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CHARACTERIZATION OF GAP JUNCTIONS BY

ELECTROPHYSIOLOGICAL AND ELECTRONMICROSCOPICAL METHODS

Dieter F. Hülser, Dietmar Paschke, Franz Brümmer and Reiner Eckert

Biology Institute, Biophysics Dept., University of Stuttgart

Pfaffenwaldring 57   D-7000 Stuttgart 80

Federal Republic of Germany

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 animals the exchange of information between cells 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 (up to a molecular weight of 900 Dalton for mammalian[1] and at least 1200 Dalton for insect cells[2]) and also connect adjacent cells mechanically.
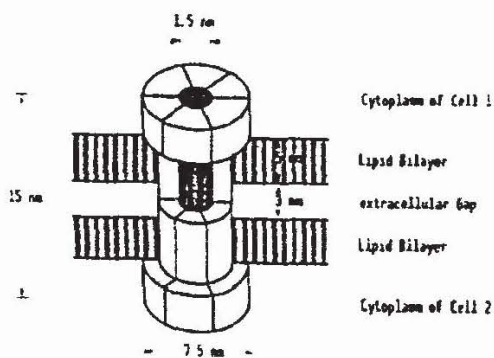

Fig. 1 Schematic model of a gap junction channel.

Gap junctions are ubiquitous in the animal kingdom from mesozoa to vertebrates. They must be discriminated from desmosomes which anchor cells together to form structural or functional units as well as from tight junctions which seal membranes of epithelial cells to each other so that the paracellular path becomes impermeable to molecules and a polarity of apical and basolateral surface is maintained. A communicating gap junction channel consists of two hemichannels, the connexons, to which both contacting cells contribute[3,4] (see Fig. 1). Each connexon consists of six protein subunits, the connexins (for review see Revel et al.[5]). Up to several hundred channels are assembled to form a typical gap junction plaque (for review see Bennett & Goodenough[6]). 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[7]).

Isolation of gap junctions and biochemical analysis revealed subunits with a molecular weight of 16k[7], and connexins with molecular weights ranging from 26k to more than 45k (for review see [8]). Each connexin consists of four α-helical regions, spanning the plasma membrane four times so that both the amino-terminal region and carboxyl-terminus are directed towards the cytoplasm[9]. The amino acid sequences of the 26k and 32k-proteins are up to 87 % homologous, which is contributed mostly by the membrane embedded and the extracellular parts of the proteins, whereas the cytoplasmic parts differ to a higher extent. This explains not only why heterologous coupling can be observed between cultured cells but also why intracellular regulation of gap junction permeability seems to be differently regulated in different cell types. Nevertheless, antibodies directed against the cytoplasmic parts of connexins 26k and 32k, are crossreactive in many tissues such as pancreas, stomach, kidney, ovary, heart, brain and uterus[10]. The gene for connexin cx32 has been identified[11] whereas for other connexins the corresponding cDNA is known (for review see[12]).

Gap junctional permeability is influenced by two endogenous signal pathways, using as second messengers either cAMP or diacylglycerol[13,14] (see Table 1 and 2). In most cells the cAMP signal pathway stimulates gap junctional communication, whereas the diacylglycerol signal pathway predominantly downregulates gap junctional 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[15]. However, not only physiological interactions by pH, Ca^{++} and retinoic acid but also non-physiological reactions by heptanol, benzhydrol, or glutardialdehyde (see Fig. 9) cause a closing of gap junctions.

Table 1. Stimulating substances for gap junctional permeability.

substance	target/function	references
db-cAMP, 8-Br-cAMP	second messenger, activation of cAMP dependent protein kinase	20,38,39
caffeine methylisoxanthine	inhibition of phosphodiesterase increase of $cAMP_i$	40,41
prostaglandin isoproterenol	increase of $cAMP_i$	42
retinoic acid	activation of cAMP dependent protein kinase	43,44,45,46
forskolin	catalytic subunit of adenylate cyclase	20,41
phenobarbital	tumor promotion	47

Metabolic cooperation

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 lightmicroscopical 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. This metabolic cooperation has first been described by Subak-Sharpe and coworkers[16], who found that Chinese hamster cells lacking the enzyme hypoxanthine guanine phosphoribosyltransferase (HGPRT) are unable to incorporate exogenously administered hypoxanthine into their nucleic acid when they grew isolated, but as soon as they made contact with wild type cells (HGPRT) they incorporated hypoxanthine. In this case, phosphoribosylpyrophosphate spreads as a signal via gap junctions into the HGPRT cells which therefore synthesize more nucleotides which are distributed via gap junctions into the HGPRT cells. Thus, the enzyme block is bypassed and the defective cells can survive.

