Electron probe X-ray microanalysis and cryoultramicrotomy of unstained myocardial sarcoplasmic reticulum, in situ and fragmented[1,2]

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Summary. Myocardial sarcoplasmic reticulum of cats in situ and fragmented sarcoplasmic reticulum (FSR) were analysed using X-ray microanalysis, cryoultramicrotomy and scanning transmission electron microscopy. 2 types of FSR vesicles can be distinguished morphologically and by their different elemental composition especially by different Ca loading. The Ca content of the sarcoplasmic reticulum can also be detected in situ.

The contribution of the sarcoplasmic reticulum (SR) to the regulation of myocardial contraction is today still unassessed. Recent suggestions point to the possible role of SR in various heart diseases involving impairment of contractility[3]. It is generally assumed that Ca^{++} crosses the surface membrane of the myocardial cell during the action potential, but that this Ca^{++} is insufficient to induce contraction directly. Rather, it induces a release of Ca^{++} from the SR[4]. In order to understand the processes of myocardial excitation-contraction coupling, more knowledge is required about the contribution of the different myocardial SR components to the regulation of calcium.

Material and methods. We investigated myocardial SR in situ and preparations of active fragmented myocardial SR (FSR) using cryoultramicrotomy, scanning transmission electron microscopy and electron probe X-ray microanalysis. For the analyses of in situ SR we used papillary muscles and trabeculae of cat left ventricle. The morphology of the cryofixed, unstained myocardial tissue is shown in fugure 1, a. The muscles were incubated in oxygenated Ringer's solution[5], and the functional quality of the strips was checked by controlling the length-tension relationship. The muscles were frozen in N_2 slush, undercooled Freon 22 or propane, cut ultrathin at -120 to $-140\,°C$ with a modified LKB cryoultramicrotome and freeze dried.

For the investigation of in vitro SR (FSR), FSR from cat ventricular myocardium was isolated by fractionated centrifugation at 0 °C with a conventional method adapted from Suko[6]. The isolation medium (pH 7.3) contained 0.3 M sucrose, 10 mM tris. HCl, 2 mM EGTA, 1.3 mM dithioerythrit, 1 mM ascorbic acid. Actomyosin extraction was performed with 0.6 M KCl + 10 mM histidine · HCl (pH 6.8). The FSR was suspended and stored at 0 °C in 0.3 M sucrose + 10 mM histidine · HCl (pH 6.8). Incubations were carried out immediately after the end of isolation. Medium for incubation of FSR for calcium uptake (30 °C, ph 6.8): 20 mM histidine · HCl, 0.1 M KCl, 10 mM $MgCl_2$, 5 mM tris · oxalate, 5 mM tris · ATP, 20–300 nM $CaCl_2$/mg FSR protein. For tests with zero calcium the same medium, but without $CaCl_2$ and with the addition of 0.5 mM EGTA, was used. After complete calcium uptake (at least 6 min), the test tubes were quickly cooled on ice and centrifuged at $60{,}000 \times g$. The FSR pellet was then treated in the same way as the tissue. For electron probe analysis we used the Siemens 102 electron microscope equipped with a KEVEX energy dispersive detector (20 mm detector-probe distance) and a Siemens scanning transmission device. The analyses of FSR preparations were performed under identical conditions: 4 μA beam current; about 20 nm spot diameter; 100 sec analysis time. As a continuum we used a white channel from 5,5–6 KeV. The analysed probes had continuum values of 400–600 counts. The measured calcium net peak/continuum ratio was converted into concentration[7] by using the calibration curve (figure 2, a). (In all graphs values are means ± SD, n = 15).

We incubated FSR with different $CaCl_2$ concentrations in the presence of oxalate and analysed elemental composition inside the vesicles. This system was used as a biological

