Gap junction distribution in adult mammalian myocardium revealed by an anti-peptide antibody and laser scanning confocal microscopy

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Summary

A polyclonal antiserum, raised against a synthetic peptide matching part of the sequence of connexin43 (a rat cardiac gap-junctional protein), was used in combination with laser scanning confocal microscopy to investigate gap junction distribution in cardiac tissues from a range of mammalian species. Comparison of the localised punctate staining patterns obtained in ventricular tissue with the distribution of intercalated disks as viewed by conventional light microscopy and electron microscopy, and with the staining observed by standard light-microscope immunofluorescence using the same antiserum, demonstrated highly specific labelling of clearly resolved individual gap junctions. Laser scanning confocal microscopy of ventricular myocardium showed the immunostained gap junctions to be confined to well-defined intercalated disks bisecting the long axis of the muscle fibre, whereas in the atrial myocardium, gap junctions were commonly distributed widely over the lateral surfaces of the myocyte body. Rat atrial gap junctions were significantly larger (as measured by the longest axial lengths of fluorescent spots), and showed a narrower spread of sizes, than their counterparts in the ventricle. Ventricular myocardium from six mammalian species including man gave similar immunostaining patterns, indicating conservation both of the epitope(s) detected by the antiserum, and of the general organisation of the cell-to-cell pathways for electrical propagation, in the mammalian heart. Optical section series obtained by laser scanning confocal microscopy permitted the quantification and mapping of the three-dimensional distribution of gap junctions in ventricular intercalated disks with high clarity over substantial specimen depths. A consistent feature of gap junction organisation within disks of ventricular myocardium in all species studied was the presence of a conspicuous ring of large gap junctions around the periphery of the disk. Immunostained gap junctions lying within the interior zone delineated by the peripheral junctions generally occurred at lower numerical densities and were significantly smaller. In all species, less than 3% of all immunolabelled gap junctions measured were >2 μm in maximal length, though a small proportion (0.06%) exceeded 4 μm. The numerical density of immunolabelled gap junctions in the disk was similar between species; however, within species there was a significant decrease in numerical density with increasing disk size. The new features of intercalated disk structure revealed in this study may have an important part to play in the intercellular communication and electrical propagation properties of the mammalian heart.

Key words: gap junction, connexin43, heart, intercalated disk, laser scanning confocal microscopy.

Introduction

Gap junctions are aggregates of intercellular channels that in the heart facilitate the transmission of electrical current between myocytes (for a review, see Page and Manjunath, 1986). By means of these channels, low-resistance pathways are formed, linking all the myocytes within the myocardium (Weidmann, 1952, 1966, 1970; Barr et al. 1965; Sommer and Johnson, 1979; De Mello, 1982; Page and Manjunath, 1986). The electrical impulses borne rapidly and repeatedly along these pathways stimulate the contraction of individual myocytes in synchrony, which in turn provides the motive force for the pumping action of the heart.

In the ventricle of the mammalian heart, the principal sites of cell-to-cell plasma membrane interaction occur at the intercalated disk. Models for the organisation and distribution of gap junctions within the intercalated disk have been derived almost exclusively from electron-microscopic studies (McNutt, 1970; Page and McCallister, 1973; Forbes and Sperelakis, 1985; Severs, 1985, 1990). From longitudinal thin sections of myocytes, the intercalated disk is commonly envisaged as a series of membrane steps following the ends and sides of the myofibril
terminations of neighbouring cells. Gap-junctional membrane is located predominantly in the lateral-facing sides of the disk steps, although small gap junctions also occur in the transverse regions abutting the myofibril ends, in association with fascia adherens junctions (Hoyt et al. 1989). The fasciae adherentes (also known as intermediate junctions or sheet desmosomes) link the myofibrils to the plasma membrane and fasten adjacent myocytes together.

Such an idealised picture of intercalated disk structure embodies features that are consistently observed, yet important details of disk architecture and gap junction organisation have remained unsettled (see, for example, Hoyt et al. 1989; Severs, 1989). One reason for this is that electron microscopy normally permits the sampling of only small portions of the disk and hence is restricted in its ability to provide comprehensive overviews of structural detail over the intercalated disk as a whole. Sectional views of disks can often reveal intricacies of geometry not encompassed in current models, but the significance of these is often difficult to evaluate. However, such features of geometry may point to the existence of patterns of organisation, other than the idealised one, that have not been identified. The advent of specific antibody probes against cardiac gap-junctional protein has recently permitted the imaging of heart gap junctions by light microscopy (Beyer et al. 1989; Yancey et al. 1989; Luke et al. 1989; Laird and Revel, 1990). This has opened up the possibility of studying gap junction distribution and intercalated disk organisation over relatively large areas of cardiac tissue compared to the spatially limited (though higher magnification) views afforded by ultrastructural observation. We have recently raised and characterised three site-specific anti-peptide antibodies to a 43K (K=10^6 M_c) rat cardiac gap-junctional protein (Harfst et al. 1990; Gourdie et al. 1990a). Here we apply one of these antibody probes, in combination with laser scanning confocal microscopy, to compare and quantify gap junction organisation in relation to intercalated disk structure in a range of mammalian species, including man. The advantage of this approach over standard immunolight microscopy is that laser scanning confocal microscopy permits optical sectioning through substantial depths of tissue (White et al. 1987; Shotton, 1989), enabling the reconstruction in three-dimensions of the disposition of the entire population of immunostained gap junctions within individual intercalated disks.

