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Invited Review

Cardiac muscle cell interaction: from microanatomy to the molecular make-up of the gap junction

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Summary. Electrical coupling between cardiac muscle cells is mediated by specialised sites of plasma membrane interaction termed *gap junctions*, one of three types of intercellular junction of the cardiac intercalated disk. Gap junctions consist of clusters of plasma membrane channels directly linking the cytoplasmic compartments of neighbouring cells. Gap-junctional channels are constructed from *connexins*, a multigene family of conserved proteins. The principal connexin isoform of the mammalian heart is connexin43; other connexins, notably connexin40, connexin45 and connexin37, are also expressed but in smaller quantities. Antibodies directed against unique sequences of these molecules allow investigation of the role of gap junctions and their component connexins in relation to the electrophysiological properties of the healthy and diseased heart. Confocal laser scanning microscopy of working ventricular myocytes immunolabelled with anti-connexin43 antibodies permits highly sensitive detection of gap junctions, allowing detailed analysis of the spatial distribution of the conduction pathways from the level of the cell to that of the tissue as a whole. Gap junction distribution, number and regional variations in the type of connexin expressed all contribute to the uniform anisotropic pattern of impulse spread characteristic of normal myocardium and the orderly, sequential contraction of the cardiac chambers. Connexin40 is preferentially expressed by myocytes of the atrioventricular conduction system and represents a specialisation facilitating fast conduction, allowing rapid distribution of the impulse throughout the working ventricle. Two major abnormalities in connexin43 gap junctions are detected in human ischaemic heart disease. First, at border zones adjacent to infarct scars, zones which are particularly prone to re-entry arrhythmia, there is marked disruption of the usual ordered distribution pattern of gap junctions. Second, a widespread down-regulation of connexin43 gap junctions occurs in myocardium distant from the infarct, a change that is also found in the hypertrophic (non-ischaemic) heart. Consequent localised heterogeneous conduction and reduced conduction velocity provide an explanation for the genesis of re-entry arrhythmias. A current working hypothesis is that reduction in connexin43 gap junctions is a general pathogenetic feature of cardiac disease which predisposes the heart to arrhythmia, and that this reduction may form part of a wider pattern of alteration in the levels of other connexin isoforms.

Key words: Cardiac myocyte, Intercalated disk, Gap junction, Connexin, Cardiac hypertrophy, Ischaemic heart disease

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Introduction - Cell interactions at intercellular junctions

All but the simplest organisms consist of communities of cells. Precise co-ordination and integration of the activities of the individual cells within these communities are essential if tissues, organs and, in turn, the entire organism are to fulfil their designated functions. Such mutual co-operation calls for various forms of direct interaction between cells, a function mediated by specialised structures termed *intercellular junctions*. Intercellular junctions are formed from connected, apposing domains of the plasma membranes of adjacent cells, and are classified into three major groups according to their structure and functions. *Occluding junctions*, also known as tight junctions, form permeability seals at the luminal edges of epithelial cell sheets; *anchoring junctions* fulfil various mechanical functions; and *gap junctions*, the subject of this review, couple cells together in such a manner that electrical, ionic and molecular signals can flow directly from cell to cell without recourse to the exterior.

The pump-like motion of the heart provides a striking illustration of the critical importance of gap junctions to the co-ordination of cellular function. Sequential contraction of the cardiac chambers is stimulated by the

orderly spread of action potentials via gap junctions acting as points of electrical coupling between all the constituent cardiac muscle cells of the myocardium (review, Page, 1992). These gap junctions are characteristically organised, together with two types of anchoring junction, the fascia adherens and desmosome, in specialised regions of the plasma membrane termed *intercalated disks* (review, Forbes and Sperelakis, 1985; Severs, 1990). The fascia adherens and desmosome are responsible respectively for attachment of the contractile filaments and the cytoskeleton to sites of adherence between the adjoining plasma membranes. Between them, the three types of intercellular junction of the intercalated disk act in concert to integrate electro-mechanical function of all the individual myocytes throughout the atria and ventricles.

The concept of the functional syncytium, through which the activities of discrete cells are integrated by means of intercellular junctions, is fundamental to our understanding of the heart in health and disease. For example, the idea of grafting healthy myocytes into the diseased heart has been advanced as a means to repair severely damaged myocardium in order to overcome the problem that the adult differentiated myocyte, unlike many other cell types, does not have the capacity to repair tissue damage by cell division. For such a cell grafting strategy to succeed, however, the full and complete integration of the grafted cells, by formation of intercalated disks with surviving, healthy host myocytes, would be required. Remarkably, it has recently been shown that such integration may indeed be achieved (Soonpaa et al., 1994), raising exciting possibilities for the eventual development of new therapies.

Microanatomy of the intercalated disk

Before discussing the cardiac gap junction in detail, we will first consider the overall structure of the intercalated disk. Intercalated disks are clearly visible by light microscopy (Fig. 1), but their fine structural details, and in particular the nature of their component junctions, cannot be resolved without the aid of electron microscopy. Early thin-section electron microscopical studies carried out by Sjöstrand and colleagues (Sjöstrand and Andersson, 1954; Sjöstrand et al., 1958; Sjöstrand and Andersson-Cedergren, 1960) established that the cardiac intercalated disk consists of closely interacting plasma membranes of separate cells, and not elements lying within a true structural syncytium of fused cells, as one school of thought had previously believed (for review see Forbes and Sperelakis, 1985). The discovery of gap junctions as we now know them

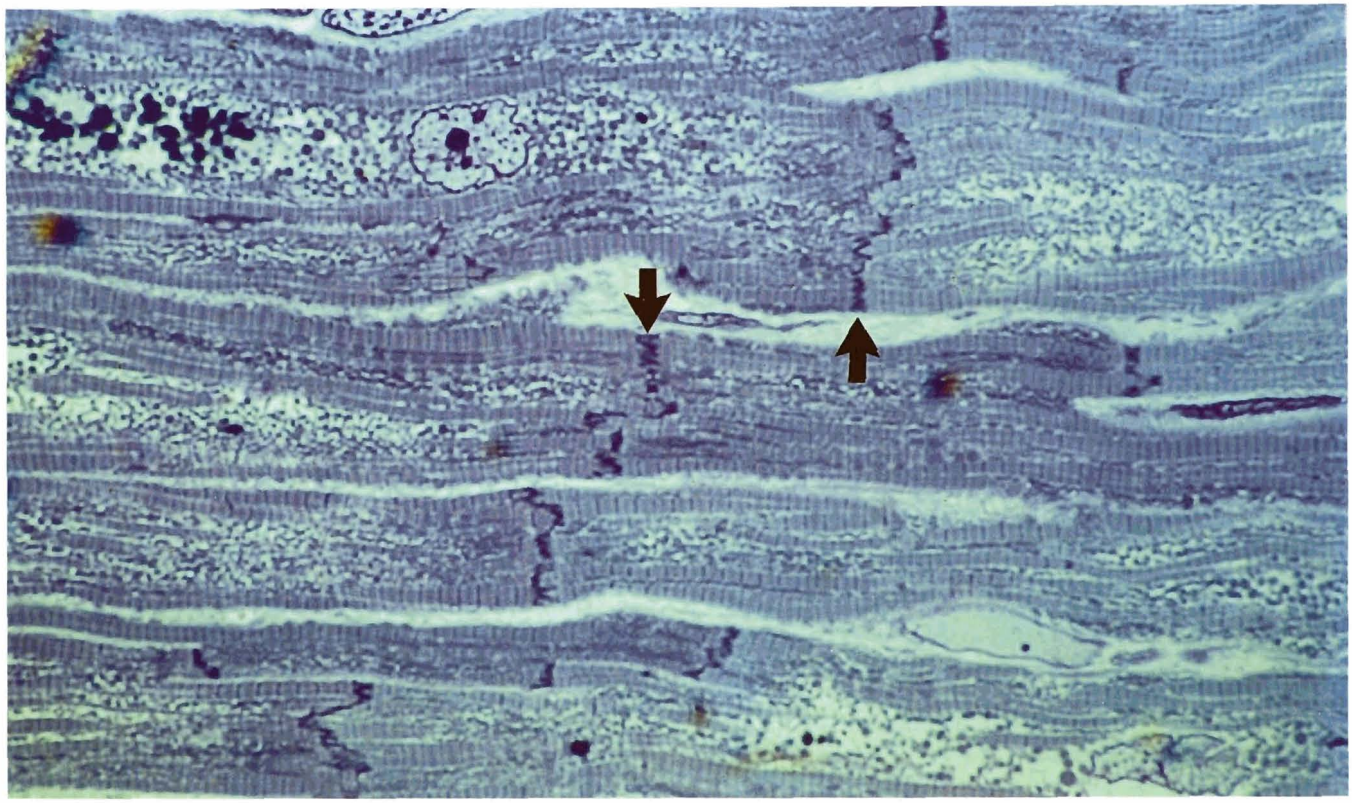


Fig. 1. Human left ventricular myocardium viewed by standard light microscopy (semithin section of toluidine blue-stained, epoxy resin-embedded specimen). Intercalated disks, marking the sites at which individual myocytes are linked together, are visible as darkly staining, somewhat irregular lines (arrows) at the transverse abutments between cells. x 2,500

came from these early electron microscopical studies.

