REVERSIBLE STRUCTURE TRANSITION IN GAP JUNCTION UNDER CA^{++} CONTROL SEEN BY HIGH-RESOLUTION ELECTRON MICROSCOPY

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ABSTRACT Deoxycholate-extracted rat liver gap junction was studied by high-resolution low-dose electron microscopy. Communicating channels between two adjoining cells supposedly form along the common axis of two apposed hexameric trans-membrane protein assemblies. These double hexamers are often arranged in large plaques on an ordered hexagonal net (8–9 nm lattice constant) and seem able to undergo structural alteration as a possible permeability control mechanism. Calcium is widely reported to uncouple gap junction, and we observed this alteration on exposure to Ca^{++} down to 10^{-4} M concentration. When EGTA was added at matching concentrations, the alteration was reversible several times over one hour, but with considerable variability. It was imaged in the absence of any negative stain to avoid ionic and other complications. The resulting lack of contrast plus low-dose "shot" noise required digital Fourier filtering and reconstruction, but no detail was recovered below 1.8 nm. In other experiments with negative stain at neutral pH, gap junction connexons were apparently locked in the "closed" configuration and no transition could be induced. However, recovery of repeating detail to nearly 1.0 nm was possible, reproducibly showing a fine connective matrix between connexons. Whether this was formed by unfolded portions of the 28,000-dalton gap junction protein is not known, but its existence could explain the observed lattice invariance during the connexon structural transition.

INTRODUCTION

"Gap junction" is now widely accepted as a name for specialized regions of cell membranes where intercellular communication is thought to occur. Gap junctions are found in a large diversity of species and tissues, and consist of patches containing an array of hexameric transmembrane proteins. According to the model of Makowski et al. (1977) each cylindrical hexamer (or "connexon") seems to form a pore down its sixfold axis through the membrane, and is in register with another connexon in the neighboring cell, providing a continuous channel for the passage of small molecules and ions between cells; this double layer is ~15 nm thick. (For review, see Loewenstein, 1975.)

Many studies have shown that gap junctions are capable of functional coupling and uncoupling under various physiological stimuli. However the correlation of functional state with changes in physical structure appears baffling; most authors show decoupling in association with highly ordered hexagonal packing of connexons in the plane of the membrane (Goodenough and Gilula, 1974; Makowski et al., 1977; Hirokawa and Heuser, 1982), while others associate decoupling with relative disorder and even dispersal of connexons about the membrane (Lee et al., 1982).

A different class of structural changes has been observed in highly ordered hexagonal arrays, in which the conformation of connexons themselves appears to change (Unwin and Zampighi, 1980). In this type of change, involving closure of the supposed communicating channels, any associated decoupling is as yet strictly presumptive, however plausible. Working entirely with negative stain (2% uranyl acetate, presumably ~pH 4.5), removal of residual detergent by prolonged dialysis resulted in a radial inward movement of connexon protein at the cytoplasmic face, causing occlusion of the central pore, but with no measurable change of the hexagonal lattice constant (8.5 nm). This structural transition (see also Zampighi and Unwin, 1979) was accompanied by apparent connexon rotation of some 30° with respect to the lattice and a conspicuous reduction in intensity of the 1, 0 crystallographic reflections, often to zero. Adding back detergent reversed the transition; intermediate stages were observed with both forms present in the same junction plaque.

Our work addresses this type of structural change by two-dimensional low-dose electron microscopy of highly ordered junction plaques. Our aim, however, was to explore the role of free calcium in causing the transition for future correlation with function, rather than to define structural details. Many studies (notably Peracchia, 1978) have shown functional decoupling of gap junction with physiological amounts of Ca^{++}, and we report a calcium-associated effect similar to the Unwin and Zampighi

structural change, but without any possible ionic complications from negative staining. We also report some structural details, at nearly 1.0-nm resolution using negative stain, which are relevant to lattice invariance during the connexon change.

MATERIALS AND METHODS

Isolation of Gap Junction

Gap junction plaques were prepared by a procedure based on that of Zampighi and Unwin (1979) using proteolysis inhibitors. Our modifications sought to decrease overall specimen handling at the expense of purity, for better preservation of original structure.