Materials and methods

Peptide synthesis and production of antisera

Peptides matching portions of the amino acid sequence of a 43K rat cardiac gap junction protein predicted from the nucleotide sequence of a cDNA clone (Beyer et al. 1987) were synthesised according to the method of Houghten (1985). These peptides were kindly provided by Dr N. B. Gilula (Research Institute of the Scripps Clinic, La Jolla, California) and were made to match residues 101–112, 131–142 and 237–246, segments of the 382-amino acid molecule deduced to be exposed on the cytoplasmic surface of the membrane (Beyer et al. 1987; Gourdie et al. 1990a). The peptides were coupled to keyhole limpet haemocyanin using gluteraldehyde, as described in detail elsewhere (Harfst et al. 1990). Antisera were raised in Sandy half-lop rabbits (two per antigen) using an initial injection of 0.4 mg of peptide linked to haemocyanin, mixed with an equal volume of Freund's complete adjuvant (total volume, 2 cm^3). The injections were subcutaneous, made at a series of sites along the animals' backs. Each animal was boosted with injections of 0.2 mg of peptide linked to haemocyanin in Freund's incomplete adjuvant at intervals that were determined by screening results. The animals were bled from ear arteries and veins after administration of 0.5 mg Hypnorm, and serum was prepared, aliquoted and frozen at −80°C. The first bleed was taken before injection of the antigen to provide pre-immune serum, the second was at 2 weeks after the initial injections, and thereafter the animals were bled weekly (or occasionally at slightly longer intervals, e.g. immediately after boosts). Screening of sera for reactivity was carried out by dot blot assay (modified from Hawkes et al. 1982), and specificity of the antisera was confirmed by dot blots using the individual peptides, and in Western blots of isolated cardiac plasma membrane and gap junction preparations (Harfst et al. 1990). Western blotting experiments showed that unpurified antisera taken from periods of peak antibody production (as determined from dot blot screening) detected the same protein bands as did affinity-purified antibodies (Harfst et al. 1990). For immunohistochemical studies, unpurified antisera to the peptide matching residues 131–142, designated 'HJ', was found to be highly effective, and so this antisera was used throughout the present study.

Experimental material and immunohistochemistry

Immunolocalisation was carried out on sections of wax-embedded or frozen myocardium from the following species: rat (Sprague-Dawley, 250 g), guinea pig (DH, 400 g), golden hamster (<90 g), mouse (Balb/c <20 g), rabbit (New Zealand White, <2.5 kg), and man. All laboratory animals were maintained on standard chow diets and tap water, available ad libitum. Human material came from samples of left and right ventricle free wall (mid-region) of the explanted hearts of patients undergoing heart transplantation. Two patients (one 60-year-old male and one 53-year-old female) were suffering from severe ischaemic heart disease; one patient (a 36-year-old male) had dilated cardiomypathy. In all, left ventricular function was significantly impaired but right ventricular function was near normal.

For wax-embedding, samples of human heart tissue and whole intact hearts from the other five species were fixed for 2–6 h in Zamboni's fixative (2% paraformaldehyde, 0.2% picric acid in 0.1 M phosphate-buffered saline, pH 7.0; Toshimori et al. 1987). The excised tissue samples from human hearts were from defined anatomical locations to enable reliable comparison. After fixation, the specimens were washed in tap water, dehydrated in ethanol, placed in chloroform and embedded in wax following standard histological procedure. Whole hearts were oriented to permit longitudinal sectioning in a mid-plane through the whole organ so as to reveal the four cardiac chambers with their surrounding myocardium (left and right ventricles and atria). These four-chambered views were taken at a sectional plane that revealed the crest of the interventricular septum and the subjacent bundle of His. Sections of ~10 µm thickness were cut, dewaxed in xylene, rehydrated in a graded ethanol series and rinsed in tap water. Incubation in buffered trypsin (0.1% trypsin (Sigma), 0.1% CaCl_2, 20 mM Trizma base, pH 7.4) then followed for 10 min at room temperature. This step is a standard precaution for unmasking epitopes that have been altered by aldehyde fixation (Harlow and Lane, 1988). The sections were washed with tap water and then blocked using 0.1 M l-lysine in phosphate-buffered saline containing 0.1% Triton X-100 (as a wetting agent). Incubation with the primary antiserum, anti-HJ, was then carried out at a 1:100 dilution in the phosphate-buffered saline overnight at room temperature. After washing with phosphate buffer, secondary antibody treatment with swine anti-rabbit conjugated to fluorescein isothiocyanate (Dako; 1:20 dilution) was given for 1 h at room temperature. The sections were then mounted using Citifluor mounting medium (a medium that reduces fading and autofluorescence). The following controls were routinely done in each staining run; (1) omission of primary antiserum; (2) use of preimmune serum in place of primary antiserum.

For cryosectioning, 25 µm thick sections were prepared from excised blocks of unfixed frozen tissue from anatomically defined locations. The sections were fixed by immersion in methanol at −20°C for 3 min and then treated with 0.1 M l-lysine and taken through the same series of immunolabelling steps as those
described for dewaxed sections. Treatment with primary antisemum was for 1 h using a 1:10 dilution. Controls using pre-immune serum, and omission of the primary antisemum were carried out in parallel.

Laser scanning confocal microscopy

Immunlabelled sections were examined using a Bio-Rad Laser-sharp 510 laser scanning confocal microscope. Gain and contrast levels were set according to procedures standardised to ensure that the image collected demonstrated a full range of grey level values from black (0 pixel intensity level) to peak white (255 pixel intensity level). All sections were initially surveyed at low magnification so that overall patterns of distribution of immunofluorescence could be compared qualitatively in cells oriented at different angles with respect to the section plane. Detailed comparisons were carried out at both high and low magnification. Confocal sections were either viewed individually, or series of optical sections (taken at 1-μm focus steps in the optical axis (z-axis) of the microscope) were digitally combined into z-axis projections or z-axis red/green anaglyphs using the laser scanning confocal microscope’s SOM software ‘maximum projection’. The SOM software’s ‘Thru-View’ sub-menu option was used to generate projections around the y-axis of optical sections steps, producing images of rotated intercalated disks.

Quantitative analysis of images

A detailed analysis of immunostained gap junction size and numerical density per disk was carried out on wax-sections from the six mammalian species. Individual fluorescent spots were assigned to correspond to discrete junctions and the longest axis dimension of the spot was taken as a measurement of gap junction size (for details on validity and limitations of this approach, see Discussion).