The architecture of the intercalated disk is best understood in relation to the overall structure and shape of individual cardiac muscle cells, exemplified in images of cells that have been isolated from ventricular myocardial tissue (Fig. 2). The typical working ventricular myocyte is an elongate, branching cell of somewhat irregular shape, but this apparent variability belies an underlying common pattern of design. The contractile myofibrils with which the cell is packed may be regarded as the basic building blocks which determine cell shape; because these cylindrical structures are of variable length, and are bunched, side-by-side, with varying degrees of displacement, a variety of

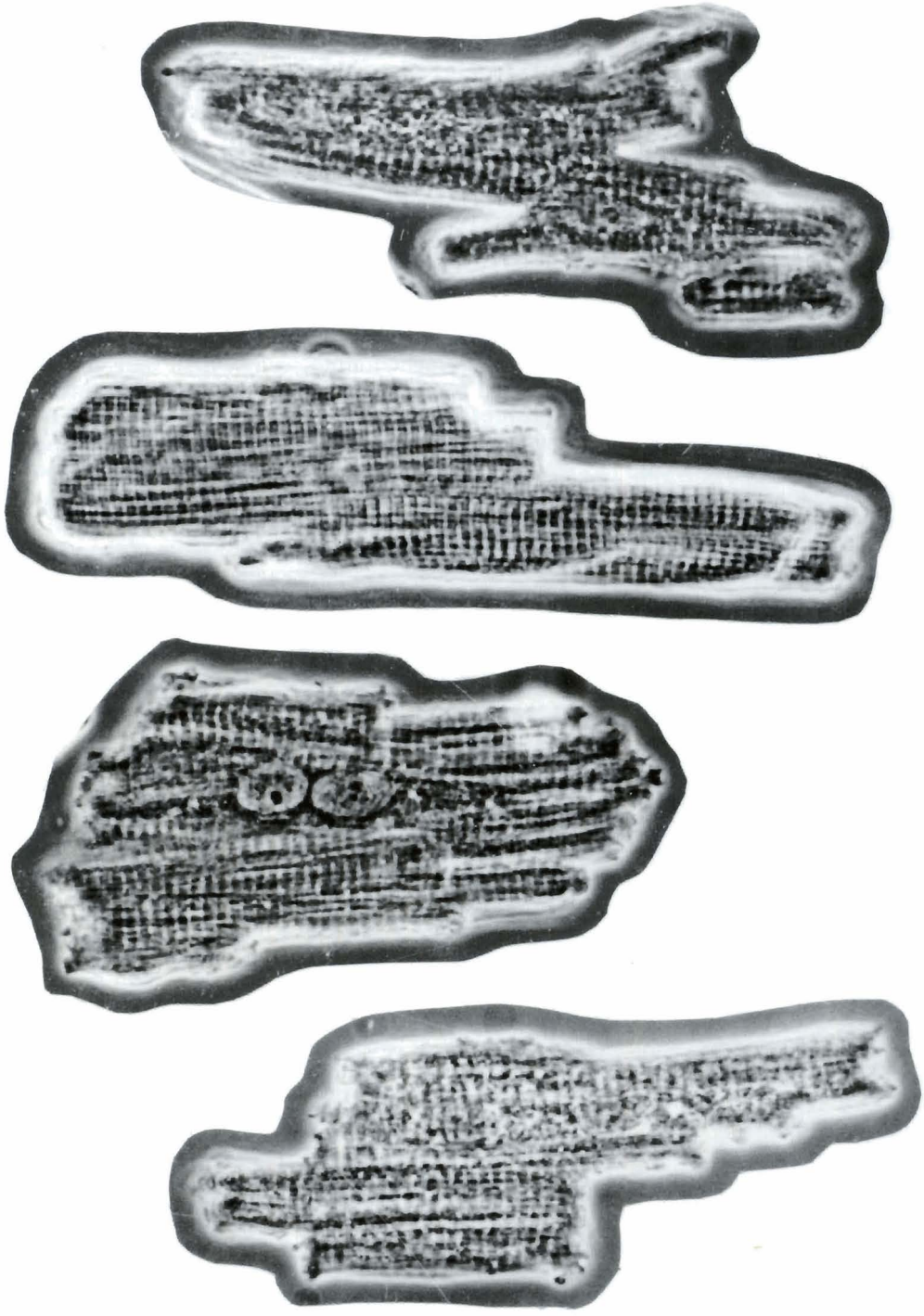


Fig. 2. Examples of isolated adult ventricular myocytes illustrating the variety of branching shapes apparent in the cell population. The overall structure and shape of the myocyte is more easily appreciated from preparations of individual cells like these than from sectional views of cells in situ in the tissue (cf Fig. 1). Light microscopy, phase contrast optics; dissociated rabbit myocytes. x 950. (From Severs (1990), with permission from Elsevier Scientific Publishers).

overall shapes and branching patterns may be generated in different cells (Fig. 2).

Intercalated disks are situated at sites of end-to-end abutment betwen myocytes. They occur not only at the ends of the main body of the cell, but also at the flattened tips of branches that arise along its length, such that, in the ventricle, each myocyte is linked to an average of ~9-12 neighbours (Luke and Saffitz, 1991; Saffitz et al., 1992; Peters et al., 1993b). In this way, a three dimensional branching network of cells is formed, making up the structure of the myofibre. The paired membranes within each intercalated disk typically interact to form a series of transverse zones of variable size which are offset from one another in staggered fashion (Fig. 3). Collar-like zones, in which the membranes lie parallel with the long axis of the cells, encircle the intervening zones, linking the transverse regions together. Accordingly in electron micrographs of thin sections cut parallel to the long axis of the myocytes, the disk shows a characteristic step-like profile (Fig. 4). The membranes of the transverse zones of the disk consist of stubby, interdigitating projections (sometimes referred to as *plicate regions)*, while the longitudinal 'collar' zones are smoothly contoured. The three types of intercellular junction are organised in a highly distinctive manner within these zones (Fig. 5). Fasciae adherentes occupy the transverse plicate zones, where they link the myofibres of adjacent cells in series. Although some desmosomes and small gap junctions are also scattered within the plicate regions, these junction types are most abundant and extensive in the longitudinal 'collar' regions.

In the ventricular myocyte, formation of the mature intercalated disk, with this distinctive pattern of junction organisation, is not completed until about 6 years of age in the human (Peters et al., 1994b,c). Other types of myocyte, even when fully differentiated, show lesser degrees of development of the intercalated disk. For example, atrial myocytes do not have the squat branching shape typical of the ventricular myocyte; they are more spindle shaped and have correspondingly smaller and fewer intercalated disks. And the various forms of myocyte that make up the atrioventricular conduction system show a series of distinct morphologies in which intercalated disks range from prominent to rudimentary or absent (Severs, 1989).

The three types of intercellular junction present in cardiac myocytes are readily distinguished from one another (Fig. 5). The fasciae adherens and the desmosome are characterised by a regular intermembrane space of ~25 nm and by the presence of particularly prominent electron-dense membrane-associated proteins. Gap junctions are less conspicuous, appearing, in transverse section, as profiles in which segments of the adjacent plasma membranes run in such close parallel contact that they appear to touch. Although it was thought in some early studies that the membranes of the gap junction are actually fused, they are, in fact, separated by a gap of 2-3 nm, clearly discernible with the electron-dense tracer, lanthanum (Revel and Karnovsky, 1967). In optimally prepared transverse thin sections, gap junctions have a septilaminar appearance, in which the narrow extracellular gap can just be discriminated at intervals along the profile (Fig. 6).

The potential functional significance of the closeness of plasma membrane interaction at the gap junction did not escape the notice of those engaged in the early morphological studies. Although the fascia adherens was at first discussed as a possible site for intercellular electrical coupling in cardiac muscle (Sjöstrand et al.,

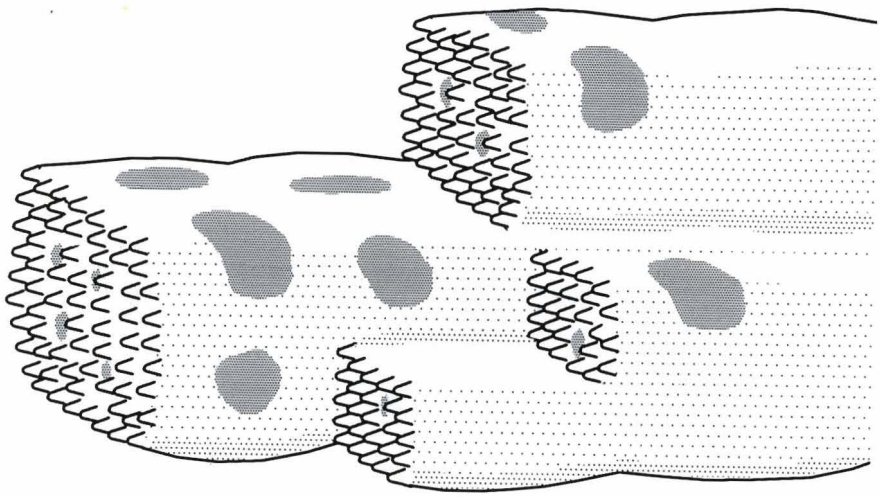
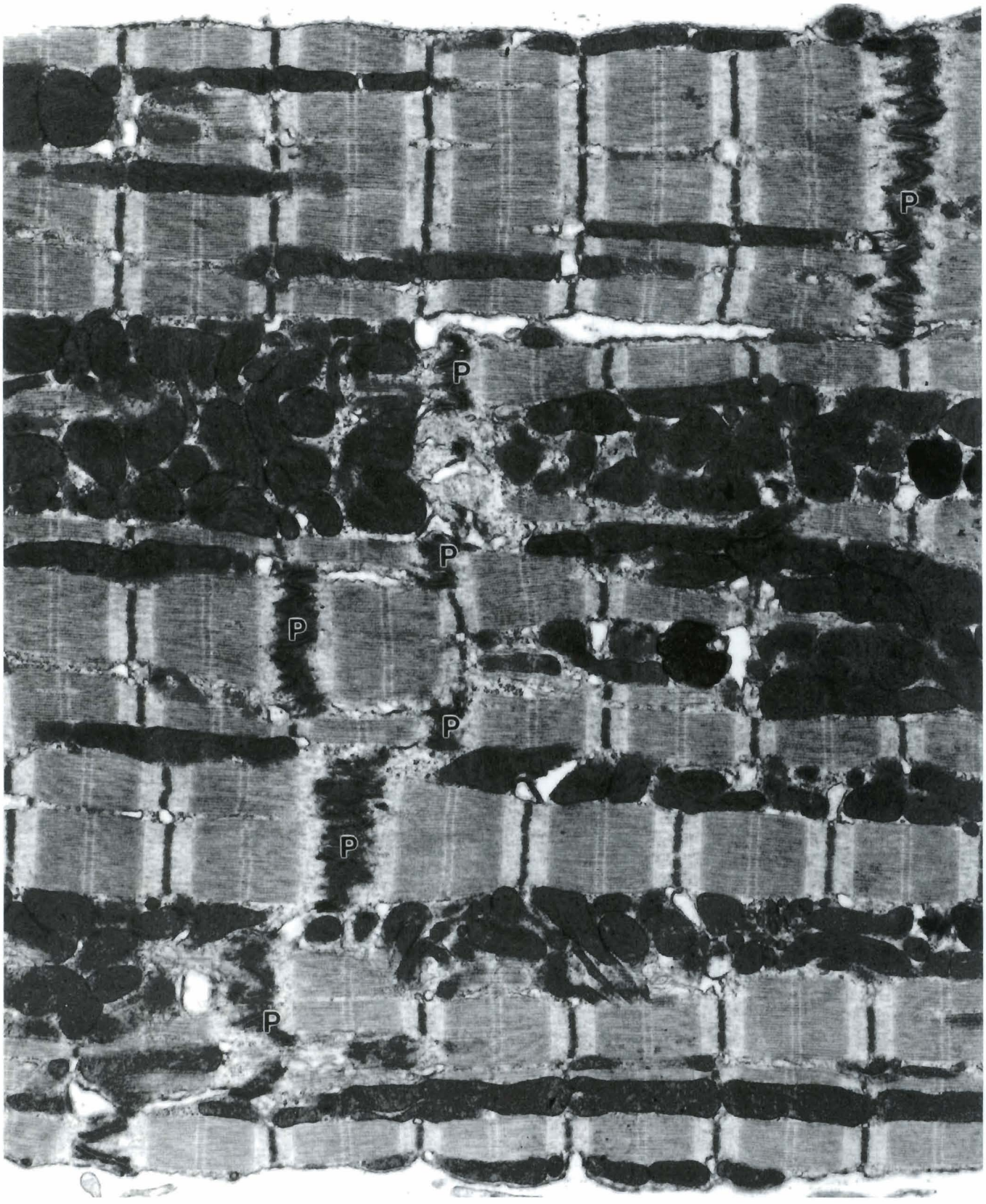


Fig. 3. Diagrammatic representation of the three-dimensional structure of the intercalated disk. The disk comprises two zones; i) transverse regions, and ii) collar-like longitudinal regions. The transverse zones are covered with projections which interdigitate with those of the neighbouring cell. The longitudinal zones wrap around the sides of the terminations of the myofibrils, and are the principal sites at which the larger gap junctions (dark plaques on the diagram) are located.