Dye coupling

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 (M_r = 457) which can be injected iontophoretically or by pressure into a cell and its spreading into adjacent cells can be followed under a fluorescence microscope[18]. When Lucifer Yellow is injected into a monolayer cell it may diffuse into all first order neighbors (N_1) and from there into the more distant second order neighbors (N_2) and so on. Thus, the intensity of the fluorescent staining in the cells varies with the distance from the injected cell for every given time interval after dye injection. Fig. 2 schematically shows the situation with the injected cell in the center and first as well as second order neighbors. The extend of coupling can be quantified by counting both the number $N1_c$ of fluorescent and the total number $N1$ of first order neighbors. A normalized coupling ratio (CR_n) is given by

$$CR_n = (N1_c / N1) \times 100$$

Table 2. Inhibitory substances for gap junctional permeability.

| substance	target/function	references
diacylglycerol	activation of protein kinase C	48
EGF, PDGF	raise of cytosolic Ca^{2+}	49
retinoic acid	inhibition of ornithine decarboxylase	20,44,50
arachidonic acid, myristic acid, lauric acid, linoleic acid	inhibition of phospholipase C, activation of guanylate cyclase, protein kinase C, adenylate cyclase	51
Na^+	raise of $[Ca^{2+}]_i$ via Na/Ca-antiporter	52
Ca^{2+}, H^+	binding sites at gap junction proteins	53,54
CCl_4	unknown	55
glycyrrhetinic acid	interaction with mineralo- and gluco-corticoid receptors	56,57
carbamylcholin GTP[S]	IP_3 production, activation of protein kinase C	58
TPA	activation of protein kinase C	45,47,56,59
mezerein	tumor promotion, activation of protein kinase C H^+ and Na^+ carrier	20,47,60,61
SV40 large and small t antigens	enhancing tyrosine kinase activity	62
retroviral oncogenes	tyrosine kinase (pp60^{src})	63
nitrobenzylalcohol, benzhydrol	membrane fluidity	20,64
chlorpromazine, tetracaine, dibucaine	calmodulin inhibitor	20,65,66
dinitrophenol	uncoupling of oxidative phosphorylation	20,63,67
heptanol	membrane fluidity	20,68,69
glutaraldehyde	protein crosslinking	20,29,70,71
A23187	Ca^{2+}-ionophore	47,72
sodium nitrite	mutagenic	47
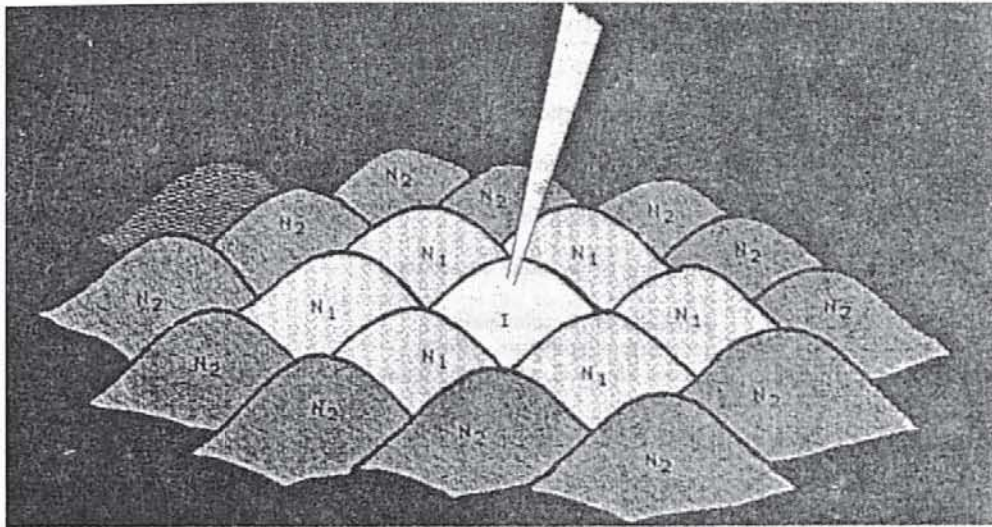
Fig. 2 Sketch of dye coupling in monolayer cells: the fluorescent dye is injected in cell I and spreads into neighboring cells N_1 and N_2 where the relative intensity of fluorescence depends on the amount of active gap junctions between the cells and the time passed after injection.