In the laboratory animal species, images of intercalated disks were recorded from immunostained sections of whole hearts prepared as described above. This was done in a defined zone – the central region of the left ventricular free wall, midway between the epicardial and endocardial surfaces. Immunostained gap junction size was also analysed in a corresponding location within the epimyocardium (which was least affected by disease) was selected for data collection. From each of the six species, hearts of three individuals were used. For each rat, guinea pig, mouse, hamster and human heart, 15 intercalated disks were recorded within the defined ventricular zone, giving a total of 45 disks per species. The subsets of 15 disks were subdivided into three groups (five disks each) according to disk size. The three size classes of disks were: (1) 0–49 μm², (2) 50–100 μm² and (3) greater than 100 μm². For the rabbits, disks were recorded only within size classes (1) and (2), as after extensive survey a total of only four intercalated disks larger than 100 μm² were found. For the recording of fluorescent spots the disk was divided into two zones, peripheral and central (details below).

In summary, then, the study design was: 6 species × 3 individuals × 5 disk sizes × 5 optically sectioned disks × 2 disk zones. A total of 1447 fluorescent spots was measured in the atrium.

Image analysis and image processing were performed on z-axis ‘maximum pixel’ intensity projections of optically sectioned disks and rat left atrial free wall fields using the PC-IMAGE (Paul Smith, Foster Findlay Associates Ltd) image analysis system. Initially a 3 × 3 median convolving filter was passed over the whole image to remove background noise. The outline of each intercalated disk, as marked by peripherally located immunostained gap junctions, was traced using a mouse. A second region was traced within each disk that excluded these peripherally located junctions. Immunostained gap junctions within this region are referred to as centrally located. Immunostained junctions external to this region, but within the mouse-traced outer border of the intercalated disk, are referred to as peripheral junctions. The number and size of immunostained gap junctions within each disk region were measured as follows. A pixel intensity threshold was adjusted such that the bright, fluorescent-labelled gap junctions were demarcated by an overlying colour binary image. The correspondence between the particles in the two images was checked by toggling back and forth between the full (256 grey levels) grey scale image and the binary image. Once correspondence was achieved, the labelled disks were inspected at high zoom to ensure that clearly separate juxtaposed junctions were not contiguous. If such artefactual merging occurred between proximate junctions, the binary image was edited to separate them. Pixels occurring singly in the binary image were rejected as noise. At the magnification used for data collection and display on the monitor, a single pixel equated to 0.60 μm. Hence, the smallest immunostained junctions measured by the technique were 90–120 nm in size.

Calibrated automatic measurement of intercalated disk area and the number and long axis length of individually labelled junctions in the colour binary image was then performed and the data were stored to disk. In some rare cases, elongate junctions demonstrated marked curvature. For the purpose of long axis measurement, these junctions were ‘straightened’ by using the PC-IMAGE ‘Binary Edit’ option. The areas of intercalated disks, as measured by the areas circumscribed by the mouse-traced outlines, should be regarded as comparative indices of the disk areas, rather than absolute values, in view of the highly undulating topology of the disk demonstrated ultrastructurally. The numerical density of fluorescent spots was calculated as the number of spots within a ventricular intercalated disk divided by the total visible area of the disk traced; hence these comparative data are likely to overestimate the true numerical density of gap junctions.

Analyses of variance (ANOVA) of immunostained gap junction length and density were performed using Minitab computer software (Minitab Corp.). ANOVAs of the lengths were performed on natural logarithms of the log-transformed data. The log-mean of the log-transformed data is the length values demonstrated marked deviation from normal distributions (see Figs 12 and 13, below). Following transform-
junctions are characteristically located at the intercalated disks adjoining neighbouring myocytes (McNutt, 1970; Page and McCallister, 1973; Forbes and Sperelakis, 1985; Severs, 1985, 1980). To explain the interpretation of the immunostaining patterns obtained, the basic features of intercalated disk and gap junction organisation, as seen by standard light microscopy and thin-section electron microscopy, are illustrated in Figs 1 and 2. In longitudinally sectioned cells viewed by phase-contrast light microscopy, the intercalated disks are seen as white lines traversing myocytes at right angles to, or diagonally across, the long axes of the cells. By thin-section electron microscopy, the characteristic stepped structure of the intercalated disk is resolved, with gap junctions located predominantly on the membrane segments lying parallel to the cell's long axis, perpendicular to the conspicuous darkly stained fasciae adherentes that link the myofibril ends of apposing cells.

In longitudinally sectioned rat ventricular myocytes examined by laser scanning confocal microscopy after immunostaining with anti-HJ antiserum, sharply defined punctate lines of fluorescence crossing the short axes of the myocytes are observed (Fig. 3). The general pattern of immunostaining in survey view (20× objective, NA 0.5) matches precisely the configuration of intercalated disks seen by the standard techniques of phase-contrast light microscopy and thin-section electron microscopy (Figs 1 and 2). Immunostained sections examined by conventional epifluorescence light microscopy show immunostaining patterns corresponding to those observed by laser scanning confocal microscopy but with the intercalated disks delineated as blurred rather than punctate lines (Fig. 9, below). Controls in which the anti-HJ antiserum treatment was omitted or substituted with pre-immune serum showed no fluorescent labelling.

Immunostained sections of rat ventricular tissue examined by laser scanning confocal microscopy show no evidence for the presence of gap junctions external to recognisable intercalated disks. This is in contrast to the pattern of localisation observed in longitudinally sectioned rat atrial myocytes (Fig. 4), where extensive punctate staining along the lateral surfaces of adjacent myocytes is common. Some atrial myocytes demonstrate punctate staining along the entire length of their side-by-side contacts. Chains of spots do also occur in intercalated disk-like structures, but these are smaller and less well defined than those of ventricular tissue.

## Results

### Immunostaining of rat ventricular and atrial tissues

In ventricular tissue from mammalian myocardium, gap junctions are characteristically located at the intercalated disks adjoining neighbouring myocytes (McNutt, 1970; Page and McCallister, 1973; Forbes and Sperelakis, 1985; Severs, 1985, 1980). To explain the interpretation of the immunostaining patterns obtained, the basic features of intercalated disk and gap junction organisation, as seen by standard light microscopy and thin-section electron microscopy, are illustrated in Figs 1 and 2. In longitudinally sectioned cells viewed by phase-contrast light microscopy, the intercalated disks are seen as white lines traversing myocytes at right angles to, or diagonally across, the long axes of the cells. By thin-section electron microscopy, the characteristic stepped structure of the intercalated disk is resolved, with gap junctions located predominantly on the membrane segments lying parallel to the cell's long axis, perpendicular to the conspicuous darkly stained fasciae adherentes that link the myofibril ends of apposing cells.

