Evidence for the Presence of Calsequestrin in Both Peripheral and Interior Regions of Sheep Purkinje Fibers

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SUMMARY. Localization of calsequestrin in sheep Purkinje fibers was determined by indirect immunofluorescence labeling of cryostat sections of sheep myocardium from the intraventricular wall. The results presented show that calsequestrin is present in discrete foci at the peripheral, as well as the interior regions of the cytoplasm. Since Purkinje fibers lack transverse tubules, the presence of calsequestrin at specific foci in the interior regions of the cytoplasm in these cells suggests that calsequestrin is localized in the lumen of peripheral junctional sarcoplasmic reticulum, as well as in the lumen of corbular sarcoplasmic reticulum present in the I band region of the myofibrils. Assuming that the function of calsequestrin is to sequester calcium into the lumen of the sarcoplasmic reticulum, these results imply that two structurally different regions of the sarcoplasmic reticulum function as calcium storage sites in mammalian Purkinje fibers and raises the possibility that calcium storage and/or release from these two sites might be regulated differently. **(*Circ Res 55: 267-270, 1984*)**

THE sarcoplasmic reticulum plays an important role in the regulation of the cytoplasmic Ca^{++} concentration in mammalian myocardial cells (Solaro and Briggs, 1974; Tada et al., 1978; Langer, 1980; Winegrad, 1982; Fabiato, 1983). Biochemical studies have shown that the Ca^{++} + Mg^{++}-dependent ATPase (Tada et al., 1978; Chamberlain et al., 1983) and calsequestrin (Campbell et al., 1983; Chamberlain et al., 1983), as in skeletal muscle, are major protein components of the cardiac sarcoplasmic reticulum. Furthermore, Jones and Cala (1981) have demonstrated that subpopulations of the canine cardiac sarcoplasmic reticulum differ with respect to protein composition and capacity for Ca^{++} uptake and release.

Immunolocalization of sarcoplasmic reticulum proteins have shown that the Ca^{++} + Mg^{++}-ATPase (Jorgensen et al., 1982) and calsequestrin (Campbell et al., 1983) are nonuniformly distributed in the different regions of the cardiac sarcoplasmic reticulum, suggesting that different regions of the sarcoplasmic reticulum are specialized to carry out specific functions. Thus, the biochemical and immunocytochemical studies support the idea that the entire surface of the network sarcoplasmic reticulum in which the Ca^{++} + Mg^{++}-ATPase is uniformly distributed actively transports cytoplasmic Ca^{++} into the lumen of the sarcoplasmic reticulum, where Ca^{++} during diastole most likely is sequestered by calsequestrin, which appears to be confined to the lumen of the peripheral and interior junctional sarcoplasmic reticulum.

Regarding the distribution of calsequestrin, this interpretation predicts that calsequestrin in myocardial cells without transverse tubules would be present in the lumen of the peripheral junctional sarcoplasmic reticulum but absent from the lumen of the sarcoplasmic reticulum in the interior regions of the myocardial cells. Immunolocalization of calsequestrin in chicken ventricular muscle cells which lack transverse tubules showed that calsequestrin, as anticipated, was localized in the lumen of the peripheral junctional sarcoplasmic reticulum, while absent from the lumen of the network sarcoplasmic reticulum. However, calsequestrin was also localized in the lumen of vesicular structures present in the I band region (Jorgensen and Campbell, 1984), which most likely corresponds to the corbular sarcoplasmic reticulum which is composed of bulbous and cisternal expansions of the network sarcoplasmic reticulum (Sommer and Waugh, 1976; Forbes and Sperelakis, 1983).

To determine whether the presence of calsequestrin in two structurally distinct regions of the sarcoplasmic reticulum (Forbes and Sperelakis, 1983) is specific to avian myocardial cells or whether similar results might also be obtained in mammalian myocardial cells without transverse tubules such as sheep Purkinje fibers, specialized cells of the cardiac conduction system (Page, 1967; Sommer and

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Waugh, 1976), calsequestrin was localized in these cells by indirect immunofluorescence labeling. The results presented suggest that as in chick ventricular muscle fibers, calsequestrin in sheep Purkinje fibers is localized in the lumen of both the peripheral junctional and the corbular sarcoplasmic reticulum.

