SUMMARY OF THE COMMUNICATIONS AND POSTERS PRESENTED AT THE EMBO WORKSHOP ON STRUCTURE, REGULATION AND FUNCTION OF THE GAP JUNCTION

erganized by

J.A.M. van den Biggelaar, P. Guerrier, M. Moreau and J.D. Pins (23rd-27th June 1985)

at the

STATION BIOLOGIQUE DE ROSCOFF

Directeur : Pr. P. Lasserre

Liste des participants

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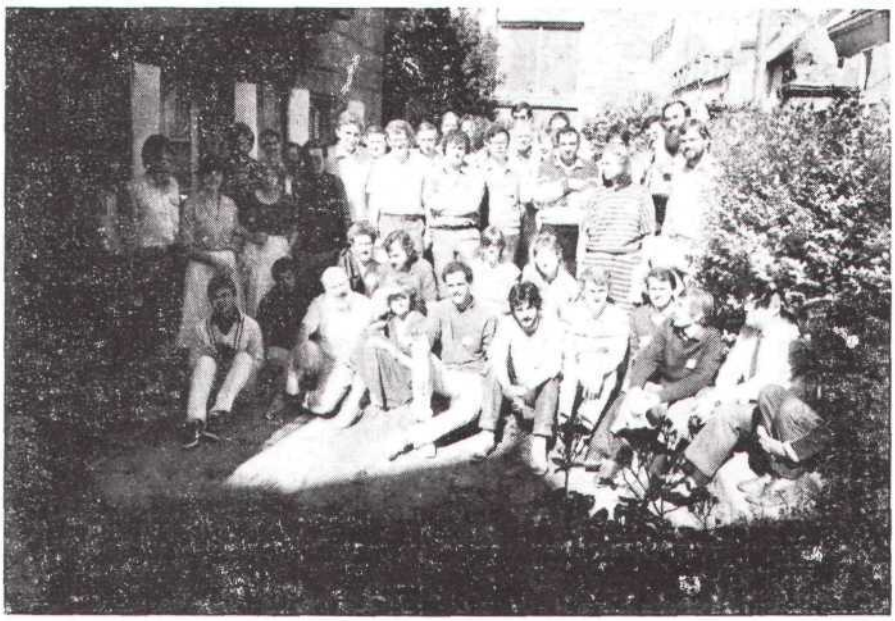
CAHIERS DE BIOLOGIE MARINE
Tome XXVII - 1986 - *pp.* 101-132.

EMBO WORKSHOP ON STRUCTURE, REGULATION AND FUNCTION OF THE GAP JUNCTION

ROSCOFF, 23-27 JUNE 1985



In the récent years, much progress have been made concorning the fine structure of gap junctions, their subunit pattern of organization and the conditions which may regulate their function. Moreover, junctional proteins have been identified and antibodies are presently available that display limited intertissue and interspecies cross-reactivity. A number of points needed to be clarified within such a potent surge of new data before one could come back to the central problem of how these highly specialized structures may play their role in intercellular communication and development. This was the main justification for organizing this interactive meeting which, we hope, will result in further progress, reducing competition in favour of cooperation.

J.A.M. van den Biggelaar, P. Guerrier, M. Moreau and J.D. Pitts.

This workshop has been financéd primarily bu the European Molecular Biology) Organization with addition support from: Centre national de la Recherche Scientifique, Paris; Cancer Research Campaing (U.K.); Unilever (The Netherlands) and Mecanex (Geneva).

I. STRUCTURE OF THE GAP JUNCTION

THE ORGANISATION OF THE PLASMA MEMBRANE-CYTOSKELETON COMPLEX IN EYE LENS FIBER DURING TERMINAL DIFFERENTIATION AND AGEING

by

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One of the key steps of thc terminal differentiation of lens epithelium into fiber is the biosynthesis of a novel membrane protein, the MP 26.

However, the mode of assembly and long and short range distribution of this protein is still questionable, and controversial results are obtained in different laboratoires as to the rôle of this polypeptide in the formation of junctional domains.

Another intriguing question is dealing with the contribution of MP 26 and its proteolytic derivatives to the intramembraneous particle patterns visualised by freeze fracture. Non geometrical assemblies of particulated entities and orthogonal lattices of repeating subunits have been described.

Other problems concern the interaction of cytoskeletal components with the plasma membrane domain formation during terminal differentiation and ageing of the fiber.

We have applied biochemical, immunochemical techniques and electron microscopy on negatively stained and freeze-fractured plasma membranes in order to eludicate the organisation of the MP 26 and its interaction with crystallins and cytoskeletal components.

We have also sludicd the reconstituted liposomes comprising MP 26 to establish the pattern that this polypeptide may form in the lipid bilayer.

INTERACTION OF CRYSTALLINS WITH THE LENS PLASMA MEMBRANE

by

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One of the still unrcsolved problems in lens rescarch is the arrangement of the crystallins in the lenticular fibers and their interaction with the cytoskeleton and/or the plasma membrane. In vitro experiments show that purified lens plasma membranes associate selectively with a very restricted number of translation products newly synthesized by lenticular |)olyribosomes. Part of this capability is retained by purified membrane junctions. It seems that in the latter case only the aA_2-crystallin subunit, aB_{1a}-crystallin and vimentin attach to the junction. The binding of a-crystallin to membranes is pH-, salt- and temperature- dependent, and is not affected by pretreatment of the membranes. This treatment releases a 4000 dalton piece from MP26 with protease. Reconstruction of MP26 into liposomes followed by binding studies of a-crystallin suggests an involvement of the major membrane protein in the association.

GAP JUNCTION PROTEIN OF LIVER, HEART AND THE PREGNANT UTERUS. EVIDENCF OF HOMOLOGY

by

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The 28kD proteins of the gap junctions of rat livcr, heart and pregnant uterus are homologous as shown by two approaches. Gap junctions were prepared from the three tissues using detergent extraction in the final steps of purification. All three tissues contained a predominant 28kD polypeptide. Two-dimensional analysis of the tryptic peptides showed that the 28kD polypeptide was similar in the three tissues. Separation of gap junctions from the three tissues using a method involving extraction with alkaline media also resulted in the production of 26kD and 20kD polypeptides; these likely proteolytic products was also shown to be homologous in the three tissues by two-dimensional analysis of the tryptic peptides.

Antigenic homology between the gap junction polypeptides of liver, heart and pregnant uterus was also demonstrated. Site-directed antibodies were prepared in rabbils against synthetic peplides from the deduced amino-terminal sequence of the rat liver gap junction protein. The immunofluorescent antibodies stained cell contact regions in thin sections of liver, uterus and heart (intercalated dises). Immunoblotting showed that 28, 26 and 20kD polypeptides resolved by SDS-PAGE bound the antibodies in gap junction fractions demonstrating that the aniino-terminal regions of the 28kD gap junctional polypeptide of heart and uterus were antigenically related to the liver protein.

A.S. Zervos thanks the Brain Research Fund of The Institute of Neurology, University of London, for support.

THE GAP JUNCTION PROTEIN

by

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We have devised a method of gap junction preparation suitable for a wide range of tissues from different species and front cultured cells. Electron microscopic analysis by thin-section and negative staining shows the preparations to be highly enriched for gap junctions. The junction preparations from vertebrate tissues, regardless of the source, contain a highly conserved (across species), tissue invariant M_r 16,000 protein. As well as the morphological criteria, a number of independent, correlative lines of evidence indicate the junctional origin of the 16k protein. The major protein present in gap junction fractions prepared from an arthropod source *(Nephrops norvegicus),* M_r 18,000, is structurally related to the 16k vertebrate proteins. Antisera raised against the 16k and 18k proteins avidly bind to isolated gap junctions.

Other laboratories have reported a 27k protein as a major constituent of rodent liver gap junctions. We have used a variety of different isolation procedures and find the 16k protein is present in all the

final junction fractions. On the other hand, the 27k protein is only present in preparations where a trypsin step is not included. The 16k and 27k proteins are unrelated hy peptide mapping and western blot analysis. Trypsin readily removes the 27k protein and yet the inclusion of a trypsin step greatly increases the purily of gap junctions in the préparations. We conclude that the 16k and 18k proteins are the structural proteins of vertebrale and arthropod gap junctions and that the 27k protein is an unrelated contaminant in some junction preparations.