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### Comparison of immunostaining patterns in longitudinally oriented ventricular myocytes in six mammalian species

Fig. 5 compares, at higher magnification, the patterns of gap junction immunostaining in longitudinally oriented ventricular myocytes in rat, guinea pig, rabbit, mouse, hamster and man, as viewed by laser scanning confocal microscopy. The general features of gap junction staining from these mammals is similar, in that it is consistently confined within clearly delineated intercalated disks. At the higher magnification afforded by the 60× objective (NA 1.4), the ability to resolve the individual spots within the disks is markedly improved. The presence of a faint background fluorescence enables the positions of the cells to be identified so that the transverse widths of the disks
can be seen in relation to the cell bodies. Staggered lines of punctate fluorescence often extend across the full widths of abutting cells; shorter lines are situated at bifurcations. In human, the linear arrays of spots tend to merge into narrow bands of fluorescence that are often terminated by distinct large spots at either end. Rabbit intercalated disks are noticeably less uniform in organisation than those of the other species, with some punctate fluorescence observed in zones of lateral interaction between cells, usually in association with recognisable disks. The rabbit also shows a higher general level of background fluorescence, attributed to non-specific binding of the secondary (anti-rabbit) antibodies used in the staining procedure.

**Optical sectioning and three-dimensional visualisation of gap junction distribution in obliquely viewed and face-on intercalated disks**

Further insights into gap junction organisation at the disk are obtained by viewing myocytes cut in a variety of section planes and by exploiting the ability of the confocal microscope to provide projections of optical section series. An optical section series taken in six 1-μm steps through obliquely oriented hamster ventricular myocytes is illustrated in Fig. 6, and a z-axis projection of the series, in which the optical sections are projected into a composite image spanning the full 6 μm depth of field, is shown in Fig. 7. Viewing the immunostaining in this section plane permits sharper visualisation of individual gap junctions than can be achieved in longitudinally sectioned cells, and disks of various shapes, sizes and orientations are clearly delineated. The ability of the confocal microscope to sample information principally in the plane of focus is well illustrated in this series. Though the punctate fluorescence from most individual gap junctions can be traced through consecutive pairs of sections, the overall pattern of punctate staining progressively changes through the series as new immunostained junctions come into view, and those formerly viewed disappear. An example is situated just above the centre of the field in Fig. 6, where the serial views combine to reveal a ring of triangularly distributed fluorescent spots, outlining a disk in face-on aspect, when viewed in Fig. 7. The densest accumulation of spots occurs at the edge of the disk. These peripherally distributed spots are larger than the less frequent, albeit well-stained spots seen within the disk.

Below the ‘triangular’ disk, interconnected myocytes are observed in oblique orientation. Here, the myocytes are organised in linear series. The main boundary between cells at their ends is marked by dense ‘collar-like’ distributions of fluorescent spots around the lateral edges of disks. Some fluorescent spots are observed within the interior zone of disks, though the frequency of these varies. However, despite the foreshortening effect of the viewing angle on obliquely oriented disks, a more prominent accumulation of large fluorescent spots appears to be present at the disk periphery.

That this pattern of immunostaining represents a consistent feature of gap junction organisation at the disk is clearly demonstrated in projections of disks viewed directly face-on at higher magnification (Fig. 8). In all species examined, the periphery of the disk is typically outlined by large fluorescent spots, often elongated in shape, which enclose numerous smaller spots within the central region. Though this feature of the disk, newly revealed by laser scanning confocal microscopy, is common to all mammalian species examined, interspecies variations in its prominence are nevertheless apparent. The pattern is most marked in man and rat, where large elongate peripherally located immunostained gap junctions often show a pronounced alignment with the outer
Fig. 3. Gap junction immunolocalisation in rat left ventricle free wall, as revealed in survey view by laser scanning confocal microscopy (20× objective; NA 0.5). This view is taken from a single optical section from a tissue slice of Zamboni's-fixed, wax-embedded heart, showing myocytes in longitudinal orientation, stretching from left to right of the field, as in Figs 1 and 2. Irregular rows of fluorescent spots marking the positions of gap junctions punctuate the end-on abutments of cells and their branches, in the same position as the disk lines seen by phase-contrast microscopy (Fig. 1). A corresponding staining pattern is seen by standard immunofluorescence (Fig. 9). The micrograph has been printed so that faint ghost-like images of the myocytes are visible, thereby enabling visualisation of disk position in relation to the cells. Note the punctate nature of the fluorescence compared with the image obtained by standard light-microscopy immunofluorescence (Fig. 9). ×300.

Fig. 4. Immunolocalisation of gap junctions in rat left atrium viewed in an optical section obtained by laser scanning confocal microscopy. As in the ventricle (Fig. 3), punctate staining representing gap junctions is seen within intercalated disk-like structures (arrows), but these disks are smaller and many immunostained gap junctions are dispersed over the lateral sarcolemmata of neighbouring myocytes. (From a wax section of Zamboni's-fixed material.) ×460.

Stereo and rotated views of reconstructed intercalated disks

Perception of three-dimensional organisation is further enhanced by viewing red/green anaglyphs of immunostained intercalated disks (Fig. 10). The example in Fig. 10 has been assembled from a series of six 1-μm optical sections from rat ventricle. An elliptical, obliquely oriented intercalated disk appearing as a 'bowl-shaped' structure is observed between two adjacent myocytes. Large spots occur around the 'lip' of the 'bowl'; smaller spots, below the 'lip', give the impression that the intercalated disk has a concave, interior surface.

