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# Associations between beta-tubulin and mitochondria in adult isolated heart myocytes as shown by immunofluorescence and immunoelectron microscopy

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**Summary.** We have investigated the associations between β-tubulin and mitochondria in freshly isolated cardiac myocytes from the rat. Beta-tubulin was identified by using monoclonal antibodies for immunofluorescence and high resolution immunogold electron microscopy. In addition, conventional transmission and scanning electron microscopic studies were performed. After chemical stabilization in a formaldehyde solution, the myocytes were shock-frozen at −150° C, cryosectioned at −70° C and subsequently processed for immunohistochemical and immunocytochemical microscopy. A characteristic of the rod shaped myocytes is the presence of a dense network of microtubules in the cytoplasm displaying a pattern of strong anti-β-tubulin reaction. The complexity of this network however varies considerably among the myocytes reflecting microtubule dynamic instability. Further, our findings demonstrate that the β-tubulin label in rod cells is confined to the perinuclear and interfibrillar spaces and, therefore, is largely colocalized with the cytoplasmic organelles. In myocytes undergoing severe contracture the distribution of β-tubulin is entirely restricted to the outer mitochondrial-containing domain. This implies that, in a cell model with marked segregation of the contractile filaments and organelles, mitochondria are codistributed with microtubules in the total absence of desmin intermediate filaments. Moreover, our immunogold preparations demonstrate anti-β-tubulin labelling in the outer mitochondrial membrane as well as of fibres in close apposition to this membrane. These results indicate the presence of a specific β-tubulin binding to the outer mitochondrial membrane that probably also involves microtubule based translocators and/or MAPs.

## Introduction

Studies of detergent permeabilized cells and in vitro organelle motility assays, have provided much useful information about components involved in organelle motility (Dabora and Sheetz 1988). Thus, a substantial part of the detailed information about microtubule motors comes from in vitro motility assays that were initially developed from extracts of the squid giant axon (Schnapp et al. 1985; Vale et al. 1986; Weiss 1987). These axons are specialized structures in which intracellular transport mechanisms are highly developed (Ball and Singer 1982). Less precise knowledge, however, exists of the control mechanisms that determine locations and movements of intracellular organelles in non-neural cells under in vivo conditions. There is a current need, therefore, for studying spatial relationships and molecular interactions between the cytoskeleton and the organelles by high resolution immunocytochemistry, microinjections of labelled proteins and related methods within the environment of non-neural cells (Schliwa 1986; Thornell et al. 1986; Mittal et al. 1989; Price and Gomer 1989). The importance of microtubules to organelle translocation has been demonstrated in a number of recent reports (Menzel and Schliwa 1986; Schliwa et al. 1987; Bayley 1990). Furthermore, direct or indirect interactions between microtubules and mitochondria have been suggested to mediate the regulation of mitochondrial motility in a number of cell types (Heggeness et al. 1978; Ball and Singer 1982; Cambray-Deakin et al. 1988). Also, an association between microtubules and the mitochondria was suggested in myocardial tissue by Watkins et al. (1987) in a study based on double-labelling of desmin and tubulin. In a recent report, we have studied the 53 kDa intermediate filament protein desmin in freshly isolated adult cardiac myocytes (Sætersdal et al. 1989) using immunofluorescence and a high resolution electron microscopic technique (Thornell et al. 1986). This particular cell preparation consists of approximately 85% rod shaped cells and 15% contracted "square cells" and "round cells". The latter cells will subsequently convert into nonviable cells if the cell membrane is irreversibly injured during hypercontraction. Isolated adult ventricular myocytes have lately become the subject of extensive experimental investigations (Jacobsen and Piper 1986), and their ultrastructure has been described in some detail (Fry et al. 1979; Russo et al.

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1981; Piper et al. 1982). With the exception of torn nexuses the rod cells of our preparations closely resemble control cells from intact hearts (Sætersdal et al. 1989). The intracellular content of square and round cells, on the other hand, is divided into two different domains during contracture. The central core consists mainly of contractile filaments, whereas the outer area consists almost exclusively of expelled mitochondria, "fibres" and membrane vesicles (Nag et al. 1977; Russo et al. 1981). The latter domain is also characterized by the formation of subsarcolemmal "blebs".

