LENS GAP JUNCTIONS AND ORTHOGONAL ARRAYS ARE UNRELATED

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1. Introduction

Gap junctions are particularly abundant in calf eye lens fibre cells [1–4], and characteristically for isolated lens gap junctions, the connexons are randomly dispersed in the plane of the junctions: thus they differ from isolated mouse liver gap junctions, where the connexons are ordered on a hexagonal lattice [5–9]. Exposure of isolated lens junctions to 5×10^{-7} M Ca^{2+} induces the randomly dispersed connexons to arrange themselves on a hexagonal lattice [10]. Similar concentrations of intracellular Ca^{2+} in *Chironomus* salivary gland cells (applied by micro-injection) cause electrical uncoupling of adjacent cells [11,12]. Therefore it has been proposed that the Ca^{2+}-induced hexagonal connexon arrangement of the isolated lens junction represents the high resistance state, when the permeable channels are closed, whilst the initial unordered connexon arrangement characterizes the low resistance (= permeable) configuration [10,13]. Conductivity measurements also have revealed that lowering the intracellular pH increases the electrical resistance between neighbouring *Xenopus* embryonic cells [14]. When isolated lens plasma membranes were incubated at pH 6–6.5, orthogonal arrays were observed in freeze-fractured preparations, and by analogy it has been proposed that these orthogonal arrays represent the lens gap junctions in another uncoupled configuration induced by low pH treatment [15].

We have investigated the nature of the orthogonal arrays which copurify with gap junctions (unordered connexon arrangement) using the basic isolation protocol for eye lens fibre junctions designed in [2]. These orthogonal arrays appear to be the same as reported to be formed from gap junctions upon incubating crude lens membrane preparations at pH 6–6.5 [15], since they have essentially the same lattice periodicity of 6.1 nm. However orthogonal arrays accumulate in our isolation procedure even when the pH is maintained above 8 and they possess only a single unit membrane structure. Our results indicate therefore, that the formation of orthogonal arrays in lens membranes is not induced by low pH and that in fact these ordered structures are not related to gap junctions.

2. Materials and methods

2.1. *Isolation protocol*

The isolation procedure for eye lens fibre junctions in [2] was used with some modifications. Lens plasma membranes were washed 3 times in 1 mM bicarbonate containing 10 mM EDTA, adjusted to pH 7.4. The crude membrane preparation (~1 mg protein/ml in 1 mM bicarbonate and 0.9% NaCl) was treated with 0.01–10 mg trypsin/ml (see sections 3.1–3.4). Proteolysis was stopped by the addition of phenylmethylsulphonyl fluoride (PMSF) to 2 mM final conc. The membranes were washed twice in 1 mM bicarbonate (30 min, 18 000 rev./min Sorvall SS34) then incubated in 1% sodiumdeoxycholate/1 mM bicarbonate at 20°C for 30 min. The detergent was removed by centrifugation and the non-solubilized membranes layered onto a 20–50% linear sucrose gradient in 1 mM bicarbonate (18 h, 35 000 rev./min Beckmann SW40). The fractions containing both orthogonal arrays and gap junctions were collected at 1.14–1.16 g/cm^3. Alternatively instead of 1 mM bicarbonate a number of experiments were performed using 100 mM Tris–acetate, all solutions being buffered to pH 8.2.

2.2. *Gel electrophoresis*

Samples were analysed by gel electrophoresis by the Laemmli procedure [16]; the gels contained 12.5%

polyacrylamide. Samples were dissolved in sample buffer (1% SDS, 3% mercaptoethanol, 10% glycerol) at 20°C for 1 h in the presence of 2 mM PMSF. Boiling in sample buffer was found to cause aggregation of some lens membrane proteins and thus prevented them from entering the gels [17].

