Chapter 3

GAP JUNCTIONS: CORRELATED ELECTROPHYSIOLOGICAL RECORDINGS AND ULTRASTRUCTURAL ANALYSIS BY FAST FREEZING AND FREEZE-FRACTURING

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I. INTRODUCTION

The evolution of complex organisms depends on the appropriate coordination of growth, differentiation, and function of the participating cells. Without intercellular communication, neither plants nor animals would have developed. In animal cells the exchange of information is maintained by three different mechanisms:

1. Indirect signal transfer, when signal substances such as neurotransmitters, hormones, growth factors or humoral antibodies are released into extracellular fluids and react with congruous receptors located in the plasma membranes of target cells. This signal is translocated across the membrane into the cytoplasm where second messengers transmit the information.
2. Direct signal transfer from cell to cell by plasma membrane bound receptors, as is the case in cell-mediated immune response or during sperm-egg-binding.
3. Direct signal transfer from cell to cell by proteinaceous channels, which provide hydrophilic paths across the plasma membranes of adjacent cells. These so-called gap junctions are bidirectionally permeable for both charged and neutral molecules, and also connect adjacent cells mechanically.

A communicating gap junction channel consists of two hemichannels, the connexons, to which both contacting cells contribute.[1,2] Each connexon can be subdivided into six subunits (for review see Revel et al.[3]). Up to several hundred channels are assembled to form a typical gap junction plaque (for review see Bennett and Goodenough[4]); the channel density in the plasma membrane may reflect their activity.[5-13] Gap junctions are ubiquitously found in the fauna, from mesozoa to vertebrates. Not only regulation of embryonic development, cell differentiation, and growth control depend on the existence of gap junctions, but also the synchronous beating of heart muscle cells and the coordinated contractions of smooth muscle cells in the intestine (for review see Pitts and Finbow[14]).

Gap junctions are very often established in permanently growing cell cultures, which adhere to the surface of plastic Petri dishes. These monolayer cultures facilitate investigations of gap junction properties, since individual cells can easily be discriminated under light-microscopical observation. They led to the detection of metabolic cooperation, which allows cells with certain enzymatic defects to proliferate without a substitution for the missing enzyme or its product, as long as they are coupled to wild type cells by gap junctions.[15] Using fluorescent dyes of different sizes, it has been detected that the upper limit for molecules which can pass intercellularly via gap junctions in vertebrate cells is about M_r = 900.[16] Since, in most cases, the intercellular signal which is exchanged between the cells is not known, artificial signals are used for the demonstration of open gap junctions. A favorite tracer is the fluorescent dye Lucifer Yellow, which can be injected iontophoretically or by pressure into a cell, and its spreading into adjacent cells can be followed under an epifluorescent microscope.[17] If Lucifer Yellow is retained in the injected cell, this block of dye coupling does not necessarily indicate a complete closure of gap junction channels, since ionic coupling still may be observed.[18,19] Thus, the gap junctional conductivity is not a constant value, but is part of the dynamic regulation processes in cells.

The opening and closing of gap junction channels is influenced by two endogenous signal pathways, using as second messengers cAMP or diacylglycerol.[20,21] The cAMP signal pathway opens gap junction channels, whereas the diacylglycerol signal pathway down-regulates the gap junction permeability. Besides a possible regulation by phosphorylation, the fine tuning may well be different in different cells, since many other substances with regulatory effects have been described.[22] However, not only physiological interactions by pH, Ca^{++} and retinoic acid, but also nonphysiological reactions by heptanol, benzhydrol, or glutardialdehyde cause a closing of gap junction.

The opening and closing characteristics of a single gap junction channel can be followed by patch clamp measurements, where the current across a channel can be determined. Depending on the type of experiment ("whole cell" recording with a cell pair, or "cell attached" measurements), only open and closed states have been determined,[23-25] or several states have been described for connexons.[26,27] In the latter case, the final proof for a gap junction channel—the test with blocking antibodies— is still missing.

Gap junctions can be demonstrated in the electron microscope after conventional fixation and staining procedures. On ultrathin sections of embedded cells they are characterized by a 2 to 3 nm wide gap between two contacting membranes, which is bridged by regularly arranged particles. When using lanthanum for membrane impregnation, the gap between the junctional proteins is filled with this electron dense material, and these proteins appear brighter than the lanthanum-filled space.[2] Larger areas of gap junction plaques can be visualized when cells are freeze-fractured and their membranes are replicated. Both the embedding procedure and the freeze-fracture techniques are conventionally performed with chemically fixed cells. In most cases, glutardialdehyde is used as a fixative, which, however, causes always irreversible uncoupling of gap junction channels.[9,12,28] It is, therefore, impossible to demonstrate open gap junction channels in such pretreated cells. The question remains, whether the *in situ* arrangement of gap junction plaques— which may consist of several hundred or thousand individual gap junction channels— is unaffected, or if the pattern of these plaques is also altered under these conditions.

Rapid freezing of biological material is considered as a method which may overcome the artifacts caused by chemical fixation and infiltration with glycerin. According to Moor[29] a freezing rate of 10^4 Ks^{-1} is necessary for the preservation of a structure in its native state. This vitrification, therefore, should be a preferential method when gap junction structures are to be investigated. Unfortunately, biological material has a poor temperature conductivity and—when cryoprotectives or high pressure cryofixation should be avoided—it can be vitrified only within a thickness of about 10 to 30 μm,[30,31] which depends on the preparation techniques (for review see Plattner and Zingsheim[32]). It is obvious that the biological probe must be very small and that a cryogen must be used which is characterized by high freezing rates.

II. MATERIALS AND METHODS

A. CELL CULTURES

Two permanently growing tumor cell lines have been used in this study; BICR/M1R_k, a neoplastic cell line in monolayer culture derived from a spontaneous mammary tumor of the Marshall rat,[33] and BT5C1, a neoplastic neurogenic cell line derived from cultures of fetal brain cells of BD IX rats.[34] These cells were transferred into monolayer culture at 90 h after an i.v. transplacental pulse of 75 μg of N-ethyl-N-nitrosourea per g body weight of the pregnant female (18th day of gestation). After an average period of 202 d, these cultures underwent malignant transformation.[34]

Cells were cultured in plastic flasks (Falcon 3024, Falcon Plastics, Oxnard, CA,) at 37°C, using modified[35] Eagle-Dulbecco medium supplemented with 10% calf serum, under an atmosphere of 5% CO_2 in air. The medium was renewed at 2- to 3-d intervals, and cultures were passaged at confluency by trypsinization [0.25% trypsin in Ca^{2+}-Mg^{2+}-free isotonic phosphate-buffered saline (PBS; pH 7.2 to 7.4)].

For the electrophysiological experiments, cells were grown in plastic Petri dishes (Falcon 3002, 60 × 15 mm), and fused by treating almost confluent monolayers with 40% (w/w) polyethylene glycol (PEG, molecular weight 1540, Koch Light Ltd., Haverhill, Suffolk, U.K.) for about 2 min at room temperature, followed by careful washing in medium and incubation at 37°C for about 4 h.[36] The homokaryons were trypsinized and transferred to

HEPES-buffered medium. Pairs of small homokaryons (about 5 nuclei/cell) were selected for the uncoupling experiments with glutardialdehyde. For the electron microscope preparations the cells were grown in Optical Tissue Culture Film Liners with Holders and Lids (Falcon 3006, 60 × 15 mm), or on plastic coverslips (Thermanox, Lux 5408, Miles Laboratories Inc., Naperville, IL,) in plastic Petri dishes (Falcon 3002).