In the present study we have used this cell preparation as an experimental model. Employing immunofluorescence and immunogold electron microscopy in combination with conventional SEM and TEM techniques, an attempt has been made to address the question of colocalization of microtubules and mitochondria. Using monoclonal antibodies we have particularly examined the subcellular distributions of $\beta$-tubulin as it appears in relationship to mitochondria in the rod shaped cells as well as in the two domains of contracted round cells. Additionally, an attempt has been made to study interactions between $\beta$-tubulin and the outer mitochondrial membrane by the use of a high resolution immunoelectron microscopy.

## Material and methods

### *Isolation of cardiomyocytes*

Suspensions of purified cardiac myocytes were obtained by perfusion of 12-week-old female (Wistar strain) rat hearts following the method of Powel et al. (1980) with minor modifications. A total of 20 hearts were perfused for subsequent immunofluorescence and immunoelectron microscopic studies. The basic perfusion medium was Joklik-modified MEM (Gibco Ltd., Paisley, Scotland, UK) with 26.7 m*M* $NaHCO_3$, 1.20 m*M* $MgSO_4$ and 1.0 m*M* DL-carnitine at pH 7.4, gassed with 5% $CO_2$ in air. Perfusion was performed in a perfusion cabinet (Vanderbilt University, Mod.VE-101, Nashville, Tenn., USA) at 37° C as previously described (Sætersdal et al. 1989). The incubated cells were filtered through a 200 nm mesh nylon gauze, twice centrifuged, and resuspended in the basic medium with 1% albumin. Finally, the calcium concentration was raised to 1.5 mM in two steps.

### *Conventional electron microscopy*

Freshly isolated cardiac myocytes were collected by sedimentation on Nucleopore (Nucleopore Corp., Pleasanton, CA, USA) filters (3 nm pore size), which constituted the bottoms of small cylinders made of BEEM capsules (Fig. 1). The cells were prepared in situ on the Nucleopore filters for both SEM and TEM studies. By placing the small containers on filter papers, all fluids could be exchanged by draining through the Nucleopore filters. Thus, any loss of cells during preparation was avoided. Cells designated for SEM studies were fixed at 20° C in a 2% glutaraldehyde solution for at least 2 h with or without postfixation for 45 min in a 1% $OsO_4$ solution. Both fixatives were made up in 0.1 *M* cacodylate buffer with 0.1 *M* sucrose (pH 7.2, vehicle osmolality = 300 mosmol/kg). After standard dehydration in ethanol, the cells were transferred to absolute acetone and critical point dried from $CO_2$. The dried cells were sprinkled on the surface of double-sided tape attached to the specimen holder, sputter-coated with gold/palladium and viewed in a JEOL 1200EX operated at 40 kV.