Fig. 4. Survey view of the intercalated disk as seen by thin section electron microscopy. In sections cut parallel with the long axis of the cells, the structure illustrated in Figure 3 is seen as a series of irregular steps. The transverse, darkly staining plicate regions (P) are prominent, while the longitudinal regions, in which gap junctions and desmosomes are frequent, are more difficult to discern at this magnification. x 16,000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1958), attention soon focused on the focal regions of apparent connection formed by gap junctions as more probable candidates, and correlative electrophysiological and structural studies soon confirmed this view (Barr et al., 1965). It should be emphasised that, apart from electrical coupling in excitable cells, gap junctions have an important role in intercellular communication and co-ordination of function in tissue systems in general. Early studies in non-excitable cells demonstrated that gap junctions form pathways for direct intercellular communication, their presence being a prerequisite for ionic coupling and the direct cell-to-cell transfer of injected marker dyes such as Lucifer yellow and small metabolites such as purines (Loewenstein and Kanno, 1964; Subak-Sharpe et al., 1966; Gilula et al., 1972).

Gap junctions - Clusters of membrane-to-membrane channels

The structural basis for the gap junction's function in intercellular electrical coupling and communication lies in the presence, in its closely apposed membranes, of

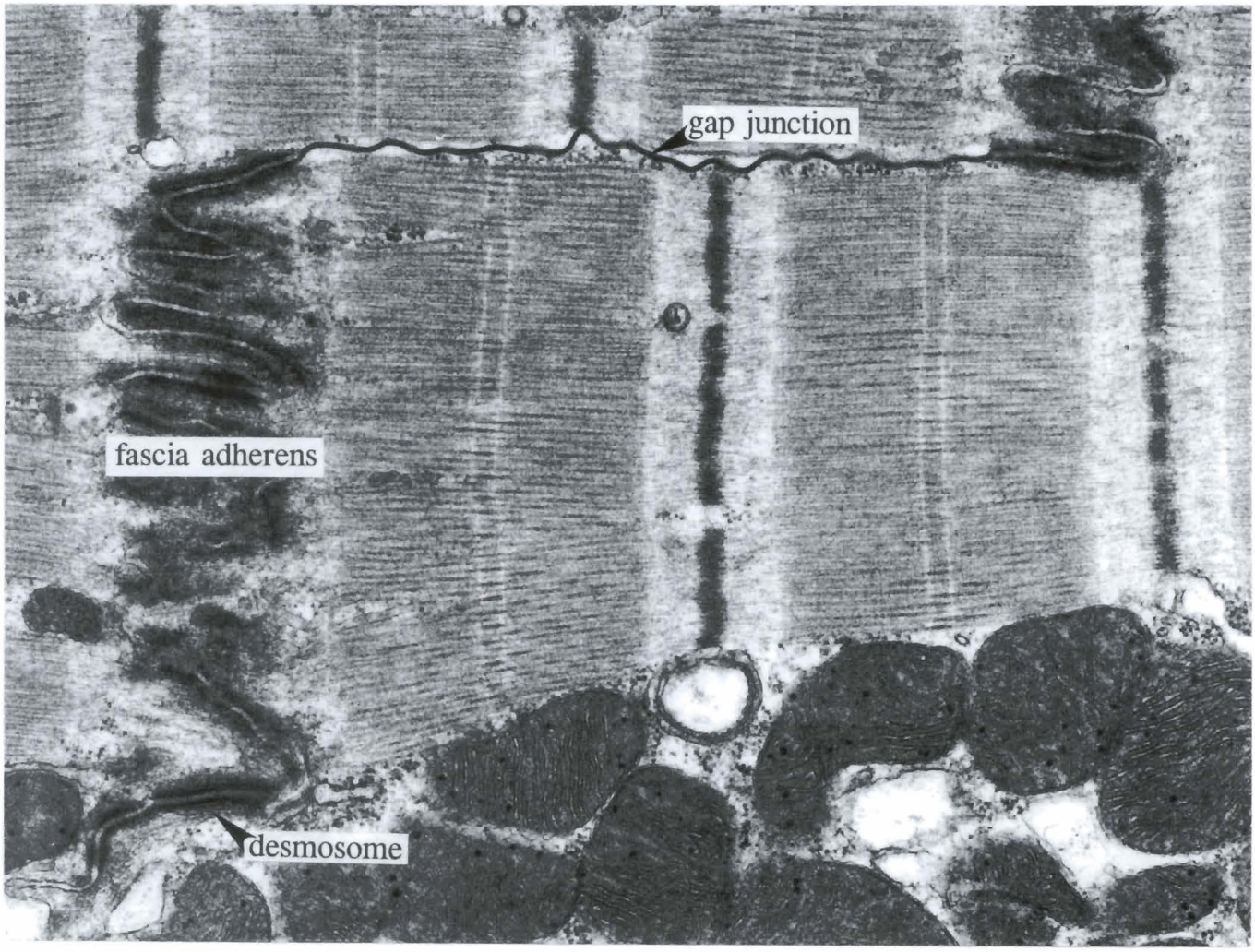


Fig. 5. Higher magnification thin-section view of part of an intercalated disk, illustrating the appearance of the three types of junction - the fascia adherens, the desmosome and the gap junction - organised in their characteristic locations within the disk steps. The fascia adherens junctions in the transverse plicate segments have a prominent electron-dense mat at their cytoplasmic surfaces. The plasma membranes of these junctions contain adhesive glycoproteins termed cadherins, which fasten the membranes tightly together at their extracellular surfaces, while actin-linking proteins of the filamentous mat attach the myofilaments to the cytoplasmic aspect of the plasma membranes. Myofibrils of consecutive cells are thus linked in series, ensuring transmission of the mechanical force of contraction throughout the heart. Desmosomes similarly act as sites at which the membranes are firmly bonded by cadherin-like molecules, and their dense plaques on the cytoplasmic side of each membrane serve as insertion sites for the intermediate filament cytoskeleton. The participating membranes of the fascia adherens and the desmosome are separated by a space of ~25 nm. Gap junctions are distinguished from both these types of anchoring junctions by the extremely close apposition of their two plasma membranes. In lower magnification views, the membranes of the gap junctiosn appear to touch, but at higher magnification, as illustrated in Figure 6, a narrow gap of ~2 nm can be discriminated betwen the membranes. x 36,250. (From Severs, 1990, with permission from Elsevier Science Publishers).