EXPERIMENTAL STUDIES ON GAP JUNCTIONS

by

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Gap junctions have been isolated from mammalian and arthropod tissues in order to generate molecular probes that can be used to study the properties of cell-cell communication during development and to provide a molecular basis for understanding the properties of the cell channel.

Thus far, antibodies prepared to the 27k rat liver junction protein have been utilized to study junctional communication properties in *Xenopus embryos* (Warner, Guthrie and Gilula, 1984, Nature *311, pp.* 127-131) and *Hydra* (C. Green, S. Frazier and N, Gilula). In addition, proteases, antibodies and calmodulin-binding have been applied to determine some lopological features of the junction protein in the intact membrane (I). Zimmer and N. Gilula) and the relevance of «reconstituted» junctional channels (D. Young and N. Gilula).

DEMONSTRATION OF TWO CLASSES OF COMMUNICATING JUNCTIONS IN THE CHICK EMBRYO LENS EPITHELIAL CELL

by

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After the lens vesicle pinches off from the overlying ectoderm, the posterior lens cells elongate into the first lens fibers, eventually closing the lumen of the lens vesicle and bringing the fibers into intimate contact with the overlying lens epithelium. The lens epithelial cells express communicating junctions whose component connexons appear crystalline in freeze-fracture replicas following aldehyde fixation; dye transfer between the epithelial cells is reversibly sensitive to a 5 min. exposure to 90 p. 100 CO_2, 10) p. 100 O_2-equilibraled culture medium. The underlying lens fibers express communicating junctions whose component freeze-fracture particles remain randomly clustered, in response to aldehyde fixation; dye transfer belween the fibers is insensitive to CO_2 exposure. At about stage 23 of ehick development, dye

transfer is observed between the epithelial cells and the lens fibers. Freeze-fracture analysis reveals that the junctions joining the heterologous cells are composed of randomly associated aggregates of connexons, indistinguishable from those joining the lens fibers to each other. The heterologous low resistance pathways, between epithelium and fiber, are intensitive to exposure to CO_2-equilibraled media. It is concluded that the lens epithelial cell is capable of forming two classes of communicating junctions: a homologous, crystallizing junction which is sensitive to CO_3, and a heterologous, non-crystallizing junction which is insensitive to CO_2.

PARTIAL SEQUENCE COMPARISON OF HEART AND LIVER GAP JUNCTION PROTEINS FROM RAT

by

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The recent characterization of junctional proteins in different tissues has revealed a surprising diversity. For some time it has been evident that the liver gap junction protein (M_r 28,000) differs substantially from MIP 26 (M, 26,000), a protein associated with junclional structures in eye lens. A protein component (M_r 28,000) of cardiac gap junctions has now also been identified. Comparisons of this protein with that of liver have produced somewhat conflicting results. lmmunologically the two proteins have been claimed in one case to be related, yet in another to show no cross-reactivity. The latter observation is supported by peptide-maps which show no homologies. Therefore a more reliable measure of the relatedness of the liver and heart junclional proteins is required to resolve this debate, and this is provided with the comparison of their sequences.

From rat heart homogenales, protein components of the sarkosyl-resistant fraction were separated by SDS-PAGE and visualized by brief Coomassie staining and destaining. The Mr 28,000 band was then excised, eleclron-eluted and electrodialysed. This junclional polypeptide was prepared in sufficient quantities for analysis by automated Edman degradation and the sequence of the aminoterminal 32 residues was obtained.

Comparison of this partial sequence with that of the liver junctional protein (which was previous publihed) reveals 43 p. 100 homology - or up to 68 p. 100 if one includes functionally conservative residue changes where an amino acid one one type (basic, acidic, polar...) is replaced by a residue of the same type. The hydrophobic portion of the sequence identified in liver as a possible transmembrane segment is also found in the heart, starting at the same point in the sequence (residue 23). The conservation of basic residues (# 's 22 and 32) within the hydrophobic region of the sequences could prove they are essential in the structure and the properties of the intercellular channel.

BIOCHEMICAL AND IMMUNOLOGICAL STUDIES OF GAP JUNCTIONS

by

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Rat liver gap junctions were isolated by a procedure we have recently developed as a higher yielding alternative to classical detergent-based isolation schemes. These gap junctions, isolated subsequent lo alkali treatment of plasma membranes, were characterized by the same 27,000 dalton polypeptide that has been shown to be the major constituent of gap junctions isolated by detergent resistance.

Specific antibodies to the gap junction polypeptide were prepared and shown to recognize 27,000 dalton polypeptides in the liver of representatives of all vertebrate classes. No crossreaction has been observed, as yet, with invertebrate samples. Crossreaction has also been observed with a number of tissue culture cell lines, including BRL and C1-1D. Interestingly, both communicating and non-communicating cultures of C1-1D appeared to contain similar levels of junction polypeptide.

The antibodies also identified, by immunocytological and immunochemical criteria, a crossreacting 27,000 dalton polypeptide in extracts of a large number of other rat tissues, including pancreas, heart, brain, kidney, stomach, adrenal gland, ovaries and uterus. Microinjection of the antibodies into cell pairs from liver, heart and neurons inhibited gap junction conductance, further supporting the notion that similar, or identical, polypeptides comprise gap junctions in these different tissues.

Patch clamp experiments using isolated rat liver gap junctions have permitted identification of channels characterized by a pH titration curve and antibody sensitivity similar to that of communication in he intact tissue. These experiments provide further evidence that the isolated gap junctions represent the same channels responsible for cell-cell communication *in vivo.*

Incubations of gap junctions with two protein kinases have demonstrated that the 27,000 dalton gap junction polypeptide can be a substrate for phosphorylation. The significance of this observation is being pursued by investigating the influence of phosphorylation on reconstitued channel activity *in vitro* and on communication competency in culture cells.

(Supported by grants from the National Institutes of Health (GM 30667) and the Robert A. Welch Foundation (Q-961)).

MP26 IN LENS JUNCTIONS: ANALYSIS WITH CHICK EMBRYO LENS CULTURES

by

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We have recently reported strong evidence for the presence of MP26 within lens junctions, based on EM immunolabeling with a monoclonal antibody for MP26. A number of questions now need to

be answered, including: (1) Does MP26 form cell-cell channels? (2) How are lens junctions related to gap junctions in other tissues? (3) What are the roles of lens junctions? (4) How are lens junctions regulated? Our approach to these issues involves cultures with highly differentiated «lentoids» which provide an excellent in *vitro* model for lens fiber cells and lens junctions. Several studies will be discussed. First, to examine the arrangement of MP26 within the membrane, lens cultures have been treated with proteases and MP26 has then been analyzed with SDS-PAGE and immunotransfers. Some MP26 molecules in these cells are cleaved to less than 20 kD and recognized by a monoclonal antibody that binds close to the original C-tcrminus (found on the cytoplasmic side in the intact membrane). We interpret this as support for the transmembrane nature of MP26, with extracellular proteases cleaving within «external» domains. These results are being compared with those derived from artificial membrane vesicles containing MP26. Second, to determine whether the lentoid cells are able to form permeable junctions with other cell types, co-culture experiments have been done with lens-heart and lens-hepatoma combinations. Functional junctions developed, consistent with the idea that MP26 can assemble with gap junction proteins from other cells into permeable junctions. Third, in an attempt to block lens junction formation in *vitro*, antibodies are being characterized which appear to bind close to the «external» protease sites on MP26 noted above. These sites may correspond to one or more of the external loops included in a recently published model for MP26 structure. Finally, we are interested in the possible regulation of MP26 by means of phosphorylation. We have found phosphorylation of MP26 in fragments of calf lenses, as well as with isolated lens membranes and exogenous protein kinases (both cyclic AMP-dependent and protein kinase C).