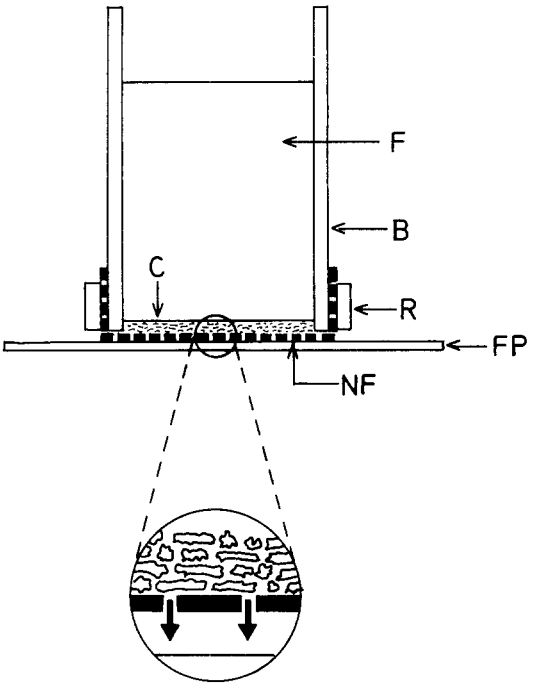


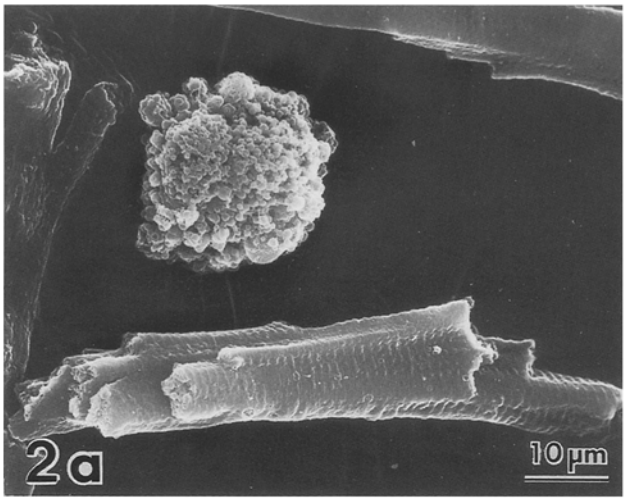

**Fig. 1.** A schematic drawing of a small container used for collecting, and, in situ processing of freshly isolated myocardial cells for comparative SEM and TEM studies. The container consists of the cylindrical portion of a BEEM capsule (*B*) and a Nucleopore filter (*NF*). The filter is tightly attached to the cylinder by a plastic ring (*R*) made of the cover of the BEEM capsule. *C* points to layers of sedimented cells, and, *FP* to filter paper. *Arrows* indicate fluid (*F*) transport through the Nucleopore filter

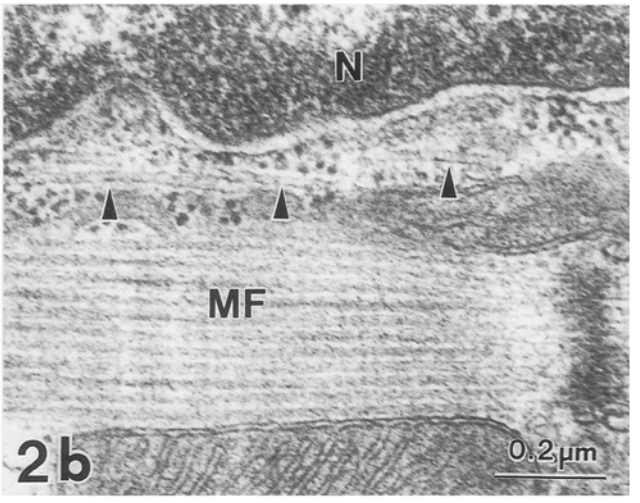
For TEM studies the cells were doubled-fixed in 2% glutaraldehyde and 1% $OsO_4$ as described above. In some experiments tannic acid (Art. 773; Merck, Darmstadt, FRG) was used in both fixatives at a final concentration of 1%. Following dehydration in rising concentrations of ethanol and embedding in Epon, thin sections were collected on piloform (BioRad, Richmond, Calif., USA) coated grids, contrasted with uranyl acetate and lead citrate and finally examined in a Philips 300TEM operated at 60 kV.