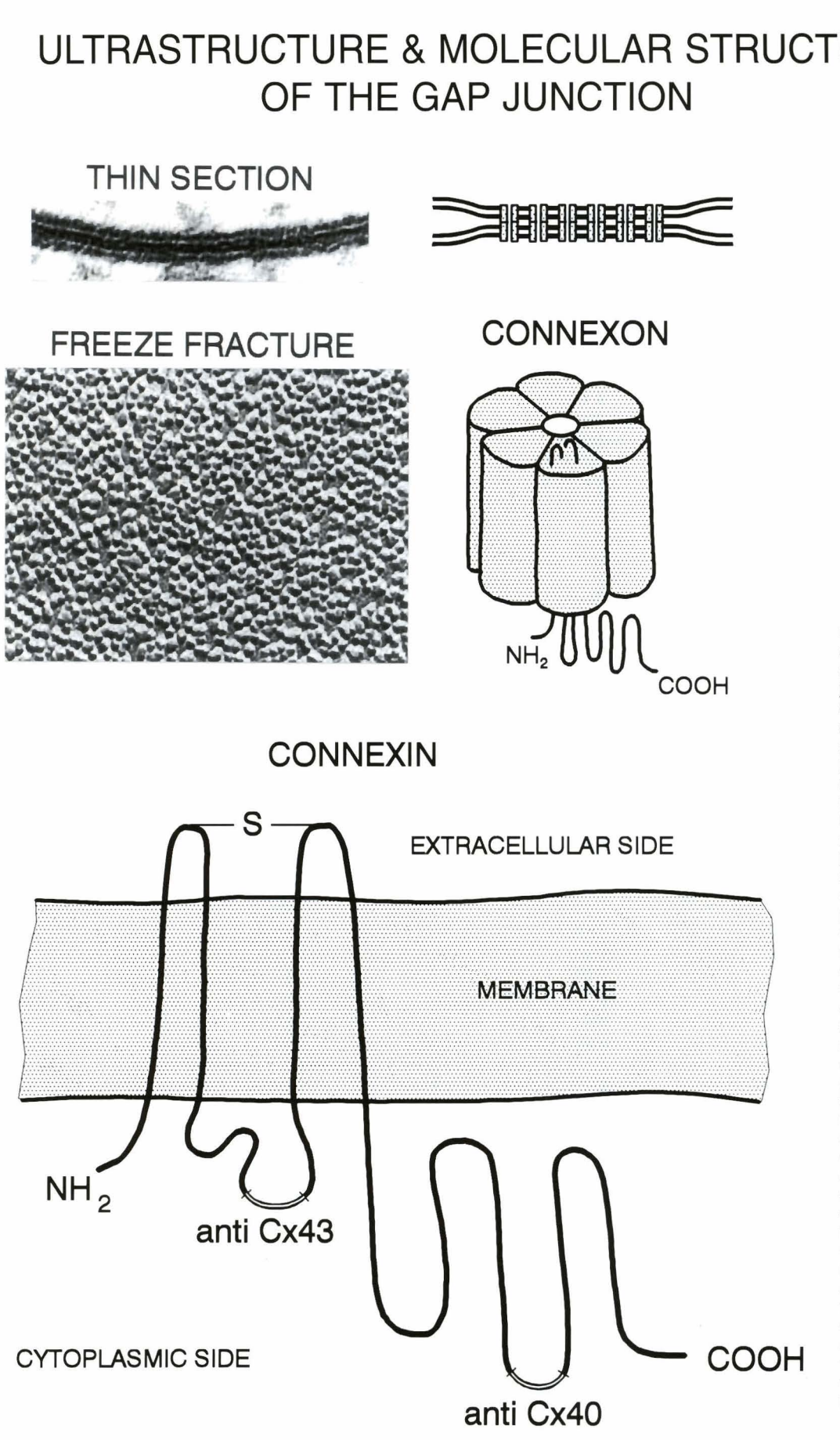


Fig. 6. Ultrastructure and molecular structure of the gap junction. In thin section electron micrographs at high magnification, gap junctions show a pentalaminar appearance, representing two side-by-side unit membranes. The junctional membranes contain clusters of connexons, visible as particles in the face-on views provided by freeze-fracture electron microscopy. Gap junctional channels extending from one cell to the next are formed from abutting connexons (top right). The connexon is a hexameric structure, each of its subunits consisting of a single connexin molecule with the topographical configuration illustrated here and explained in the text. Note positions of the amino and carboxyl termini, intramolecular disulphide bond (S) and segment to which the specific antibodies used for imunolabeling in Figures 9-15 were raised.

clusters of protein channels which extend between the adjacent cells, directly linking their cytoplasmic compartments (for reviews see Bennet et al., 1991; Peracchia, 1991; Page, 1992; Beyer, 1993; Hall et al., 1993). These channels are formed from pairs of hemichannels termed connexons (Goodenough, 1975), which, as illustrated in Figure 7, can be imaged as striking arrays in the membrane by the technique of freeze-fracture electron microscopy (Kreutziger, 1968; Goodenough and Revel, 1970; McNutt and Weisntein, 1970; Chalcroft and Bullivant, 1970).

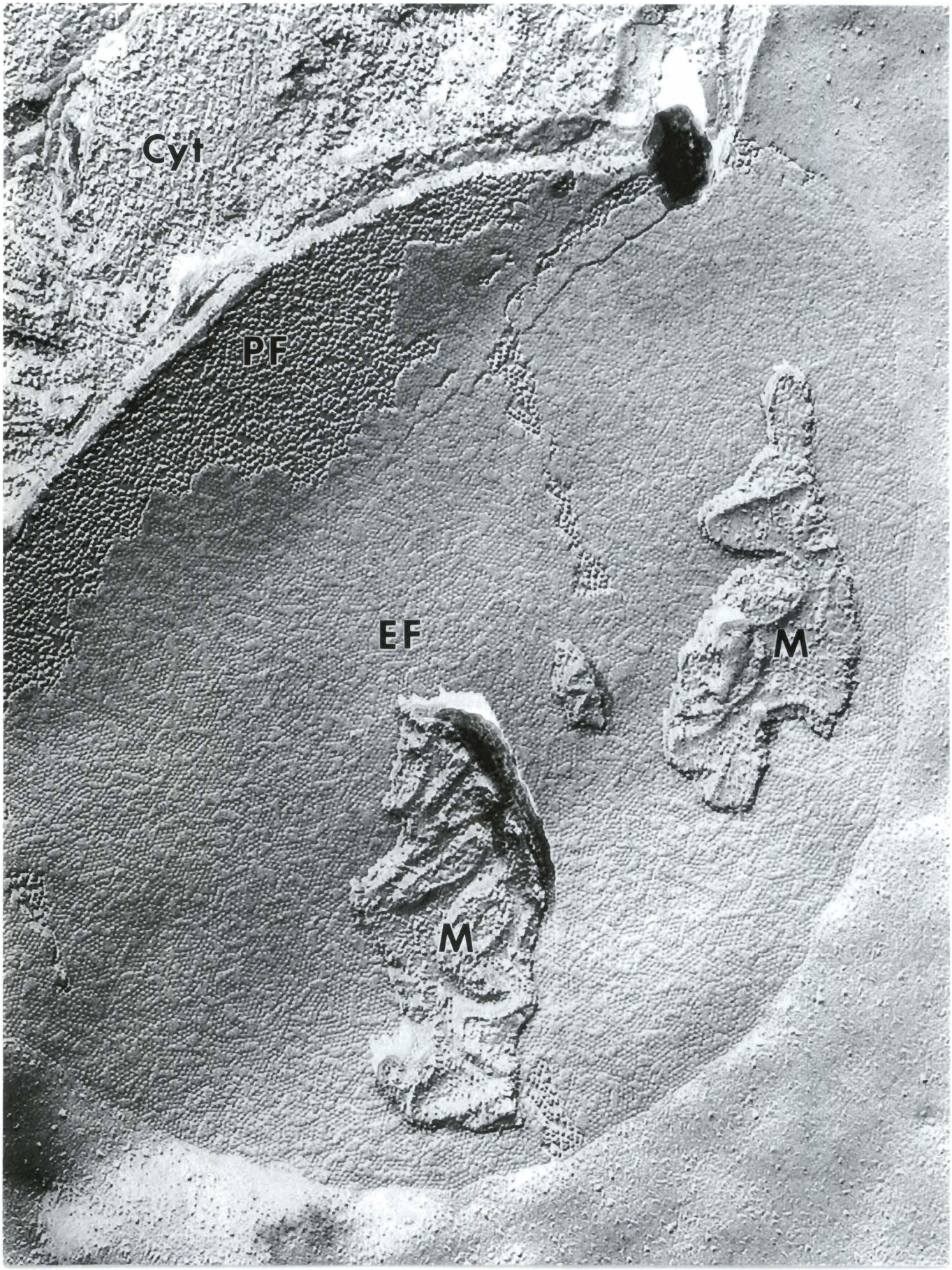
In freeze fracture, membranes are split apart down their hydrophobic interior by fracturing the sample at low temperature; structural detail is then visualised by vacuum-evaporation of a fine layer of platinum at an oblique angle to the surface of the specimen, followed by a further layer of carbon deposited from above to strengthen the replica (review, Severs and Shotton, 1995). What the replication process does is translate the uneven topography of the specimen surface into variations in platinum thickness which, when viewed in the transmission electron microscope, make sense to the eye as recognisable and meaningful structure. By means of membrane splitting, freeze-fracture electron microscopy gives unique *en face* views of the internal organisation of biological membranes, allowing study of the in-plane distribution of integral proteins that span the lipid bilayer (Pinto da Silva, 1987). It is because the connexons of gap junctions are transmembrane proteins that they are so clearly displayed as distinctive aggregates of intramembrane particles in freeze-fracture replicas (Fig. 7). The introduction of freeze-fracture electron microscopy in the 1970s significantly broadened the scope of gap junction research, allowing, for example, studies of connexon arrangement in relation to the functional properties of gap junctions, and of the mechanisms of gap junction formation and disassembly under different experimental and physiological conditions (e.g. Baldwin, 1979; Peracchia, 1980; Green and Severs, 1984; Larsen and Risinger, 1985). When used at low magnification, freeze fracture can be particularly helpful in revealing three-dimensional aspects of cellular organisation, and Figure 8 illustrates how, in this mode, our model of gap junction organisation in the longitudinal zones of the intercalated disk (Figure 3) is borne out.

From freeze fracture of intact tissue, negative staining and X-ray diffraction of isolated membrane fragments, and subsequently, low dose electron microscopy and atomic force microscopy, a detailed picture of gap junction channel structure emerged (review, Severs, 1995). The salient details, relating the ultrastructural features to molecular structure, are summarised in Fig. 6. Clusters of connexons are packed closely together, side-by-side in each membrane, spanning its full depth. Each connexon has a central channel of ~2.5 nm at its widest point, and is coaxially aligned, across the narrow extracellular gap, with its partner from the apposing membrane. The resultant complete channel extending between the two cells has a pore size large enough to admit small molecules of the size of second messengers such as cyclic AMP and inositol triphosphate, as well as ions such as K^+, Ca^{2+}.

Connexins - The protein constituents of gap junction channels

The connexon is a hexameric structure (Unwin and Ennis, 1984; Manjunath and Page, 1985) each of its six subunits consisting of a single connexin molecule (Fig. 6). The connexins are a multigene family of conserved proteins, different membranes of which are expressed in different cell types, tissues and species (Beyer et al., 1990; Willecke et al., 1991a). Connexins are divided into two major classes, designated α and ß, according to their genetic and primary sequence relationships (Kumar and Gilula, 1992; Kumar, 1992) but individual members are conventionally identified by a suffix denoting their predicted molecular mass. The first gap-junctional protein to be characterised by molecular cloning was connexin32 (or $ß_1$), the major component of liver gap junctions; connexin43 (or α_1), the principal connexin of the mammalian heart, was identified soon after (Kumar and Gilula, 1986; Paul, 1986; Beyer et al., 1987). cDNAs for 12 distinct mammalian connexins have now been cloned and sequenced. More than one connexin can be simultaneously expressed by the same cell type, and differentiation, development and adaptive physiological processes involve distinctive spatiotemporal patterns of connexin expression (e.g. Fishman et al., 1991; Van Kempen et al., 1991; Fromaget et al., 1992; Gourdie et al., 1992, 1993a; Minkoff et al., 1993). Electrophysiological studies of gap junctions formed between cells transfected with cDNAs encoding different connexins indicate that voltage dependence and unitary channel conductance vary according to the constituent connexin (Veenstra et al., 1992; Spray et al., 1992; Moreno et al., 1993).