2.3. *Electron microscopy*

Isolated membranes were adsorbed from solution onto carbon/collodion coated grids which had been glow-discharged in air at reduced pressure shortly before use. Uranyl acetate or uranyl formate were used as negative stains. Monolayer freeze-fracturing was done basically following [18]. There were two modifications of this technique. Membranes were adsorbed onto glass cover slips pre-sonicated in ethanol and dried, and subsequently air glow-discharged but not acid-cleaned or coated in polylysine. The specimens were sandwiched between the cover slip and a similarly cleaned piece of glass slide (rather than a copper disc); then the sample was frozen and fractured under liquid nitrogen. Shadowing with platinum/carbon at 45° was carried out with the specimen enclosed in a simple shielded cold block device equipped with tunnels to allow shadowing [19–21]. Freeze-fracturing of lens tissue was done following [21]. Embedding and sectioning of membrane pellets was performed following [9]. Micrographs were taken on Kodak 35 mm fine grain release positive film using a Philips EM 301 at a calibrated magnification of 20 000. Micrographs from shadowed preparations have been printed with reversed contrast, heavy metal appearing white and shadows black.

3. Results

3.1. *Isolation of orthogonal arrays from lens membrane*

Orthogonal arrays (fig.1) accumulate together with lens gap junctions (fig.2) at 1.14–1.16 g/cm^3 in the final isopycnic gradient of the isolation protocol described in section 2.1. The arrays consist of a single membrane, a fact which can be deduced from the observation that they always split along a single fracture plane in freeze-fracture preparations (fig.3). Isolated lens junctions however are built from two membranes (micrographs of thin sectioned lens junctions are in [2]). Characteristically for freeze-fractured lens junctions with irregular ordering of the connexons the fracture plane frequently jumps between the two junctional membranes as in [10]. This is visualized in fig.4, where small islands (smooth areas) of the extracellular half leaflet (E-face, nomenclature [22]) of one membrane cast their shadows onto the particulate cytoplasmic half leaflet (P-face) of the other membrane (for both fig.3,4 the shadowing directions are from top, shadows appearing black).

The isolation protocol includes the treatment of the crude plasma membranes with trypsin. Whereas samples treated with low concentrations of trypsin (0.01–0.1 mg/ml, 30°C, 40 min) yielded a mixture of about equal numbers of both orthogonal arrays and gap junctions, those treated with high concentrations of trypsin (1–10 mg/ml) yielded almost exclusively gap junctions (negatively stained specimens were examined). This observation was confirmed by examining in thin sections pellets of each type of material: those exposed to low concentrations of trypsin exhibited both single and double membrane structures but those exposed to high concentrations of trypsin contained only double membrane structures. These results together with the observed large differences in morphology suggest that in fact the orthogonal arrays and the gap junctions are distinct structures. This is different from the proposition in [15] that orthogonal arrays are derived from gap junctions at pH 6–6.5 by way of a conformational rearrangement. Since the lattice repeat of our orthogonal arrays is 6.1 nm and thus very similar to that

Fig.1. Isolated orthogonal arrays, uranylacetate-stained. Fig.2. Isolated gap junction, uranylformate-stained. Both structures were prepared for microscopy from the same final sample of the isolation protocol (0.1 mg trypsin/ml 40 min 30°C). Fig.3. Isolated orthogonal arrays, monolayer-freeze-fractured. The single fracture-plane represents the P-face of the membrane. Fig.4. Isolated gap junction, monolayer freeze-fractured. The fracture-plane frequently jumps between the two junctional membranes thus revealing islands of E-face of one membrane above the particulate P-face of the other membrane. Fig.5. Freeze-fractured lens tissue kept at 37°C for 1 h before fixation with glutaraldehyde. Note the small orthogonal arrays in the freeze-fractured membranes. Fig.1–5 are displayed at the same magnification; the shadowing directions are from top and shadows appear black in fig.3–5. Fig.6. Thin-sectioned lens gap junctions from a sample treated with 10 mg trypsin/ml at 37°C for 1 h.