The 'Thru view' facility of the SOM software permits rotation of disks, allowing face-on views to be correlated directly with the edge-on perspectives normally viewed in longitudinally sectioned cells. A group of projections of the human intercalated disk in Fig. 8, taken at six 15°-angle increments around the y-axis of a stack of 10 optical sections, is shown in Fig. 11. The rotations suggest that the structure is composed of two shallow bowl-shaped disks skewed slightly with respect to one another in three-dimensional space. In Fig. 11E, where the lower smaller disk is seen oriented edge-on, with its interior obscured, the larger disk is tilted slightly, so that its centrally located junctions are still visible. As the disks are rotated from face-on to edge-on aspects, the characteristic peripheral ring arrangement becomes progressively less obvious. At the same time, the apparent shape of many of the peripheral junctions alters. Junctions that appear elongate or strip-like, with their long axes aligned around the circumference of the face-on disk, progressively broaden out as the disk is rotated to edge-on view, eventually appearing as irregular or rounded domains. This suggests that such junctions are viewed edge-on in face-on disks, and face-on in edge-on disks, i.e. they lie on lateral-facing zones of the cells. When viewed in this way, it becomes clear that immunostained junctions are seldom markedly elongate, and where they have a noticeably longer axis, this shows no preferential orientation.

Quantitative analysis of immunostained gap junctions

Table 1 summarises data on numerical density of immunostained junctions (number of junctions μm⁻² 'disk') in ventricular myocardium of the six species. Overall, the absolute number of immunostained junctions per intercalated disk increases with size of the disk, but the numerical density decreases as disk size increases. This trend is significant within all species (P<0.05), except guinea pig (Table 1). When disks of all sizes are pooled there is no significant difference (P>0.05) in the overall
Fig. 5. Comparison of gap junction immunolabelling patterns in left ventricular myocardium of six mammalian species, as viewed by laser scanning confocal microscopy at higher magnification (60× objective, NA 1.4). The long axis of the myocytes runs from left to right so that the intercalated disks are viewed in edge-on aspect. All species demonstrate a similar pattern of punctate fluorescence specifically located within intercalated disks. In rabbit, some lateral staining is present, reflecting a tendency for more extended disks whose precise limits are more difficult to define. The higher general level of background fluorescence in the rabbit results from non-specific binding of the anti-rabbit second antibody used in the staining procedure. Note that in the example from man, the visible ends of the disk are marked by particularly large, bright spots (arrows). (All examples are from wax sections of Zamboni's-fixed material, except those from guinea pig and rabbit, which are from -20°C methanol-fixed cryosections.) ×792.
Fig. 6. A laser scanning confocal microscopy optical section series through obliquely oriented myocytes in hamster left ventricle. (Prepared from a 25 μm cryosection fixed in -20°C methanol before immunostaining.) Each of the six optical sections is separated by a 1-μm step in the optical axis of the microscope. Note how the pattern of punctate staining of the individual intercalated disk changes from section to section as one moves through the 6 μm depth of tissue. In this way, the entire complement and distribution of gap junctions within whole intercalated disks can be viewed. ×450.

Numerical density of immunostained gap junctions between the species.

Histograms illustrating the frequency distributions of fluorescent spot longest axis for the six species are shown in Figs 12 and 13. In all the myocardia examined, the distributions of this index of junction size are asymmetric, being skewed towards the lower end of the range, with the majority of immunostained junctions (>97%) being less than 2 μm long. The small fraction of immunostained junctions above this size extended up to a maximum of 7.9 μm (mouse ventricle), but only 13 out of the 15,345 immunostained junctions measured in all species were greater than 4 μm in length.

The frequency distribution in the rat atrium shows a flatter curve and lower spread of values than that of rat ventricle (Fig. 13), with the longest immunostained junctions measuring 2.9 μm and 5.4 μm, respectively. There is a highly significant difference ($F=131.0, P<0.01$)
in size between atrial and ventricular immunostained junctions, those in the atrium being on average 33% longer than those in ventricle (mean spot lengths for atrium and ventricle, 0.56 \mu m and 0.42 \mu m, respectively).

Table 2 summarises the results of ANOVA of immunostained gap junction size in the six species. Though overall there was significant variation between species in the mean size of immunostained junctions; means in five of the six species were similar. The cause of the apparent variation between species was that the rabbit had significantly smaller junctions (P<0.01) than the other five species. Small differences in spot size between disks of different size were found within species but, though some of these differences reached statistical significance, no consistent trend between these two variables was found across the species examined.

The major result revealed by ANOVA is a pronounced, highly significant difference (P<0.01) in size between immunostained junctions located in the central regions of disks compared with those at the periphery in all species. The ANOVA F levels determined for this source of variation were consistently an order of magnitude greater than all other sources of junction length variation examined in this study (Table 2).

**Discussion**

In the present study, we have exploited a specific cardiac gap junction antiserum in combination with laser scanning confocal microscopy to extend our understanding of the distribution and arrangement of gap junctions between different types of cardiac muscle cell.

**Table 2. Gap junction size as measured by fluorescent spot longest axis (nm) in ventricular myocardium of the six species: summary of the results from ANOVA**

<table>
<thead>
<tr>
<th>Disk size class</th>
<th>Rat</th>
<th>Guinea pig</th>
<th>Rabbit</th>
<th>Mouse</th>
<th>Hamster</th>
<th>Man</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small (&lt;50 \mu m²)</td>
<td>480</td>
<td>460</td>
<td>450</td>
<td>450</td>
<td>420</td>
<td>440</td>
</tr>
<tr>
<td>Medium (50–100 \mu m²)</td>
<td>420</td>
<td>430</td>
<td>370</td>
<td>420</td>
<td>450</td>
<td>440</td>
</tr>
<tr>
<td>Large (&gt;100 \mu m²)</td>
<td>420</td>
<td>490</td>
<td>–</td>
<td>430</td>
<td>420</td>
<td>410</td>
</tr>
<tr>
<td>F</td>
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<td>7.4</td>
<td>12.3</td>
<td>0.6</td>
<td>5.0</td>
<td>3.9</td>
</tr>
<tr>
<td>P</td>
<td>*</td>
<td>**</td>
<td>NS</td>
<td>**</td>
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<table>
<thead>
<tr>
<th>Disk zone</th>
<th>Central</th>
<th>Peripheral</th>
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<td>270</td>
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<tr>
<td>520</td>
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</tr>
<tr>
<td>F</td>
<td>243.0</td>
<td>55.2</td>
</tr>
<tr>
<td>P</td>
<td>**</td>
<td>**</td>
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| Overall means | 420 | 440 | 390 | 430 | 430 | 420 |

F (for species pooled)=5.28.
P (for species pooled)=**.