Fig. 7. A gap junction viewed en face by freeze-fracture electron microscopy. The freeze-fracture process splits membranes along an interior hydrophobic plane. A fine platinum-carbon replica is made, and, after removal of the biological material, the replica is viewed in the transmission electron microscope. Because the gap junction consists of two closely apposed membranes, the fracture plane may travel back and forth between the two, revealing portions of each. In this example, portions of the protoplasmic fracture face (P face; PF) of the lowermost of the two membranes making up the junction is seen toward the upper left. The major area seen is of the external half-membrane leaflet (E face; EF) belonging to a cell that extends above the plane of the micrograph. The P-face view shows particles which represents connexons; the E-face view shows numerous pits, which represent the imprints of connexons that have been fractured out with the apposing half-membrane leaflet during the fracture process. Rabbit left ventricular myocardium. Cyt: cytoplam; M: fragments of the fractured membranes of mitochondria lying adjacent to the gap junction. For further details see, Severs, 1990. x 106,000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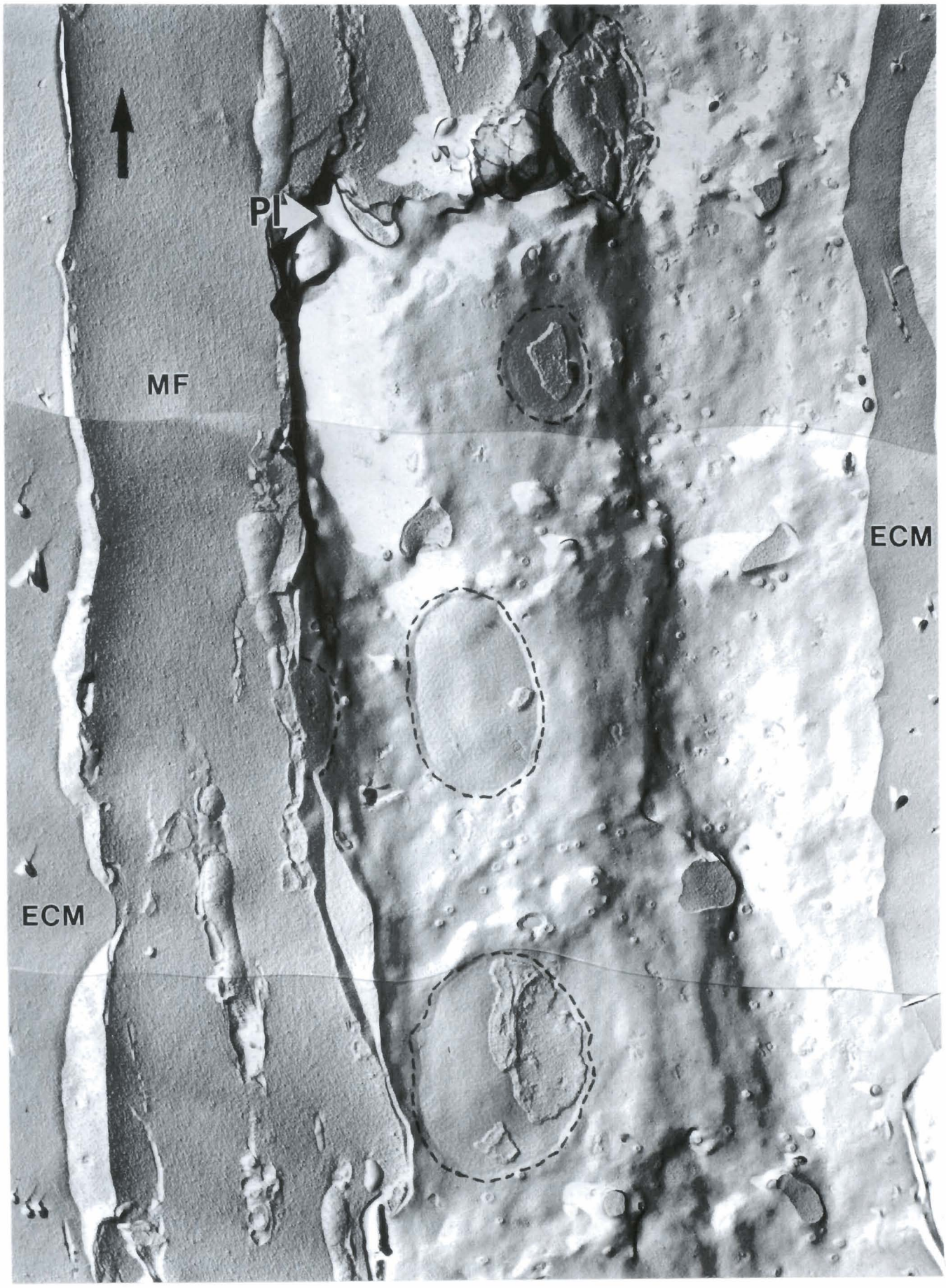
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Figure 6 (lower diagram) depicts the topology of the connexin molecule in the membrane. Four membrane-spanning segments are present, with two extracellular loops, one cytoplasmic loop and the amino and carboxyl tails located on the cytoplasmic side of the membrane (Zimmer et al., 1987; Milks et al., 1988; Yancey et al., 1989). Intramolecular disulphide bonds may link the two extracellular loops, which are highly conserved regions of the molecule thought to be involved in docking connexons of apposing membranes (Rahman and Evans, 1991; John and Revel, 1991). The third membrane-spanning segment is thought to form the interior lining of the channel. The various connexin types differ principally in the amino acid sequences of the cytoplasmic loop and carboxyl tail, these domains being the likely sites responsible for determining the different functional properties of different connexins (Dunham et al., 1992; Becker et al., 1995).

Individual gap-junctional channels made from connexin43 have unitary conductance values in the order of 60 pS (Burt and Spray, 1988; Fishman et al., 1990). Because of the abundance of channels within each plaque, there is a sufficiently large overall gap-junctional conductance to achieve low-resistance impulse

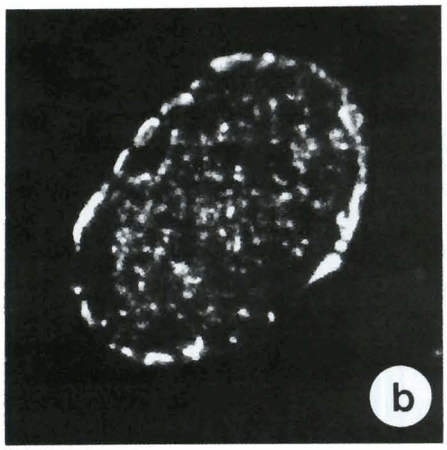

Fig. 9. Confocal microscopy of connexin43 antibody-labelled gap junctions in normal left ventricular myocardium. **(a)** shows a single optical section of longitudinally sectioned myocardium. The lines of punctate staining represent gap junctions organised in the familiar pattern of the intercalated disk (cf Fig. 1). **(b)** projection constructed from multiple optical sections of transversely sectioned myocardium showing the complete population of gap junctions, all in focus, within an en face-viewed disk. (a) x 140; (b) x 1,950. (From Severs N.J., et al. J. Microsc. 169, 299-328 (1983), and Green C.R. and Severs N.J. Histochemistry 99, 105-120 (1993); with permission from Blackwells Scientific Publishing, and Springer-Verlag respectively).

Fig. 8. Freeze-fracture replication, by virtue of its ability to reveal extensive expanses of en face-viewed membranes, not only permits viewing of structural detail within the gap junction - it can be used at lower magnification to reveal three-dimensional aspects of cellular organisation such as the distribution of gap junctions at the intercalated disk. This montage shows a series of gap junctions (encircled) over a segment of face-fractured longitudinal disk membrane. The expanse of membrane viewed is an E-face, belonging to a cell situated above the page before freeze fracture. To the left is the cross-fractured view of the interior of a second myocyte, which extends beneath the membrane expanse of the first cell, connecting to it at the gap junctions visualized. ECM: extracellular matrix; MF: myofibril in cross-fractured portion of cell; PI: position of plicate region, which extends above the plane of the picture. Arrow shows direction of the long axis of the cell. x 30,000

propagation (Weingart and Maurer, 1988; Page, 1992). Voltage clamp studies reveal that junctional conductance is reduced at transjunctional voltages exceeding ±50 mV, but that a residual voltage-insensitive component is apparent even at large transjunctional voltages, indicating a voltage insensitive substate of the connexin43 channel (Moreno et al., 1994a). The most important physiological factors in the regulation of gap junction channels are intracellular calcium concentration, pH and connexin phosphorylation by protein kinases (Spray et al., 1985; Sáez et al., 1986; Lau et al., 1991; Moreno et al., 1992; Ek et al., 1994). The commonly observed ~60 pS unitary conductance is now known to correspond to a phosphorylated form of the connexin43 channel, and dephosphorylation results in a higher unitary conductance, in the order of 90-100 pS (Moreno et al., 1994b). Apart from regulatory mechanisms operating at the level of the channel, cells may also control the overall quantity of gap-junctional membrane at the cell surface. Junctional plaques may be assembled or disassembled by aggregation or dispersal of connexons in the membrane plane. Where more gap-junctional membrane is required than can be met by assembly of pre-existing connexons in the plasma membrane, new gap-junctional protein may be synthesised at the rough endoplasmic reticulum, assembled into connexons (Musil and Goodenough, 1993), and then delivered to the plasma membrane. Where entire gap junctions are no longer needed, they may be internalised by endocytosis for degradation within the cell (Larsen and Risinger, 1985).

Connexin-specific antipeptide antibodies

Antibodies raised to peptides matching sequences that are unique to different types of connexin molecule are proving to be invaluable as experimental probes in gap junction research. Among their various applications, these probes have helped: i) establish the topological arrangement of the connexin molecule as depicted in Figure 6, ii) identify the trafficking routes of connexins to the cell surface, and iii) pinpoint sites of the molecule involved in regulating permeability (Evans et al., 1992; Rahman et al., 1993; Becker et al., 1995).