GAP JUNCTIONS IN ARTHROPODS

by

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Gap junctions in a range of arthropod species and tissues have been examined with regard to their structure and biogenesis. Although they appear very similar in ultra-thin cross-sections, tangentially-cut gap junctions impregnated with tracers such as lanthanum frequently reveal relatively loose connexon spacing. Freeze-fracture replicas, made after conventional fixation and cryoprotection also exhibit a range of connexon clustering. Very loose aggregates of connexons are particularly prevalent in insect, arachnid and myriapod tissues; yet even on the same membrane face, closely packed and loosely packed aggregates may be found side by side. Some of these loose aggregates bear similarities to the arrays seen during gap junction assembly in developing embryonic tissues and suggest the possibility of junctional turnover. Although gap junctions often appear on the same membrane face as do tight junctional structures, tne two are distinguishable in arthropods on the basis of the size and fracturing characteristics of their component intramembranous particles; in embryonic tissues they appear to become assembled independently. Freeze-fracturing after fast-freezing by both propane jet and plunge

or by rapid impaci against a liquid helium-cooled copper block, produces connexon arrays which are also variable in their state of aggregation.

Gap junctions ran be isolated from the hepatopancreas of erustacea such as the lobster to produce a high yield of relatively pure junction-hearing membrane. The junctional membranes from adjacent cells adhere together in the form of plaque-like discs which in thin-section reveal the characteristic reduced intercellular cleft. The features of these isolated junctions can also he visualized in freeze-fracture replicas and negatively-stained preparations. Immunocylochemical studies on fractions of isolated lobster gap junctions show that antibodies raised against them will bind specifically to junctional membrane, as revealed by subsequent Protein-A-gold labelling. In *situ* immnnocytochemical labelling of gap junctions using these antibodies is in progress on a range of arthropod tissues at both the light and electron microscopical level. In the latter, ultra-thin sections, fracture-labelled thin sections and labelled replicas are being investigated.

IMMUNOLOGICAL ANALYSIS OF GAP JUNCTION STRUCTURE AND FUNCTION

by

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Five different types of anti-sera have been raised: rabbit against chicken gap junctions, rabbit against *Xephrops norvegiens* gap junctions, mouse against chicken gap junctions, mouse against *Xephrops norvegicus* gap junctions and rabbit against an octapeptide synthesised according to the N-terminal sequence of the mouse 10k gap junction protein. These sera have been used to show (i) the immunolocalization of the 16k protein to mouse gap junctions and an 18k protein to *Sephrops noroegicus* gap junctions, (ii) the immunologieal cross-reactivity between all gap junctions proteins examined (from six different species of three different phyla), (iii) the localization of the X-terminus of the lfik protein on the cytoplasmic surface of the mouse gap junction in a pronase accessible conformation, (iv) the presence of other antigenic sites on the cytoplasmie face, (y) the blocking of channel permeability by intracellular injection of antibodies and (vi) the role of the N-terminus of the 16k protein in the blocking reaction.

STRUCTURAL AND MOLECULAR ANALYSIS OF INTERCELLULAR COMMUNICATION CHANNELS

by

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Essentially all animal cells are linked to each other by membrane specializations, «gap junctions» (GJ), which permit them to exchange small molecules or ions. By EM, gap junctions are recognizable

throughout the animal Kingdom by their characteristic appearance. There is also an apparent consensus on ca.26-28 kd for the molecular weight of the protein, although there is now evidence for other sizes as well, and the issue is far from settled at this point. The wide phylogenetic range through which antibodies raised against the junction protein from rodent liver can react also fosters the idea that gap junctions are basically similar throughout the animal Kingdom. On the other hand, information based on one- and two-dimensional peptide maps as well as sequence data does not provide support for the idea that junction proteins are identical «everywhere». Recently obtained information on the sequence of the protein isolated from heart, and the complete sequence of the lens MIP, now offer a way to reconcile these apparent discrepancies. The heart sequence data, while fragmentary, points to a 40 p. 100 or so identity between heart and liver. The MIP data, while revealing no significant sequence identity, allows one to deduce that MIP could be a channel-forming protein, a finding compatible with the idea it is a junction molecule, although incontrovertible evidence for the latter idea is lacking. It can nevertheless be argued that sufficient sequence homology exists to allow cross reactivity of antibodies, in spite of differences responsible for widely differering results on peptide mapping. All gap junction protein may have a quaternary structure in common, which is not necessarily expressed as sequence identity.

QUATERNARY STRUCTURE OF THE PROTEIN FORMING THE GAP JUNCTION CHANNEL

by

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Several low resolution (~ 20 Å) three-dimensional maps of the gap junction protein (connexon) have now been determined by electron microscopy and diffraction analysis, using material isolated from rat hepatocytes. They all indicate that this membrane protein is an oligomer composed of six 70-80 Å long rod-shaped subunits, which protrude 15-20 Å into the extracellular space (the gap) and somewhat less into the cell interior. Within the lipid bilayer their cross-section, visualized in frozen-hydrated specimens, is 400 Å^2 (equal to that formed by four closely packed α-helical rods) ; within the extracellular space their cross-section is slightly smaller and they produce a more rounded outline, indicating that the secondary structure in this region is different. The channel is widest in the extracellular space and narrowest at the cytonlasmic surface. Structural changes produced by different treatments (low and high Ca^{2+}) are most pronounced in the cytoplasmic region and involve coordinated changes in inclination of the subunits tangential to the channel axis.

The details visualized suggest that switching between open and closed states of the channel may occur by an allosteric mechanism in which a localized change induced by ligand binding triggers a long range concerted rearrangment of the subunils. Progress is now being made in visualizing these details at higher resolution and under conditions closer to that in live tissue.

EXPRESSION OF THE 26 K GAP JUNCTION PROTEIN IN HEPATOCYTES AND HEPATOCARCINOMAS

by

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Rabbit affinity purified antibodies and a rat monoclonal antibody to the liver gap junction 26 k protein have been used to study the expression of this protein in mouse or rat hepatocytes. While proliferating in defined serum-free medium the hepatocytes were labelled with ^{35}S methionine or ^{32}P phosphate. The half life of the 26 k protein in these cells was determined by pulse/chase and immunoprecipitation. Phosphorylation of the 26 k protein in cultured hepatocytes can be increased by addition of dibutyryl cAMP and inhibitors of phosphodiesterase. Thus the 26 k protein is presumably phosphorylated in response to hormon signals which use cAMP as second messenger.

The rat monoclonal antibody reacts with the liver 26 k gap junction protein after immunoblot and recognizes the cytoplasmic domain of gap junction plaques as shown by electron microscopy. This antibody detects gap junction plaques in cultured proliferating hepatocytes from rat or mouse embryos. Specific immunofluorescence first appeared on apposed plasma membranes of contiguous hepatocytes in the center of the corresponding clones. This suggests that cell to cell contact and/or proliferation may regulate the assembly of gap junction plaques in primary embryonic hepatocytes.

Several chemically induced rat hepatocarcinomas exhibit decreased immunofluorescence of gap junction plaques and contain less 26 k protein than livers from control rats.

II. FUNCTION AND REGULATION OF THE GAP JUNCTION

QUANTITATION OF JUNCTIONAL CHANGES ASSOCIATED WITH VIRALLY INDUCED CELL TRANSFORMATION

by

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We and our colleagues have reported that NRK cells infected with a temperature-sensitive RSV mutant show a decrease in junctional permeance to Lucifer Yellow when the cells are grown at the transforming temperature. The effect develops within 15 min of temperature downshift and is reversed equally rapidly after temperature upshift. Now we have carried out experiments designed to address two questions: 1. Are the permeance changes associated with structural changes in the gap junctional area or particle arrangements? 2. Is junctional permeance decreased when NRK cells are infected with other transforming viruses carrying different oncogenes? Freeze-fracture studies of LA25-infected NRK cells have demonstrated that junctional areas are not detectably changed after acute temperature shifts. However, the junctional particles in fixed material are more ordered for cells displaying normal junctional permeance and are more disordered for those with decreased permeance. Thus, the structural evidence suggests that the changes in permeance reflect closing or opening of channels rather than changes in number of channels. Junctional permeance has been measured using a SIT camera and computer assisted analysis of Lucifer Yellow transfer between pairs of cells. A decrease in junctional permeance has been demonstrated for NRK cells infected with the ts-110 mutant of a murine sarcoma virus when the cells are grown at the transforming temperature and for NRK cells infected with a Kirsten sarcoma virus. These results are consistent with a more general association between cell transformation and junctional defects than previously demonstrated by more qualitative studies.