Information on the differences in junction size between species, and for different disk size classes within species is presented. The variation of junction size between central and peripheral disk zones within species is also shown. Means have been retransformed from log, to real nm values. The level (F) and significance (P) of the variance between the means is derived from ANOVA performed on log, transformed data. F, ANOVA F ratio; ** variation between log, means significant at the 1% level of confidence; * variation between log, means significant at the 5% level of confidence; NS, variation between log, means not significant at the 5% level of confidence (sample sizes (N) for each of the species were as follows; rat, 2832; guinea pig, 2586; rabbit, 935; mouse, 2467; hamster, 2856; man, 2222).
Fig. 8. The typical patterns of gap junction organisation as seen in face-on views of entire intercalated disks prepared from projections of optical section series taken in ventricular myocardium of six mammalian species. Large bright patches of immunofluorescence characteristically mark out the disk periphery; smaller immunostained junctions are situated interiorly. This pattern is most prominent in man and rat, and least pronounced, though still discernible, in guinea pig and rabbit. In rabbit, a paucity of well-defined intercalated disks larger than 100 \( \mu m^2 \) in area was observed, with punctate fluorescence occurring external to the 'disk', thus confirming the distribution observed in longitudinally sectioned cells (Fig. 5). (All examples taken from ventricular free wall in Zamboni's-fixed, wax-embedded hearts.) x 1640.

The antiserum used, referred to as anti-HJ, is one of three polyclonal antisera that we have raised to synthetic peptides whose amino acid sequences match different putative cytoplasmically exposed portions of the connexin43 molecule, a cardiac gap junction protein identified in molecular cloning studies (Beyer et al. 1987). Our demonstration that in longitudinally sectioned ventricular myocytes, anti-HJ gives a localised punctate staining pattern that mirrors precisely the positions of intercalated disks seen by standard light microscopy and electron microscopy, suggests very strongly that specific gap junction labelling is being achieved. When anti-HJ is omitted from the staining procedure, or replaced with pre-immune serum, no immunofluorescence is observed, demonstrating that the staining observed in the experimental samples is due specifically to the binding of anti-HJ. Direct evidence that gap junctions and not some other component of the intercalated disk are indeed the specific target for anti-HJ binding comes from a detailed characterisation of the antisera (Harfst et al. 1990). In brief, each
Fig. 9. Immunolocalisation of gap junctions in rat ventricle by standard light-microscope epifluorescence combined with oblique back lighting to reveal cellular detail, viewed using an Olbrich 4000 prototype research microscope. Intercalated disks at the end-on boundaries of myocytes are clearly resolved, showing a similar distribution and appearance to those seen by standard phase-contrast light microscopy. Despite clear localisation, it is not possible to obtain the very precise punctate pattern representing individual gap junctions that is achieved by laser scanning confocal microscopy. x380

Fig. 10. Red/green stereo anaglyph of a laser scanning confocal microscopy projection reconstructed from six optical sections taken at 1-μm steps through a disk. This example comes from an immunostained wax section of Zamboni’s-fixed rat ventricle. A large intercalated disk, spanning 27.7 μm at its long axis, is seen in oblique aspect. When viewed in three dimensions, the disk appears as a bowl-shaped structure. Large, bright fluorescent spots are located as a peripheral ring on the lip of the ‘bowl’, with many smaller spots, and less frequent large ones, visible on the ‘bowl’s’ interior surface. Some of the larger fluorescent patches appear to be composed of groups of more than one discrete spot lying close together. x1500.

Fig. 11. A human intercalated disk is shown in projections taken at six 15°-angle increments about the central y-axis of the optical section stack. The image has been coloured falsely using the SOM software ‘geog’ colour look-up table (highest intensity red, lowest blue). Two bowl-shaped disks are seen in face-on view in (A). Many of the junctions that at one angle of viewing appear elongated, are larger and no longer elongated in the same axis, when viewed at other angles. These changes result from viewing the immunostained junctions at a variety of angles from edge-on to face-on. The example indicated by the arrow shows a large immunostained junction that is seen edge-on in (A), its longer axis lying parallel to the disk periphery. As the disk is rotated, the shorter axis progressively increases through (B)–(D), reaching maximum dimensions in (E). Thus, a stained junction that appears markedly elongate in a face-on disk is seen not to be so when the junction itself is viewed face-on. Inspection of numerous junctions in this way shows that the principal surfaces of the junctions lie parallel or near-parallel to the long axes of the myocytes they interconnect. x1450.

of the antisera binds specifically: (1) to the peptide to which it was raised; (2) to the proteins of isolated preparations of enriched cardiac gap junctions separated by SDS–polyacrylamide gel electrophoresis; and (3) to ultrastructurally recognisable cardiac gap junctions, as determined by dot blotting. Western blotting and immunogold cytochemistry, respectively (Harfst et al. 1990).

The different patterns of immunostained gap junction localisation observed between atrial and ventricular tissues can be explained by reference to the characteristic morphological features of these two myocyte types. Atrial myocytes are slender (5-10 μm width) compared with their counterparts in the ventricle (25–35 μm width) and they are less uniformly oriented, forming ramifying rather than parallel muscle fibres (McNutt and Fawcett, 1969; Simpson et al. 1973; Severs, 1989a). Although classical step-like intercalated disks are seen by electron microscopy of atrial tissue, they occupy correspondingly shorter widths across the cell. Often, the differentiation between intercalated disk and general (non-disk) sarcolemma is less clear-cut than in the ventricle, and consequently lateral-facing segments of disk membrane may stretch for long distances along the cells’ sides (Forbes and Sperelakis, 1985; Severs, 1985, 1990). This occurs not

Fig. 12. Histograms showing frequency distributions of gap junction size, as measured by fluorescent spot longest axis, in ventricular myocardium of six mammalian species. In all species, a similar distribution, skewed towards the lower values of the range, is found. Note that few immunostained junctions above 2 μm in length occur, although occasional large junctions are detectable. The largest immunostained junctions measured in each of the species were as follows: rat, 4.2 μm; guinea pig and rabbit, 3.8 μm; mouse, 7.9 μm; hamster, 3.0 μm; human, 5.3 μm. Such large junctions are, however, extremely scarce; junctions >4 μm accounting for less than 0.1% of the total population of junctions.