B. ELECTROPHYSIOLOGY

Electrical coupling was determined by measuring the intercellular electrical signal transfer across the gap junctions with three glass microelectrodes.[37] They were pulled out of glass capillaries (outer diameter 1 mm, with inner filament, Hilgenberg Glas, Malsfeld, F.R.G.) in a vertical pipette puller (700 C David Kopf Instruments, Tujunga, CA, U.S.A.), and backfilled with 3 *M* KCl. A high-impedance amplifer (WPI 750, World Precision Instruments, New Haven, CT,) was used to measure the membrane potentials in two cells, and into one of them rectangular current pulses (generated by a GX 239 pulse generator, ITT-Metrix, Paris, France) were injected via a WPI 701 amplifier. These signal pulses V_1 were superimposed onto the cell's membrane potential and—when the cells were electrically coupled—signal pulses V_2 could be recorded in the neighboring cell, indicating the extent of coupling by the ratio V_2/V_1. These measurements were registered together with the membrane potentials pd_1 and pd_2 on a strip-chart recorder (Servokass 600, Gebr. Laumann, Selb, F.R.G.). In addition, this method also allows the determination of the cellular input resistance, which is the effective transmembrane resistance seen by the membrane potential recording electrode. The coupling capacity of the cells was also determined by iontophoretic injection of the fluorescent dye Lucifer Yellow (Sigma L-0259, St. Louis, MO; 4% in LiCl) into one cell, and observation of the dye spreading into the coupled cells.[17] These measurements were performed with electrically driven micromanipulators (Märzhäuser, Wetzlar, F.R.G.) under inverted microscopes (Epivert, Leitz, Wetzlar, F.R.G., or IM 35, Zeiss, Oberkochen, F.R.G.).

Patch clamp experiments[38] were carried out for the characterization of the connexon conductivity,[26] as well as for the measurement of intercellular coupling in isolated cell pairs.[23-25] Patch pipettes were pulled out of soft glass (Micro-Haematocrit-tubes No. 564, Bardram, Birkerod, Denmark) with a vertical electrode puller (L/M-3P-A, List, Darmstadt, F.R.G.), and filled with a patch solution (120 m*M* KCl, 5 m*M* EGTA, 10 m*M* HEPES, adjusted to pH 7.3 with KOH). Cell attached- as well as whole cell-recordings were measured with patch clamp amplifers (L/M-EPC 7, List). Observed channel activities were digitized with a pulse code modulator (PCM-501 ES, Sony, Japan), stored with a video recorder (Ultravideo 6A10 VHS, Saba, Villingen-Schwenningen, F.R.G.), and analyzed by an IBM-PC AT compatible computer with a program designed according to McCann et al.[39] using Asyst as a scientific software (Asyst, Keithley Instruments GmbH, München, F.R.G.).

C. ELECTRON MICROSCOPY

In situ freeze fracturing of cell monolayers was performed by a procedure modified after Pauli et al.,[40] including some advice given by Dahl[41] and Dermietzel.[42] Eighty ml PBS (pH 7.45) and 20 ml of 100% glycerol (Merck, Nr. 4093, Darmstadt, F.R.G.) were mixed at room temperature, and under continuous stirring, 20 g polyvinyl alcohol (15,000, Fluka, Buchs, Switzerland) were added slowly. The stirring was continued over night at room temperature till the mixture was centrifuged at 27,000 g for 30 min. The supernatant dimethylsulfoxide (DMSO, Serva, Nr. 20385, Heidelberg, F.R.G.) was added to a final concentration of 2%; fungal growth was prevented by a crystal of thymol. This polyvinyl alcohol solution was heated to 90°C for 40 min, portioned into Eppendorf tubes, frozen, and stored at −20°C.

For freeze fracturing a portion was slowly warmed to room temperature, and a droplet

of polyvinyl alcohol was allowed to polymerize on a gold specimen carrier (3.0 mm diameter, Balzers, Liechtenstein) for about 5 min. Falcon Film Liners or Thermanox coverslips were cut to 2 × 2 mm squares, adhesive monolayer cells were washed carefully with PBS, the remaining fluid was drained with a filter paper, and the coverslip was placed with the cells on the highly viscous polyvinyl alcohol droplet. This sandwich was immediately plunged into liquid propane (about 90 K); after about 10 s the specimen carrier was mounted on a liquid nitrogen (LN_2) cooled object table (BB 172 058-U, Balzers), and transferred into a Balzers 301 instrument on the 123 K cold rotary stage (BB 176 312-T, Balzers).

When a vacuum of about 130 μPa was reached, the cooled (123 K) microtom knife was placed under an edge of the plastic square and lifted till the coverslip separated from the polyvinyl alcohol. Thus, preferentially, cell membranes sticking to polyvinyl alcohol were fractured and immediately replicated by shadowing with platinum/carbon under 45° to about 2 nm thickness by a high voltage evaporation device (EVM 052A, Balzers) under thickness control with a quartz crystal thin film monitor (QSG 201D, Balzers), and stabilized by perpendicular carbon evaporation (~20 nm). Replicas were separated from the polyvinyl alcohol by immersing the specimen carrier into 0.9% NaCl solution. They were cleaned overnight in 12% sodium hypochloride solution (Hedinger, Stuttgart, F.R.G.), washed three times in distilled water, and transferred onto copper grids (G 300 HS, SCI, Science Services, Munich, F.R.G.). Replicas were investigated in a Zeiss EM 10 transmission electron microscope; pictures were taken at magnifications between 20,000 and 40,000 on High Speed Clear Negative Film (HS-7 Cronalar Dupont Ltd., St. Neots, Huntington, U.K.).

For comparison, cells were also fixed with 2% glutardialdehyde (electronmicroscopy grade, Nr. 4239, E. Merck, Darmstadt, F.R.G.) in PBS for 30 min, washed several times in PBS, and infiltrated with glycerol solutions in PBS with increasing concentration to a final glycerol concentration of 30%. After infiltration over night at 4°C, the cells were either sandwiched with polyvinyl alcohol, as described above, or collected with a rubber policeman, pelleted, sandwiched between two gold specimen carriers (4.6 mm diameter, Balzers), frozen in LN_2, and fractured on a double replica table (BB 172 137-T, Balzers).

D. IMAGE ANALYSIS

For the analysis and quantitative evaluation of gap junction replicas, a computerized image processing system was used. These programs have been established at the Institut für Physikalische Elektronik (University of Stuttgart), and have been adapted for the pattern analysis of gap junction plaques.[12] The electron microscopical micrograph negatives were digitized by an A/D converter, resulting in images with a resolution of 256 gray levels, which were stored on tape. These data were then analyzed on a minicomputer (VAX, DEC, Maynard, MA). Local gray level maxima were determined in selected gap junctional areas. By shrinkage of these local maxima to single points, a matrix was formed which contained all spacings between these points, i.e., particles on PF-face leaflets and pits on EF-face leaflets. These parameters characterize the gap junction pattern in a computer-applicable mode. The center-to-center spacings between all skeletonization points were classified by 1 nm step, and spacings between 5 nm and 25 nm were included for subsequent determination of frequency distributions and statistical anaylsis, which revealed nearest neighbor distances as well as particle densities.