Working rapidly in a cold room with all vessels and solutions at 4°C, two adult female Sprague-Dawley rats were killed and their livers removed. Each whole liver was dropped into a 50-ml homogenizer ("Uni-Form," Jencons Ltd., Leighton Buzzard, UK) containing ~40 ml of medium made up as follows: 10^{-2} M $NaHCO_3$; 4% sucrose; 5×10^{-4} M ethylene glycol-bis(beta-amino-ethyl ether) *N,N'*-tetra acetic acid (EGTA); 10^{-3} M $CaCl_2$; 5×10^{-5} M phenyl methyl sulfonyl fluoride (PMSF); and 10^{-3} M DL-dithiothreitol (DTT). This medium was made up using deionized water and adjusted to pH 8.0 with NaOH; the last three items were from Sigma Chemical Co., St. Louis, MO, and the last two were added freshly each time more medium was used. Using loose and then tight PTFE pestles, each liver was coarsely, then finely, homogenized, and the pooled homogenate diluted to ~800 ml with fresh medium. This was then filtered twice through four layers of gauze and once through two layers of 50-mesh nylon. The filtrate was then sedimented at 5,500 rpm (1,500 *g*) for 10 min at 4°C (J14 rotor, Beckman J21C centrifuge). The precipitate was resuspended in a minimal volume of fresh medium, the sucrose concentration raised to 43.5% (wt/wt) using a 65% solution in 10^{-2} M $NaHCO_3$, pH 8, and the mixture evenly divided among six 35-ml tubes. These were carefully overlayered with medium until full and then centrifuged at 25,000 rpm (78,000 *g*) for 75 min (SW27 rotor, Beckman L8).

A leathery yellow membrane fraction formed at the sucrose/medium interface; these layers were removed, pooled, homogenized in fresh medium, and sedimented at 11,000 rpm (10,000 *g*) for 10 min (J20 rotor, Beckman J21C). The pellet was whitish uppermost on a yellower underlayer with a brownish patch at the bottom. The top layer was gently washed off with 10^{-2} M $NaHCO_3$, and homogenized. This was repeated two more times, with the final pellet taken up in a minimal volume of the $NaHCO_3$ for total protein estimation (usually ~30 mg from two livers). A solution of sodium deoxycholate (DOC, Calbiochem-Behring, La Jolla, CA) in 10^{-2} M $NaHCO_3$ was added to a final concentration of 2% at 3 mg DOC/mg protein and the mixture was left for 30 min. It was then washed twice in ~15 vol of 10^{-2} M $NaHCO_3$ by sedimentation for 15 min at 16,500 rpm (20,000 *g*) in the J20 rotor, and the final gap junction pellet was taken up in 1–2 ml of the bicarbonate containing 0.02% sodium azide. This material was used for all electron microscopy after being fully resuspended overnight.

The purity of this final material was not high; despite the absence of collagen there was a significant proportion of unidentified cell debris, including nonjunctional and presumably mitochondrial membrane. However gap junction plaques were present in large quantities as recognized by morphological criteria, and Fig. 1 shows a clear protein component of ~28,000 daltons (Finbow et al., 1980; Nicholson et al., 1981; Traub et al., 1983). Conveniently, junction plaques were always easy to find away from other material and therefore free of local contamination.

Electron Microscopy

Micrographs were taken in a JEM 100C electron microscope operated at 100 kV. It was fitted with a top-entry stage (1.8 mm objective) and equipped with beam deflection systems above and below the specimen.

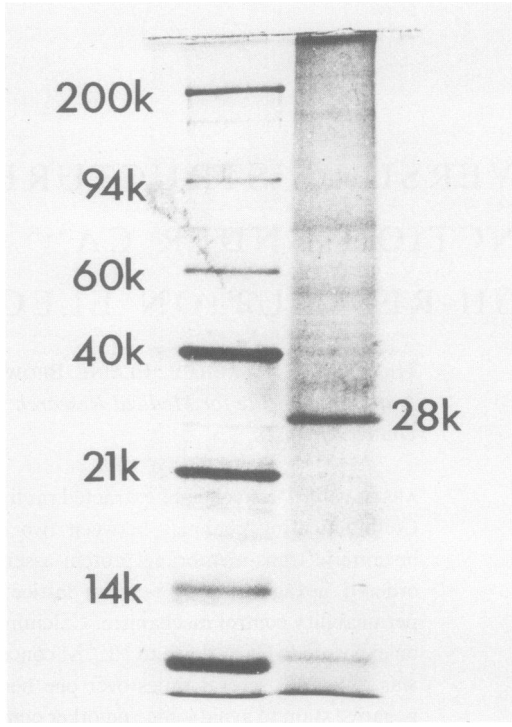

FIGURE 1 SDS-polyacrylamide gel (15% acrylamide, 0.086% bis) of gap junction isolate (*right*), showing a principal band of 28,000 mol wt. The standards (*left*) are, from the top: myosin , phosphorylase *a*, catalase, creatin kinase, soya bean trypsin inhibitor, and lysosyme.

