The primary structure of segment S4 includes 4 to 8 positively charged amino acids (arginine, lysine) which are regularly arranged in the sequence, each at every third position. This structure is highly conserved in voltage-gated channel proteins. However, searching for such a sequence pattern in the whole protein sequence database (SWISS-PROT) reveals that it is not characteristic for channel proteins. The unique pattern for S4 segments of voltage-gated channel proteins, as found according to the rules outlined by Bairoch[11], is defined as follows:

R-X-[FILV]-R-[LVAI]-[LVAI]-R-X-X-[RK].

This characteristic pattern of segment S4 consists of 10 amino acids. It contains 4 charged amino acids (arginine, lysine) and some strongly hydrophobic amino acids between them. This pattern appears in all the 40 known S4 segments; only one, the potassium channel protein, DRK1, has a methionine in position 3 of the pattern. There is one non-channel protein, cystatine, with such a pattern (data bank entry: CYTC$BOVIN).

S4 segments from repeats I and II of Na-II-channel proteins were the objects of extensive studies involving the combined use of site-directed mutagenesis and patch-clamp recordings[12]. These investigations provided evidence that the positive charges in S4 segments are involved in the voltage-sensing mechanism for activating of the channel. We calculated hydrophobicity values, using the window of 13 amino acids, for the wild-type and for mutants of segment S4. We have found that the substitutions of amino acids did not lead to significant increases of hydropathy values. It appears that rearrangements of the secondary structure are not responsible for the electrophysiological effects observed in this case. Lately Auld et al.[13] observed a dramatic change in the gating properties of voltage-dependent Na-II-channel protein, after replacement of a neutral amino acid (phenylalanine in place of leucine) in segment S4 of repeat II. This finding can be explained as being due to conformational changes, but only a minor increase of the hydrophobicity value was found in this case. Thus small conformational alterations cannot be excluded, and both the charge and the conformation of S4 segments have to be considered in attempts to elucidate the voltage-dependent gating mechanism of channel proteins.

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Some extracellular matrix elements as markers of 'capillary tunnels' in hypertrophied rat heart

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Abstract. We studied the distribution of the extracellular matrix proteins fibronectin (FN) and laminin (LM) in the hypertrophied hearts of spontaneously hypertensive rats (SHR), using an immunofluorescence method with specific antibodies. The immunohistochemical reaction was positive in the cytoplasm of some hypertrophied cardiomyocytes. The results showed that FN and LM can be used as markers for tunnels, i.e. intracardiocytic invaginations of the sarcolemma. The tunnels observed contained capillaries.
Key words. Heart hypertrophy; spontaneously hypertensive rats; tunnel capillaries; fibronectin; laminin; immunohistochemical staining.