III. RESULTS

A. GAP JUNCTION PERMEABILITY

Under normal culture conditions it is difficult to increase the gap junctional conductivity of BICR/M1R_k- or BT5C1-cells by substances which are effective in primary cultures or *in vivo*.[19,43,44] These substances are known to interfere with the synthesis of the intracellular

second messenger cAMP (3′,5′-cyclic adenosine monophosphate), e.g., forskolin or methylisoxanthin, or they substitute its function, as does dBcAMP (dibutyryl-cAMP). As monolayer cultures, these two cell lines seem to be completely upregulated; their gap junctional conductivity, however, can be downregulated by retinoic acid, intracellular pH-shifts to values lower than pH 6.8, and by unphysiological substances such as benzhydrol, heptanol, and glutardialdehyde.[19]

The extent of gap junctional coupling in BICR/M1R$_k$ cells is demonstrated with a dye-spreading experiment, where cells were in contact only with some fine cellular protrusions, as is shown in Figure 1a. Two min after injection of Lucifer Yellow into the cell located in the center of this group, the dye had spread into all cells which were in direct contact with the injected one, and into some indirect neighbors, as is revealed from the same spot under epifluorescent illumination (Figure 1b).

As most other channels in the plasma membrane, individual gap junction channels are not permanently open, but may open or close in response to cellular regulation processes. Thus, *in situ*, an active gap junction plaque consists of channels which can be open or closed at a given time. It is difficult to follow this dynamic process by voltage measurements or by dye spreading experiments, as both determine the total conductivity of gap junction plaques. It can be detected, however, in the cell attached mode of the patch clamp technique, which resolves the current through one or few individual channels. Examples for channel characteristics are shown in Figure 2, where two different conductivity levels of an individual channel (connexon?) were measured with a BICR/M1R$_k$-cell (Figure 2a), and four different levels with a BRL-cell (Figure 2b). Their maximum conductivity of about 250 to 320 pS is of the same order as has been described for gap junction channels,[24,25] which consist of two hemichannels.

Conventional preparation methods for freeze fracturing or embedding in epon requires chemical fixation of the biological material. Very often glutardialdehyde is used, which, however, closes the gap junction channels irreversibly within 1 or 2 min. This uncoupling effect of glutardialdehyde is demonstrated with homokaryons of BICR/M1R$_k$-cells in Figure 3. The lower trace shows the rectangular current pulses which were fed into cell 1 (middle trace), where they caused voltage changes V_1 (proportional to the input resistance) superimposed to the cell's membrane potential (pd_1). Cell 2 (upper trace) was electrically coupled to cell 1, as can be seen by the voltage changes V_2 superimposed to the membrane potential of cell 2 (pd_2). The arrow indicates the timepoint when 0.5 ml of 2% glutardialdehyde was added to the dish so that a final concentration of about 0.5% was reached. The effect of glutardialdehyde can be seen about 1 min later (start of reaction: timepoint "0"), when it had diffused across the impaled cells. Due to a reaction of glutardialdehyde with the plasma membrane, the input resistances of both cells increased, which is indicated by higher amplitudes of both V_1 and V_2. The coupling ratio V_2/V_1, however, remains unaffected at about 0.65. Shortly thereafter (1.84 ± 0.21 min; mean $\pm$ standard error; $n = 70$) the cells started to uncouple, as can be seen from the V_2-amplitudes, which decreased continuously because less current passed when gap junction channels closed. Consequently, this uncoupling from neighboring cells further increased the input resistance in cell 1, as is indicated by the growing V_1-amplitude. Finally, when the coupling ratio V_2/V_1 was below 0.08, the cell membranes suddenly became very leaky, as is indicated by the collapse of the membrane potentials.

The same uncoupling effect of glutardialdehyde is also demonstrated using the patch clamp technique for whole cell recordings with a pair of BT4C1-cells (Figure 4). The upper trace represents cell 1, where the voltage was pulsed between -5 and -15 mV; the lower trace represents cell 2 where the voltage was clamped to a constant value of -10 mV. For both cells only the currents necessary for maintaining the preset voltage clamp conditions are shown. The arrows indicate the time when the medium was replaced by 0.2% glutar-