CONTROL OF COMMUNICATION VIA GAP JUNCTIONS

by

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Gap junctional communication can be regulated by a number of gating mechanisms that open or close the channels. Differences among tissues exist which may reflect differences in the junctions themselves or in associated molecules. Increases in cytoplasmic H or Ca ions decrease junctional conductance g_j. H may give good titration curves close to the resting pH where physiological changes might affect g_j (blaslomeres, cardiac muscle) or far from resting pH where g_j is probably insensitive to physiological changes (livers). Unless application is brief, decreases in g_1 induced by H may be only poorly reversible suggesting secondary changes. Available data suggest low sensitivity to Ca such that only pathological Ca levels would affect g_j, as for example following cell rupture. Voltage dependence varies in

kind and sensitivity. Rectifying synapses are moderately sensitive to transjunction voltage, unidirectional and very fast. Amphibian blastomeres are sensitive to transjunctional voltage, bidirectionally and slowly. Constancy of selectivity over wide ranges of g_j suggests that channels are either open or closed. Some junctions in invertebrates are sensitive to the potential between cytoplasm and exterior as well as to transj unctional voltage. Some junctions are voltage insensitive. These gating processes appear analogous to those of voltage and chemically sensitive channels of ordinary excitable membranes. Aldehydes and octanol reduce g_j, irreversibly and reversibly respectively, but these effects are not easily related to gating processes. A polyclonal antibody to liver gap junctional proteins reduces g_j in a number of tissues, implying at least partial homology and providing a specific reagent for blocking junctional communication. Increase in cytoplasmic cAMP increases or decreases g_j depending on tissue. In a neuronal system the increase involves protein synthesis. Junctions can form under acid conditions in which g_j is low. Possibly junctional formation always proceeds through the closed state.

Phosphorylation of g_j junctions can be obtained in vitro, but it and relations between gating, formation and removal remain to be determined.

ON THE CORRESPONDENCE BETWEEN DYE-COUPLING COMPARTMENTS AND MORPHOGENETIC COMPARTMENTS IN THE MOLLUSCAN EMBRYO

by

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The molluscan embryo is generally known as mosaic embryo. The whole ectoderm derives from the first three quartets of micromeres, the entoderm derives from the fourth quartet of micromeres and the macromeres; the mesodermal bands derive form one out of the four macromeres, 3D. The ectoderm is divided into two parts with a different prospective fate: the pretrochal ectoderm formed by the first quartet of micromeres and the posttrochal region developped by the second and third quarted of micromeres. In between lies a circular ring of larval cells provided with cilia for the swimming movements of embryo.

In both ectodermal regions a subdivision in compartments with a specific developmental fate can be distinguished. This mosaic of sharply separated morphogenetic compartments makes the molluscan embryo very usefull for an analysis of the possible role of gap junctions within and between compartments.

It appears that before the formation of developmental compartments, from the 32-cell stage all blastomeres are dye-coupled. In the larva, however, the dye-coupling is restricted to compartments, the boundaries of which appear to correspond with the developmental compartments. This restriction of dye spread to specific regions may indicate that the formation of developmental comparments is associated with closure or reduction of gap junctionnal communication

between regions with different developmental fates. The intercellular communication within the compartments may be a prerequisite for the coordination of cell activities.

More difficult to denote is the significance of overall cell coupling in the 32-cell embryo. One possibility might be the formation of a voltage gradient in animal-vegetal direction, which could be involved in the progression of cytoplasmic segregation.

THE ROLE OF THE CYTOSOL IN THE REGULATION OF SOLUTE DIFFUSION WITHIN CELLS AND THROUGH INTERCELLULAR CHANNELS

by

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Fickian diffusion models have been developed which depict the diffusion of a solute in an array of cells linked in series by gap junctions. The models can be used to calculate cytoplasmic diffusion coefficients (D_c), junctional membrane permeability (P_m), and plasma membrane permeability (Pm) for a solute. The diffusion of fluorescent probes within the septate giant axon of earthworm are used as a source of experimental data which a 3 cell model fits to determine D_c, P_j, and Pm.

A multicellular diffusion model has also been developed to show how D_c and P_j can affect the longitudinal spread of a solute (morphagen) over µm to mm distances. Two cases have been solved for the 10 cell case. The first sets the initial concentration in one cell at t=0 and then allows the concentration to fall within the cell with time. Therefore a finite number of molecules are instantaneously placed uniformly within a cell and no replacement of solute with time is allowed. The second case sets the initial uniform concentration in one cell at t=o but with time for every molecule with diffuses out of the cell another is introduced to maintain the initial concentration. Both of these cases have potential in describing how morphagens, or for that matter any solute, might spread through out a tissue. The possible effects of solutes on junctional membrane conductance can also be illustrated with the double voltage clamp. While highly speculative, the data collected to date using long clamp times (5-60s) indicate a possible involvement of solutes in affecting junctional conductance under the influence of a transjunctional potential.

REGULATION OF CELL COMMUNICATION IN A GROWING EPIDERMAL COMPARTMENT

by

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The epidermis of each insect body segment is a developmental compartment that develops to some extend independently of its neighbours. Within the segmental epidermis the cells are strongly coupled

by membrane channels that allow inorganic ions and organic tracers to pass rapidly from cell to cell; at the segment margin special cells retard selectively the movement of organic tracers between compartments. As the segment grows in size during larval development, the rate at which microinjected tracers spread within the epidermis rises. This results from an increase in the permeability of the junctions connecting the cells, and is not due to a decrease in cell density, which remains constant. Over a period when the area of the segment increases 9-fold, the effective diffusion coefficients for the issue movement of carboxyfluorescein and lissamine rhodamine B, and the junctional conductance to inorganic ions, rises 3-fold. This suggests that a mechanism in the epidermis regulates junctional coupling in relation to a linear dimension of the segment, and this may be important in growth regulation and size invariance in the segment pattern. The coordinate increase in the rate of movement of molecules of different size implies that more channels are incorporated into the junctional membrane, or are open at any moment, as the segment grows, and that these channels are likely all-or-none in their opening and closure. Junctional coupling may be further raised by the hormone 20-hydroxyecdysone, or lowered by several treatments, at any stage in epidermial growth, and here, too, a coordinate increase or reduction in the transfer of small and large molecules is seen. The epidermis appears to have a single population of all-or-none channels whose number and physical state are carefully modulated during development.

Supported by NSERC of Canada.

REGULATION OF GAP JUNCTIONAL COUPLING IN THE MOUSE PREIMPLANTATION EMBRYO

by

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Extensive gap junctional coupling arises during the 8-cell stage in the early mouse embryo but appears not to transmit the signals that mediate the proximate induction of cell polarity. The onset of this coupling, as assessed by transmission of injected carboxyfluorescein or ionic current, does not require protein synthesis beyond the mid-to-late 4-cell stage onwards, nor is it regulated by the simultaneous progression of cell flattening. However, this onset does require the presence of extracellular Ca^{2+} but not a functional homotypic Ca^{2+} -dependent adhesion molecule, uvomorulin. Once established, junctional coupling cannot be reversed by removing extracellular Ca^{2+} nor by the use of a polyspecific antiserum that also blocks its onset. Towards the end of the 8-cell stage, junctional coupling becomes reduced and finally disappears as blastomeres within the embryo enter mitosis. The blockade of embryos in mitosis by the microtubule disrupting drug nocodazole, holds them in a non-coupled state but nocodazole itself neither prevents junctions from forming not does it cause uncoupling. Following division to the 16-cell stage the blastomeres recouple. Preliminary experiments using lucifer yellow injections into trophectoderm cells, during the early blastocyst stage (approximately 32 cells), indicate no preferred gap junctional passage of dye through

other trophectoderm cells rather than through cells of the inner cell mass, despite the fact that the two cell layers hy now have embarked on different paths of development. However direct coupling via cytoplasmic bridges remaining from the last mitotic event is always confined within the same cell subpopulalion.