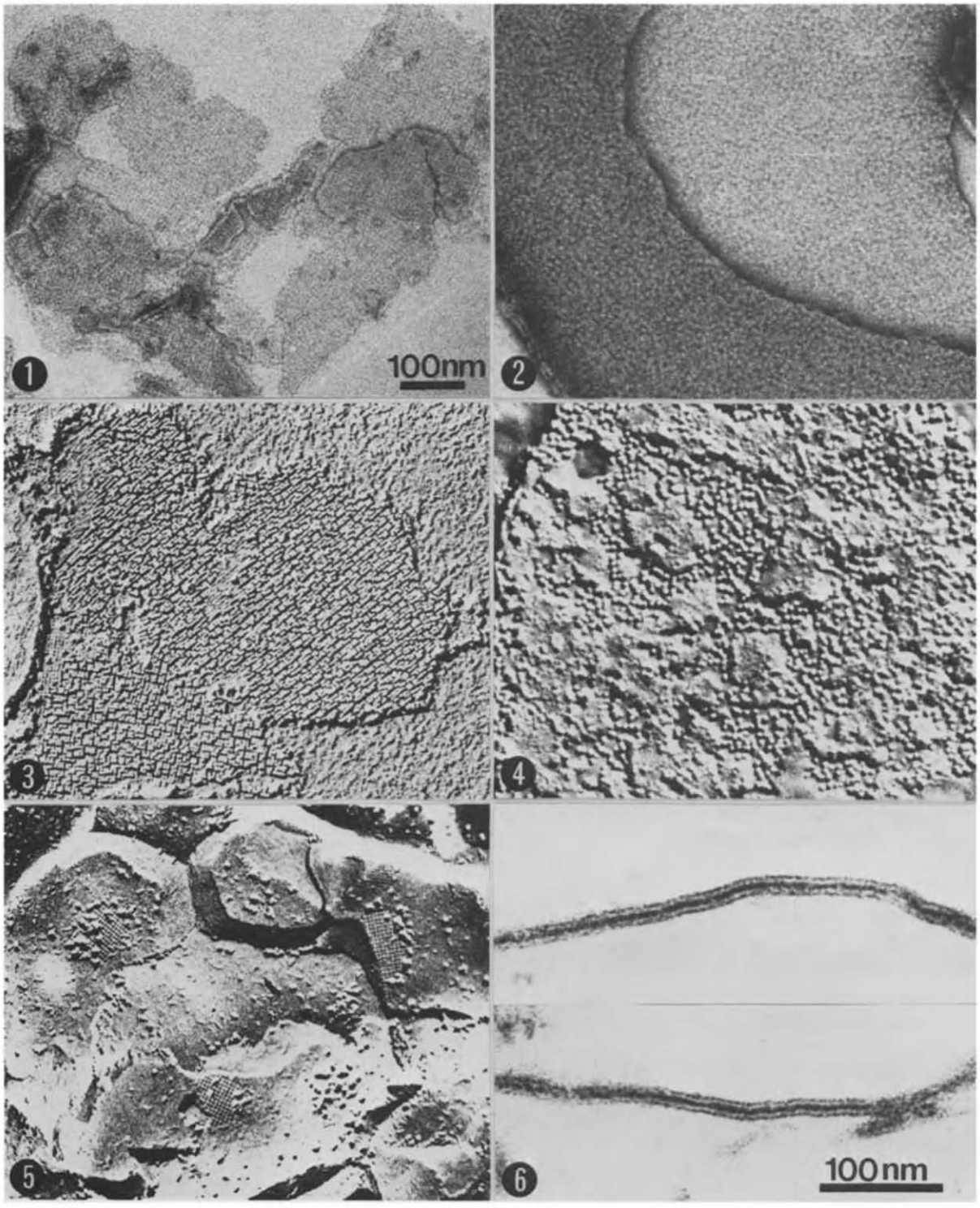

Fig.1–6

of those in [15], we believe that almost certainly they are the same structure. However our results indicate that their formation is not induced by lowering the pH, since in fact these arrays accumulate in the final samples of the isolation procedure when all solutions are buffered at pH 8.2.