This allowed low-dose searching at magnification of 1,000–2,000, very accurate focus off-axis using high magnification (× 300,000), and hence any desired defocus for picture-taking at × 30,000 (Wrigley et al., 1983). Defocus, as checked by optical diffractometry, was always chosen such that the first zero of the phase-contrast transfer function fell comfortably beyond the limit of expected resolution. Thus with negatively stained specimens showing detail preservation approaching 1.0 nm we had to work close to Scherzer defocus; with unstained specimens showing no detail below 1.8 nm we could work further below focus and gain marginally in phase contrast.

Preparation of Specimens

All specimens were mounted on carbon-coated 400-mesh supports. A drop of gap junction suspension was deposited on the grid and left for ~1 min before floating for a further minute on a bath containing the same reagents as the specimen. For negative staining (when used) the grid was floated for a third minute on a bath of 1% (wt/vol) sodium silicotungstate ($Na_4[Si(W_3O_{10})_4] \cdot 20H_2O$, MW 3327, Hopkins & Williams, Essex, England) which has a convenient pH of 7.0 in aqueous solution. Finally, all grids were blotted and air dried.

Picture Processing

The magnification of micrographs was calibrated to 2% using the 17.5 nm spacing of cross-linked catalase crystals. After screening for correct defocus, absence of astigmatism, and quality of crystallographic reflections by laser diffractometry, suitable micrographs were digitized (using a modified Nikon microdensitometer) in 512 × 512 arrays with a sampling distance equivalent to 0.7 nm (unstained junction) or 0.35 nm (stained junction). Translational Fourier filtering (3 × 3 masking of reflections) and reconstruction were performed on a DEC 2060 computer. No space

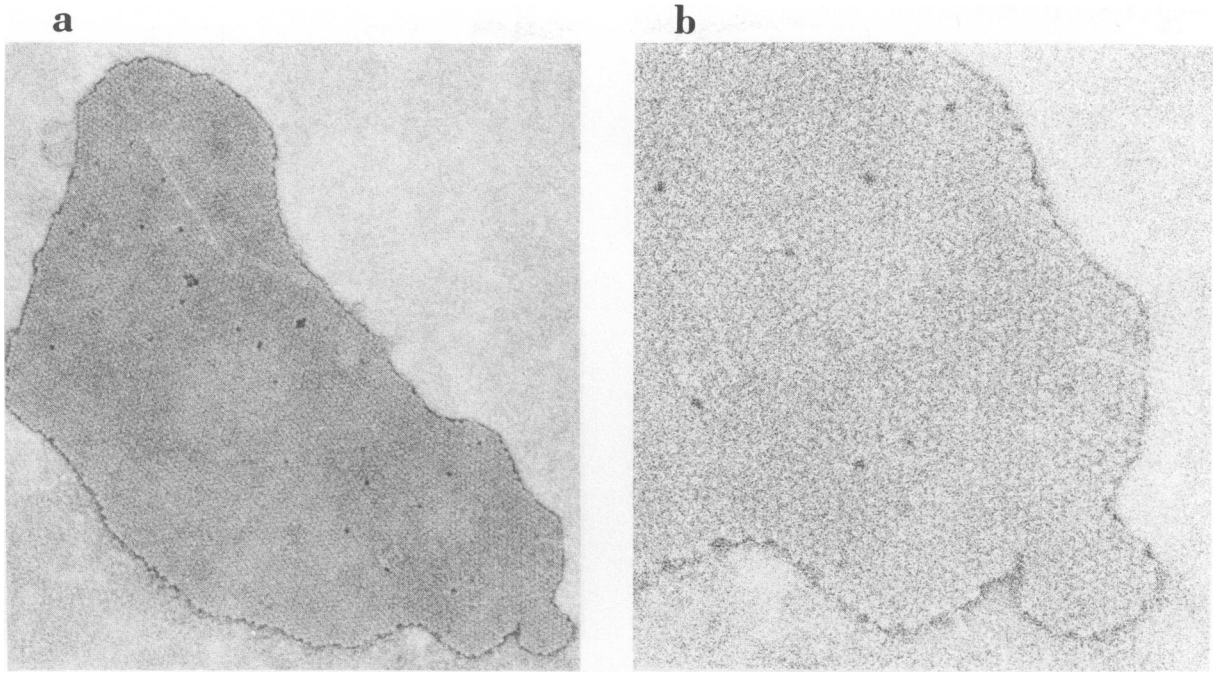

FIGURE 2 *a*, typical gap-junction plaque in negative stain (× 100,000). *b*, the same at × 250,000 showing the hexagonal connexon lattice with some discontinuities.