A major breakthrough brought about by means of connexin-specific antibodies has been in the field of gap junction imaging. In particular, immunofluorescent labelling in combination with the technique of confocal laser scanning microscopy has permitted visualisation of gap junctions with hitherto unprecedented sensitivity (Gourdie et al., 1991). Among the new developments made possible with this approach are the facility for reconstruction of complete views of the organisation of gap junctions within the entire intercalated disk, the ease with which gap junction distribution may be mapped through large tissue volumes, and the ability to determine which connexins are expressed when, where and to what extent during disease and developmental processes (Gourdie et al., 1990, 1991, 1992; Peters et al., 1993b). As a result, significant advances have recently been made in the study of gap junction distribution and connexin expression in the healthy and diseased heart (reviews, Green and Severs, 1993; Severs et al., 1993;

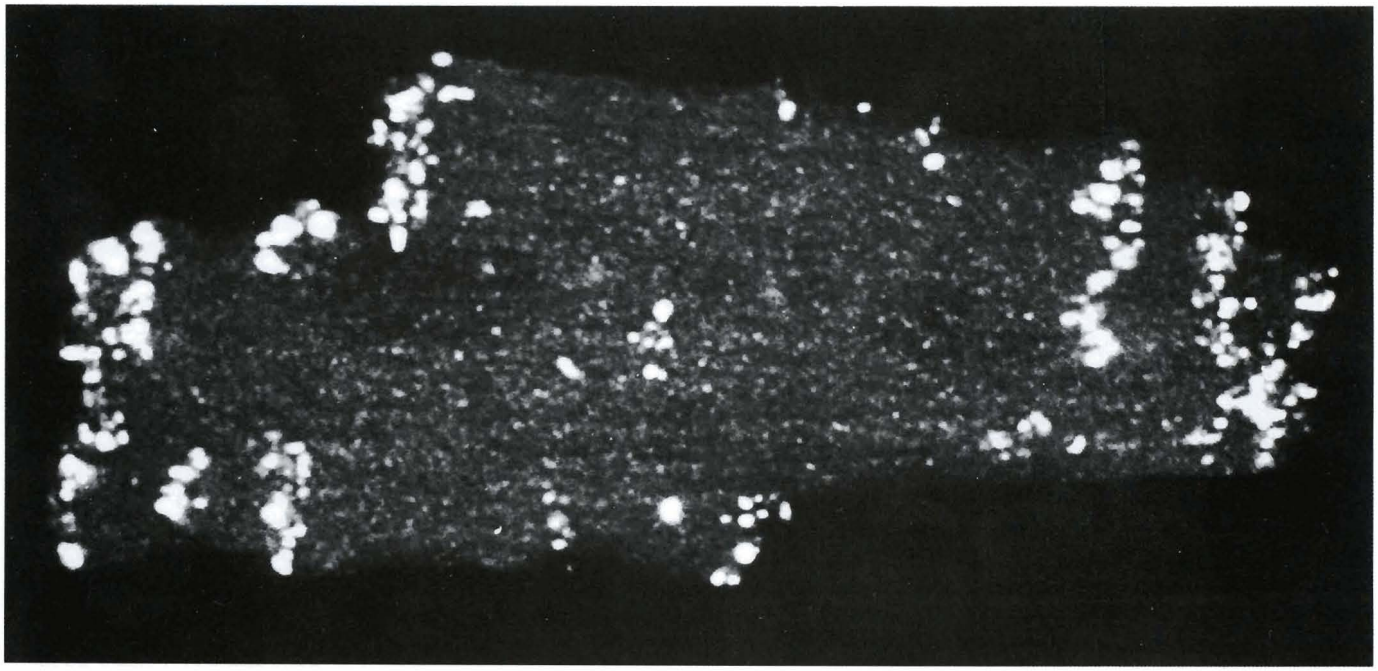

Fig. 10. Confocal microscopy of an isolated Guinea pig myocyte after immunofluorescent labelling using connexin43 antibody. Here we are able to view the localisation of gap junctions, seen as clearly defined punctate label, over a single intact myocyte (cf Figures 1, 2). x 2,1000. (From Peters N.S. et al. Circulation 88, 864-875 (1994), with permission from the American Heart Association.

Severs, 1994a,b, 1995).

Confocal microscopy of immunolabelled gap junctions in normal myocardium

Confocal images of longitudinally sectioned ventricular working myocardium after immunofluorescent-labelling using anti-connexin43 primary antibody reveals gap junctions in the familiar pattern reflecting the positions of the intercalated disks (Fig. 9a). When serial optical sections of transversely cut myocardium are integrated, complete disks, with the entire population of gap junctions in focus, can be viewed en face (Fig. 9b). These images typically reveal a ring of large gap junctions circumscribing the periphery of the disk with smaller gap junctions in an interior zone, an arrangement common to a range of mammalian species including humans (Gourdie et al., 1991). The organisation of gap junction clusters over the cell as a whole is dramatically visualized in confocal projections of immunolabelled isolated myocytes (Fig. 10), especially with the aid of stereo imaging techniques (Fig. 11). Confirmation that the fluorescence labelling observed in these isolated myocytes really does represent that of gap junctions is demonstrated by post-embedding immunogold electron microscopy of thin sections using the same primary antibody. The gold label in these preparations is specifically associated with directly visualised gap junctions, and not with other intercellular junctions or other structures in the cell (Fig. 12).

The highly specific patterns of gap junction distribution revealed in these ways represent the pathways which give rise to the normal uniform anisotropic pattern of impulse spread of healthy myocardium, in which conduction parallel to the long axis of the cells is approximately three-fold more rapid than that in the transverse direction (Delmar et al., 1987; Dillon et al., 1988). Because of the distribution patterns of gap junction clusters in the tissue as a whole, which are related to the size, shape and degree of branching of the cells, as well as to the arrangement and distribution of gap junctions within the disks themselves, the longitudinally propagating impulse encounters less

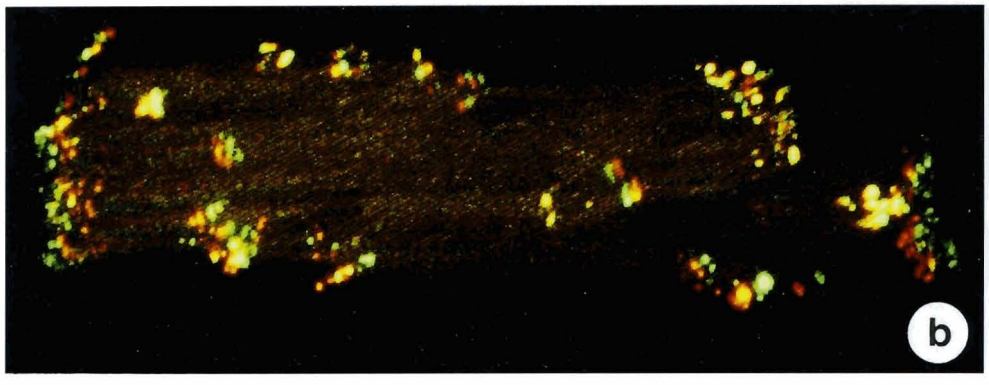

Fig. 11. Three-dimensional imaging of a guinea pig isolated myocyte in which gap junctions have been labelled using anti-connexin43 antibody. **(a)** This myocyte has been optically sectioned by confocal microscopy (13x2 μm steps) with rotation of the image stack to allow viewing from different angles. The first two images (top and middle panels) are viewed 63° apart; the cell is then rotated through a further 42° for viewing in the bottom panel. In this set of images, false colouring is used to highlight the gap junctions. x 720. **(b)**. This view shows the same immunolabelled myocyte as shown in a. This time the myocyte optical series, with the myocyte seen from the side, is presented as a red/green stereo image to allow the three-dimensional arrangement of the gap junctions delineating the intercalated disks to be appreciated. To be viewed using red/green stereo spectacles. x 1,300. (From Severs N.J. et al. J. Microsc. 169, 299-328, 1993). With permission from Blackwells Scientific Publshing.

resistance to flow than does the transversely propagating impulse (Luke and Saffitz, 1991; Saffitz et al., 1992, 1994; Spach, 1994; Hall and Gourdie, 1995). A further factor influencing the patterns of current transfer through the heart is gap junction *quantity*. Distinctive variations in gap junction number and abundance are apparent in different functionally specialised zones of the heart. In the atrioventricular node, where conduction is slowed to ensure sequential contraction of atria and ventricles, gap junctions are sparse and small, whereas in Purkinje fibres and working myocardium, where rapid spread of depolarisation throughout the ventricles is required, gap junctions are abundant and large in size (Gourdie et al., 1991, 1992; Fromaget et al., 1992; Dolber et al., 1992).

Apart from the influence of gap junction distribution, number and size, however, recent evidence has shown that electrophysiological specialisation within sub-populations of cardiac muscle cells is determined by yet another factor - the particular *type of connexin* expressed.

Expression of different connexins in the normal heart

Although connexin43 is the major connexin of the heart, messenger RNA transcripts encoding a series of other connexins, namely connexin40, connexin45, connexin 46 and connexin 37 have recently been reported in mammalian hearts, and the expression of the corresponding protein has so far been demonstrated for connexin 40 and connexin45 (Willecke et al., 1991a,b; Haeflinger et al., 1992; Kanter et al., 1992, 1993a, 1994; Reed et al., 1993; Gourdie et al., 1993b,c). As noted earlier, electrophysiological studies on transfected cell pairs indicate that different connexins confer specific electrophysiological characteristics on the assembled channel protein (Veenstra et al., 1992; Spray et al., 1992; Moreno et al., 1993). Connexin42, the avian homologue of mammalian connexin40 (Haeflinger et al., 1992; Kanter et al., 1992), has a greater unitary conductance that does connexin43 (Veenstra et al., 1992) and connexin40 in the rat and dog heart, and connexin42 in the avian heart, are preferentially expressed in the specialised myocytes of the atrioventricular conduction system, where they are implicated in fast conduction (Bastide et al., 1993; Gourdie et al., 1993a,b,c; Kanter et al., 1993a; Gros et al., 1994). Examples of connexin40 and connexin43 localization in the rat conduction system are illustrated in Figures 13 and 14, taken from the work of Robert Gourdie (Gourdie et al., 1993b,c). Both connexin40 and connexin43 are expressed in low quantity in myocytes of the atrioventricular node. In rat and human heart, connexin40 appears to be the sole connexin of the atrioventricular bundle and proximal parts of the bundle branches, and is expressed in progressively greater quantities along the conduction axis toward the working ventricle, reaching a maximum in the Purkinje fibres where connexin43 is co-expressed in large quantities (Bastide et al., 1993; Gourdie et al., 1993c; Gros et al., 1994). Connexin40 has also been reported in relatively high concentration in the guinea pig atrium (Gros et al., 1994) and in the crista terminalis of canine right atrium (Saffitz et al., 1994). Although, under some preparation conditions it can be difficult to detect, connexin40 is expressed in some myocytes of the working myocardium, though overall in markedly lower quantities than that found in the atrioventricular conduction system (Bastide et al., 1993; Kanter et al., 1993a; Gourdie et al., 1993a,c). The picture with connexin45 is less clear, though some reports suggest that this connexin is present in both working myocardium and Purkinje fibres (Kanter et al., 1992; Saffitz et al., 1994).