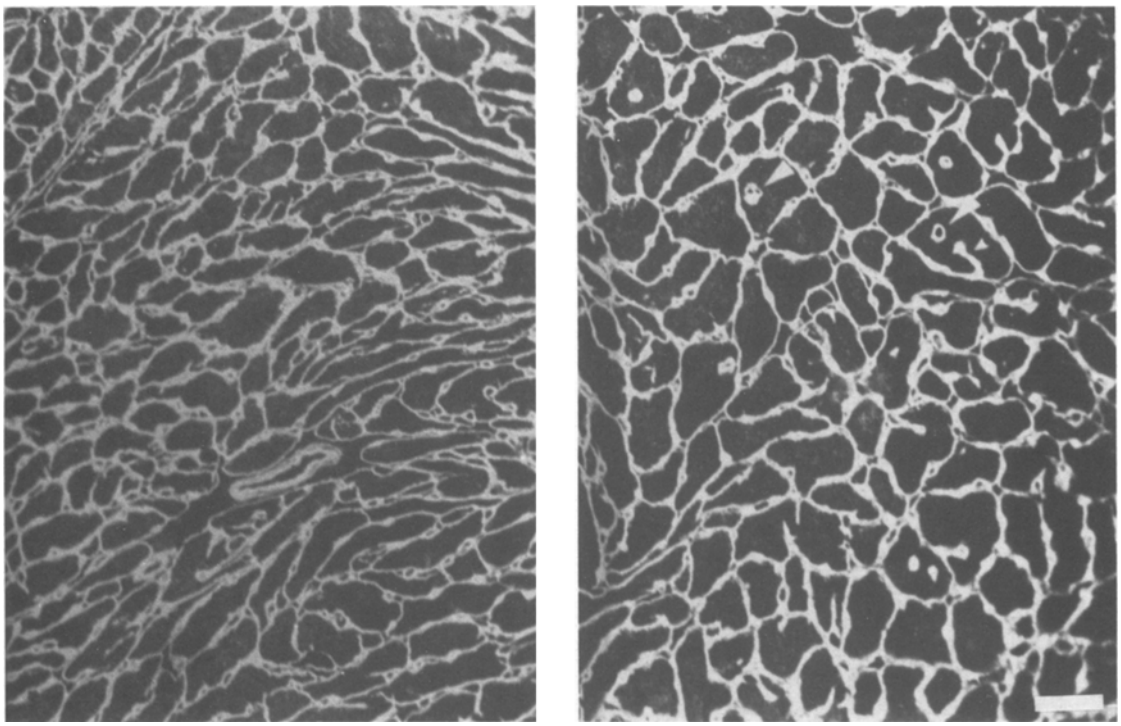

Figure 1. Immunofluorescent staining with antilaminin antibodies in myocardial cryostat sections. *A* WKY rat aged 4 months. Antibodies are situated along the basement membranes. *B* SHR aged 4 months. Deposition of antibodies in some cardiomyocytes identifies cross-sectioned tunnels (arrowheads). The basement membrane invaginations into the cytoplasm demonstrate cardiomyocyte splitting (small arrowheads). Bar = 100 μm.