group was assumed for the junction lattice and consequently no rotational operations or symmetrization of phases and amplitudes were imposed. Final contrast was inverted in the case of unstained junction to preserve the convention of showing protein as white, for direct comparability with negatively stained specimens.

RESULTS

Fig. 2 shows a gap-junction plaque typical of our preparations, negatively stained to show its characteristic hexagonal lattice and 8.2-nm center-to-center spacing between connexons. Such plaques typically contained several thousand connexons, though seldom in a single coherent lattice. More often each plaque consisted of a mosaic of ordered patches with high ordering extending over only some hundreds of connexons. Nevertheless, general outline characteristics, plus extremely uniform density and occasional lattice holes, made it very easy to distinguish these plaques from other material, whether stained or unstained, at × 1,000 magnification in our low-dose search routine. Differences of intensity between 1, 2 and 2, 1 diffraction maxima were often evident in either case; as there may be twofold symmetry axes in the junction plane relating each connexon to its apposed pair (Baker et al., 1983), these differences could reflect differential staining and/or distortion between the top and bottom halves of the plaque. However, the use of glucose embedding had no effect on this phenomenon, so the differences may be real.

Calcium-EGTA Experiments

Gap junction suspensions containing no added reagents except the final 10^{-2} M sodium bicarbonate at pH 8 and mounted without negative stain had the appearance shown in Fig. 3. Individual connexons were clearly defined with their centers 8.2 nm apart and showing six subunits some 2.5 nm in diameter. If these are approximately cylindrical and 7.5 nm long (half the junction thickness) perpendicular to the picture plane, then each subunit would have a molecular weight near 30,000; this crude estimate is consistent with known data. A central hole of similar diameter was also very clear, and corresponds in position to the supposed intercellular communicating channel. With some exceptions, a characteristic of such unstained junction images was absence or extreme weakness of 1,0 optical diffraction maxima, a feature noted by Zampighi and Unwin (1979) with their "B" junction after exhaustive removal of detergent. We found that addition of calcium chloride to our material in any concentration from 10^{-5} to 10^{-2} M had no effect in producing these 1, 0 reflections; on the contrary, when they were occasionally present at the outset, addition of Ca^{++} weakened or abolished them completely.

To reverse this situation we attempted chelation of any calcium by addition of EGTA to gap junction suspensions with either nothing or 10^{-4} M $CaCl_2$ added. This resulted in appearance of 1, 0 reflections from plaque images at a concentration threshold of 10^{-4} M EGTA (10^{-2} M $NaHCO_3$, pH 8 was present throughout these experiments). The effect is shown in Fig. 4, where differences compared to Fig. 3 are apparent. The hexagonal lattice constant remained virtually unchanged at 8.2 nm, yet the connexons appear relatively condensed; the central hole is much less obvious.