Methods

Fixation, Dissection, and Sectioning

Small tissue blocks of regions of the intraventricular wall that contain Purkinje fibers were dissected from adult sheep myocardium (Hunnisett Abatoire/Canada Packers) less than 30 minutes after the sheep had been killed. The tissue blocks were fixed first in 2% paraformaldehyde in buffer A (0.1 M Na cacodylate, pH 7.2, and 4.5 mM $CaCl_2$ at 4°C for 3 hours, and then in a mixture of 2% paraformaldehyde and 0.3% glutaraldehyde in buffer A for an additional 3 hours. Subsequently, the tissue was rinsed in buffer A for 15 minutes, treated with buffer B (0.2 M Tris-HCl and 0.8 M lysine-HCl, pH 7.4) for 3 hours, and finally left in buffer A overnight. The fixed tissue blocks were infused with 0.6 M sucrose, frozen in isopentane, cooled in liquid N_2, and 4- to 8-μm cryostat sections were cut as previously described (Jorgensen et al., 1979).

Indirect Immunofluorescence Labeling

Indirect immunofluorescence labeling of cryostat sections of sheep myocardium with affinity-purified antibodies to dog myocardial calsequestrin (Jorgensen and Campbell, in press) was carried out as previously described (Jorgensen et al., 1979). The primary antibody reagent, affinity-purified antibodies to canine cardiac calsequestrin, was used at 10 μg/ml in buffer C (PBS and 0.5%, Triton X-100). The secondary antibody reagent, the fluorescein-conjugated immunoglobulin fraction of goat anti-rabbit IgG was used at 0.5 mg/ml of phosphate-buffered saline. Sections were observed with a Zeiss photomicroscope with an Epi-fluorescence attachment and a phase contrast condenser. Photographs were taken on Kodak 2415 film and developed in Kodak developer D-19.

Results

Longitudinal and transverse sections of sheep myocardium from the intraventricular wall containing both working myocardial cells and Purkinje fibers, were labeled with antibodies to canine cardiac calsequestrin by immunofluorescence labeling. Examination of these sections showed that the intensity of calsequestrin labeling in Purkinje fibers (P) was at least as intense as that of the working muscle cells (M) (Fig. 1a).

Regarding the distribution of calsequestrin in the Purkinje fibers, examination of a longitudinal section through one of these fibers showed that calsequestrin labeling was localized in small discrete foci at the periphery of the cell, as well as throughout most of the interior regions of the cytoplasm (Fig. 1b). Comparison between the immunofluorescence staining pattern (Fig. 1b) and the phase-contrast view of the same fiber (Fig. 1d) showed that the fluorescence labeling corresponded to the regions of the cytoplasm where the myofibrils are localized, whereas the cytoplasmic regions free of immunofluorescence staining (S; Fig. 1b) corresponded to regions of the cytoplasm lacking retractile material (S; Fig. 1d), presumably, areas where glycogen was localized in situ.

Similarly, the distribution of calsequestrin labeling in transverse sections of Purkinje fibers also showed that immunofluorescence foci were present both at the peripheral and the interior regions of the cytoplasm (Fig. 1, c and e). Although immunofluorescence labeling was not visible in all cells at one particular plane of focus (star; Fig. 1e), focusing through any section clearly demonstrated that calsequestrin labeling was always present in the interior regions of all Purkinje fibers (arrowhead; Fig. 1c).

The specificity of the calsequestrin labeling was demonstrated by labeling sections of Purkinje cells with calsequestrin antibodies that had been adsorbed with canine calsequestrin prior to the immunofluorescence labeling. As shown in Figure 1f, the specific fluorescence staining pattern shown in Figure 1, c and e, was greatly diminished after adsorption.

Discussion

Ultrastructural studies of goat Purkinje fibers have shown that the sarcoplasmic reticulum in these cells is composed of at least three structurally distinct regions (Nunez-Duran, 1980). These include the network sarcoplasmic reticulum, the peripheral junctional sarcoplasmic reticulum, and the corbular sarcoplasmic reticulum. The network sarcoplasmic reticulum is composed of an anastomosing network of sarcotubules with an electron lucent lumen which surrounds the myofibrils. Both the peripheral junctional sarcoplasmic reticulum and the corbular sarcoplasmic reticulum are continuous with the network sarcoplasmic reticulum and contain electron dense material in their lumen (Sommer and Waugh, 1976; Forbes and Sperelakis, 1983). The obvious structural difference between these two specialized regions of the sarcoplasmic reticulum is that the peripheral junctional sarcoplasmic reticulum is closely apposed to the sarcolemma to which it is connected, by junctional processes called feet, whereas the corbular sarcoplasmic reticulum, bulbous and cistemal expansions on the network sarcoplasmic reticulum, is not close to the sarcolemma.