Cardiac gap junction localisation
Fig. 13. Histograms comparing frequency distributions of gap junction size, as measured by fluorescent spot longest axis (µm) in rat ventricular and atrial myocardium. Atrial junctions have a flatter distribution and smaller range of values than in the ventricle. The largest sizes of immunostained junctions found in the atrial and ventricular tissues were 2.9 and 5.4 µm, respectively.

only at the cell tips, but also in association with small, often poorly distinguished, transverse disk segments located at various points along the cell body. The net result is a 'spreading' of the gap junctions over the sides of the cell, rather than their being confined solely to sharply defined regions in the manner of the ventricular myocyte. The distribution of immunostained atrial gap junctions that we report here is thus consistent with the existing observations and data but, without the benefit of laser scanning confocal microscopy it has not previously been possible to appreciate the full extent of such side-to-side coupling.

Our comparison of gap junction localisation in the ventricular myocardium of different species demonstrates that the general plan of gap junction distribution and intercalated disk architecture, and the epitopes of the component gap-junctional protein detected, are common to and conserved within a range of mammalian species, including man. This basic similarity encompasses overall immunostaining pattern, numerical density and size distribution of immunostained junctions, and differentiation of a border of larger junctions at the disk periphery. Within this plan, distinct species differences are nevertheless discernible. Most notably, in the rabbit a small proportion of immunostained gap junctions was detectable at apparent regions of lateral contact between myocytes. These contacts were far less numerous and extensive than those observed in the rat atrium, but show some similarity to the junction distribution observed in the ventricular myocardium of immature rats (Gourdie et al, 1990b). The apparent lateral staining in the rabbit may represent a population of gap junctions that is quite separate from the usual form of intercalated disk or, bearing in mind the smaller overall apparent disk sizes measured in the rabbit, they may simply reflect the presence of disks that have a more extended organisation, with less clearly defined limits than those found in other species.

Although the results obtained in human paralleled those from the other species, any detailed conclusions as to their similarity have to be qualified in view of the source of the material examined. The human specimens came from areas of ventricle that appeared histopathologically unaffected in three patients whose hearts were removed at surgery for two different disease conditions (ischaemic heart disease and dilated cardiomyopathy). The samples were not age-matched with those from the other species, and right rather than left ventricle was analysed (the right ventricle being less affected by disease). These variables may well influence some of the data obtained (e.g. hypertrophy is a common response in cardiac disease, and this might be expected to lead to an increase in size of the intercalated disk). Clearly, the use of such material is far from ideal in attempting to establish the characteristics of gap junction organisation in the normal human heart, but this was the only practicable approach available. It seems reasonable to conclude, however, that features present in the human material examined that are also common to all the other species, notably the peripheral ring arrangement, and the data on size and numerical density of immunostained junctions, are in all likelihood features of the normal human heart too.

A major finding of the present study is our demonstration that the intercalated disk in mammalian ventricular myocardium is characterised by a conspicuous peripheral ring of large gap junctions. In larger disks, the rings of brightly stained junctions demarcate the boundaries of the principal ends of the main cell body, and the diameter of the ring thus often corresponds to that of the cell itself. Smaller, and less abundant gap junctions are typically located in the interior zone of the disk, though some large bright spots may also be present in this zone. The gross morphology of the large disks, as revealed by laser scanning confocal microscopy, is more accurately likened to a stack of two bowls than to the classically envisaged irregular step-like structure. The organisation of immunostained gap junctions in smaller disks, located at the interacting ends of stub-like branches of the main cell body, resembles that of the larger disks, though numerical density of immunostained junctions is inversely related to disk size.

The perspective of gap junction organisation at the intercalated disk thus revealed by confocal microscopy differs substantially in emphasis from the existing concepts and models derived from standard light- and electron-microscopical observations. Thin-section and freeze-fracture electron microscopy (e.g. see Page and McCullister, 1973; Rössle and Sperelakis, 1985; Hoyt et al, 1988; Severs, 1985, 1990) provide multiple highly detailed
views of small portions sampled within disks, but in the absence of complete overviews of entire individual disks, a recurrent feature at a more gross level of organisation is easily overlooked, particularly in structures (like the disk) that are of large size and have a complex, variable three-dimensional topology. Visualisation of these new features in the present study stems from the ability of the laser scanning confocal microscope to detect the detailed three-dimensional distribution of fluorescent labels through substantial depths of tissue (White et al. 1987; Shotton, 1989). Conventional immunohistochemical techniques have also been used previously to visualise gap junctions at the light-microscopical level (Beyer et al. 1989; Yancey et al. 1989; Luke et al. 1989) but as is also apparent from the present study, this approach has inadequate resolution and is unable to provide clear views of immunostained junctions free from out-of-focus blur over sufficiently large depths of focus. Moreover, the thinner tissue sections normally required (usually <10 μm) for standard light microscopy as compared with confocal microscopy lessen the probability of including entire intercalated disks within the width of the tissue slice examined.

In addition to the integrated overviews of gap junction organisation and intercalated disk structure made possible by confocal microscopy, the images of en-face oriented disks integrated from optical section series are well-suited for morphometric analysis. An alternative morphometric approach, involving silver-enhanced gold immunostaining and standard light microscopy of longitudinally oriented canine ventricular myocytes, has recently been proposed (Luke et al. 1989). However, this method has the drawback that when intercalated disks are viewed edge-on (the predominant orientation of disks in longitudinally sectioned myocytes), larger gap junctions at the disk periphery will appear superimposed upon one another, and obscure the numerous smaller junctions present in the disk central zone. Owing to the ability of the confocal microscope to reduce out-of-focus blur from fluorescently labelled structures (White et al. 1987; Shotton, 1989), sharper visualisation of discrete immunolabelled gap junctions is achieved in confocal images, and so measurements are correspondingly more precise than those obtained from standard light microscopy. Moreover, the laser scanning confocal microscope enables more comprehensive data to be collected and superior sampling as the total population of immunolabelled gap junctions within complete intercalated disks can be analysed.