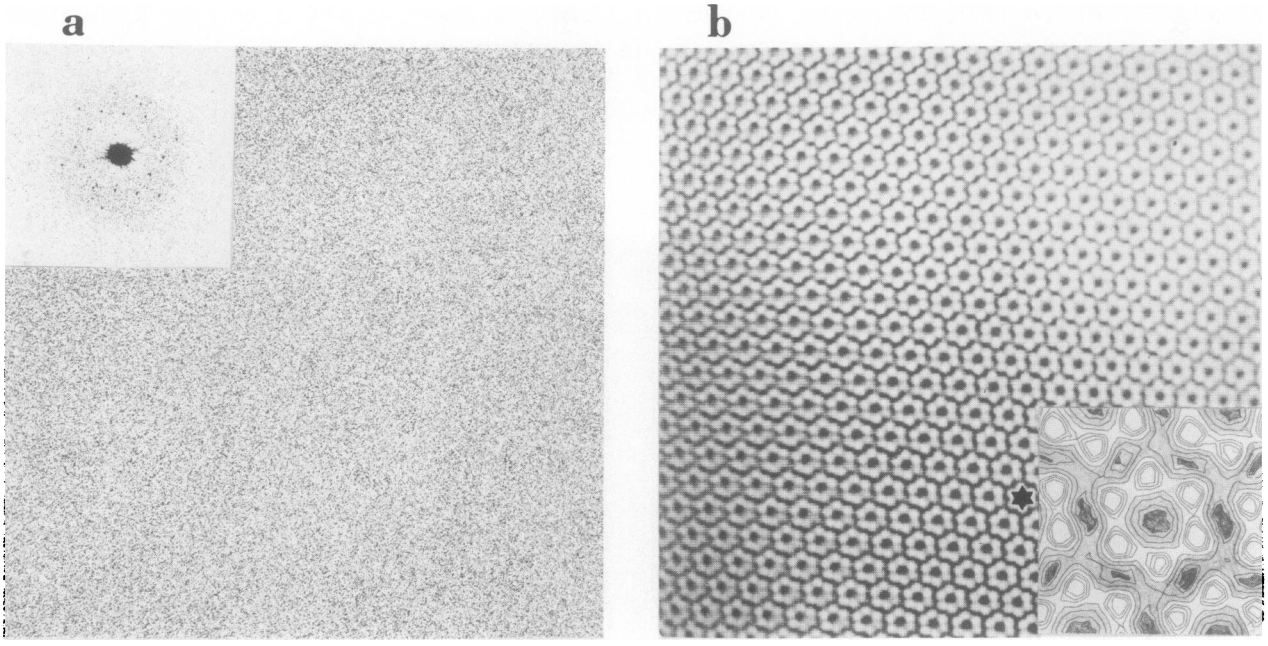

FIGURE 3 *a*, unstained gap junction with 10^{-4} M Ca^{++} added; *inset*: its diffractogram showing absence of 1, 0 reflections. *b*, reconstruction after Fourier filtering (3 × 3 mask) showing "open" connexon configuration, 8.2 nm center-to-center. (× 500,000; contrast inverted so that protein is white); *inset*: the same, displayed as density contours.

This effect was observed in four separate gap-junction preparations and in repeated trials with each, up to eight weeks after each preparation was made. It was also reversible two or three times upon sequential addition of Ca^{++} and then EGTA to the same sample aliquot, allowing ~10 min reaction time each way; longer times had no additional effect.

We did not Fourier filter all micrographs but used the status of the 1, 0 optical diffraction maxima as an index of conformation. (Incidentally, there was a tendency for a

