Ultrastructural Localization of Calcium in the Pigeon Papillary Muscle as Demonstrated by Cytochemical Studies and X-Ray Microanalysis

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Summary. The intracellular localization of calcium as an antimonate precipitate is studied in myocardial cells of a non-mammalian vertebrate. Pigeon papillary muscles are pretreated in a calcium-free potassium solution containing 60 mM K$_2$Sb(OH)$_6$, and fixed in 1% OsO$_4$ containing the same concentration of antimonate. Calcium is chelated by K-EDTA or K-EGTA, in part separating it from the sodium-calcium antimonate. Atomic absorption spectrophotometry is employed to study the precipitate formation when sodium and calcium ions are added to a pH controlled antimonate solution. The chelating effect of K-EDTA and K-EGTA on the precipitates is studied by the same method. Both sodium and calcium cations are heavily precipitated by the antimonate anion. More calcium ions are precipitated when sodium ions are also present in the solution. K-EDTA and K-EGTA do not redissolve more than about 50% of the calcium antimonate from a sodium-calcium antimonate precipitate. When calcium cations only are added to the antimonate solution, K-EGTA redissolves about 95% of the calcium antimonate precipitate. A direct evidence for the presence of calcium in the tissue precipitates is given by X-ray microanalyses of 2500 Å thick sections.

Calcium antimonate is located to the sarcoplasmic reticulum (SR), the myofibrils, the mitochondria and the nuclei. In the SR, calcium antimonate is consistently found in the subsarcolemmal cisternae of the peripheral couplings (Sommer and Johnson, 1969) and in the Z line cisternae (Sætersdal and Myklebust, 1975) or extended junctional SR (Sommer and Johnson, 1970). Along the myofibrils, calcium antimonate is found at the overlap of thick and thin filaments. In sarcomeres with short I bands, a dense antimonate precipitate consisting of large granules is found at the A-I junction. No calcium is found at the Z lines. The calcium antimonate granules along the myofilaments seem to be related to the length of the sarcomeres. The significance of these findings is discussed in relation to functional aspects of the myocardial cell.

Key words: Cardiac ultrastructure — Sarcoplasmic reticulum — Myocardial calcium localization — EDTA/EGTA — X-ray microanalysis.

Introduction

Calcium is believed to be sequestered in the sarcoplasmic reticulum of the resting muscle cell (Hasselbach, 1964; Constantin et al., 1965; Ebashi and Endo, 1970).
The initiation of contraction is thought to occur as Ca\(^{2+}\) are released to take part in operation of the ATP-ase activity of the actomyosin. Subsequently, the calcium is reaccumulated by the sarcoplasmic reticulum during relaxation (Hasselbach, 1964; Ebashi and Endo, 1968). In skeletal muscle, it is almost generally accepted that the intracellular calcium movements during relaxation depend on the sarcoplasmic reticulum (Winegrad, 1965a, 1965b; Kübler and Shimbhorne, 1971). In the myocardial cell, however, the accumulation of calcium by the sarcoplasmic reticulum seems much less than in the skeletal muscle cell (Katz and Repke, 1967; Harigaya and Schwarz, 1969; Nayler and Merrillees, 1971; Schwartz, 1971). Also, studies relating to the intracellular distribution of calcium in cardiac muscle cells (Revel, 1962; Constantin \textit{et al.}, 1965; Pease \textit{et al.}, 1965; Langer, 1968; Katz \textit{et al.}, 1971; Nayler and Merrillees, 1971) have indicated that the distribution of calcium deposits is more complex than that described for skeletal muscle fibres, and that other subcellular structures, i.e. mitochondria, myofibrils, nuclei and sarcolemma, may interfere with the functional cycle of calcium.

Recently, contributions to the understanding of the calcium distribution in cardiac muscle cells, have been given by cytochemical investigations of mammalian heart tissues at the ultrastructural level (Legato and Langer, 1969; Diculescu \textit{et al.}, 1971; Yeh, 1973; Yarom \textit{et al.}, 1974). It is well known that the myocardial cells of the mammalian heart contain transverse tubules and internal couplings similar to those found in skeletal muscle cells. In the myocardial cells of non-mammalian vertebrates, however, transverse tubules are absent (Sommer \textit{et al.}, 1972, Sætersdal \textit{et al.}, 1974) and the only specialized sites of contact, therefore, between the sarcoplasmic reticulum and the sarcolemma, are at the peripheral couplings (Sommer and Johnson, 1969).

No exact information exists concerning the intracellular distribution of calcium in the myocardial cells of non-mammalian vertebrates. Also, the path of excitation-contraction coupling is not clear. The main object of the present investigation, therefore, has been to study the localization of calcium in these cells. The present study has been based on a previous investigation from this laboratory (Sætersdal and Myklebust, 1975) on the ultrastructure of the pigeon papillary muscle.

**Material and Methods**

**Chemical Procedure**

Atomic absorption spectrophotometry is employed to study the precipitate formation when sodium and calcium ions are added to a pH controlled antimonate solution. The chelating effect of K-EGTA and K-EDTA on the precipitates is studied by the same method.

The **basic antimonate solution** contains 60 mM K\(_2\)Sb(OH)\(_6\), 20 mM sucrose and 40 mM glycine at pH 7.8 adjusted with KOH. In the refrigerator this solution is stable for months, as is also the pH value. Glycine is chosen as the main buffering agent as the log of the apparent stability constant for calcium in glycine at pH 7.8 is 0.65, indicating that the chelating calcium complex is negligible. The \(pK_a\) for glycine is 9.8 but the stability of the pH in the antimonate solution may indicate that the \(pK_a\) for glycine is lowered.

Separate stock solutions are prepared from spectrograde quality NaCl and CaCl\(_2\) salts. Aliquots of these solutions are combined to form an intermediate solution of 1 mM Ca\(^{2+}\) and 25 mM Na\(^{+}\) in the basic antimonate solution. Solutions containing either 1 mM Ca\(^{2+}\) or 25 mM Na\(^{+}\) in antimonate solutions are also made. All experiments are performed at +4°C in 5 ml polypropylene tubes. The precipitates are allowed to settle for 30 minutes, the tubes are then