How far are these theoretical advantages of laser scanning confocal microscopy translated, in practice, to accurate data on junction number and size? Given that the measured sizes of immunostained junctions are often less than the 1-μm optical section steps used in this study, it might be argued that counts made on intercalated disks reconstructed from those optical sections will fail to reveal all the junctions present in the disk. In practice, however, this is not the case. Although the confocal microscope reduces out-of-focus blur from above and below the focal plane, a limited spread of collected light about focus nevertheless occurs (Inoue, 1989). This is demonstrated practically in Fig. 6 where punctate fluorescence from the same individual gap junctions can be seen in register from one 1-μm stepped optical section to the next. Although in some regions of ventricular intercalated disks in which the labelled epitopes of the gap-junctional protein were more limited in the interior regions of the disks than in the peripheral zones, then a differential pattern of the type we describe might be produced artificially. However, our preparations show clear and consistent staining at all levels in the tissue slice, and where large gap junctions are detected in the central regions of disks, they fluoresce as brightly as those at the periphery. These two observations suggest that the trypsin treatment used in the procedure, aided by inclusion of detergent as a wetting agent in the lysolecithin blocking step, are adequate in ensuring even penetration of the immunostaining reagents both throughout the thickness and within the individual cells of the entire tissue slice.

It thus appears justified, in the light of the present findings, to reconsider some aspects of gap junction organisation and intercalated disk morphology deduced from electron microscopy. Longitudinal steps of membrane bearing gap junctions in the interior zone of the intercalated disk may, on the basis of our observations, either be less frequent or less extensive than previously supposed, or their gap junctions may be substantially smaller and spread of light intensity along the z-axis would be expected between sequential 1-μm spaced optical sections. Other potential sources of error are: that dumbbell-shaped junctions may be analysed as two separate spots, or that two closely adjacent junctions may appear as a single spot, though careful procedures to deal with such eventualities were devised for the data collection procedure.

The measurements of immunostained gap junction longest axis correspond well with the dimensions of cardiac gap junctions reported in previous electron-microscopical studies (e.g. see Shibata and Yamamoto, 1979; Gros et al. 1991; see, however, Hoyt et al. 1989). In particular, our data on rat ventricle match closely those published by Shibata and Yamamoto (1979) from freeze-fracture replicas. These authors found that slightly greater than 50% of junctions were less than 0.1 μm² in area, equivalent to 0.36 μm in diameter (assuming circularity of smaller maculae). Similarly, in the present work, 55% of immunostained gap junctions measured in the rat ventricle had long axes of 0.4 μm or less. The mean length of immunostained gap junctions in rat atrium was slightly larger than that for rat ventricle as a whole, but was comparable to that of the peripherally located ventricular junctions. The difference between the overall mean sizes for the two tissues may reflect constraints on the dimensions of junctions located within the central regions of ventricular intercalated disks, imposed by the presence of the fascia adherens junctions that occupy a major portion of these regions. In all species, only a small fraction of ventricular gap junctions (3%) were longer than 2 μm, though in the mouse a single junction of 7.9 μm was recorded. Thus, we found no evidence for a major population of very large ribbon-shaped gap junctions oriented with their long axes (5.1 μm mean length) transverse to the long axis of the cell, as proposed in the model described by Hoyt et al. (1989). By rotating disks through 90°, immunostained junctions can often be seen to have an irregular rather than a perfectly rounded shape, but such junctions are seldom markedly elongated, and where a longer axis is apparent, this shows no favoured orientation.

Could the immunodetection of a prominent ring of large gap junctions at the periphery of the ventricular intercalated disk be an artifact of the staining technique? If access of the labelling reagents to the cytoplasmically located epitopes of the gap-junctional protein were more limited in the interior regions of the disks than in the peripheral zones, then a differential pattern of the type we describe might be produced artificially. However, our preparations show clear and consistent staining at all levels in the tissue slice, and where large gap junctions are detected in the central regions of disks, they fluoresce as brightly as those at the periphery. These two observations suggest that the trypsin treatment used in the procedure, aided by inclusion of detergent as a wetting agent in the lysolecithin blocking step, are adequate in ensuring even penetration of the immunostaining reagents both throughout the thickness and within the individual cells of the entire tissue slice.
less common than those of the outermost longitudinal-facing segment circumscribing the cell periphery. Some of the large gap junctions described by Hoyt et al. (1989) situated in a lip of membrane overlapping the cell periphery could correspond to the peripheral ring of gap junctions we describe, though these authors did not distinguish peripheral from interiorly located longitudinal segments of disk membrane as the preferential site for large gap junctions. The few large bright spots that we detect in the central zone of the disk most likely represent large gap junctions. The few large bright spots that we detect in the central zone of the disk most likely represent large gap junctions. The few large bright spots that we detect in the central zone of the disk most likely represent large gap junctions. The few large bright spots that we detect in the central zone of the disk most likely represent large gap junctions. The few large bright spots that we detect in the central zone of the disk most likely represent large gap junctions. The few large bright spots that we detect in the central zone of the disk most likely represent large gap junctions.

What might be the functional significance of the gap junction organisation we describe? One possibility is that a peripheral ring of large gap junctions represents the most efficient arrangement for rapid longitudinal spread of electrical impulses between myocytes. If two rod-shaped ventricular myocytes are envisaged, interconnected end-to-end by an intercalated disk, then the large peripheral gap junctions can be imagined as lining the inside surface of an exterior rim of membrane created by the abutment of the two cells. The principal gap junction ring thus lies directly in the path of the depolarising current moving along the surface of one cell to its neighbour, thus increasing the rapidity of electrical current propagation in the direction of the long axes of the myocytes. The role of the smaller gap junctions in the disk interior is less certain; whether these junctions differ in function from their counterparts at the disk periphery is an intriguing possibility for future investigation.

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