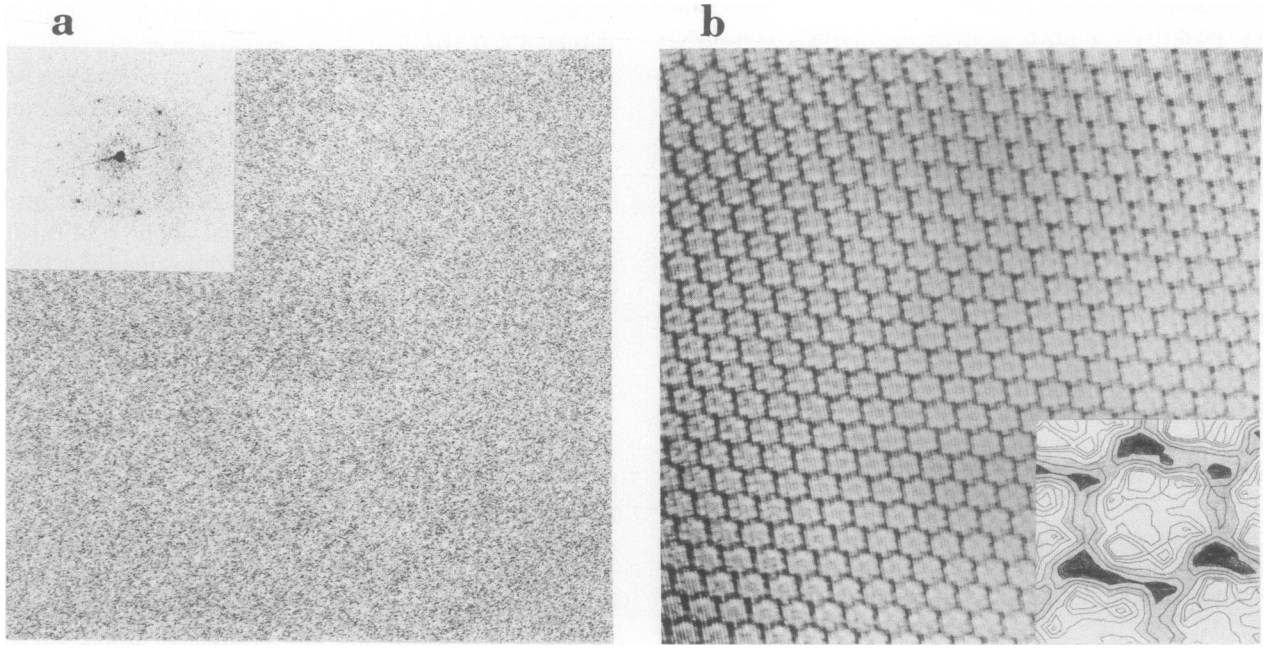

FIGURE 4 *a*, unstained gap junction after addition of 10^{-4} M Ca^{++} followed after 10 min by 10^{-4} M EGTA; *inset*: diffractogram showing presence of 1,0 reflections. *b*, filtered reconstruction showing relatively "closed" connexon configuration, again 8.2 nm center-to-center. (× 500,000; contrast inverted so that protein is white); *inset*: the same, displayed as density contours.

presence of 1, 0 reflections to be accompanied by absence of 2, 0 reflections, and vice versa.) We took micrographs of some dozen junction plaques in each cycle of each experiment, and the presence or absence of 1, 0 reflections was never 100% within each dozen. Sometimes 1, 0 intensities merely increased and decreased. Furthermore, threshold concentrations of Ca^{++} and EGTA also varied up to 5 $\times 10^{-4}$ M; occasionally, no effect at all was obtained even above this concentration, and never below 10^{-4} M. These variations came and went randomly, without relation to junction preparation, individual experiment, or even cycle within each experiment. Nevertheless the trend was always the same: Ca^{++} abolished 1, 0 reflections and produced the "open" connexon appearance of Fig. 3; EGTA restored 1, 0 reflections and condensed connexons shown in Fig. 4.

The uncontrolled factor(s) giving the above variability remains unknown. In case any free Ca^{++} remained from the preparation procedure, or more was released from sequestration in mitochondrial or other debris, we attempted closer control by using EDTA-calcium buffer, with 10^{-4} M and 10^{-3} M free Ca^{++}. More work is required, but in our first trial with the lower concentration, weak 1, 0 reflections remained; at the higher concentration they virtually disappeared.

Negative Stain Experiments

All our gap junction isolates were initially checked with negative staining (1% sodium silicotungstate, pH 7). Such preparation resulted, without exception or variation, in strong 1, 0 reflections and the highly condensed connexon appearance shown in Fig. 5, yet the 8.2 nm lattice remained constant. Several different stains, some at low pH, had the same effect, and no amount of added calcium succeeded in abolishing the 1, 0 reflections or in altering the apparent connexon structure. They appeared locked in the condensed configuration similar to Fig. 4, though far more detail was visible thanks to greatly increased contrast. Resolution usually extended to 5, 1 reflections, and occasionally included a very weak 6, 1 set (corresponding to 1.08 nm), at or near the extreme limit available from negative stain.

The interesting new feature revealed at this high resolution was a fine connective matrix, visible in the large spaces opened between the condensed connexons. We do not seek to specify the structural details of this matrix, but point out that its existence provides an explanation for lattice invariance during structural transition of the connexons, whose condensation would otherwise cause the whole lattice to contract. We have no information on the relation of this lattice to normal junctional membrane with its full lipid complement, and we can only speculate that it consists of unfolded portions of the main junctional protein, or an additional low molecular weight protein.

DISCUSSION

Structural Transition

Any discussion of these results must recognize the fact that our junction material bears an unknown relation to native functional gap junction. Our junction plaques were pre-