Ionic coupling

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[19,20,21]. Thus, the gap junctional conductivity is not a constant value, but is part of the dynamic regulation processes in cells. Ionic coupling is determined by measuring the membrane potentials of the probed cells with glass-microelectrodes and injecting current pulses into one of these cells (see Fig. 3). The resulting hyper- or depolarizing pulses are registered in the same (U_1) and in the coupled (U_2) cell together with the membrane potential of both cells. The ratio $CR_a = U_2/U_1$ is a measure for the amount of ionic coupling as long as cell pairs are probed.
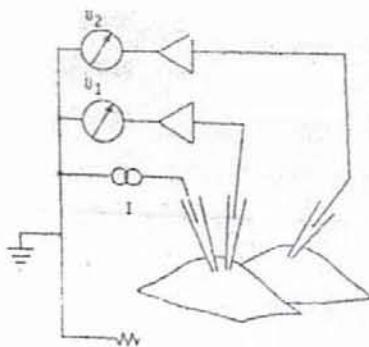
A measurement of ionic coupling between extremely well coupled brain tumor cells (BT5C1) is demonstrated in Fig. 4. These cells grow as monolayers and are interconnected by many gap

Fig. 3 Determination of ionic coupling with three intracellular electrodes.

junctions so that an injected current spreads into all cells in the monolayer. This is the reason why a rather steep decrement of the coupling pulses is observed which would not be the case if cell pairs were probed.

Different cell types from different species can be interconnected by gap junctions (heterologous coupling), as has been shown for several cell cultures[22,23]. Since human cells can be coupled to chicken cells and form heterologous gap junction channels the outer region of the connexon must be considered as very conservative. Both endogenous signals such as action potentials from chicken heart muscle cells and exogenously generated injected signals such as Lucifer yellow could be passed into murine (EMT6/Ro) and rodent (BICR/M1R$_b$, C6) cells[23].

Gap Junction Channels

Tight seal whole cell recording[24] with patch pipettes is an alternative to the conventional voltage clamp techniques,
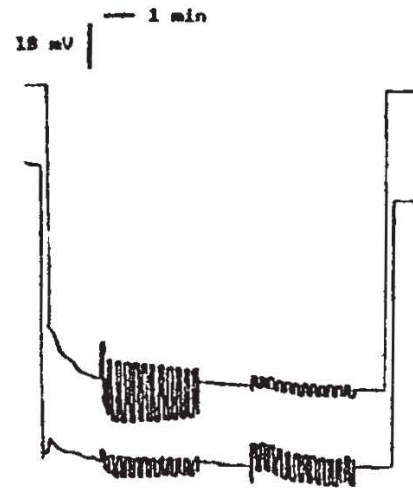

Fig. 4 Ionic coupling between BT5C2 cells. After insertion of the recording electrodes in neighboring cells, stable membrane potentials of about -65 mV are registered after 1 min. After insertion of the current electrode in cell 1 (upper trace), voltage pulses are superimposed to the membrane potential of both cells, due to the injection of 10 nA current pulses. When the current electrode was withdrawn from cell 1 and inserted into cell 2, voltage pulses with different amplitudes than before are measured. In both cases, however, a coupling ratio $CR_{el} = 0.35$ can be calculated. Upon withdrawal of the current electrode, the membrane potentials remained stable and after retraction of the recording electrodes from the cells the base lines (0 mV) are again registered.
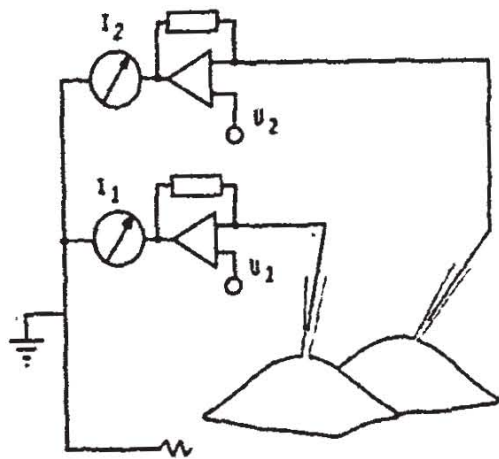
when applied to small cells as are often found in permanently growing mammalian cell lines. Small cells are not adequate for standard voltage clamp procedures due to cell damage caused by the insertion of two microelectrodes. A derivative of this procedure - the double whole cell recording technique - has been developed by Neyton & Trautman[25] to study the properties of gap junction channels. The opening and closing characteristics of a single gap junction channel can be followed by this type of experiments, where the current across an individual channel can be determined. A current will only flow through gap junction channels if a transjunctional potential difference is maintained between the cytoplasm of two cells. Experimentally this is