Precisely how these different connexins interact at the molecular and cellular level, and how such interaction influences electrophysiological properties, is an area of active research. In myocytes coexpressing connexin43 and connexin40, linkage could be by junctions consisting exclusively of the same connexin isoform (i.e. a given pair of cells joined by some junctions consisting only of connexin43 and other junctions consisting only of connexin40), or by junctions containing both

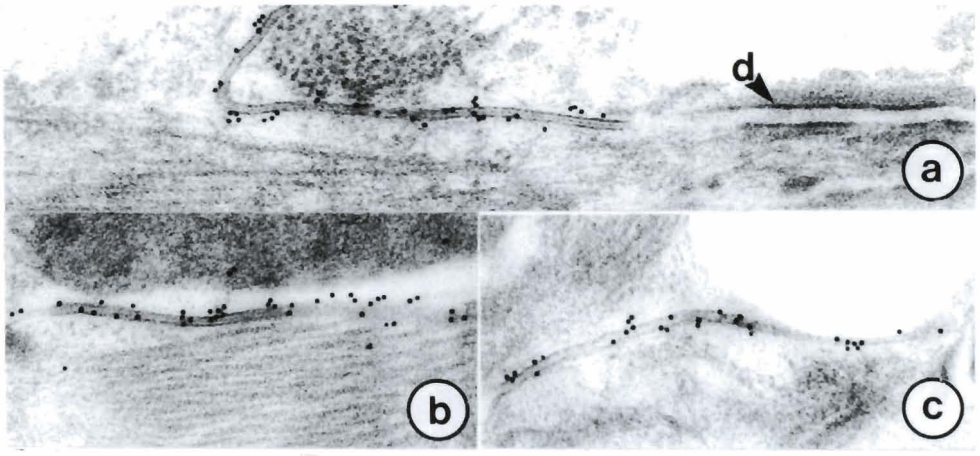

Fig. 12. Immunogold labelling of cardiac gap junctions using the same anti-connexin43 primary antibody used for immunfluorescence-confocal imaging. Results from Lowicryl-embedded human **(a)** and rat **(b, c)** left ventricular myocardium. Gold label is specifically associated with profiles of gap-junctional membrane. Other cellular structures, including a nearby desmosome (d) remain unlabelled. (a) x 81,400; (b) x 78,400; (c) x 86,500. From: Green C.R. and Severs N.J. Histochemistry 99, 105-120, 1993 (with permission from Springer-Verlag).

connexin isoforms (Kanter et al., 1993b). Evidence that both these possibilities may occur has been presented by confocal microscopy of double immunolabelling experiments in guinea-pig atrial myocytes (Gros et al., 1994). To establish whether individual junctions containing more than one connexin are formed by one myocyte expressing one isoform joined to a neighbour expresing the other, or from mixtures of both connexins present at each side of the junction, will require the application of higher resolution techniques. The importance of defining the nature of such interactions is illustrated by work *in vitro* expression systems, in which it appears that only some specific (heterotypic) combinations of connexins form functional (i.e. permeable) channels (Swenson et al., 1989; Barrio et al., 1991). Heterotypic channels formed from connexin40 and connexin43 have been reported to be non-functional, whereas those formed from either one of these connexins with connexin37 do appear to form functional channels (Bruzzone et al., 1993). Such discriminatory properties, if applied *in vivo*, would open a range of possible mechanisms for differentially regulating electrical properties between subpopulations of myocytes. For example, it might be hypothesized that connexin37 could mediate functional connection between connexin40 and connexin43. So far, however, no subsets of connexin37-expressing myocytes have been reported.

Gap junctions, connexins and cardiac disease

Given that gap-junctional coupling of myocytes is an important determinant of intercellular conductance, might gap junctions play a role in abnormalities of electrical rhythm associated with cardiac disease? Arrhythmias are a serious manifestation of cardiac disease, accounting for a major part of the mortality due to coronary heart disease. The underlying mechanism of most clinical arrhythmias is a reentrant electrical circuit, arising from reduced conduction velocity, heterogeneity of conduction and undirectional block (Hoffman and Dangman, 1987; Kleber, 1987; Wit and Janse, 1992). Although alterations in active membrane ionic properties are widely implicated in these processes, reports that myocyte resting membrane potentials and other electrophysiological characteristics can be essentially normal in the presence of manifest cardiac arrhythmias

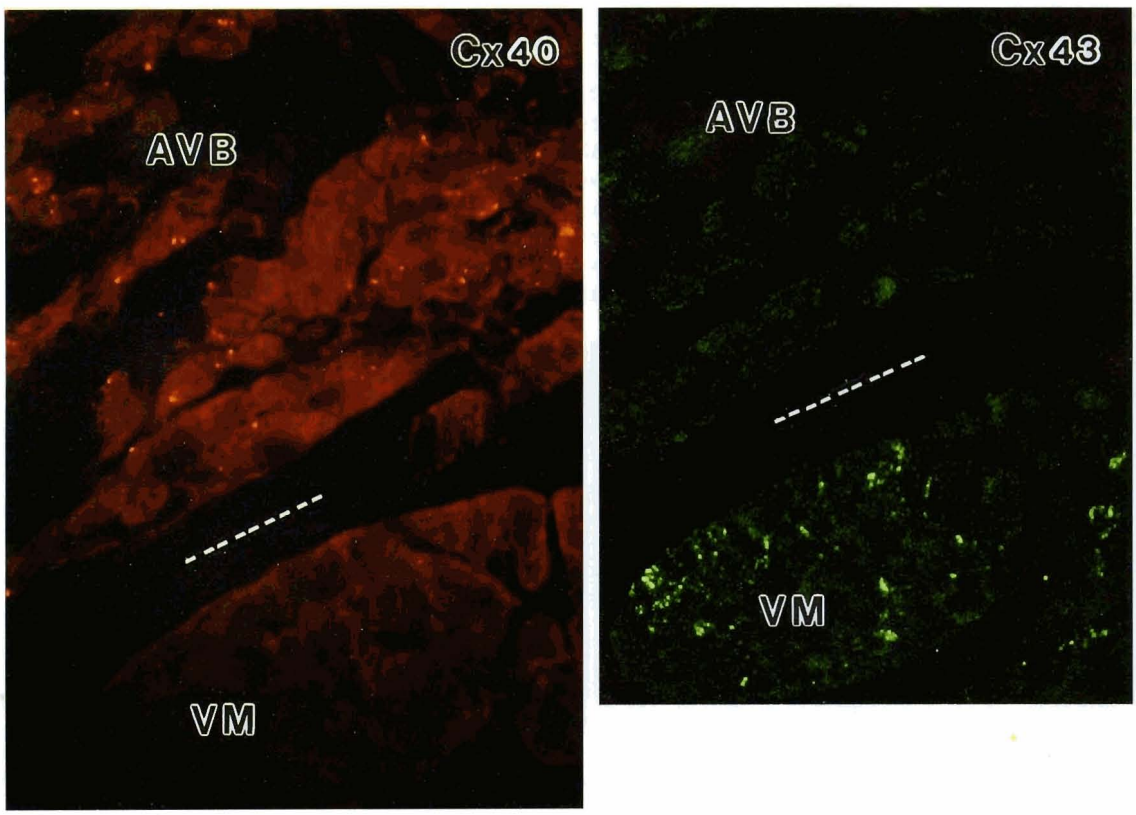

Fig. 13. Comparison of connexin40 and connexin43 gap junction immunolabelling of atrioventricular bundle (AVB) and subjacent working ventricular myocardium (V) in serial tissue sections from Zamboni-fixed rat heart imaged by confocal microscopy. Connexin40 gap junctions are localised in the distal atrioventricular bundle tissue, but are absent from subjacent working ventricular myocardium; connexin43 gap junctions, by contrast, are abundant in working myocardium, but not in atrioventricular bundle myocytes. These images are projections from 5 x 1 μm z-steps (For details of antibody detection, see legend to Figure 14). x 730. (Micrographs by courtesy of Dr. Robert Gourdie, from: Gourdie R.G. et al. J. Cell Science 105, 985-991, 1993c), with permission from the Company of Biologists).

(Ursell et al., 1985; Dillon et al., 1988; Spach et al., 1988) have increasingly drawn attention to the possibility that abnormal cellular coupling may also play an important role (Dolber et al., 1992; Saffitz et al., 1992; Spach and Heidlage, 1993). A series of recent articles has thus increasingly focused on the possible roles of the spatial distribution of gap junctions and changes in the patterns of expression of their constituent connexins, as causes of electrophysiological dysfunction in cardiac disease (Smith et al., 1991; Saffitz et al., 1992, 1993; Peters et al., 1993b, 1995; Severs, 1994a,b).

Alteration in gap junction distribution: The border zone of mycoardial infarct scars

One site that is particularly susceptible to malignant arrhythmia is the myocardium that borders the scar tissue of myocardial infarcts. To investigate gap junction distribution at these sites, we applied immunoconfocal microscopy to: i) hearts removed from patients with endstage ischaemic heart disease who were undergoing cardiac transplantation, and ii) biopsies from coronary by-pass patients with previous clinically documented myocardial infarction (Smith et al., 1991). Confocal imaging of this material after immunolabelling with anti-connexin43 antibody reveals that myocardium distant from scar tissue which is free from histologically-detectable structural damage has a gap junction distribution that qualitatively resembles that of normal myocardium, i.e. the junctions are organised in normal, well-defined intercalated disks. At the borders of healed infarcts, however, a striking disturbance in gap junction distribution is apparent. Instead of being confined to intercalated disks, the junctions are widely dispersed over the cell surfaces (Fig. 15). Myocytes showing this abnormal feature are found to a distance of several hundred micrometers from the infarct scar. Electron microscopy reveals that the cells involved have abnormal patterns of cellular interactions (Smith et al., 1991; Severs et al., 1992). Generalised disorganisation of gap junction distribution of this type would likely result in complex, heterogeneous pathways of wave-front propagation, thus providing a substrate for the genesis of arrhythmias in the infarcted heart. Apart from disorganisation of gap junction distribution in human infarct border zones, a variety of other types of abnormality have been reported in border zone myocytes in a canine model of infarction analyzed by quantitative thin-section electron microscopy, notably reduced frequency of gap junctions per unit length of disk membrane, decreased gap junction size and fewer intercalated disk contacts per myocyte (Luke and Saffitz, 1991).

While these findings suggest one possible link between gap-junctional electrical communication pathways and arrhythmogenesis, gap junction distribution alone clearly cannot provide an adequate explanation for all clinical ventricular arrhythmias. The occurrence of arrhythmias in clinical settings such as hypertrophy, for example, raised the question as to whether the diseased heart is afflicted with a more widespread gap-junctional abnormality. One form this could theoretically take is altered expression patterns of connexins. If, for example, there were a generalised reduction in the quantitiy of connexin43 gap-junctional protein, the predicted consequent decrease in conduction velocity might in itself be sufficient to give rise to a proarrhythmic state.