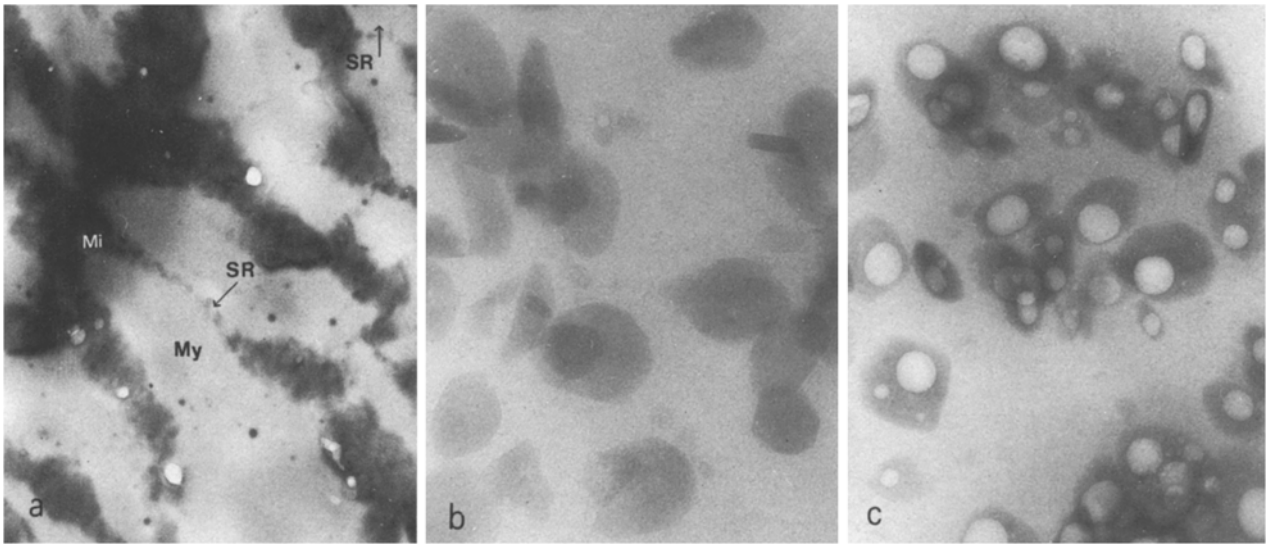

Fig. 1. Morphology of cryofixed, unstained myocardial tissue and of components of FSR. *a* Transverse section of papillary muscle. M = mitochondria; My = myofibrils; SR = chains of transversally cut longitudinal tubuli. *b* Vesicles of fragmented sarcoplasmic reticulum. Electron resistant vesicles. The preparations were unfixed and unstained. *c* Calcium oxalate-filled vesicles, which become damaged by exposure to the electron beam. Magnification: *a* × 12000, *b* and *c* × 180000.

standard for Ca quantification[8] (More details of method in Wendt-Gallitelli et al.[9]).

Results and discussion. 2 types of FSR vesicles are distinguishable: 1 population is resistant to the electron beam (figure 1, b). The 2nd population (figure 1, c) becomes damaged by exposure to the electron beam. This latter type of vesicle was described by Carsten et al.[10] as a Ca-oxalate filled type. We found the 2 populations in all FSR preparations, but mostly in well-separated regions of the pellet. Because it is known that oxalate precipitates only occur in SR cisternae and not in longitudinal tubuli[10,11], we can also distinguish these 2 components of FSR in the in vitro preparations. The concentration of calcium in the electron resistant vesicles (originally longitudinal tubuli?) increases nearly linearly up to 60 nM $CaCl_2$/mg FSR protein in the medium (figure 2, a) (corresponding to the range of exchangeable Ca[9,11]). At concentrations in the medium higher than 80 nM $CaCl_2$, calcium decreased in the inside of these vesicles. This decrease of calcium concentrations inside the electron beam resistant vesicles at Ca^{++} concentrations in the medium above the physiological range (estimated about 70 nM $CaCl_2$/mg FSR protein[4]) could be due to stronger Ca accumulation ability of Ca oxalate precipitating FSR under the condition or/and to a increased permeability of FSR membranes, at least of the electron resistant vesicles.

The concentrations of K^+ and Cl^- (figure 2, b) are very high in the Ca-free vesicles (treated with EGTA) and remain relatively low in the range between 20 and 200 nM $CaCl_2$/mg SR protein. With maximally Ca-loaded oxalate vesicles (300 nM $CaCl_2$/mg FSR protein) the concentrations of K^+ and Cl^- in the vesicles suddenly become very high. The extremely different concentrations of these elements in the vesicles at different Ca-loadings can also indicate Ca-dependent changes in the permeability of the FSR membrane for these ions as well.

In order to test whether the FSR vesicles maintain the calcium gradient between their interior and the surrounding matrix despite preparation, freezing and cryotomy, point analyses were made in the inside of single vesicles and 0.1 µm to their exterior (figure 2, c). This surrounding matrix, in which the vesicles accumulate, probably consists of fragments of organelles and membranes. Only calcium is clearly sequestered in the interior. The concentrations of all other specific probe elements do not differ significantly from those in the surrounding regions.