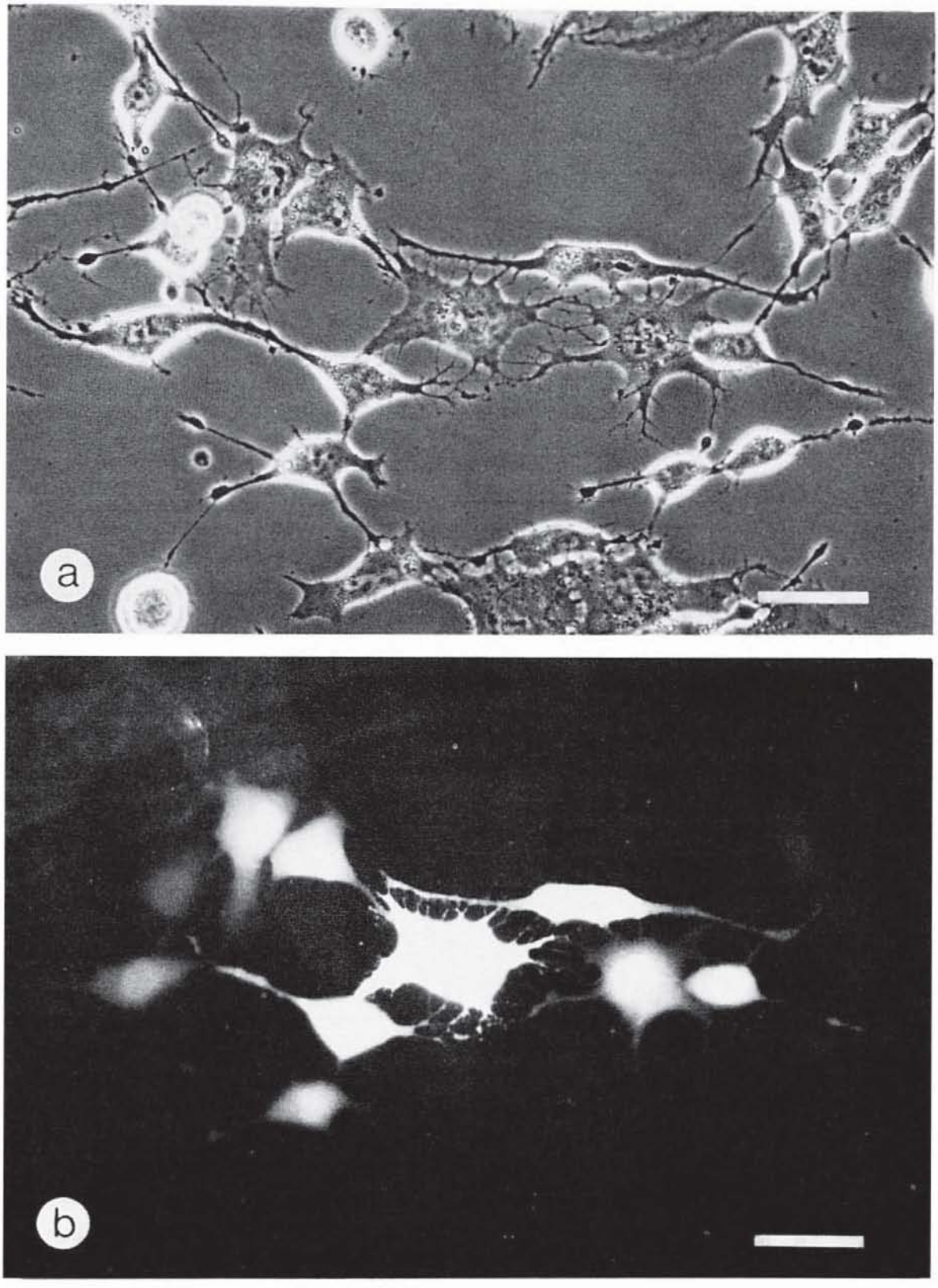


FIGURE 1. Intercellular communication between mammary tumor cells BICR/M1R$_k$ in culture demonstrated with Lucifer Yellow. The fluorescent dye was iontophoretically injected with a glass micropipette into the cell located in the center. Spreading of Lucifer Yellow through gap junctions into all directly and into some indirectly coupled cells was documented 2 min after the injection. (a) phase contrast, (b) epifluorescent illumination. Bar: 50 μm.

dialdehye in PBS. This led to a decrease in junctional coupling, as can be seen from the reduction of the current in cell 2. Assuming a conductivity of about 150 pS[24,25] for a fully open gap junction channel, these two cells were originally coupled by at least 220 open channels, whereas 3 min after the addition of glutardialdehyde, a conductivity of about 2

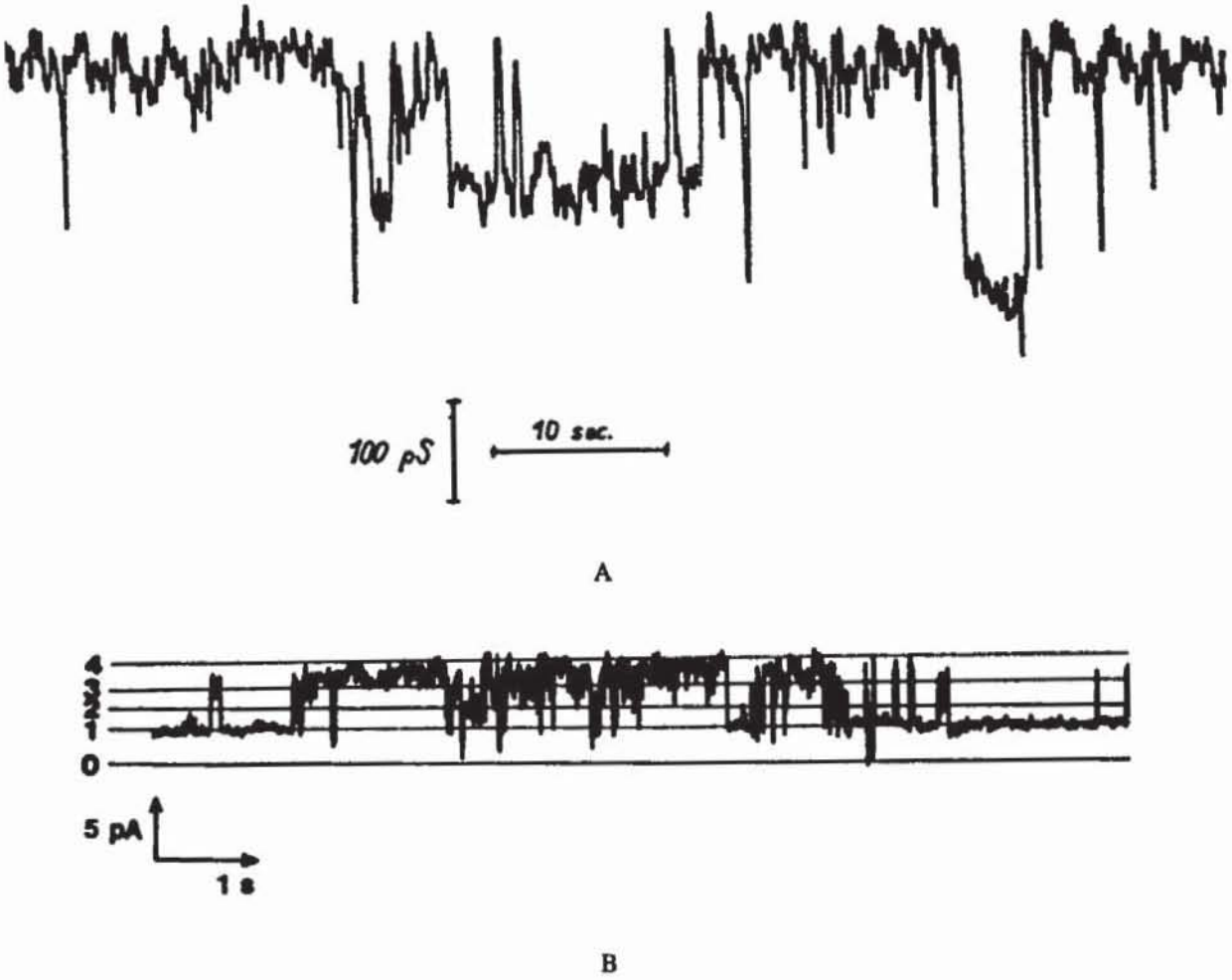


FIGURE 2. Patch clamp measurements of channels (connexons?) with different conductivity levels. (A) Mammary tumor cell BICR/M1R$_k$ (B) buffalo rat liver (ATTC CRL 1442).

nS was measured. This corresponds to about 10 to 15 fully open channels, or accordingly, more channels with reduced conductivities.

B. MORPHOLOGY OF GAP JUNCTION PLAQUES

Glutardialdehyde does not preserve gap junction channels in their open state, and it cannot be excluded that the gross morphology of gap junction plaques is also affected. Rapid freezing without preceding addition of chemical fixatives should, therefore, give some information about the *in situ* structure of gap junction plaques.