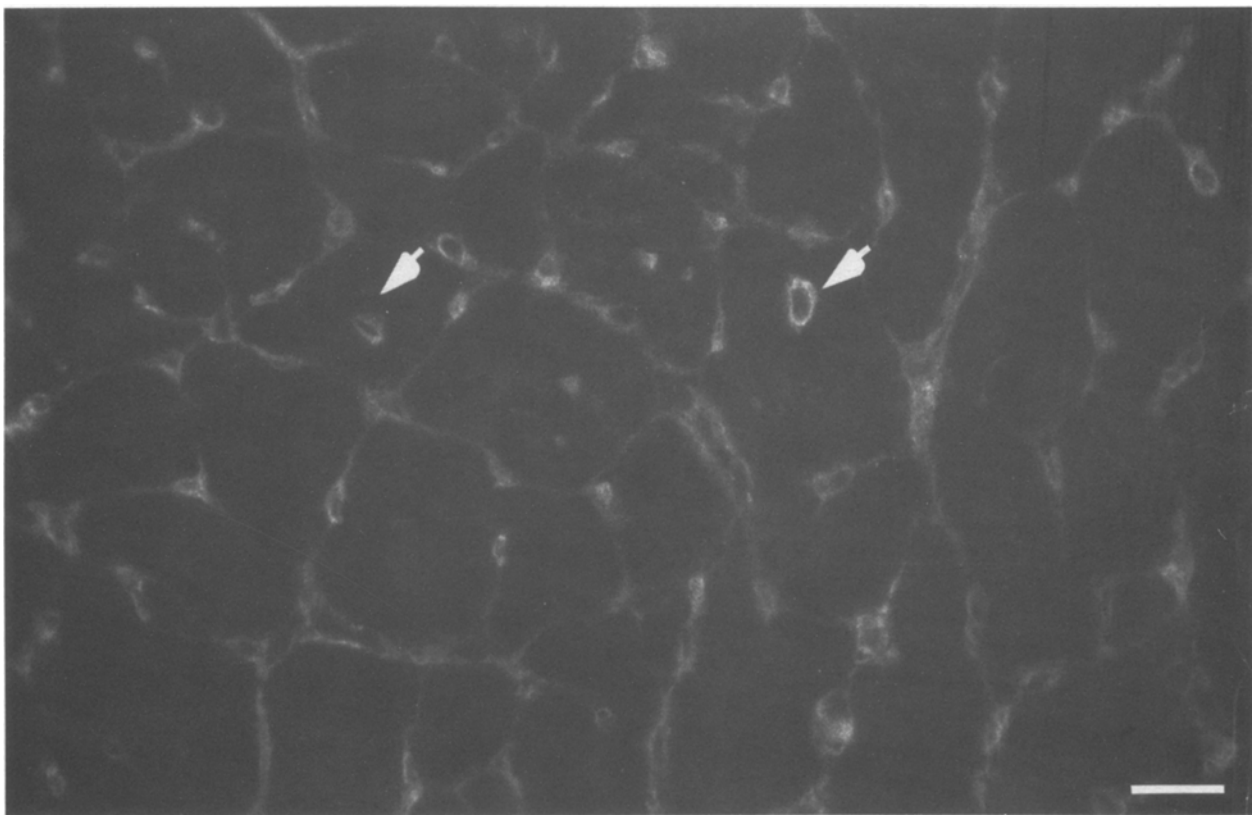

Figure 2. Immunofluorescent staining with antibodies to fibronectin in the myocardium of SHR aged 7 months. Cross-sectioned tunnels are visible in some cardiomyocytes (arrows). Bar = 20 μm.

FN and LM are major extracellular matrix glycoproteins which bind other extracellular matrix macromolecules, basement membrane macromolecules and some cell surface receptors [1-3]. These glycoproteins have been shown to play a vital role in many developmental and pathological processes such as tissue differentiation, wound healing, and tumor metastasis [4, 5]. Their involvement in angiogenesis, by making a scaffold for capillary tube formation (LM) [6], and promoting endothelial cell growth (FN) [7], has been described recently. During heart hypertrophy the cardiocyte enlargement is accompanied by more or less intensive capillary proliferation and interstitial tissue remodeling, in which some extracellular matrix glycoproteins might be involved. Therefore we studied the distribution of FN and LM hypertrophied rat heart.

Materials and methods

Hearts taken from adult spontaneously hypertensive rats served as a model of myocardial hypertrophy [8]. SHRs aged 4, 7, and 12 months were used. Hearts from Wistar Kyoto rats (WKY) and Wistar white rats of the same age and sex served as controls. Each experimental group consisted of 3 rats.

For immunofluorescence studies, frozen myocardial sections were stained with rabbit anti-FN (Behring) or anti-LM (Calbiochem) antibodies, and then with fluoresceinated anti-rabbit goat antibodies. Each step of staining was followed by thorough washing with phosphate buffered saline (PBS), pH 7.6. Then the sections were mounted in glycerol-PD medium [9] and viewed under a Zeiss-Fluoval fluorescence microscope.

For electron microscopical investigations, small tissue specimens were cut from different regions of the left ventricle, and immersed in 2% glutaraldehyde with 1% tannic acid in 0.1 M cacodylate buffer. Then they were postfixed in 1% osmium tetroxide, and embedded in Epon 812. Semithin sections were stained with toluidine blue and viewed under a light microscope. Ultrathin sections were viewed under a transmission electron microscope after counterstaining with lead citrate and uranyl acetate.

Results and discussion

Cryostat sections of control myocardium stained with anti-FN and anti-LM antibodies revealed a typical endomysial staining pattern: the reacting material is deposited along the basement membrane of capillaries (LM, FN), along the basement membranes of the cardiocytes (LM, FN) and in the interstitial tissue (FN) (fig. 1 a). In sections of the myocardium of SHRs aged 4, 7, and 12 months the immunofluorescent reaction appeared additionally to be located in the cytoplasm of some hypertrophied cardiomyocytes (fig. 1 b, arrowheads). Analysis of the pattern of the immunofluorescence reaction in cross-sectioned cardiocytes allowed these structures to be identified as tunnels with walls made of the basement membrane materials (LM, FN) and some extracellular matrix materials (FN) (fig. 2, arrows).