OPENING AND CLOSING OF GAP JUNCTION PORES

by

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Mammary carcinoma cells of the Marshall rat (BICR/MlR-k) grow fibroblastioid when cultured as monolayer cells in petri dishes. These cells are well coupled as has been shown by both electrical current and Lucifer yellow injection. They can be fused with polyethylene glycol to giant cells without loosing their coupling capacity. Two homocaryons were manipulated into close contact and the coupling signal was measured in the docking cell with a high voltage resolution: the signal increased continuously in amplitude.

BICR/MlH-k cells remain in a very well coupled state when a monolayer is formed and when the stationary growth phase is reached. However, cultivating these cells as thrcedimensionally growing multiceli spheroids leads lo a continuous decrease in junctional conductivity. In spheroids grown for four days, Lucifer yellow is retained in the injected cell, electrical current, however, still passes into other cells. When the spheroids are eight days old, the cells reach a completely uncoupled state.

A mathematical simulation based on our electrophysiological data, including input resistance, input currents, resistance of a single pore as well as parameters such as channel configurations, channel opening characteristics and lateral interactions revealed best fits for our measured curves when a cooperative effect of channels within a gap junction plaque is taken into account.

A computer controlled image processing system allowed pattern analysis of freeze-fractured gap junctions of these BICR/MlR-k cells. In unfixed propane-jet and freon-dip preparations both loosly and tightly packed gap junction plaques were found. The loosely packed gap junction plaques may represent the open state of gap junction channels.

JUNCTIONAL COUPLING AND SECRETORY FUNCTION OF THE ADULT INSULIN-PRODUCING B-CELLS

by

P. Meda

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The insulin-producing B-cells of the pancreatic islets of Langerhans have been studied to investigate the possible function(s) of gap junctions and coupling in an adult, nonproliferating cell system which

is highly differentiated for secretion. B-cells are interconnected by clusters of numerous minute gap junctions and are dye, ionically and melabolically coupled. Under control conditions, coupling appears to be spatially restricted and to be heterogeneous between adjacent B-cells of a given communication territory. It also does not appear obligatory between all B-cells. To assess whether junctional coupling is involved in the secretory function of B-cells, we first studied the effects on gap junctions and coupling of conditions known to modify insulin secretion. These experiments showed that different insulin secretagogues, including the physiologic stimulus glucose, increase the amount of gap junctions and/or the extent of dye and ionic coupling between B-cells, in vitro and in vivo. In vivo, inhibition of insulin release also led to increased junctional coupling when the level of circulating glucose was elevated. To further elucidate the relationship between the changes in junctional coupling and those in B-cell functioning, we studied the effects on insulin secretion of conditions expected to abolish junctional coupling either by disrupting gap junctions (following B-cell isolation) or by decreasing the permeability of existing gap junctions (with uncouplers). Isolated (uncoupled) B-cells had an abnormally elevated basal insulin release and specifically lost their ability to respond to glucose. Using a reverse hemolylic plaque assay to detect secretion from individual cells we directly observed that uncoupled B-cells exposed to glucose released insulin in much smaller amounts and/or at a much lower rate than clustered (coupled) B-cells. Incubations of intact isolated islets with either heptanol or octanol further confirmed that B-cell uncoupling (verified by dye injection) was associated with an elevation of basal insulin release and with a marked inhibition of glucose-induced secretion. Both effects were rapidly reversible upon recovery of normal coupling, following removal of the alkanols. Taken together, these studies indicate that close relationships exist between the control of gap junctional coupling and of insulin secretion in the adult endocrine pancreas. Coupling could be a system by which the highly differentiated B-cells coordinate their secretory function and, in particular, their physiological response to nutrients.

POSITIONAL SIGNALLING AND MORPHOGENESIS IN CHICK LIMB DEVELOPMENT

by

L. Tickle

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During limb developmen, organized arrays of dierentiated cells are generated from a population of apparently homogenous mesenchyme cells. This process of pattern formation depends on cell interactions in the developing limb bud. A signal from tissue at the posterior margin of the bud, the polarizing region, appears to control the pattern of cellular differentiation across the antero-posterior axis. This signal can be mimicked by local application of retinoids, such as retinoic acid. Pattern formation across the antero-posterior axis is linked to bud morphogenesis by the activity of the apical eetodermal ridge, the thickened rim of epithelium at the tip of the bud. The apical ridge mediates bud outgrowth and its extent is modulated by

retinoid application. Gap junctions are found between the mesenchyme cells of the bud and link the epithelial cells of the apical ridge. These potential pathways for cell-cell communication may be involved in positional signalling and the control of bud outgrowth.

ANTIBODIES TO GAP JUNCTION PROTEIN AS PROBES FOR STUDYING CELLULAR INTERACTIONS DURING DEVELOPMENT

by

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Antibodies raised against the major 27kD protein electrophoretically eluted from isolated rat liver gap junctions (Gilula *et al.*, Ms in preparation; Warner, Guthrie and Gilula, 1984) inhibit both dye transfer and electrical coupling between cells of the early *Xenopus laevis* embryo but have no effect on cleavage or cell membrane resting potential (Warner, Guthrie and Gilula, 1984). These antibodies have been used to generate clones of communication incompetent cells in different regions of the early amphibian embryo and so explore the developmental consequences of inhibiting direct cell to cell communication. The results show that blocking communication through gap junctions has profound effects on embryonic patterning and induction.

III. POSTERS

1.

MEASUREMENT OF JUNCTIONAL DYE PERMEANCE IN NOVIKOFF HEPATOMA CELLS

Rebecca Biegon

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A procedure has been developped recently for quantitating the permeance of gap junctions to the fluorescent dye Lucifer Yellow. In pairs of cultured rat (Novikoff) heptoma cells, junctional permeance, defined as the product of junctional permeability coefficient (P) and total cross-sectional area of open junctional channels (A), increase in rough proportion to cell volume. The relationship between dye permeance (PxA) and cell size is currently being studied in pairs of freshly reassociated Novikoff cells.

2.

HOMOLOGOUS AND HETEROLOGOUS JUNCTIONAL COMMUNICATION IN PRIMARY CULTURES OF RAT EMBRYONIC TISSUE WITH MAMMALIAN TUMOR CELLS

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Intercellular communication in primary cultures of rat embryonic nerve and glial cells was studied using micro-iontophoresis of Lucifer yellow as well as freeze-fracture-techniques. Junctional communication between glial ceils could be detected by extensive dye spreading. Freeze-fracture replicas revealed numerous gap junctions, arranged in large plaques. In order to study intercellular adhesion and communication in tumor cell invasion of normal brain tissue, primary cultures of rat embryonic brain were confronted with several coupled and non-coupled mammalian tumor cell lines. Dye coupling measurements were applied to these heterogeneous cell cultures.

3.

CHANGE OF THE PLASMA MEMBRANE-CYTOSKELETON COMPLEX IN GERMINATIVE AND EQUATORIAL CELLS OF THE BOVINE LENS EPITHELIAL

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It is possible that subtle topographical modifications in organization in vivo between the lens capsule and the plasma membrane-cytoskeleton complex play a role in controlling the differentiation of bovine epithelial lens (B.E.L.) cells in the equatorial lens region. A technique based on the Triton X-100 insolubility of some cytoskeleton-associated membrane components has been employed to establish whether the appearence of the detergent-resistant complex in the central epithelial region differs from that in the germinative and equatorial one. Our results are consistent with the idea that the organization of at least 4 surface proteins and 4 glycoproteins connected to actin microfilaments is contingent on the topographical localization of BEL cells on the lens capsule.

4.