After conventional fixation with glutardialdehyde, and with 30% glycerol as a cryoprotectant, gap junctions between BICR/M1R$_k$-cells and between BT5C1-cells showed a typical clustered arrangement of tightly packed channels within a plaque (Figure 5). This crystalline-like structure normally identifies gap junctions, especially when both PF-face particles and EF-face pits are preserved and replicated at the same plaque. Nonjunctional membranes are characterized by a homogeneous distribution of membrane particles in PF-face leaflets, and by a low particle density in EF-face leaflets. When the cells were rapidly frozen by dipping a sandwich of plastic, polyvinyl alcohol, and gold specimen carrier into liquid propane, the fractured membranes were less smooth; PF-face leaflets revealed unevenly distributed particles, but also particle-free areas, and EF-face leaflets appeared rather particle-free. Under this condition, tightly packed gap junction plaques also have been found (Figures 6a and 8b), which, however, were always smaller than in fixed preparations. In addition, many loosely packed clustered gap junction plaques could be detected (Figures 6b, 7a, and 8a), where the particle density and cluster size varied considerably. Whereas all these structures

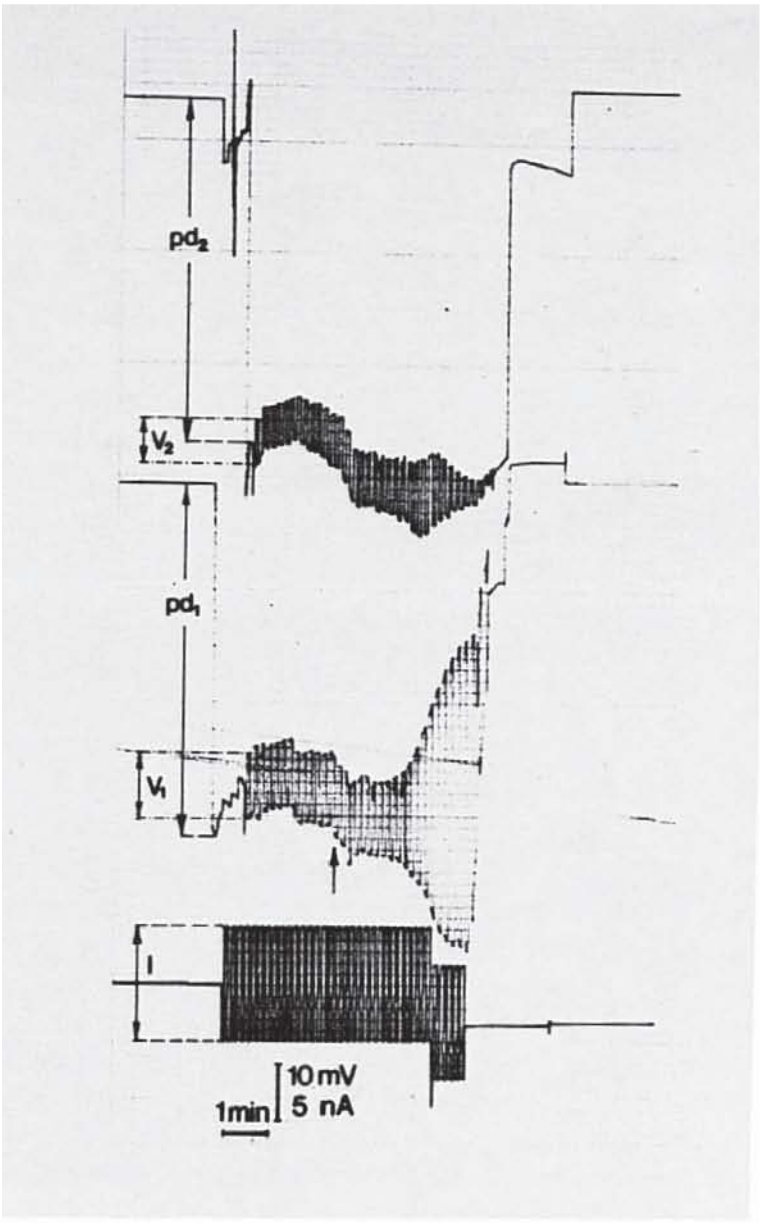


FIGURE 3. Uncoupling effect of glutardialdehyde. Electrically coupled homokaryons of BICR/M1R$_k$-cells were treated with 0.2% glutardialdehyde (arrow), which resulted in an increase of the membrane input resistance, an uncoupling of cell 2 (upper trace), and a collapse of the membrane potentials, within 3 min. For details see text. pd_1 and pd_2: membrane potentials of cell 1 and 2; V_1 and V_2: voltage changes in cells 1 and 2, due to the injected current pulse I in cell 1.

may well represent gap junctions, it is difficult to decide whether the particle aggregations shown in Figures 7b and 8c also consist of gap junction proteins.

A quantification of the different gap junction plaques has been performed by a pattern analysis for both fixed and unfixed BT5C1-cells. The mean particle density ($\pm$ SE) measured on PF-replicas for conventionally treated and fixed cells is 5,408 $\pm$ 340 μm^{-2}, and 2,685 $\pm$ 340 μm^{-2} for unfixed cells. Comparison with frequency distributions of trigonal, tetragonal, and hexagonal patterns revealed a rather tetragonal packing of gap junction proteins with mean values for the nearest neighbor distance ($\pm$ SE) of 7.08 $\pm$ 0.12 nm for the fixed, and 9.33 $\pm$ 0.49 nm for the unfixed preparations. BICR/M1R$_k$-cells have only been analyzed for fixed and conventionally frozen monolayer pellets, where the nearest neighbor distance was 6.70 $\pm$ 0.18 nm, and the particle density 6,990 $\pm$ 476 μm^{-2}.

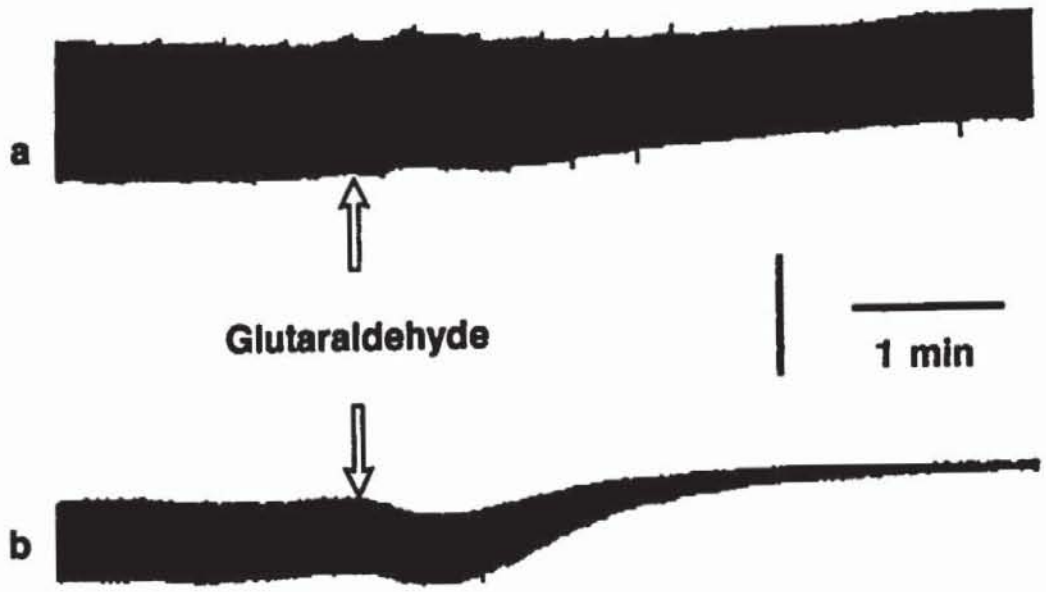


FIGURE 4. Uncoupling effect of 0.2% glutardialdehyde in BT5C1 cells measured with the patch clamp technique in whole cell configuration. Trace a: current in cell 1, trace b: current in cell 2; the amplitude is proportional to the degree of coupling. Horizontal bar: 1 min. Vertical bar: 1 nA (trace a), 0.5 nA (trace b).