3.2. *Mild proteolysis induces formation of orthogonal arrays*

The appearance of calf eye lens tissue in freeze-fracture preparations has been described in [1,4,23] but none report observing orthogonal arrays. In agreement with [1,4,23] we have not detected orthogonal arrays in freeze-fractured lens membranes when lens tissue blocks were fixed with glutaraldehyde at 4°C immediately after dissection. However upon storage of lens tissue blocks at 37°C for 1 h prior to fixation and freeze-fracture, small orthogonal arrays were observed (fig.5). To test the hypothesis that endogenous proteolysis may be involved in the formation of these small orthogonal arrays, we exposed isolated lens plasma membranes to buffers of both various pH (5.5–8) and various ionic strengths (1–100 mM) in the presence or absence of 0.1 trypsin mg/ml (samples without trypsin were kept at 0°C to prevent endogenous proteolysis). Twenty independent experiments were performed, each testing a set of conditions with and without proteinase and the samples analysed for the occurrence of orthogonal arrays by monolayer freeze-fracturing (in some experiments samples were examined before detergent-treatment, in others after). Our consistent finding throughout all experiments was that orthogonal arrays were formed only in those plasma membranes pre-treated with trypsin. In fact mild proteolysis (0.01 trypsin mg/ml 40 min at 0°C) was found to be sufficient to induce the formation of these arrays. We conclude that limited proteolysis of a lens membrane protein species leads to the formation of orthogonal arrays. This process occurs independent of pH at least for pH 5.5–8.

3.3. *Orthogonal arrays are not related to lens junctions*

To exclude the possibility that orthogonal arrays may arise from lens junctions by limited proteolysis of the junctional protein, we have treated the lens fibre plasma membranes with increasing amounts of trypsin during the isolation procedure. Samples were then analysed by electron microscopy. The rationale of this experiment was that if a precursor–product relationship existed between gap junctions and orthogonal arrays one might anticipate that increased proteolytic digestion would reduce the concentration of gap junctions whilst increasing the number of orthogonal arrays.

However isolated lens junctions revealed the characteristic unordered connexon arrangement by negative staining, irrespective of the pre-exposure trypsin concentrations whereas at high concentrations of trypsin the orthogonal arrays disappeared (section 3.1). In fig.6 the undisturbed double-membrane morphology of lens junctions treated with 10 trypsin mg/ml at 37°C for 60 min is confirmed by sectioning a pellet of the sample. From the structural integrity of the lens junctions following trypsin digestion we conclude that the orthogonal arrays, which are induced upon mild proteolysis, are not related to the junctions but are formed from a cleavage product of another intrinsic membrane protein species.

3.4. *Which membrane protein constitutes the orthogonal arrays?*

To further characterize the orthogonal arrays we have analysed the samples in section 3.3. by SDS gel electrophoresis (fig.7). With increasing proteolysis by trypsin a stable 21 000 mol. wt protein accumulates which is the cleavage product of the 26 000 mol. wt

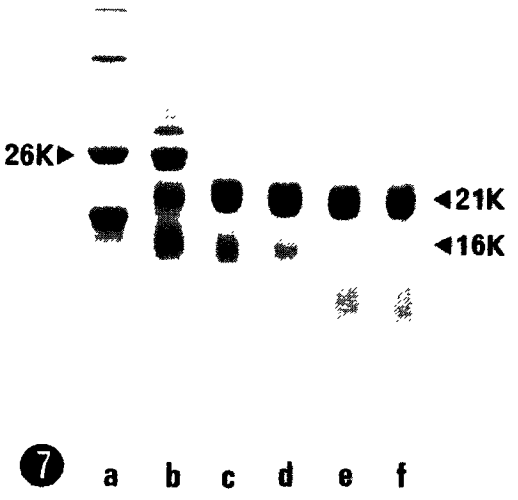

Fig.7. SDS gel electrophoresis of the final samples of the isolation protocol in section 2.1: (a) no trypsin; (b) 0.01 trypsin mg/ml 5 min 30°C: (c–f) all incubations with trypsin for 40 min at 30°C, (c) 0.01 mg/ml, (d) 0.1 mg/ml, (e) 1 mg/ml, (f) 10 mg/ml.