GAP JUNCTION FORMATION IN THE UTERINE EPITHELIUM OF THE RABBIT

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The uterus epithelium has been used as a model for studying alterations of gap junction formation in the preimplantation phase of non pregnant, pregnant and pseudopregnant animals. Morphological investigations using freeze-fracture-methods have shown, that a considerable number of gap junctions are found only at day 7 of pregnancy on the lateral membrane. In non pregnant and pseudopregnant animals, however, at this stage only small gap junctions are detectable. By iontophoretic injections of Lucifer yelow we can demonstrate intercellular spreading via open gap junctions exclusively in pregnant animals and here only in the implantation chamber but not in the blastocyst free segments of the same uterus.

5.

IMMUNOCYTOCHEMICAL AND FUNCTIONAL STUDIES USING RABBIT ANTISERA TO LOBSTER (NEPHROPS NOVERGICUS) GAP JUNCTIONS

T.E.J. Buultjens, J. Pitts, E. Kam and M. Finbow
(Glasgow)

On immunoblots a rabbit antiserum to isolated lobster gap junctions recognizes the 18,000 dalton junctional protein (and its multimeric forms) and a 52,000 glycoprotein. The anti-52,000 activity can be removed by three rounds of affinity purification using a modification of the Olmsted procedure (Olmsted, J.B., J. Biol. Chem., *256*, 11955, 1981). The affinity purified anti-18.000 antiserum binds strongly to isolated lobster junctions as shown by (1) the antiserum-induced agglutination of lobster junctions detected by phase-contrast light microscopy and (2) the extensive decoration of lobster junctions by protein A-Gold following incubation with the antiserum. When microinijected into cells of the rat liver (BRL) line the antiserum rapidly blocks dye transfer. This antiserum has enabled to localize the 18,000 dalton protein to gap junctions and serves as a useful reagent for the evaluation of the role of junctional communication both *in vitro* and *in vivo.*

6.

STUDIES ON MECHANISM OF TPA INHIBITION OF JUNCTIONAL COMMUNICATION

J.S. Davidson, I.M. Baumgarten and E.H. Harley
Dept Chemical Pathology, University of Cape Town

Intercellular communication between argininosuccinate lyase-deficient fibroblasta and V79 cells, measured by citrulline incorporation is completely inhibited by TPA. Treatment of co-cultures with exogenous phospholipase C or OAG aiso caused communication inhibition, suggesting that protein kinase C activation leads to communication inhibition. However, co-cultures which had been made refractory to TPA inhibition by prolonged exposure to high concentrations, remained sensitive to inhibition by phospholipase C and OAG. This surprising result may indicate that refractoriness to TPA is not simply due to downregulation of protein kinase C.

7.

MP 26 AS A MARKER FOR TERMINAL DIFFERENTIATION AND AGEING OF LENS FIBER PLASMA MEMBRANE

I. Dunia, S. Manenti, D. Louvard* and E.L. Benedetti
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*** Institut Pasteur, Paris - France**

The lens fiber plasma membrane comprises a major component MP 26 which is cotranslationally inserted in plasma membrane domains.

Freeze fracture and immunolnbelling techniques are applied for the study of the long and short range distribution and stabilisation of MP 26 in the plane of the plasma membrane, in developping and ageing fibers, and in reconstituted lipid vesicles comprising MP 26.

8.

INHIBITION OF INTERCELLULAR COMMUNICATION (IC) IN CULTURED CELLS BY DIACYGLYCEROL, A FUNCTIONAL ANALOGUE OF PHORBOL ESTERS

U. Frixen, T. Enomoto and H. Yamasaki
International Agency for Research on Cancer, Lyon, France

We studied IG of BALB/c 3T3 and Syrian hamster embryo (SHE) cells treated with diacylglycerol that substitudes for 12-0-tetradecanoyl-phorbol-13-acetate (TPA) binding to protein kinase C. An almost complete inhibition of dye-coupling in 3T3 and SHE cells was observed after 0.5 and 1.5 h incubation, respectively, with 200 µg 1-oleoyl-2-acetyl-glycerol (OAG, synthetic diacylglycerol) per ml culture medium. In 3T3 cells IC returned to control level after 8 h culture with OAG and was not inhibited after further addition of OAG within 24 h.

9.

THE PROJECTION STRUCTURE OF GAP JUNCTIONS ; EFFECT OF LATTICE DIMENSION

Edward Gogol and Nigel Unwin
Stanford University

We have examined the projection structure of rat liver gap junctions over a range of lattice dimensions (covering approximately 80 to 92 A), using minimum-dose electron microscopy of uranyl-stained specimens. The major effect of shrinking the lattice spacing (by treatment with increasing concentrations of detergent) is an apparent skewing of the channel protein with respect to the lattice vectors. This packing variability must be better understood and controlled in order to properly probe the three-dimensional structure of the junctions in a minimally perturbed state.

10.

Sarah C. Guthrie
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In the early amphibian embryo, disruption of gap junctional communication with antibodies can have profound effects on subsequent development. Such antibodies can also be used to study a range of other interactions during amphibian embryogenesis, including axis formation and neural

induction. Organisation of the axis may be initiated by specific cells at early stages; the consequences of blocking junctions in this region will be discussed. A procedure for observing neural induction in culture and investigating communication between inductive mesodermal and induced ectodermal cells will also be described.

11.

GAP JUNCTIONS IN THE EYE PRIMORDIA OF AMPHIBIAN EMBRYOS

J. Kohonen and Tuula Jalonen
Department of Biology, University of Turku, SF-20500 Turku, Finland

In the amphibian neurulae homotypic cells in the lens ectoderm and in the optic vesicle are coupled by gap junctions. During optic cup formation the gap junctions in the prospective retina are endocytosed, and appear as annular formations in the cell interior. Gap junctions of the lens ectoderm persist through lens primordium formation and differentiation of the lens.

Gap junctions probably facilitate the synchronization of the early events of the retinal differentiation but are replaced by other means of intercellular communication as the sensory system of retina begins to develop.

12.

GAP JUNCTION FORMATION BETWEEN CULTURED HEART CELLS

HJ. Jongsma, M.R. Rook and B. de Jonge
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When isolated heart cells in tissue culture grow together they synchronize their beat rate most often from one beat to another. Between the contraction moments of the synchronized cells a delay is observed which tends to shorten with time. Already 45 s after synchronization fairly large gap junctions are observed between the cells. These results suggest that gap junctions either develop very fast or that they are formed before they become functional.

13.

COMMUNICATION COMPARTMENTS IN NEW BORN MOUSE SKIN

E. Kam, D. Morgan, L. Melville and J. Pitts
(Glasgow)

The aim of this project is to study the organisation of the skin in normal and tumourigenic conditions with respect to cell-cell communication. We have chosen to use mouse skin because of the relatively well-defined histology, easy accessibility and large amount of information on tumour induction in this organ. Using microinjection of Lucifer Yellow CH into excised strips of new born mouse skin, and dye-injection and nucleotide transfer in mixed cultures derived from mouse skins, we are able to demonstrate several kinds of communication compartment. It appears cells in the epidermis and dermis, although they can communicate with other homologous cells, generally fail to communicate with heterologous cells. The compartmentalisation does not seem to be caused by the basement membrane because in mixed cultures, communication compartments exist between fibroblasts and epithelial cells despite the absence of discernable basement membrane. In the stratified squamous epidermis, epithelial cells form small communication compartments consisting of 6 or 7 cells which might correspond to the reported proliferative units. In the hair follicle, again, small groups of cells form communication compartments

which might be related to developing sebaceous glands. Some epithelial cells at the external root sheath of the hair follicle, but not the stratified squamous epithelial cells, communicate with the neighbouring fibroblasts. This phenomenon may be related to the growth of the hair follicle which might require epithelial-fibroblastic interaction.

On-going experiments in our laboratory will continue to examine the pattern of communication in the developing mouse skin as well as during the process of tumourigenesis.

14.