Although in general Ca and P concentrations change in a similar way to one another (figure 2, d), the P concentration is significantly higher in the electron resistant vesicles at Ca-concentration in the medium of 60 nM $CaCl_2$/mg FSR protein (297 ± 30 mM/kg dry wt) than in the oxalate vesicles (126 ± 28 mM/kg dry wt).

The results of these investigations demonstrate that the application of the methods used to in vitro preparations of FSR can yield new information about the Ca-dependent changes in the elemental composition of different components of FSR, especially of their different Ca loading ability. In sections of cryofixed, unstained myocardial tissue we found that, as is the case for skeletal muscle[12], these methods are sensitive enough for the quantitative detection of calcium in different compartments of the myocardial cell. However, the problem of these in situ investigations is a morphological one: the myocardial subcellular structure is recognizable and elemental analysis possible only if the

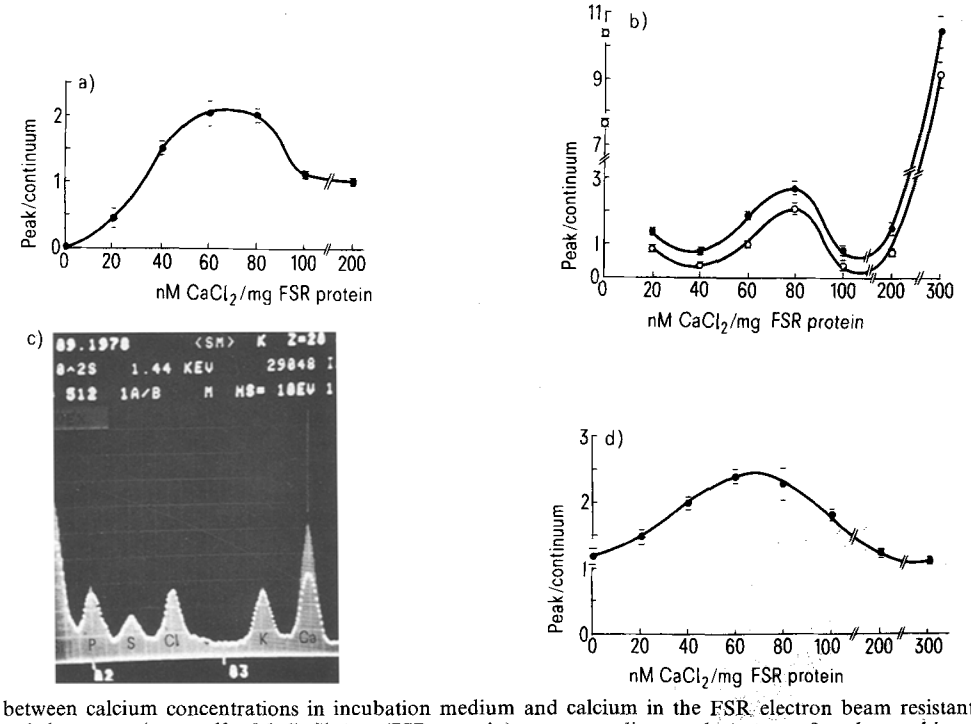

Fig. 2. *a* Relation between calcium concentrations in incubation medium and calcium in the FSR electron beam resistant vesicles. The ascending portion of the curve (up to 60 nM $CaCl_2$/mg FSR protein), corresponding to the range of exchangeable calcium (about $\frac{1}{3}$–$\frac{1}{4}$ of maximal FSR capacity for calcium[2]), was used as the calibration curve for calcium quantification. *b* K^+ (–●–) and Cl^- (–○–) in vesicles loaded with different concentrations of calcium. At a calcium concentration of zero the K^+ and Cl^- concentrations become very high as a consequence of the EGTA treatment which influences the permeability of the FSR membranes. Similar high values were found in oxalate vesicles, which accumulate calcium up to 300 nM/mg FSR protein. Between 20 and 200 nM $CaCl_2$/mg FSR protein, K^+ and Cl^- concentrations in the vesicles remain relatively low. *c* Superimposed spectra of elemental composition inside electron resistant vesicles (loaded with 60 nM $CaCl_2$/mg FSR protein) and at a distance of 0.1 µm from the vesicles. Dotted line corresponds to the spectrum outside the vesicles, full column to the spectrum inside the vesicles. The Si peak is an instrumental peak. *d* The plot of the detected phosphorous peak/continuum ratios is dependend of calcium concentrations.

tissue is really free from ice crystals in the superficial zone, and if the sections are thin enough. In longitudinal sections of relaxed papillary muscle in the region of the cisternae we found concentrations of 67.2 mM Ca/kg dry wt ±2.8 (= 16.8 mM/l SR) and in the sarcomere 6.8 mM Ca/kg dry wt ±0.8.