protein identified as the junctional component [2,17,24]. A further protein with app. mol. wt 16 000 appears under mild proteolysis conditions but is further digested at higher levels of trypsin. The intensity of the 16 000 mol. wt band alters in parallel with the occurrence of orthogonal arrays in negatively stained preparations of the final samples of the isolation procedure when increasing amounts of trypsin were used (section 3.1). Therefore we speculate that the 16 000 mol. wt protein is a component of the orthogonal arrays. Based on the data in [17] for the analysis of lens membrane proteins, we consider their protein 1B component to be the only species present in sufficient amounts to give rise to the amount of cleaved product which we observe on our SDS gels.

4. Discussion

Interest in lens gap junctions focusses on the fact that they can be isolated with the connexons randomly dispersed in the junctional membrane, a configuration believed to represent the low resistance state of the junction [10]. Exposing lens plasma membranes to pH 6–6.5 at 37°C induces the formation of orthogonal arrays, and by analogy to the experiments in [14] these orthogonal arrays were proposed to represent the lens junctions in their high resistance configuration induced by low pH [15]. Our results demonstrate that the orthogonal arrays are unrelated to gap junctions since their formation is induced by treating isolated lens membranes with low levels of trypsin, a treatment which leaves the gap junction structure intact even at higher levels of trypsin.

We have excluded the possibility that the orthogonal arrays are induced by exposure to low pH. However we consider it likely that in [15], endogenous proteolysis may have occured upon incubating the membranes at 37°C, and that the formation of these arrays has been induced by the cleavage of an intrinsic lens membrane protein other than the junctional component. This conclusion is supported by the experiment in which small orthogonal arrays appear in the lens plasma membranes upon storage of lens tissue blocks at 37°C for 1 h but not in tissue fixed immediately.

Other evidence supports the view that the orthogonal arrays are unlikely to be gap junctions:

(i) Isolated orthogonal arrays are single-layered; i.e., the two adjacent 'junctional' membranes would be separated from each other in the low pH-induced high resistance state which is unlikely in view of the reversibility of the uncoupling process [14];

(ii) We do not consider it likely that subunit conformational changes induced by Ca^{2+} and low pH may arrange the same randomly dispersed connexons into alternatively hexagonal and orthogonal arrays. In fact the differing symmetries of these two types of lattices imply completely different types of bonding between the individual protein subunits.

(iii) The stain penetrated centres of the connexons (thought to represent the trans-membrane channels) are typically found to be present in negatively stained specimens of gap junctions having the connexons arranged either randomly or hexagonally but are not observed at all in specimens of the orthogonal arrays.

Finally we point out the resistance of the cleaved junctional, 21 000 mol. wt protein to further degradation by proteolysis. Under the same conditions (10 mg trypsin/ml 1 h at 37°C) the 26 000 mol. wt protein of lens junctions is cleaved to 21 000 mol. wt and then remains stable, whereas the 26 000 mol. wt junctional protein from mouse liver is cleaved further to 13 500 mol. wt [9]. This may be accounted for by some differences in the amino acid composition of the two 26 000 mol. wt species, revealed by comparison between the data in [17] lens, and [9] mouse liver. However the difference in the resistance to proteolysis may also be related to the fact that isolated lens junctions basically exhibit the unordered connexon arrangement whereas isolated mouse liver junctions exhibit only the hexagonal arrays of connexons. The observed difference in proteolytic cleavage thus may represent a conformational change of the junctional protein associated with the transition from the low resistance to the high resistance state. We are now following up this idea.

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