BREAKDOWN OF FOLLICLE CELL-OOCYTE DESMOSOME JUNCTIONS INDUCED BY MATURATION-PROMOTING FACTOR (MPF) IN STARFISH OVARIAN FOLLICIES

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Immature starfish oocytes are each surrounded by an envelope consisting of follicle cells, the processes of which are connected to the oocyte surface with desmosomes. Such follicular envelopes break down at the lime of oocyte maluration. In the present study, maturation-promoting factor (MPF), which is produced and induces maturation in oocyte cytoplasm after 1-methyladenine addition, was found to act from the inside of oocytes to disrupt desmosomes between follicle cells and oocytes, resulting in the breakdown of follicles.

15.

DIFFERENCES IN TRANSPORT BEHAVIOUR OF LOW- AND HIGH MOLECULAR WEIGHT SUBSTANCES IN MOLLUSCAN EMBRYOS

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The low molecular weight tracer Lucifer Yellow (LY) and the high molecular weight tracer horseradish peroxidase (HRP) have been injected in embryos of several species of equal cleaving molluscs. Transport of LY was found to occur earlier than of HRP. In addition to peripheral spreading, positive HRP was found to be transported directly from the central macromere to the animal micromeres during 32-cell stage. This phenomenon was not found for negative HRP or LY.

16.

FILIPIN DIGITONIN STUDIES OF MEMBRANE CHOLESTEROL IN FROG ATRIAL FIBERS WITH THE UNUSUAL GAP JUNCTION CONFIGURATION

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The distribution of cholesterol in frog atrial fibers was studied with filipin a polyene antibiotic and digitonin as probes. The filipin/sterol complexes were randomly distributed on the P-frncture face outside the mature gap junction but were never found inside the smooth area circumscribed by the circle of junctional particles. The same results was obtained with digitonin. At the level of junction formation zones almost no filipin/sterol complexes were found.

17.

THE FATE OF NEXUS COMPLEXES IN ADULT CARDIOMYOCYTES DURING ISOLATION FROM TISSUE AND IN PRIMARY CULTURE

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In low Ca^{2+} perfused myocardium the intercalated discs separate. While fasciae and maculae adhaerentes split, nexus complexes are teared out from adjacent cells in toto. Subsequently, part of these complexes are incorporated into the cell body and their remnants disappear in 24 h primary culture. These terminally differentiated cells do not divide and show only minimal spreading. However, closely adjacent cells form new cell-cell contacts, e.g. nexus, often in an end-to-side configuration of the two cells that does not occur in vivo.

18.

GAP JUNCTION REDUCTIONS PRECEDE TISSUE HYPERPLASIA IN THE RDOSOPHILA ts-MUTTANT c43

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Reductions in gap junction number and density precede the onset of tissue hyperplasia (as defined by an increase in cell number and abnormal morphology) in wing discs of the *Drosophila* ts-mutant *1(3) c43 hsl*, following upshift to the restrictive temperature of 28°C. The finding that reductions in gap junctions precede rather than coincide with or follow the initiation of tissue overgrowth supports the idea that gap junctions are causally involved in regulating growth and pattern formation in normal development.

19.

REGULATION OF CELL-TO-CELL DIFFUSION VIA GAP JUNCTION CHANNELS

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Rates of diffusion of co-injected fluorescent dyes (expressed as effective diffusion coefficient, De) have been measured among the developing epidermal cells of a larval insect. The molting hormone, ß-ecdysone, raises De for the smaller dye carboxyfluorescein (CF) from 3.7 to 4.9 $\times 10^{-7}$ cm^2/s and for the larger dye lissamine rhodamine (LRB) from 1.5 to 2.0 $\times 10^{-7}$ cm^2/s. The anticalmodulin drag chlorpromazine lowers De for CF and LRB (over 20 min) from 3.4 to 1.6 and from 1.5 to 0.6 $\times 10^{-7}$ cm^2/s respectively. The ratio of the diffusion rate of the smaller dye to the diffusion rate of the larger dye remains constant which suggests that channels open/close in an all-or-none manner.

20.

F. Serras, M.R.L. Krul, W.M. Kühtreiber and J.A.M. van den Biggelaar
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In the early molluscan embryo Lucifer Yellow is transported aspecifially. In later developmental stages dye transport is restricted within specific compartments which correspond with presumptive areas.

21.

SINGLE CHANNEL AND MACROSCOPIC PROPERTIES OF RAT LIVER GAP JUNCTIONS

D.S. Spray*, J.C. Saez* and El. Hertzberg**
•Einsten **Med., Bx. N.Y. and **Baylor Colls.** Houston, Tx.

Junctional conductance (g,) between voltage clamped pairs of rat hepatocytes is not voltage dependent, is pH sensitive (pK_a c. 6.4, Hill coefficient c. 8), is blocked reversibly by octanol and irreversibly by injected antibody. Open times of single channels (recorded dipping patch electrodes into isolated gap junctions through a surface of lipid) are not voltage dependent, are largely reduced between pH 6.5 and 6.0 and are blocked by antibody. Unitary conductance is c. 100 pS in .15 m KC1 or NaCl and somewhat less in Cs SO_4. Similar physiological properties of intact and isolated junctional membranes suggests that gating is intrinsinc to the gap junction macromolecule.

22.

IONTOPHORESIS OF DEVELOPING LEPIDOPTERAN WINGS

Ruth Taylor
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Dye injection is often used to demonstrate intercellular communication boundaries. Weir and Lo have shown that in *Drosophila* wing disks, boundaries shown by iontophoresis and cell lineage studies correspond. In Lepidoptera, the colour patterns in each wing-cell apparently develop independently. This study is to show whether boundaries exist (1) at the edges of each wing-cell, or (2) at the positions of each pattern element, and whether the dynamics of the system indicate the passage of a determination wave over the wing.

23.

PHOSPHORYLATION OF THE 26K GAP JUNCTION PROTEIN IN CULTURED MOUSE HEPATOCYTES

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The incorporation of ^{32}P phosphate into the 26k gap junction protein in cultured mouse hepatocytes is stimulated 2 to 4 fold by addition of dibutyryl cAMP or of forskolin to the culture medium. Under these conditions at least two serine residues of the 26k protein become labelled. When cultured hepatocytes reached confluence and ceased to proliferate about 90 p. 100 of these cells were coupled as shown by intercellular diffusion of microinjected Lucifer Yellow. Possibly phosphorylation of the 26k protein is necessary for optimal function of the intercellular gap junction channel.

IV. CONCLUDING REMARKS

The workshop provided an opportune forum for the discussion of a number of controversial issues which have arisen during the past year or so. Of these the most fundamental is the arguement over the identity of the structural protein of the gap junction.

The established view (since 1979) supports a 27k protein but more recent work (since 1983) favours a 16k protein. Finbow presented data based on peptide mapping and Western blot analyses showing that the 16k protein is unrelated to the 27k and that the 16k protein **is** present in gap junction preparations made by all of the published methods. He argued that the 27k protein cannot be of junctional origin because it is lost when junction preparations are treated with trypsin even though the junctional morphology is unaffected. This dilemma, which has been seen for some time, was previously explained when Revel published data showing that a 10k band seen in trypsin treated preparations is a fragment (or two comigrating fragments) of 27k. Finbow, however, presented evidence in direct contradiction showing that 10k is not derived from 27k but from an unrelated 24k protein seen in some junction preparations. He also showed that the 16k protein is the major component in junction fractions prepared from 4 different tissues, that it is highly conserved between vertebrate species and that it is related to the 18k protein which is the major component of arthropod gap junctions.

There is as yet no disagreement in the literature over the 18k arthropod junctional protein but Gilula presented some new data showing that crayfish nepatopancreas contains structures which look similar to gap junctions in that they have double membranes but are distinct because they are thinner and have an intracellular location. Gilula reported that the major protein component of these structures also has a M_r of 18k. In discussion Lane said she had also seen these structures in lobster hepatopancreas and measurements showed they were thinner than the in situ or isolated gap junctions. There was therefore no reason to believe that the arthropod protein isolated by Finbow came from these structures.