Fig. 5 Determination of gap junctional conductance with double whole cell recording.

achieved by clamping cell A to a fixed transmembrane potential while cell B is kept at its resting potential. If the two potentials differ from each other, there will also be a potential difference across the gap junction which generates a current through gap junction channels. Every change in the amplitude of this current, which can be measured in both cells, must be attributed to an opening or closing of these gap junction channels (for further details see also Eckert et al.[26]).
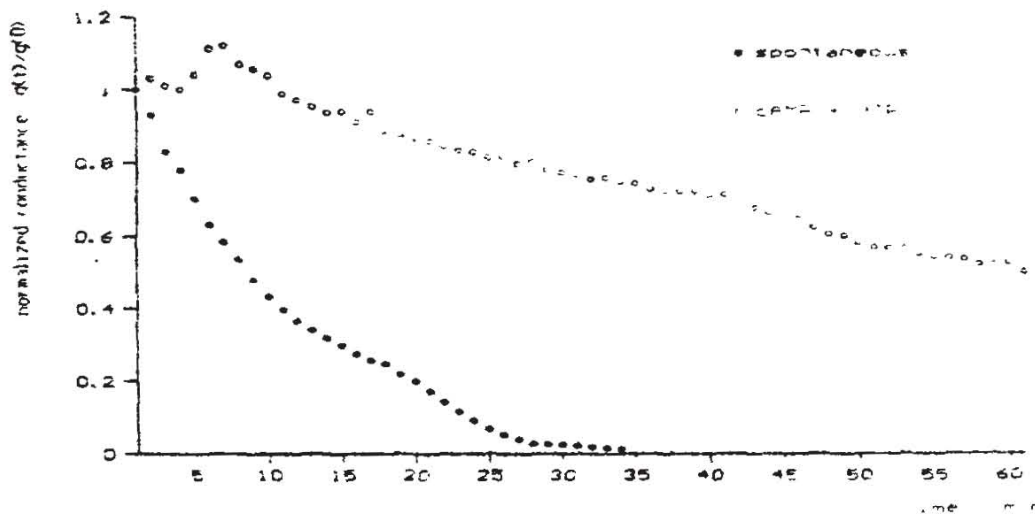

Fig. 6 Spontaneous uncoupling and stabilization of junctional conductance in pairs of BRL (Buffalo Rat Liver) cells. Stabilization was achieved with 1 mM db-cAMP and 5 mM ATP in the pipette saline.

Due to the wide opening diameter of patch pipettes a conceivable exchange of the pipette solution with the cytosol as well as a diffusion of higher molecular components into the pipette will occur[24]. This offers the possibility of rapid internal perfusion of cells with defined media, called a "dialysis" of the cell interior against the pipette filling, but also causes inhomogeneities of channel behavior during the recording interval. With gap junctions this effect can be seen in what is called "spontaneous uncoupling", i.e. the time dependent decrease in junctional conductivity during normal double whole cell