IV. DISCUSSION

BICR/M1R$_k$- and BT5C1-cells are always very well coupled when grown as monolayer cultures (Figures 1, 3, and 4), but even with their gap junctional conductivity completely upregulated, individual channels may well vary in their conductivity levels. This cannot only be seen from patch clamp experiments where different conductivity levels of large channels are resolved (Figure 2), but also from simultaneous measurements of dye spreading and ionic coupling,[18,19] where ionic coupling can still be detected when dye spreading is already blocked.

With an open bore size between 1 and 2 nm[45] for a channel, it can be doubted that open channels in a plaque could be discriminated from closed ones when the membrane is replicated by Pt/C evaporation. From this point of view, it should be irrelevant whether fixed or unfixed material is used for the demonstration of gap junctions. However, when two cells initiate intercellular coupling, the gap junction conductivity increases with a cooperative characteristic, which indicates that more than one channel is involved in the formation of a stable contact.[46] These electrophysiological measurements correspond very well with the often documented ultrastructural morphology of freeze fractured gap junctions, where many individual channels are always arranged to aggregates.

The question arises whether the particle packing within a plaque may give some information on the coupling state of the cell, or of the conductivity of the gap junction plaques. Several experiments have been carried out to correlate the structure of freeze fractured gap junctions with their functional state;[5-13,47-50] however, the results were equivocal. In retrospect, this may be explained by the preparation techniques used before, which did not always preserve the *in situ* situation. Conventional preparation methods require fixation with glutardialdehyde, which causes irreversible uncoupling of the cells, as can be seen in Figures 3 and 4.

The morphology of unfixed gap junctions ranges from loosely to tightly packed plaques, whereas in fixed cells, loosely packed gap junction plaques are missing. The particle diameter is similar under both preparation techniques. Do these structures represent different dynamic states of gap junctional activity, or have artifacts been induced? The electrophysiological measurements revealed that it takes some time before the cell depolarizes after the addition

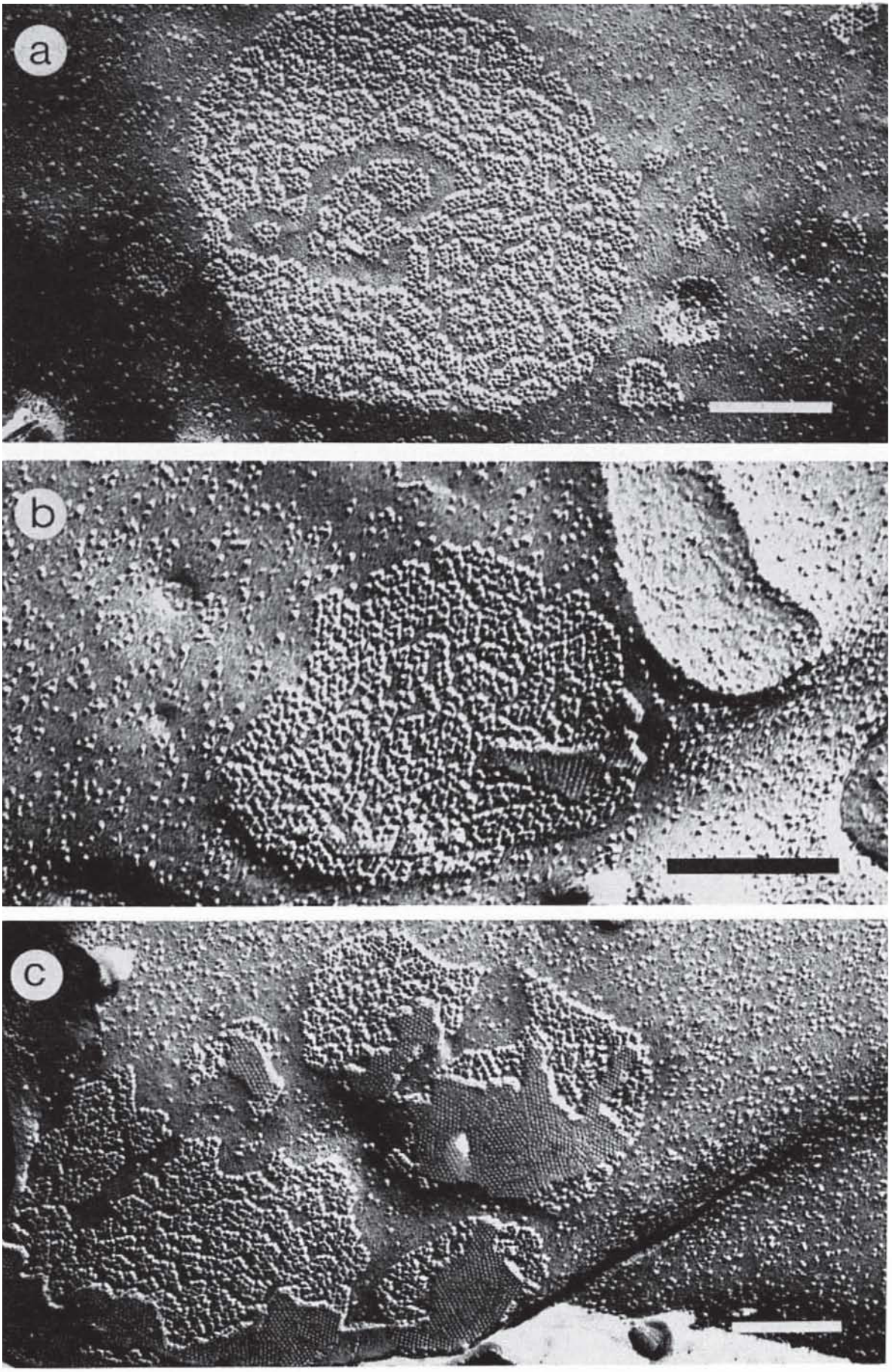


FIGURE 5. Gap junctions between glutardialdehyde-fixed cells. (a) BT5C1-cells, (b) and (c) BICR/M1R$_k$-cells. Bar: 0.2 μm.