Much of the recent evidence in favour of the 27k protein is based on new work with antisera raised against proteins in junction preparations. Conflicting data were presented by Hertzberg and by Willecke. Whereas Hertzberg finds an immunologically related 27k protein in all junction containing tissues **so** far studied, Willecke's

antiserum shows only limited tissue cross-reactivity. Gros also presented evidence, based on peptide mapping, for differences between the 27k liver protein and his 28k heart protein. Gilula added to the confusion by presenting evidence for phylogenetic conservation based on immunological cross-reactivity of 54k proteins using an antiserum which also recognises rat liver 27k (see below). Some of these polyclonal antisera contain activities which bind to what appear to be, by fluorescence microscopy, membrane domains (Hertzberg, Willecke) but at this resolution it is not possible to decide if these are gap junctions. Results with two interesting site specific antisera were also presented. Evans, using an antiserum raised against the N-terminal peptide of; the 27k protein, gave evidence showing the N-terminus is on the extracellular face of the membrane while Pitts' group, using one raised against the N-terminal octa-peptide, find the N-terminus of the 16k protein is on the cytoplasmic face. The site specific antiserum against 16k, but not that against 27k, binds to isolated gap junctions (as shown by gold EM immunocytochemistry) and blocks junctional communication when injected into cultured rat liver cells. This serum also cross-reacts with a cytoplasmic determinant of the lobster 18k junctional protein. • Hertzberg's polyclonal anti-27k serum also blocks functional communication (Spray) but it has been shown (Hertzberg, Finbow) that this antiserum also contains anti-16k activity.

The main antagonists in this 27k-16k arguement still maintained their positions at the end of the meeting but it was agreed that their was no longer an «established» view.

Most people accept that lens fibre junctions are different from the communicating gap junctions found in other tissues and, before the meeting, that the structural protein of these junctions is the main intrinsic protein (MIP26) of lens membranes. Johnson, using poly- and monoclonal antibodies, showed with peroxidase EM immunocytochemistry that MIP 26 is localized in the junctions. Goodenough, on the other hand, showed his poly- and monoclonal sera only decorate the non-junctional regions of the fibre membrane. He therefore suggested that MIP26 is not the junctional protein and went on to present unpublished data of Kirstler and Bullivant who can label lens junctions with a monoclonal antiserum which only recognises a 70k protein in Western blot analyses. This new disagreement further illustrates the difficulties of identifying membrane structural proteins which have no known associated activity to follow during isolation.

Reconstitution studies could be helpful in a number of ways for both lens junctions and other gap junctions. Benedetti and Bloemendal presented some data on the reconstitution of MIP26 into lipid vesicles. They find the protein aggregates into domains which are reminiscent of the junctions seen between fibre cells in freeze-fractured lens tissue.

In contrast to the disputes over the proteins, general agreement is emerging about the 3-dimentional maps of the gap junctional channel obtained by EM and X-ray diffraction analyses of both fixed and frozen preparations. The presently available resolution shows the approximate size and positions of the six subunits which surround the central hole of the channel and shows the apparent closure of the hole in the presence of added calcium (Unwin). Gogol showed that the skewing of the subunits which accompanies channel closure can also be induced by adding detergents, a treatment which shrinks the lattice. Detailed discussion centred, among other things, on the size estimates which can be made for the junctional subunit from these approaches. This is usually quoted as 27,000 but this choice has been influenced by the belief in the 27k protein. Volume estimates can

only provide a guide of between 20k and 30k and each subunit could contain more than one polypeptide chain. These uncertainties, coupled with the difficulties in estimating the M_r of very hydrophobic proteins by SDS-PAGE (estimates can be too low by as much as 30 p. 100), means that as yet physical analysis cannot resolve the protein issue.

Gilula presented data to show that a 54k band sometimes seen in gap junction preparations is due to contaminating B-fibrinogen, and then confused the picture by explaining that the 54k protein used to show phylogenetic conservation and the 54k present in Xenopus embryos are not B-figrinogen but are related to the 27k protein. This conclusion is based on immunological cross-reactivity using a double affinity purified polyclonal antiserum. Pitts however showed the results of a similar double afinity purification carried out in an attempt to remove an anti-52k activity from an antiserum raised against lobster junctions. This was unsuccessful unil an additional absorption step with 52k was added between the two affinity steps against 18k.

Warner presented her recent data showing the effect of injecting Gilula's double affinity purified antiserum into one of the cells of 8-cell stage Xenopus embryos. She also presented new data showing that the same developmental abnormalities (tadpoles with missing or deffective right eyes) could be induced by injecting short mixed antisense oligonucleotides made according to the amino acid sequence of the 27k protein. This approach to understanding the functions of junctional communication in development depends on the specificity of the antisera or oligonucleotides for blocking junctional channels. The absence of any detectable 27k in Xenopus 8-cell embryos and the lack of direct evidence relating the 54k band to 27k, on top of the other uncertainties about the identity of the junctional protein gave cause for concern during discussion. A further complication also came to light in the form of as yet unpublished experiments carried out by de Robertis which were described by Warner. He has injected cloned antisense DNA to the Xenopus homeobox and to E. coli gal into the same cells of Xenopus embryos and gets very similar one eyed tadpoles.

During the general discussion it was agreed that specific antibodies and perhaps oligonucleotides with demonstrated inhibition specificity would be important reagents for future work if it could be shown that inhibition of junctional communication did not have general affects on cell growth which interferred with the normal expansion of a developmental clone. It was also pointed out that reagents which must be injected might be particularly valuable for the analysis of early embryos, but for later development where junctional communication must be inhibited in many cells, other reagents are required which can be added extracellularly and which will interfere specifically either with junctional permeability or with junction formation.

Work on the molluscan embryo presented by van den Biggelaar provides some of the best evidence to date for communication compartments. At the 32 cell stage all the blasomeres are dye coupled but after the onset of the larval stage, the spread of injected dye is restricted to sharply defined regions which correspond to morphogenetic compartments. It is possible that junctional communication within compartments plays some important role in the distribution and coordination of cell activities. The general idea of junctions providing a pathway for cellular coordination was also dealt with by Meda. He presented a detailed study of communication within pan-

creatic islets and suggested that B-cells coordinate their secretory functions and physiological responses to nutrients through junctional coupling. These studies on intact tissues are providing a satisfying extension to the work on metabolic cooperation and other phenomena resulting from the partial syncytial state produced by junctional coupling which have already been well studied in cultured systems.

Van den Biggelaar also described a new phenomenon, the spread of peroxidase from cell to cell in early molluscan embryos. The spread between blastomers is rapid, clear-cut and follows specific patterns. It cannot be explained by residual bridges left behind after cell division or by gap junctions. The most likely mechanism may involve exo- and endo-cytosis between closely adjacent cells but as yet there is no evidence to support this.

Caveney provided data showing that in the insect epidermis the number of junctional channels per cell appears to be regulated to allow increased coupling as the cell sheet increases in size. The approximately 3-fold increase in junctional conductance which accompanies a 9-fold increase in epidermal area suggests communication times within the population may remain fairly constant. The mechanism may simply be related to the increased depth of the cells providing increased area for lateral membrane contact. Goodall described the regulation which occurs in the early mouse embryo and the effects of mitotic inhibitors and antibodies which interfere with compaction (the time at which gap junctions first appear). It appears that junctional communication is not required for the establishment of polarity or for the subsequent commitment to trophectoderm and inner cell mass.

A debate has rumbled on for many years about the possible role of junctional communication (or its loss) in the onset or maintenance of the transformed state. Perhaps the most direct evidence for an association is provided by recent work of Sheridan who described the changes in junctional communication which accompany the switching of a temperature sensitive ppSrc60 gene. Coupling is reduced in the transformed state.

Finally the discussion turned to rates of movement (Brink) of ions and small molecules through coupled cell populations and to the control of channel permeability. The gap junction channel does have some form of gate as it can be closed by low intracellular pH, high intracellular calcium, retinoic acid and octanol (a group with no obvious common element). The available antibodies may also operate a gating mechanism but as the blockage produced in this way is incomplete (Pitts) these reagents may just operate through steric hindrance. The demonstration by Unwin that calcium can gate purified junctions suggests there must be a direct interaction which is inconsistent with the reported binding of calmodulin (Hertzberg, Gilula) to the isolated 27k protein.

John Pitts, 19-8-1985