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Transplanted leg imaginal discs establish nerve connections with the appropriate neuromeres of the host's thoracic ganglion in the fly *Sarcophaga bullata*[1]

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Summary. Ectopic transplantations of prothoracic leg imaginal discs of mature 3rd instar larvae of *Sarcophaga bullata* on to young prepupae resulted in the development of supernumerary legs. The nerves that connected these legs with the host's CNS projected in the appropriate neuromere of the thoracico-abdominal ganglion.

The central question of developmental neurobiology is how the growing axons find their appropriate target cells on which to synapse. Though regenerating axons usually form proper connections with very high accuracy[2,3], the mechanism of formation of specific connections during normal development is far from clear. Studies with homeotic mutants of *Drosophila*, where an antenna is transformed into a leg structure, have revealed that the sensory neurons of these homeotic legs project to the antennal glomerulus of the brain and not to the leg projection areas of the thoracic ganglion[4,5]. In this report, I have taken advantage of a special transplantation technique[6] to study the development of nerve connections between the peripheral structures and the central nervous system in the fleshfly, *Sarcophaga bullata*.

Material and methods. Imaginal leg discs of mature 3rd instar larvae of the fleshfly *Sarcophaga bullata* were dissected out in sterile *Drosophila* Ringer's solution[7] and surface transplantations were made as described elsewhere[6,8]. Less than 3-h-old prepupae were used as hosts. When the host pupae metamorphosed, the transplanted leg discs also everted and differentiated as supernumerary legs on the host flies. The leg nerves were back-filled with 150 mM cobalt chloride solution[5,9], and the filled ganglia were processed and mounted in Canada balsam.

Results and discussion. Since the intent of this report is to locate the general projection area of the nerves of the transplanted legs, no attempt was made either to identify the individual neurons involved or to distinguish between the sensory and motor components in the projected area. Immersion of the cut ends of the in situ legs of the control flies into cobalt chloride solution resulted in the deposition of cobalt sulfide precipitate in the entire neuromere of the respective leg. There was no diffusion of cobalt chloride beyond the boundaries of the particular neuromere concerned. For the studies on the nerves of the supernumerary legs, transplantations of prothoracic leg discs were made on the posterior ventral surface of host prepupae. This resulted in the development of supernumerary legs on the ventral surface of the mid-abdomen of host flies. Of the 16 fully everted supernumerary legs that were filled with cobalt chloride, in 11 cases there was definite precipitation of cobalt sulfide in one of the prothoracic neuromeres; and in no instance were both the right and left prothoracic neuromeres simultaneously filled. There was no deposition of cobalt sulfide in the mesothoracic, metathoracic or abdominal neuromeres. The filling was always restricted to the prothoracic neuromeres, and that to only 1 of the 2 prothoracic neuromeres. In the remaining 5 thoracico-abdominal ganglia, the filling was so poor that definite localization of cobalt sulfide could not be noticed in any area of the ganglion.

The results of the present report demonstrate that the supernumerary legs differentiated from the ectopic transplantation of imaginal discs develop nerve connection with the host's CNS and that these nerves project in the appropriate areas of the thoracico-abdominal ganglion. The adult nerves of normal in situ legs, as well as those of homeotic mutants, have been suggested to develop on the principle of contact guidance by following the larval nerve route[10]. In the case of supernumerary legs, no such larval nerve connection exists between the transplanted imaginal disc and the host's CNS. How then the growing axons from these transplanted imaginal discs find the host's thoracico-abdominal ganglion which is far from the site of transplantation, is not clear at present. They may very well have followed some pre-existing abdominal nerve of the host or they might have reached the thoracico-abdominal ganglion by some kind of general attraction, independent of contact guidance as it happens with the pioneering embryonic nerves[11]. Upon reaching the vicinity of the host's CNS, the nerve from the prothoracic supernumerary leg always enters the ganglion close to its site of projection – the prothoracic neuromere.

Although the nerves from the homeotic wing or leg of the double mutant bithorax post bithorax of *Drosophila* do project in their proper area, namely, mesothoracic neuropile, these nerves enter the ganglion at the sites appropriate for haltere nerve or metathoracic leg nerve respectively[10,12]. In these studies, it was suggested that the growing axons follow certain specific 'trails' within the CNS in order to form proper projections. In the case of the growing nerves of the supernumerary legs reported here, no such suggestion need be proposed because these nerves enter the ganglion close to the site of their projection area.