The results presented showed that, in sheep Purkinje fibers, calsequestrin is localized in small discrete foci at the periphery as well as in the interior regions of the cytoplasm, suggesting that calsequestrin in these fibers, as in chicken ventricular muscle fibers (Jorgensen and Campbell, 1984), is localized in the lumen of the peripheral junctional sarcoplasmic reticulum and in the corbular sarcoplasmic reticulum, while absent from the lumen of the network sarcoplasmic reticulum.

Assuming that calsequestrin sequesters calcium

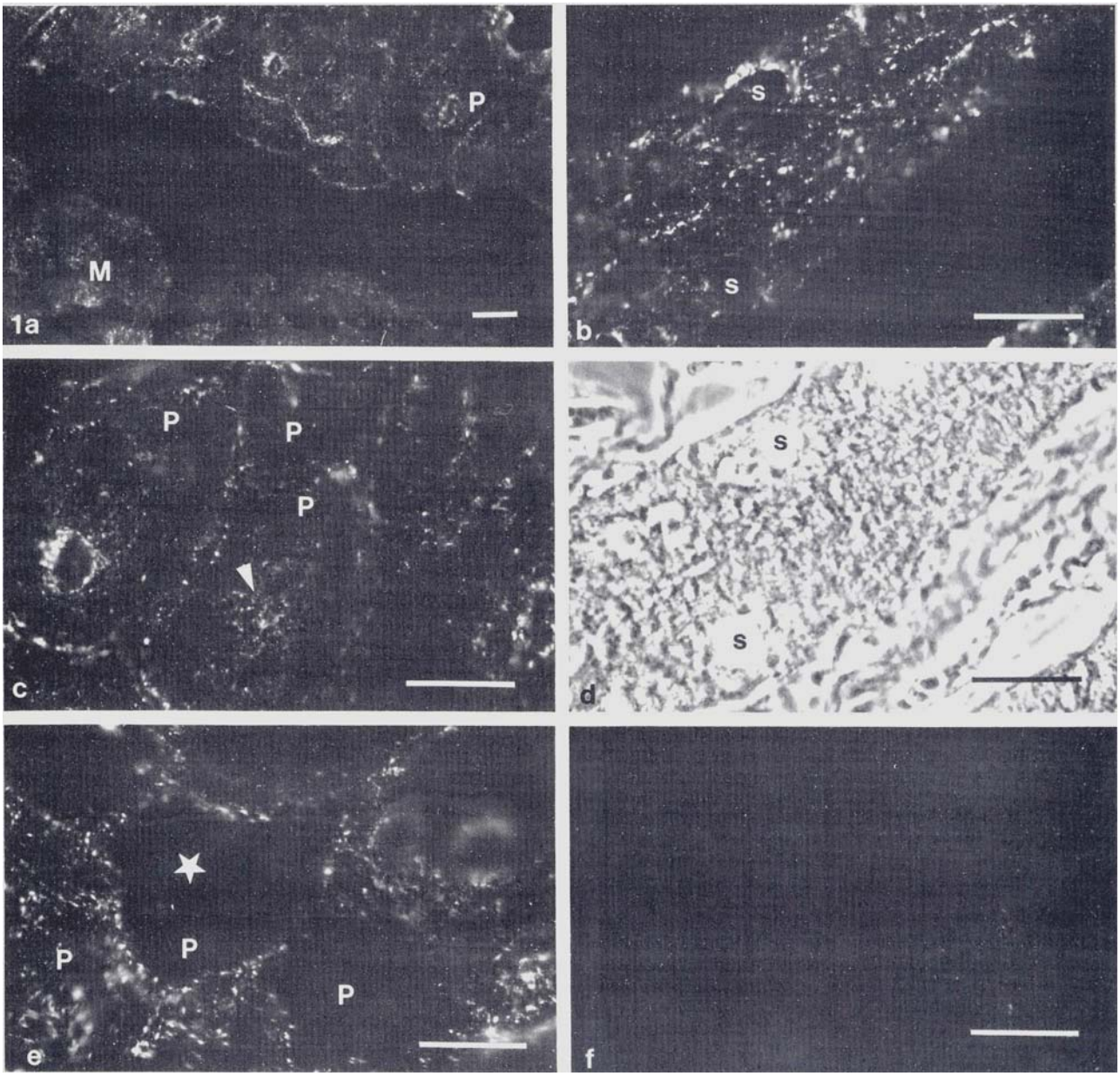

FIGURE 1. *Immunofluorescence localization of cardiac calsequestrin in sheep Purkinje fibers. Light micrographs of transverse (parts a, c, e, and f) and longitudinal (parts b and d) sections of fixed sheep myocardium with bundles of Purkinje fibers were labeled with cardiac calsequestrin antibodies (parts a, b, c, and e) and calsequestrin antibodies adsorbed with canine cardiac calsequestrin (part f). The fluorescent staining pattern in part b was compared with the position of the outline of the Purkinje fiber in part d, a phase-contrast micrograph of the same field. Note that the fluorescent punctate foci in parts b, c, and e are distributed throughout the cytoplasm. In a particular plane of focus, some Purkinje fibers would often appear to be labeled only at the celt periphery (part e, white star), however by changing the plane of focus, it became evident that most cells were indeed labeled in the interior regions of the cytoplasm (part c, arrowhead) as well as at the cell periphery. Bar 20 μm.*

transported into the lumen of the sarcoplasmic reticulum these results raise the possibility that Ca^{++} storage in and/or release from these two structurally different regions of the cardiac sarcoplasmic reticulum occur in response to different stimuli during the excitation-contraction-relaxation cycle. Thus, Ca^{++} release from the lumen of the peripheral junctional sarcoplasmic reticulum might be directly triggered by depolarization of the sarcolemma, whereas Ca^{++} release from the corbular sarcoplasmic reticulum, which cannot be directly stimulated by the depolarization event, might be triggered by a diffusible agent. This possibility is