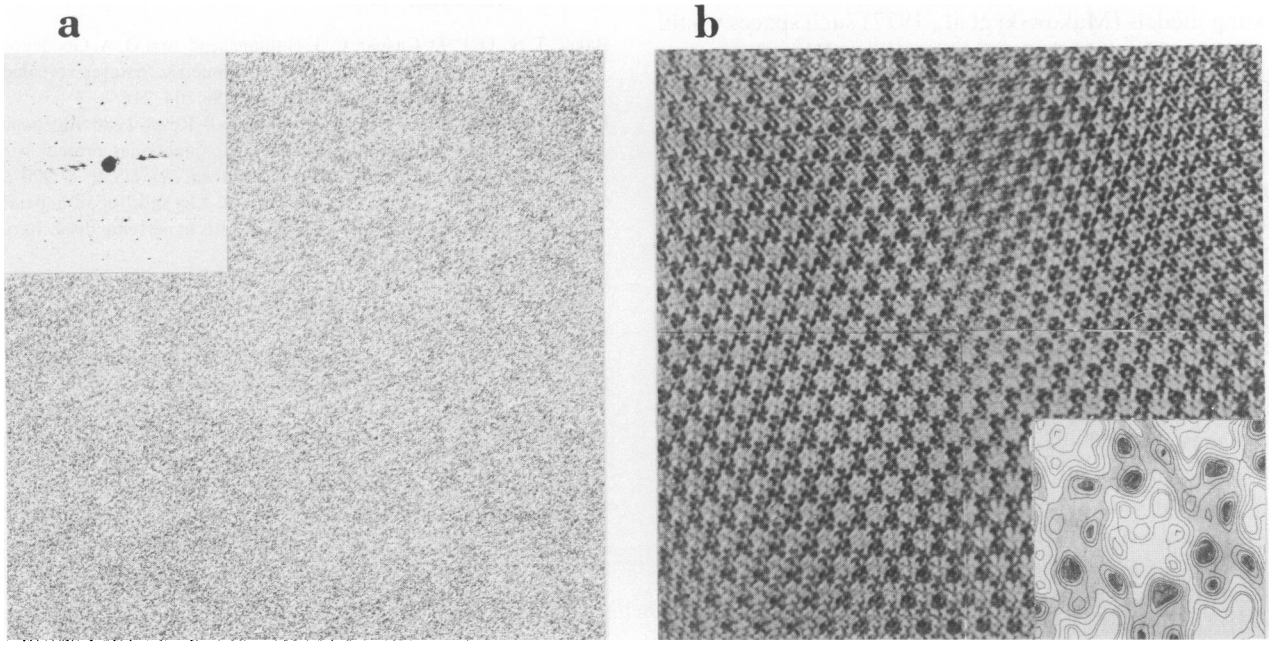

FIGURE 5 *a*, gap junction with light negative staining. Optical diffraction (*inset*) included the following independent reflections: 0,0; 1, 0; 1,1; 1,2; 1,3; 1,4; 1,5; 2,1; 2,2; 2,3; 3,0; 3,1; 3,2; 3,3; 4,0; 4,1; 5,1; 6,1. *b*, filtered reconstruction showing "closed" connexons (8.2 nm center-to-center) with fine connective matrix in the gaps between them. ($\times$ 500,000; contrast not inverted, so that protein is white); *inset*: the same, displayed as density contours.

sumably different at least in having no—or very low—lipid content; the whole basis of isolating them from all other membrane rests on lipid removal. However the protein content remains very similar to native junction in its overall organization so our results may not be irrelevant to function in vivo. We report for the first time an attempt to correlate a structural transition of connexons with a known physiological effector, Ca^{++}, though we have not yet shown the effect at physiological concentrations (10^{-5} M), nor tightly defined the conditions prevailing in our work.

The structural transition we observe is clearly capable of interpretation as a coupling/decoupling mechanism independent of junction dispersal about the membrane. If this is the mechanism—and our future program is designed to explore this directly with native junction—then we must discover which conformation corresponds to cellular coupling and which to decoupling. In our work the Fig. 3 appearance suggests "open" channels along connexon six-fold axes perpendicular to the junction plane; in the absence of stain (which sometimes shows only surface layers) we must suppose that the channels extend right through the junction, since all "depth" information is integrated into two-dimensional projections. Yet this "open" configuration occurred in response to raised Ca^{++}, which has been universally associated with junction decoupling. Conversely our condensed, or "closed" connexon appearance of Fig. 4 resulted from removal of Ca^{++}. We must therefore recognize the possibility that intercellular communication occurs via some route other than through the connexon pores. The large spaces which appear between connexons when they condense (Fig. 5) are an obvious candidate for this other route, though according to existing models (Makowski et al., 1977) such spaces would be filled with lipid in native junction. We venture no further with this conundrum at the present time.

