figure 5. Relationship between the permeability of the gap junction (P_j) to large molecular substances and their molecular weight. Closed circles correspond to the values obtained from multicellular preparations, calculated from the following equation (Weingart[37,38]):

$$P_j = \frac{D_{cell} \cdot D_{apparent}}{D_{cell} - D_{apparent}} \cdot \frac{1}{h \cdot q}$$

where h = intercalated disk distance, 10^{-2} cm; and q = ratio of gap junctional membrane to cross sectional area, 0.3. Values of P_j for K^+ (at. wt 39) were provided by Weidmann[35], for TEA^+ (mol. wt 130) by Weingart[37], for $cAMP^-$ (mol. wt 329) by Tsien and Weingart[33], for fluorescein$^-$ (mol. wt 332) by De Mello[5] and Pollack[25], for Lucifer Yellow^{2-} (mol. wt 457) by De Mello[5], for db-$cAMP^-$ (mol. wt 469) by Tsien & Weingart[33], for Procion Yellow^{3-} (mol. wt 697) by Imanaga[11], and for Digoxin (mol. wt 781) by Hess and Müller[9]. Open symbols were obtained from experiment on ventricular cell pairs[12]. A_j was assumed to be 30% of cross sectional area, and V_{cell} to be 12% (□), 30% (○), and 50% (△) of the total cell volume.

Optical examination revealed the following sequence of diffusion rates: 6CF > Lucifer Yellow > LRB-200. 6CF was detectable in the non-injected cell within 9 s, and Lucifer Yellow within 30s. However, for LRB-200 it required more than 50 s to produce a recognizable signal in the adjacent cell. This suggested that intracellular as well as intercellular diffusion of LRB-200 is much slower than that of 6CF or Lucifer Yellow. Because of the slow diffusion of LRB-200, it was frequently possible to observe the pathway of the dye across the nexal membrane. In the lower panel of figure 3, it can clearly be seen that cell-to-cell diffusion took place over a narrow region of apposed cell membranes, presumably the gap junction (see arrows G).

On the basis of these experiments, it was possible to calculate the diffusion coefficients in cytoplasm, D_{cell}. The values obtained are summarized in the table. They suggest that the cytoplasmic diffusion of LRB-200 was somehow impaired. For comparison, the diffusion coefficient of the fluorescent substances was determined in agar rods as well (D_{agar}). The table shows that the three dyes showed almost identical diffusion coefficients in this medium. The relatively large ratio D_{agar}/D_{cell} for LRB-200 suggests that this compound was presumably bound intracellularly to some structures.

Diffusion coefficients determined in agar rods (D_{agar}) and ventricular cells (D_{cell}). The values of D_{cell} were calculated on the basis of a multicompartment model[10,12,14] using experimental data gained from ventricular cell pairs[12].

	D_{agar} cm^2/s	D_{cell} cm^2/s	D_{agar}/D_{cell}
6CF	7.5×10^{-6}	5.8×10^{-6}	1.3
Lucifer Yellow	5.0×10^{-6}	3.0×10^{-6}	1.7
LRB-200	6.0×10^{-6}	8.6×10^{-7}	6.9

Permeability of the gap junction

In order to estimate the permeability of the gap junctional membrane, two photodiodes were focussed on the fluorescence images displayed on a TV screen. Progressive changes of fluorescence in the injected cell (cell 1) and the adjacent cell (cell 2) were measured simultaneously and registered continuously using a pen recorder. Figures 4Aa and 4Ba demonstrate that the increase in fluorescence in cell 2 was much slower for LRB-200 than for 6CF. Moreover, the time to saturation in cell 2 was much larger for LRB-200 than for 6CF.

These findings suggest that the gap junctional membrane is more permeable to 6CF than to LRB-200. The rationale is that both the coupling ratio and the saturation time reflect the ease with which the dyes penetrate the gap junction.

Using a multicompartment model[10,12,14] for the quantitative analysis, the permeability of the gap junction (P_j) may be calculated from the expression

$$P_j = \frac{V_{cell}}{A_j} k_j$$

where V_{cell} is the cell volume accessible to intracellular diffusion, A_j the area of the junctional membrane, and k_j the rate constant of transnexal diffusion.

It can be calculated from morphometric studies performed on ventricular tissue that A_j contributes 17% to 30% of the cross sectional area[16,23]. The main myoplasmic components such as myofibrills, sarcoplasmic reticulum, mitochondria, and nucleus occupy about 88% of the total cell volume. However, since diffusion occurs within the interfibrillar space, V_{cell} must be somewhat greater than 12% of the total cell volume. Assuming an A_j of 30%, and V_{cell} to be 12%, 30%, or 50% of the total cell volume, P_j was calculated to be 4×10^{-4}, 1.0×10^{-3}, and 1.7×10^{-3} cm/s for 6CF; 1.6×10^{-4}, 2.3×10^{-4} and 6.7×10^{-4} cm/s for Lucifer Yellow, and 4.2×10^{-5}, 1.0×10^{-4} and 1.7×10^{-4} cm/s for LRB-200, respectively (fig. 5, open symbols).

Figure 5 compares the calculated values of P_j obtained from the cell pair measurements (open symbols) with values previously reported from experiments on multicellular cardiac preparations (closed symbols; for references, see Weingart[38]). The graph shows a plot of P_j versus molecular weight of the probing substances. It indicates that the data extracted from the two different types of preparations are rather similar. However, figure 5 also shows that the values of P_j obtained from ventricular cells are larger than those observed in Purkinje fibers [38]. This suggests that these tissues possess different junctional membranes. The permeability of the gap junction to various substances is inversely correlated with the molecular weight. However, probably not only is the molecular weight important but also the net charge of the molecule, since the channels of the gap junctions are regarded as negatively charged.

Critical diameter of the gap junctional channel

Experiments performed on ventricular cell pairs revealed

that the tetraglycine conjugate of LRB-200 (mol. wt 859 Da) also passes through the gap junction[12], but not Chicago Blue[11] (mol. wt 1000 Da). This suggests that there exists a critical size limit for junctional permeation. Assuming spherical molecules[28], the diameter of the conjugated LRB-200 and Chicago Blue may be calculated to be 12 Å and 13 Å, respectively. On the other hand, morphometric studies yielded a diameter of 10 to 15Å for the cardiac gap junctional membrane channel[16,18]. Thus, the values obtained from the diffusion experiments are in good agreement with those determined morphometrically.

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