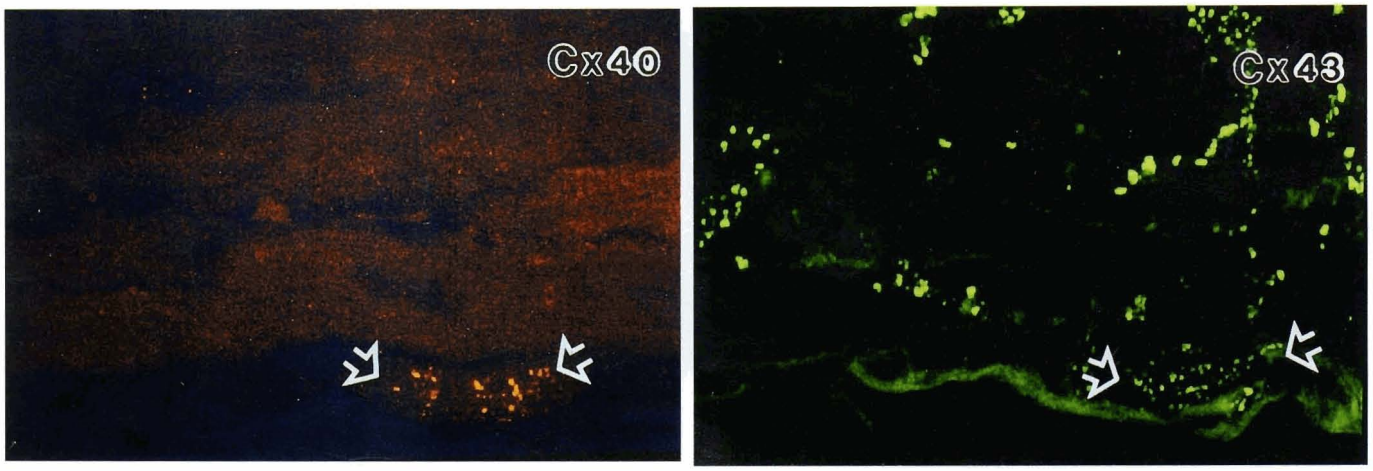

Fig. 14. Demonstration that connexin43 and connexin40-containing gap junctions are co-localised in the same Purkinje fibre (indicated by double arrows). Connexin43 immunolabelling is abundant in the adjacent working left ventricular myocardium, but little connexin40 labelling is apparent. Rat heart; double-labelling experiment using rabbit polyclonal connexin40 antibodies and mouse monoclonal connexin43 antibodies. Connexin40 detected by biotinylated anti-rabbit secondary antibodies and streptavidin conjugated to Texas red; after laser-photobleaching, connexin43 labelling was carried out with detection by biotinylated anti-rabbit secondary antibodies and streptavidin conjugated to fluorescein. x 630. (Micrographs by courtesy of Dr. Robert Gourdie, from Gourdie R.G. et al. J. Cell Science 105, 985-991, 1993c; with permission from the Company of Biologists).

Reduced expression of connexin43 gap junctions in heart disease

To investigate this hypothesis, we carried out quantitative analysis of connexin43 gap-junction content using confocal images of immunolabelled human myocardium in structurally well-preserved regions distant from scar tissue in patients with: i) chronic ischaemic heart disease, and ii) non-ischaemic hypertrophy (Peters et al., 1993b). The former specimens came from left ventricular biopsies of recurrently ischaemic myocardium in patients with triple-vessel coronary artery disease who were undergoing by-pass operations; the latter were from patients undergoing surgical replacement of a stenosed aortic valve. To provide control data on undiseased hearts, biopsies were obtained from cardiac transplant donor hearts and myocardium identified as normal from patients with the Wolff-Parkinson-White syndrome. Gap junction surface area per unit cell volume was estimated to be reduced by 47% in the ischaemic hearts and by 40% in the hypertrophied hearts (Peters et al., 1993b). Cell size was increased in both sets of pathological samples, markedly so in the hypertrophied hearts. Estimates of the content of connexin43 gap junction per cell derived from these data indicate a significant decrease in the ischaemic samples, involving a uniform reduction in the numbers of gap junctions of all size classes. The number of intercalated disks per cell, as determined by the number of clusters of gap junctions identified in isolated myocytes, remained unchanged.

A similar pattern of down-regulation of connexin43 has been reported in transgenic hypertensive and post-infarct rats using quantitative immunoblotting techniques (Bastide et al., 1993, 1994). Furthermore, reduced levels of histochemically-detectable connexin43 at the cell surface repeteadly correlate with decreased gap-junctional coupling in cultured myocytes infected with *Trypanosoma cruzi*, the unicellular parasite responsible for Chagas' disease (the most common cause of heart disease in South America) (Campos De Carvalho et al., 1992, 1994). Taken together, these observations raise the possibility that a reduction in the quantity of connexin43 gap junctions may be a general pathogenetic feature of cardiac disease that could account for conduction disturbances in diverse disease settings.

Future directions - are there disease-related changes in the expression of a range of connexin types?

In view of the recent data revealing differential expression of connexins in functionally specialised zones of the normal heart, the question arises as to

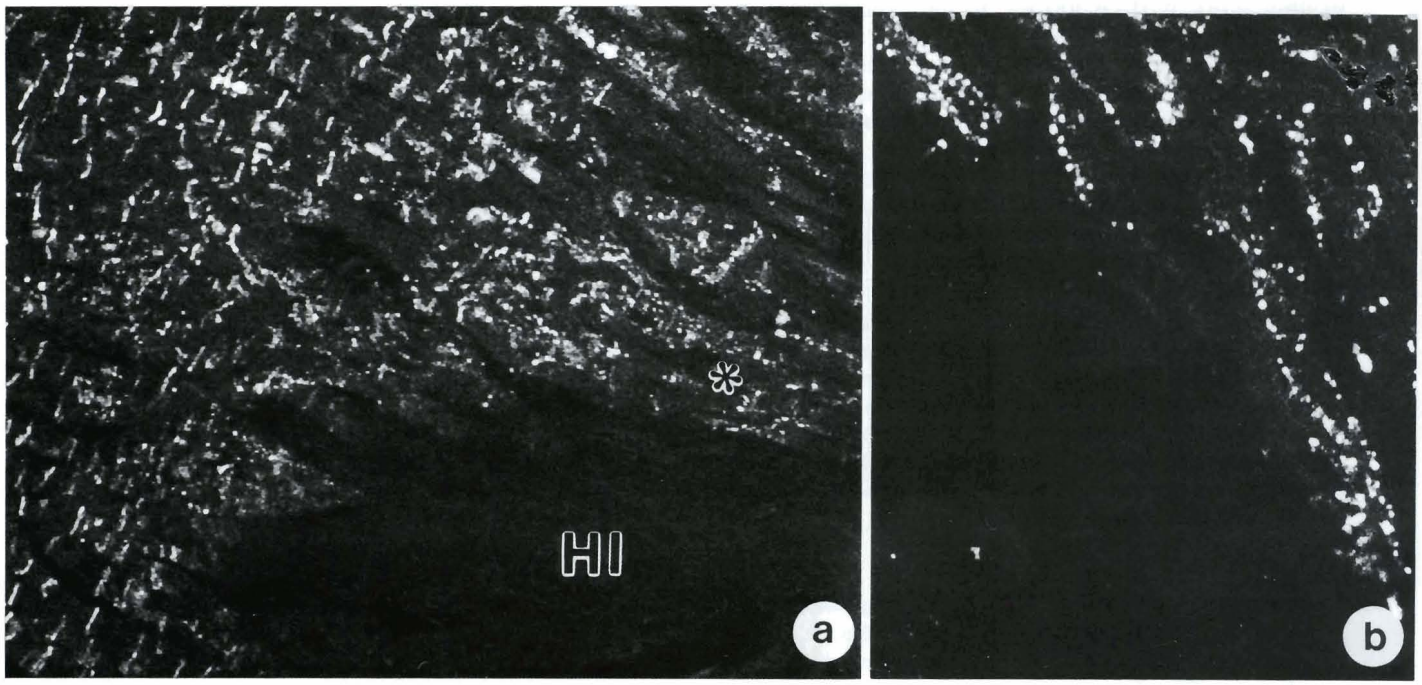

Fig. 15. Disturbance in the distribution of connexin43 gap junctions in surviving myocardium bordering scar tissue after myocardial infarction, as seen by connexin43 immunolabelling and confocal microscopy. **(a)** survey view; **(b)** detailed view of border zone myocytes at higher magnification. The fibrotic healed infarct (HI) is immunonegative. In (a) gap junction immunolabelling in intercalated disks of normal appearance is observed toward the top and bottom left of the field, but the immunolabelled gap junctions in myocardium at the border of the infarct scar show a highly disordered arrangement towards the right (*), with many located over the lateral surfaces of the cells. This lateralisation of the gap junctions is shown clearly at higher magnification in (b). (a) x 220; (b) x 800. (From Severs N.J. et al. J. Microsc. 169, 299-328 (1993), with permission from Blackwells Scientific Publishing)

whether changes in connexin43 tell the whole story. Few data are yet available on the expression of connexins other than connexin43 in the diseased heart. Concomitant with the down-regulation of connexin43 reported in left ventricular myocytes in the hypertensive rat heart, up-regulation of connexin40 was reported (Bastide et al., 1993). From the electrophysiological data and spatial patterns of distribution detected in the healthy heart, up-regulation of connexin40 might be predicted to increase conduction velocity, thus raising the intriguing hypothesis that raised connexin40 levels might represent a compensatory response, in the overloaded ventricle, to the consequences of reduced connexin43 (Bastide et al., 1993). If this were so, down-regulation of connexin43 might form just part of a wider, generalised pattern of altered expression of different connexin types in the diseased heart (Severs, 1994a). This attractive hypothesis is currently under active investigation, but whether it will be confirmed must at present remain open. Preliminary indications are conflicting. In the early phases of myocardial remodelling following infarction in the rat, decreased connexin43 appears to be associated with no clear-cut change in connexin40 (Bastide et al., 1994), while another preliminary study of the very early stages of cardiac hypertrophy induced by renovascular hypertension in the guinea pig suggests a paradoxical increase in connexin43 (Peters et al., 1993a, 1994a). Further work will be needed to determine whether a generalised expression pattern common to a range of diseases exists, or whether different expression patterns are associated with different diseases. With the pace of current research, the picture will undoubtedly soon be clarified.

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