In some places, deep invaginations of the plasmalemma together with the basement membrane into the cardiocytes could be seen (fig. 1 b, small arrowheads). These invaginations could be the counterpart of cardiocyte splitting. Linzbach was the first to describe the longitudinal splitting of cardiocytes which accompanies the hypertrophy of human heart [10]. The morphological appearance of the structures observed by immunohistochemistry inside some cardiocytes is reminiscent of the capillaries or single endothelial cells found in the cytoplasm of cardiocytes in ultrastructure studies (fig. 3).

Our data are consistent with the previous ultrastructural report on tunnel capillaries, which enter some cardiocytes of SHRs [11, 12]. Bishop et al. confirmed the presence of tunnels by showing deep grooves on the surface of isolated cardiocytes in SHRs in the scanning electron microscope [13]. In the present report we showed the plasmalemmal tunnels by means of an immunohistochemical staining method, using antibodies to some extracellular matrix components. This method allows tunnels to be studied in whole myocardial sections. We thus demonstrated that the localization of tunnels is not equally distributed throughout the myocardium of SHRs. During the developmental stage of heart hypertrophy (at the age of 4 months), tunnels appear focally in the subendocardial region, in the lateral side of the left ventricle (fig. 4). Then during the established phase of heart hypertrophy (7 months) they appear additionally around the coronary arteries and in the subepicardial area. After 12 months, tunnels were randomly distributed in the myocardium (fig. 4).

By making serial cryostat sections we were able to reconstruct the tunnels. They entered the cardiocytes as side invaginations of the sarcolemma, and then oriented longitudinally to the long axis of the cardiocyte. According to our evaluations, the tunnels can be longer than 10 μm. This is contrary to Imamura's suggestions [12], which were made on the basis of semithin toluidine blue serial sections.

In spite of marked proliferative activity of blood capillaries during heart hypertrophy, capillary density decreases because of enlargement of the cardiocytes [14]. Thus the appearance of tunnels is an adaptative response to heart hypertrophy [10]. It is, however, difficult to establish whether the formation of tunnels is merely the result of cardiocytes growing around the adjacent capillaries or the result of active ingrowth of the endothelial cells, together with interstitial tissue, into the cardiocytes. The role of LM and FN in tunnel formation during heart hypertrophy remains to be established.

Formation of capillary tunnels has been noted in human dystrophic skeletal muscle [15] and in hypertrophic skeletal muscle in experimental animals [16]. The immunohistochemical staining method presented by us offers a screen-