supported by studies demonstrating a correlation between the ability of ryanodine to inhibit, in part, the excitation-contraction coupling and its ability to separate the transverse tubules from the junctional sarcoplasmic reticulum (Penefsky, 1974a, 1974b). These findings support the idea that the physical contact between these two structures is essential for the depolarization of the sarcolemma to result in the release of Ca^{++} from the lumen of the junctional but not from the corbular

sarcoplasmic reticulum. Furthermore, studies by Fabiato (1982) have shown that Ca^{++} can induce Ca^{++} release from the sarcoplasmic reticulum of mechanically skinned canine Purkinje fibers. Assuming that skinned Purkinje fibers lack junctional sarcoplasmic reticulum, as previously suggested by Fabiato (1982), the results presented are consistent with the idea that Ca^{++} release from the calsequestrin-containing corbular sarcoplasmic reticulum is induced by Ca^{++}.

Perfusion studies of $^{45}Ca^{++}$-labeled rat hearts (Hunter et al., 1983) suggested that at least two different Ca^{++} storage pools in cardiac sarcoplasmic reticulum could be distinguished on the basis of the stimuli required for Ca^{++} to be stored in or released from these two pools. Thus, whereas Ca^{++} pool A sequesters Ca^{++} at both normal and increased inotropic states of the heart and requires extracellular Ca^{++} for the subsequent release of Ca^{++}, Ca^{++} pool C sequesters Ca^{++} only at increased inotropic states of the heart (e.g., catecholamine stimulation) and does not require extracellular Ca^{++} to release Ca^{++} from this Ca^{++} pool.

Although this study supports the idea that two differently regulated Ca^{++} pools are present in mammalian sarcoplasmic reticulum, it would be necessary to determine the distribution of Ca^{++} in the different regions of the sarcoplasmic reticulum in hearts at normal and increased inotropic states by, for example, electronspectroscopy or electron microprobe x-ray analysis, in order to know whether Ca^{++} pools A and C correspond to the calsequestrin-containing corbular and junctional sarcoplasmic reticulum of mammalian cardiac muscle cells.

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