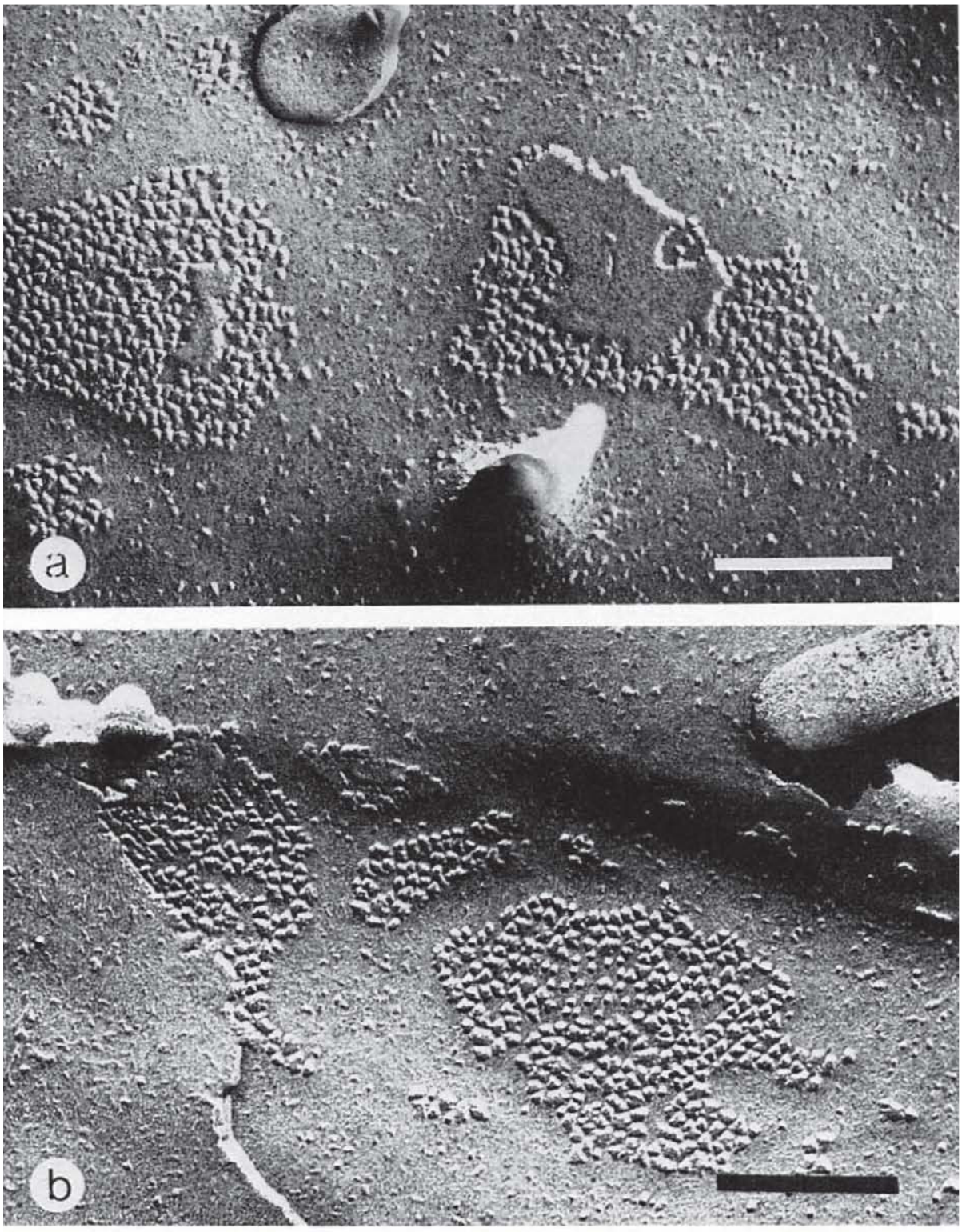


FIGURE 6. Gap junctions between unfixed BT5C1-cells, rapidly frozen by dipping a plastic-polyvinyl-sandwich in liquid propane. (a) tightly packed, (b) loosely packed and clustered. Bar: 0.2 μm.

of glutardialdehyde, even when some channels may close immediately. The depolarization may be accompanied by a collapse of electrical charges of proteins, which—in addition to the crosslinking property of glutardialdehyde— may result in a closer aggregation of the gap junction particles. On the other hand, a 5 min fixation with glutardialdehyde and freezing of BICR/M1R$_k$-cells by dipping them as a sandwich with 2 gold specimen carriers into liquid Freon® revealed gap junction patterns which were indistinguishable from propane jet preparations, i.e., loosely as well as tightly packed gap junction plaques. However, fixation