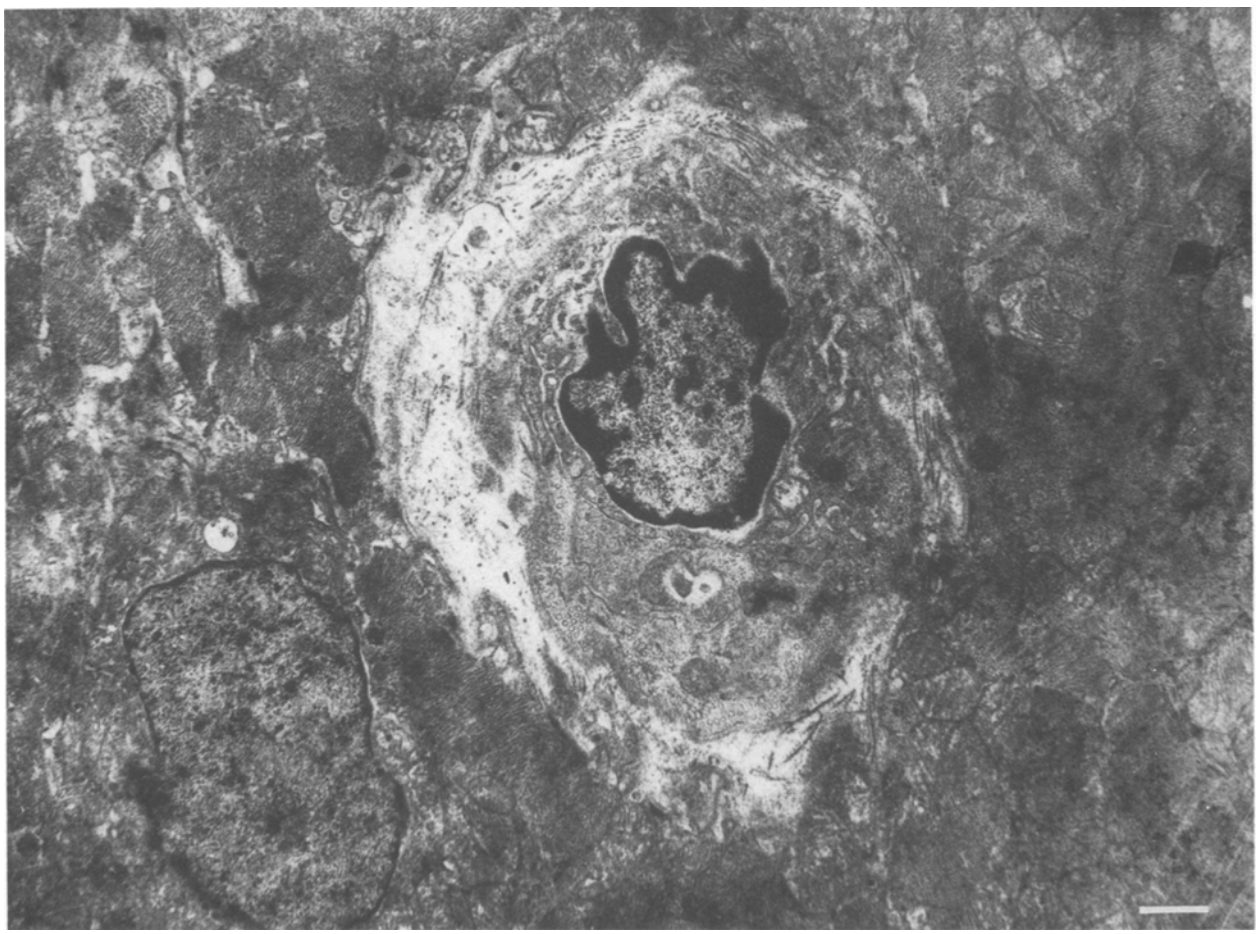

Figure 3. An electron microscope image of an endothelial cell and collagen interstitial tissue entrapped by a cardiocyte from a 12-month-old SHR. Bar = 2 μm.

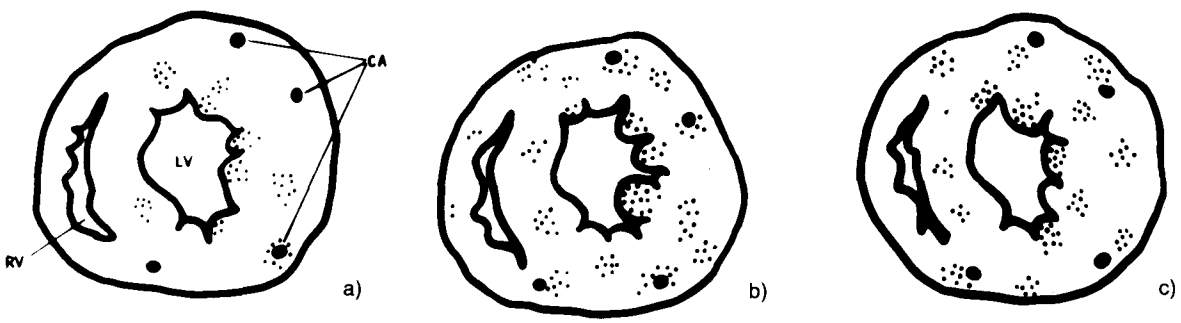

Figure 4. Schematic representation of regional distribution of the capillary tunnels (dotted areas) in the cross-sectioned myocardium of SHR aged 4 months (a), 7 months (b), and 12 months (c). LV, left ventricle; RV, right ventricle; CA, coronary arteries.

ing method for detecting tunnels in other pathologic conditions of heart and skeletal muscle.