experiments[a,b]. Pipette media for whole cell recordings, therefore, are often supplemented with additional components such as ATP and cAMP to reduce this uncontrolled dialysis and "stabilize" gap junctional conductance as is shown in Fig. 6.
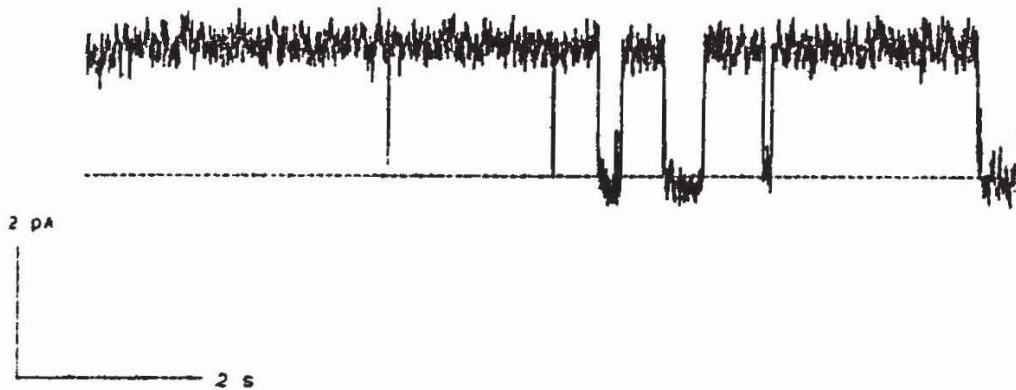

2 pA

2 s

Fig. 7 Current fluctuations due to opening and closing of individual gap junction channels recorded from a PLC (human hepatoma) cell pair at 40 mV transjunctional voltage. The trace shown is from the cell which is clamped to its resting potential to reduce membrane noise. The dashed line indicates the baseline of the record, i.e. the zero conductance level.

In most cell lines studied so far current fluctuations through individual gap junction channels may only be seen at the end of a 30 min period of spontaneous uncoupling, when the total junctional conductance is low enough to resolve current steps in the range of some pS as is shown in Fig. 7. From these records single channel conductances of 60 and 95 pS for BRL as well as for FL and of 26 and 51 pS for PLC cell pairs have been determined[a]. Assuming a pore length of about 15 nm, an inner pore diameter of 1-1.5 nm, and a cytoplasmic resistivity of about 0.64 Ωm (Frank & Hülser, unpublished data) a maximal conductance ranging from 77 to 170 pS can be predicted for gap junction channels which fits well to the values observed from single channel records.

Antibodies directed against connexins may bind to gap junctions in situ so that the passage of molecules is blocked or the channels may close as a consequence of this binding. The specificity of antibodies can, therefore, be tested when they are added to the pipette medium and thus diffuse into the cytoplasm of the attached cell. An example of uncoupling in the presence of antibodies is demonstrated in Fig. 8 for a BRL cell pair with anti-cx32-antibodies. Similar conductivity levels of individual gap junction channels in different cell lines, however, do not necessarily indicate the same specificity for antibodies[a].
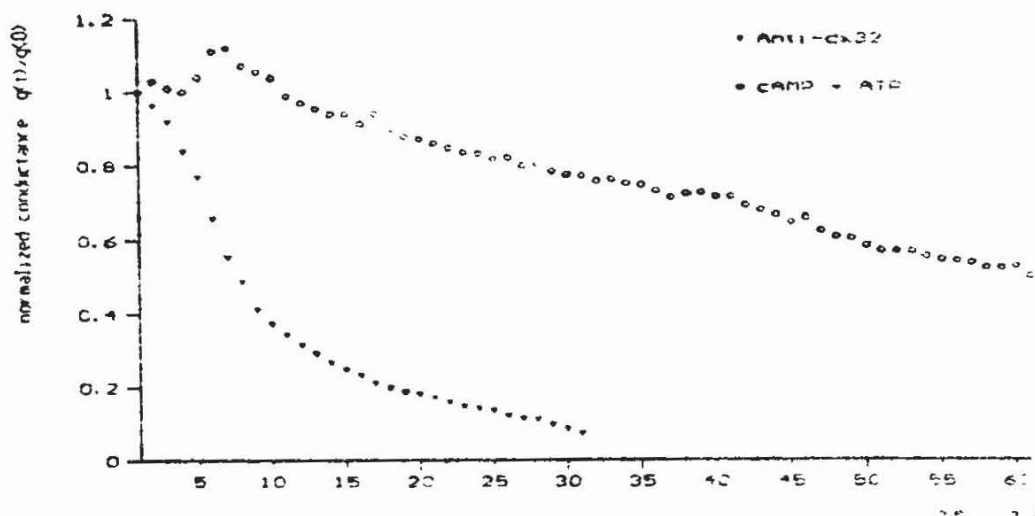
Fig. 8 Addition of anti-cx32-antibodies to the pipette saline of one electrode results in an acceleration of uncoupling (closed triangles) compared to control (open circles) in BRL cell pairs. In both experiments the cells were stabilized by 1 mM db-cAMP and 5 mM ATP in the pipette saline.