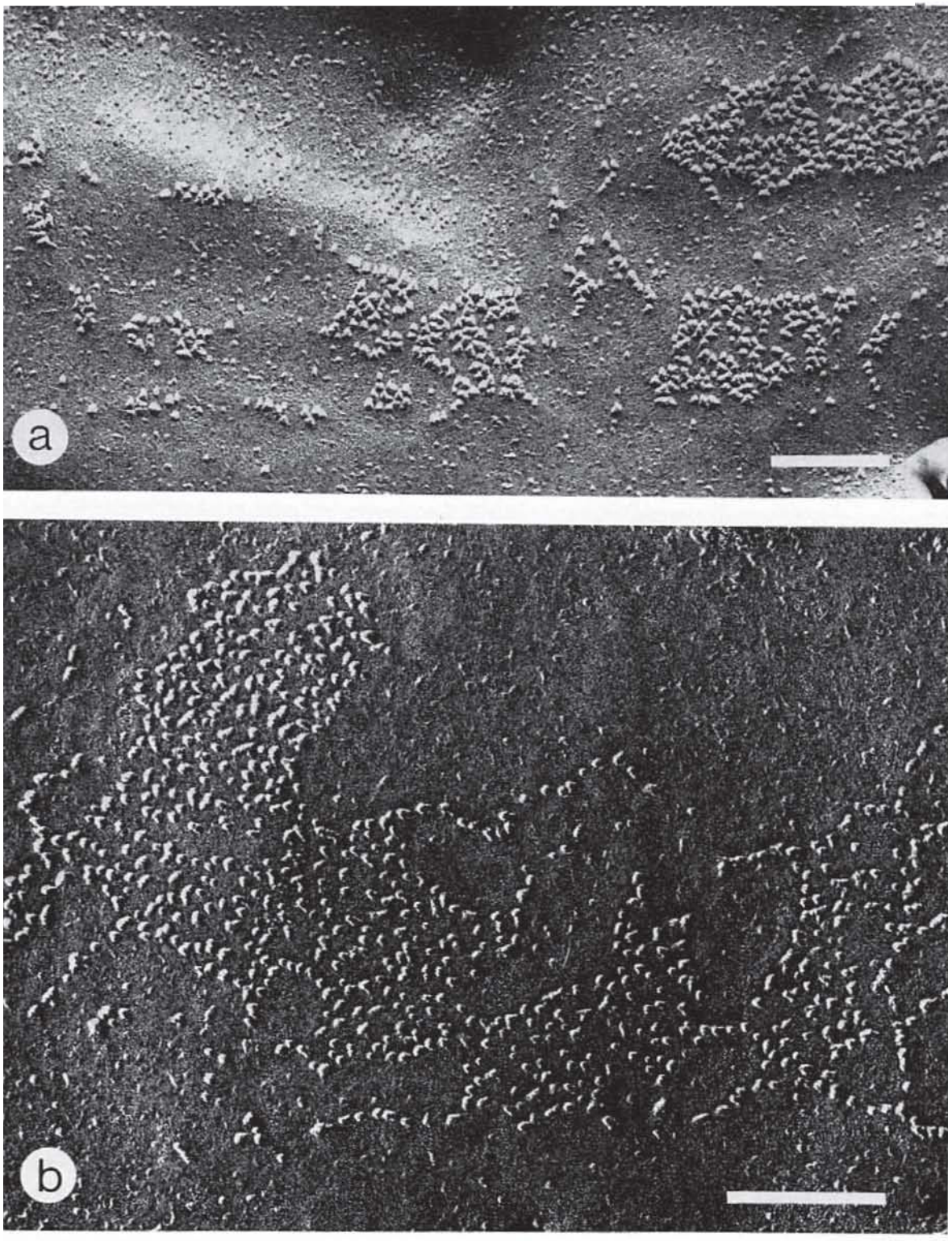
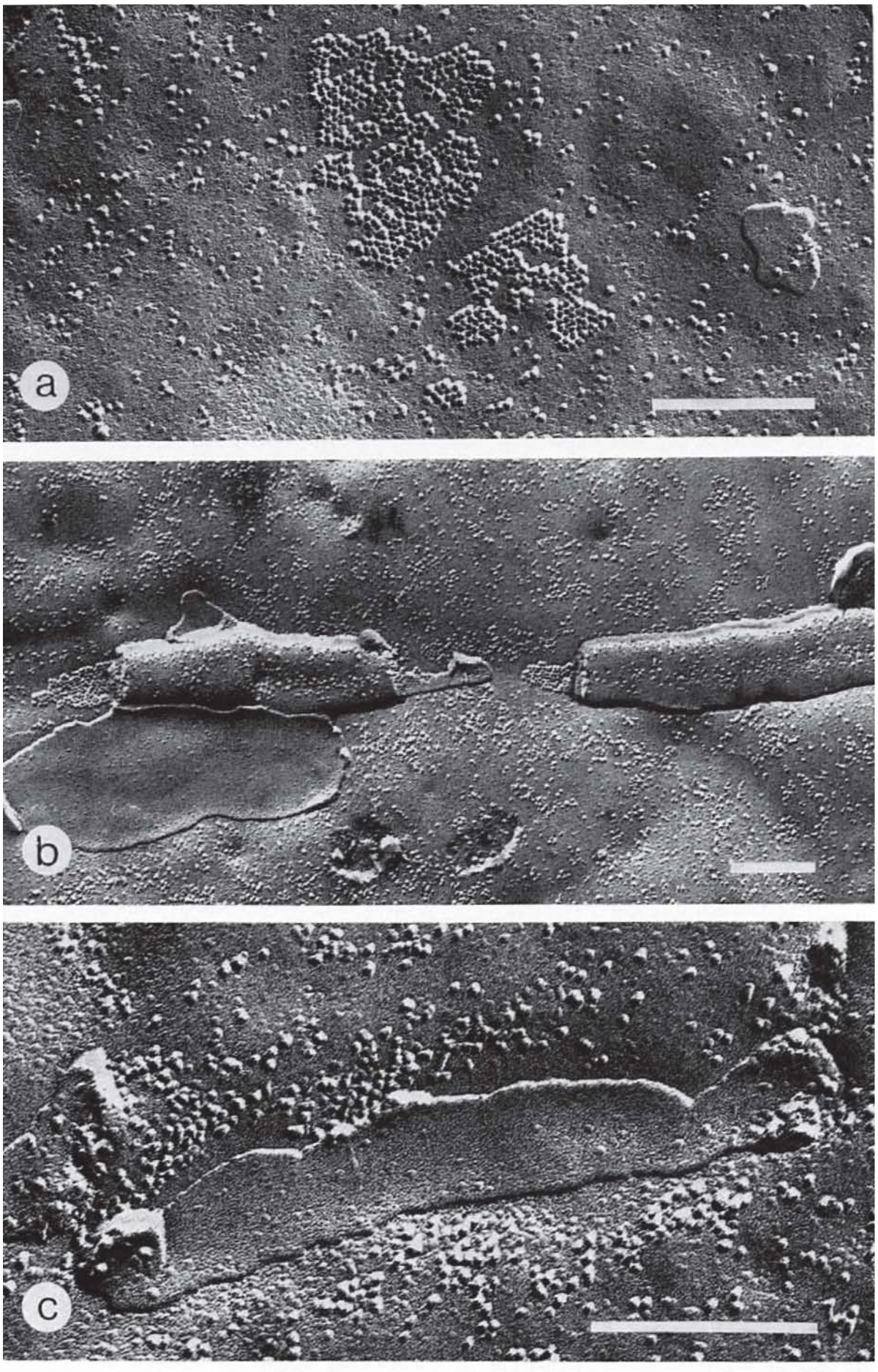


FIGURE 7. Gap junctions between unfixed BT5Cl-cells, rapidly frozen by dipping a plastic-polyvinol-sandwich in liquid propane. (a) Clustered and loosely aggregated particles, (b) loosely aggregated particles (gap junction?). Bar: 0.2 μm.

for 30 min and infiltration with glycerol increased the number of tightly packed gap junction plaques considerably.[12] Similar results have been reported for pancreatic acinar cells[47] and for heart cells.[7,49,50] Thus, a closing of most gap junction channels within 3 min is not necessarily accompanied by changes of the gap junction plaque morphology.

But why then are tightly packed gap junction plaques also found in unfixed cells? Interestingly, in thin sections of BICR/M1R_k- and BT5Cl-cells sometimes internalized gap junctions can be detected,[51] as have been found in many other tissues.[51,52] These internalized



IGURE 8. Gap junctions between unfixed BICR/M1R$_k$-cells, rapidly frozen by dipping a plastic-polyvinol-sandwich ı liquid propane. (a) Loosely packed and clustered, (b) tightly packed, (c) loosely aggregated particles (gap junction?). ar: 0.2 μm.

gap junctions might provide a clue for the interpretation of unfixed gap junction plaques: if one assumes that gap junction proteins have a limited life span and will be recycled, then the densely packed structure is the final state of a gap junction plaque before the proteins are internalized, as membrane receptors are collected in coated pits. Consequently, the loosely arranged particles may represent active gap junction plaques, where channels open and close to regulate the intercellular signal and metabolite transfer. Furthermore, it explains why both tightly and loosely packed gap junction plaques are found in unfixed material. Under these circumstances, i.e., with fluent transitions in the morphology of gap junction plaques, a pattern analysis cannot be very helpful in discriminating different functional states of gap junctional activities.

Rapid freezing should cause fewer alterations in biological membranes than fixation with glutardialdehyde and subsequent addition of glycerol as a cryoprotectant. According to Knoll et al.,[53] the cooling rate for a copper-Thermanox-sandwich is several times lower when dipped by hand into propane than with a propane jet application, which reaches a cooling rate of about 10^4 Ks^{-1} for monolayer thicknesses. This condition is certainly not fulfilled for the polyvinyl alcohol technique with monolayer cells grown on thin Film Liners or on Thermanox coverslips. The electron microscopic experiments show, however, that the obtained cooling rate is sufficient for the preservation of plasma membranes, since the formation of ice crystals is obviously suppressed to an extent that they do not interfere with the investigated gap junction structures. Our electrophysiological experiments reveal that this cooling rate is certainly fast enough to detect different functional gap junction plaques.

V. CONCLUSIONS

The effect of glutardialdehyde on the dynamic organization of gap junctions cannot only be seen by electrophysiological measurements where the uncoupling of cells occurs within 3 min, but also by freeze fracturing the cells. Gap junctions from unfixed cells rapidly frozen by dipping into liquid propane appear polymorphic; loosely packed and clustered plaques are found, as well as tightly packed aggregates, which are mainly found in fixed preparations. Whether these different structures correspond with different functional states, or whether they depend on the local configuration of the contacting membranes, is difficult to decide. The presented results, however, support the idea of active (coupling competent) gap junctions with loosely packed channels and nonactive (permanently closed) gap junctions where the channels are tightly packed.

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