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An improved test for Africanized honeybee mitochondrial DNA

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Abstract. Mitochondrial DNA derived from *Apis mellifera scutellata*, the ancestor of the Africanized bees of the New World, lacks a *Bgl*II restriction site found in other types of honeybee [1,2]. We present primers allowing amplification of a 485-bp section of the cytochrome b gene containing this site, using the polymerase chain reaction. Digestion of the amplified product with *Bgl*II yields contrasting patterns between Africanized and other honeybees.
Key words. Africanized honeybee; *Apis mellifera scutellata*; mitochondrial DNA; restriction fragment length polymorphism; *Bgl*II; polymerase chain reaction.

The introduction of *Apis mellifera scutellata* from Africa into the New World at São Paulo, with the aim of improving apiculture, led to the dramatic spread of these bees [3,4]. The genetical evidence so far supports the notion that the Africanized bees have spread almost wholly by ecological displacement of the commercial, European-derived strains, rather than by superior mating success, in that a strong association between nuclear and mtDNA RFLPs remains even in the northernmost populations of the advancing front [1,2,5]. It has been argued [1,5] that the introduced bees have interbred sufficiently little with other strains as to be termed African and not Africanized, but we prefer to retain the latter term in this paper.

As noted by Taylor and also McDowell (Hall [5]), Africanized bees are aggressive, difficult to manage, and liable to disrupt bee-based pollination and other agricultural activities. The economic impact of Africanized bees is therefore liable to spread considerably beyond the effects on honey production. A rapid, easy, and reliable test for Africanized bees would be valuable not only for testing reports of incursions of these bees into new areas, but also for continuing research into the evolutionary dynamics of the spread of this colonising insect. We report a test based on mtDNA and PCR which would permit easy identification of Africanized matrilines.

The restriction enzyme *Bgl*II cuts the mtDNA of Africanized bees into one and that of non-Africanized bees into two fragments [1,2]. *Eco*RI digestion patterns also discriminate between bees of different origins, but not as simply [1,2].

Published [6,7] and our unpublished sequence studies show that the rRNA genes and the genes encoding the proteins cytochrome oxidase subunits I, II, and III, cytochrome b, and ATPase subunits 6 and 8, are in the same relative positions as for *Drosophila yakuba* [8], but that there have been shifts in tRNA genes. We have therefore identified the position of the diagnostic *Bgl*II site within the cytochrome b gene (fig. 1) and designed appropriate primers to amplify a 485 bp portion of this gene containing the site.

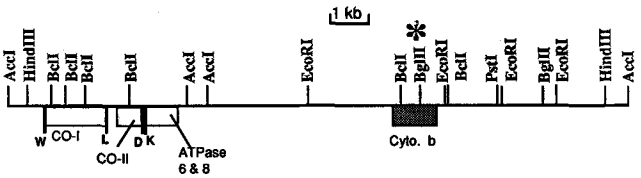

Figure 1. Restriction map of *Apis mellifera ligustica* mtDNA showing the location of the cytochrome b gene and of genes completely sequenced so far (CO-I and CO-II: genes for cytochrome c oxidases subunits 1 and 2, W, L, D, K: tRNA genes as denoted by the one-letter amino acid code). The asterisk indicates the *Bgl*II site whose absence characterises Africanized bees.

Materials and methods

Africanized and non-Africanized adult and larval bees were collected into 95% ethanol and sent to us by H. G. Hall [5] at ambient temperatures. Adult bees were dried to remove ethanol, while larval specimens were equilibrated overnight [1] before total DNA extraction using a modification of the CTAB method of Boyce et al. [9]. Individual bees were quick frozen in liquid N_2, ground using mortar and pestle and digested at 65 °C for 2 h in 500 µl of 2 × CTAB buffer (0.1 M Tris-HCl (pH 8.3), 0.02 M EDTA (pH 8), 1.4 M NaCl, 0.2% betamercap-