Connective Matrix

Our observations of a connective matrix visible in the spaces between condensed connexons in negative stain (Fig. 5) seem at odds with recent work of Hirokawa and Heuser (1982), showing that junctional interiors invariably appear extremely smooth. However our two-dimensional pictures give no indication of the depth within the junction at which the matrix might lie; this could be a plane not revealed by their freeze-fracture experiments. Although our highest-order Fourier terms were too weak to contribute significantly to the filtered reconstructions, the remaining terms still extended to much higher resolution than any yet reported—1.4 nm at least—and we found this detail repeatedly in negative stain. There can be little doubt of the existence of this matrix, and no other way to explain how the junction lattice constant remained fixed at 8.2 nm within 0.2 nm while connexons themselves condensed to leave 2.0 nm spaces between them. This could suggest a hitherto-unrecognized protein of low molecular weight forming the matrix. It could also suggest matrix formation from unfolded portions of the known gap junction protein. Current work (Nicholson et al., 1983) indicates such a moiety of some 2–4,000 daltons at the COOH-terminus, probably exposed at the cytoplasmic face of connexons, which may often be removed during preparative procedures.

Some Technical Notes

Recovery of high-resolution detail from our unstained junction fell significantly short of recovery from the stained material. Since the low-dose procedure was identical in both cases, we attribute the shortfall to sheer lack of phase contrast rather than added radiation damage. Although stain may have been intrinsically more stable, the highest reflections (3,0 corresponding to 2.4 nm) obtained from unstained junction showed no additional broadening and degradation.

We used the status of 1, 0 reflections as an index of configuration as described; other reflections, notably 2, 0, also showed systematic changes, but we have not yet studied their significance.

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REFERENCES

Baker, T. S., D. L. D. Caspar, C. J. Hollingshead, and D. A. Goodenough. 1983. Gap junction structures. IV. Asymmetric features revealed by low irradiation microscopy. *J. Cell. Biol.* 96:204–216.

Finbow, M., S. B. Yancey, R. Johnson and J.-P. Revel. 1980. Independent lines of evidence suggesting a major gap junctional protein with a molecular weight of 26,000. *Proc. Natl. Acad. Sci. USA.* 77:970–974.

Goodenough, D. A., and N. B. Gilula. 1974. The splitting of hepatocyte gap junctions and zonulae occludentes with hypertonic disaccharides. *J. Cell Biol.* 61:575–590.

Hirokawa, N., and J. Heuser. 1982. The inside and outside of gap-junction membranes visualized by deep etching. *Cell.* 30:395–406.

Lee, W. M., D. G. Cran, and N. J. Lane. 1982. Carbon dioxide disassembly of gap-junctional plaques. *J. Cell Sci.* 57:215–228.

Loewenstein, W. R. 1975. Permeable junctions. *Cold Spring Harbor Symp. Quant. Biol.* 40:49–63.

Makowski, L., D. L. D. Caspar, W. C. Phillips, and D. A. Goodenough. 1977. Gap junction structures II. Analysis of the x-ray diffraction data. *J. Cell Biol.* 74:629–645.

Nicholson, B. J., M. W. Hunkapiller, L. B. Grim, L. E. Hood, and J.-P. Revel. 1981. Rat liver gap junction protein: properties and partial sequence. *Proc. Natl. Acad. Sci. USA.* 78:7594–7598.

Nicholson, B. J., L. J. Takemoto, M. W. Hunkapiller, L. E. Hood, and J.-P. Revel. 1983. Differences between liver gap junction protein and lens MIP26 from rat. Implications for tissue specificity of gap junctions. *Cell.* 32:967–978.

Peracchia, C. 1978. Calcium effects on gap junction structure and cell coupling. *Nature (Lond.).* 271:669–671.

Traub, O., P. M. Drüge, and K. Willecke. 1983. Degradation and resynthesis of gap junction protein in plasma membranes of regenerating liver after partial hepatectomy or cholestasis. *Proc. Natl. Acad. Sci. USA.* 80:755–759.

Unwin, P. N. T., and G. Zampighi. 1980. Structure of the junction between communicating cells. *Nature* (*Lond.*). 283:545–550.

Wrigley, N. G., E. Brown, and R. K. Chillingworth. 1983. Combining accurate defocus with low-dose imaging in high resolution electron microscopy of biological material. *J. Microscopy* (*Oxford*). 130:225–232.

Zampighi, G., and P. N. T. Unwin. 1979. Two forms of isolated gap junctions. *J. Mol. Biol.* 135:451–464.