Electron microscopy

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 - 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[.] 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 un-coupling of gap junction channels[8,29,30] as can be seen from Fig. 9. It is, therefore, impossible to demonstrate open gap junction channels in such pretreated cells. The question remains, if 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. With an open pore size between 1 and 2 nm[31] 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
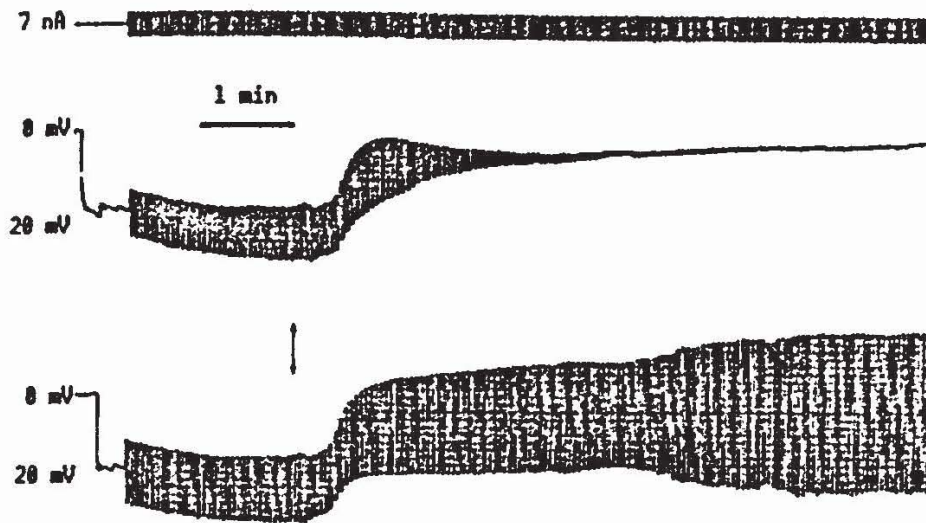
7 nA ——

1 min

0 mV

20 mV

0 mV

20 mV

Fig. 9 Glutardialdehyde induced uncoupling between homokaryons of mammary tumor cells of the Marshall rat (BICR/M1R$_k$). The upper trace shows the rectangular current pulses of 7 nA which were fed into cell 1 (lower trace) where they caused voltage changes U$_1$ (proportional to the input resistance) superimposed to the cell's membrane potential of about -20 mV. Cell 2 (middle trace) was electrically coupled to cell 1 as can be seen by the voltage changes U$_2$ superimposed to the membrane potential of cell 2. The arrow indicates the time point when the phosphate buffered saline (PBS) was washed out and replaced against 0.2% glutaraldehyde in PBS. The effect of glutardialdehyde can be seen about one minute later when it had diffused across the impaled cells. Due to a reaction of glutardialdehyde with the plasma membrane both cells were depolarized and their input resistances increased which is indicated by higher amplitudes of both U$_1$ and U$_2$. The coupling ratio CR$_{ab}$, however, remained unaffected for a short time till the cells started to uncouple, as can be seen from the U$_2$-amplitudes which decreased continuously because of the closure of gap junction channels. Consequently, this uncoupling from neighboring cells further increased the input resistance in cell 1 as is indicated by the growing U$_1$-amplitude.

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[32]. 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.

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[33] a freezing rate of 10^4 K·s^{-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 vitrificated only within a thickness of about 10 - 30 $\mu m^{34,35}$ which depends on the preparation techniques (for review see Plattner & Zingsheim[36]). 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.

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[29]. 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. Non-junctional 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, EF-face leaflets appeared rather particle free. Under this condition, also tightly packed gap junction plaques have been found which, however, were always smaller than in fixed preparations. In addition, many loosely packed clustered gap junction plaques could be detected where the particle density and cluster size varied considerably. Our electron-microscopical investigations[29,37] support the idea of active (coupling competent) gap junctions with loosely packed channels and non-active (permanently closed) gap junctions where the channels are tightly packed.

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