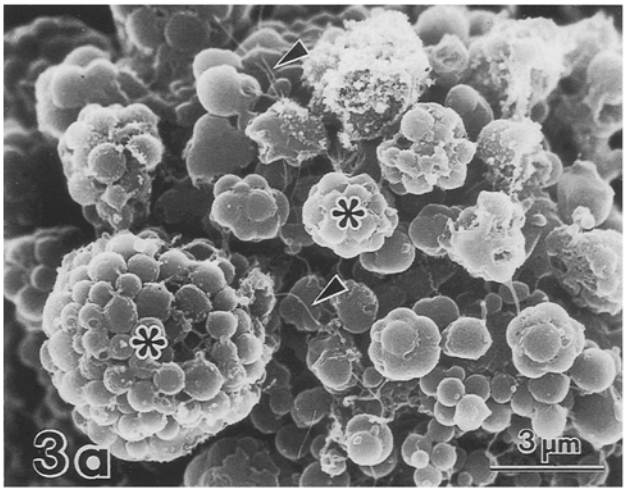
### *Immunofluorescence microscopy*

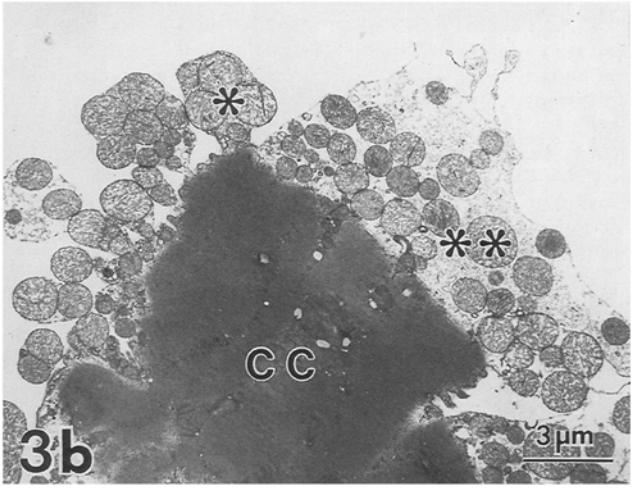
Cells were rinsed at 20° C in 0.1 *M* PBS with 1.5 m*M* $CaCl_2$ and thereafter washed twice in a calcium free microtubule stabilization buffer (100 m*M* PIPES, 5 m*M* EGTA, 2 mM $MgCl_2$; pH 6.8; Soltys and Borisy 1985), then fixed for 15 min at 20° C in a 3.7% formaldehyde solution in stabilization buffer and finally washed in the stabilization buffer. Some of the samples were frozen without cryoprotection. Most samples, however, were either infused in a 1.5–2.3 *M* sucrose solution, or, in a 25% glycerol solution for 30 min prior to freezing. Suspensions of cells were then mounted on copper pins and quick-frozen at −150° C in Freon cooled with liquid nitrogen ($LN_2$).

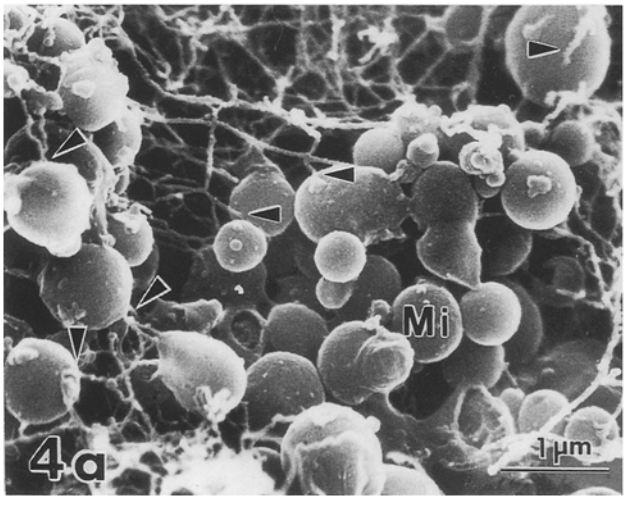
Cryosections at thicknesses of 0.1, 1, and 2 µm were cut with dry wolfram coated glass knives in a Reichert (Wien, Austria) cryoultramicrotome at kn −90° C/sp −70° C (Sætersdal et al. 1978). The temperature in the cryochamber was −90° C. The frozen sections were collected on the surface of a droplet of either 1.5 *M* sucrose or 25% glycerol and then transferred to the glass slides. Sections were immersed in 1% newborn calf serum (Gibco) in 0.01 *M* PBS for 30 min, then in 0.2 *M* glycine, ultra pure (Bethesda Res Lab, Gaithersburg, MD, USA) in stabilization buffer for 16 min and thereafter in 5% swine serum and 0.8% BSA made

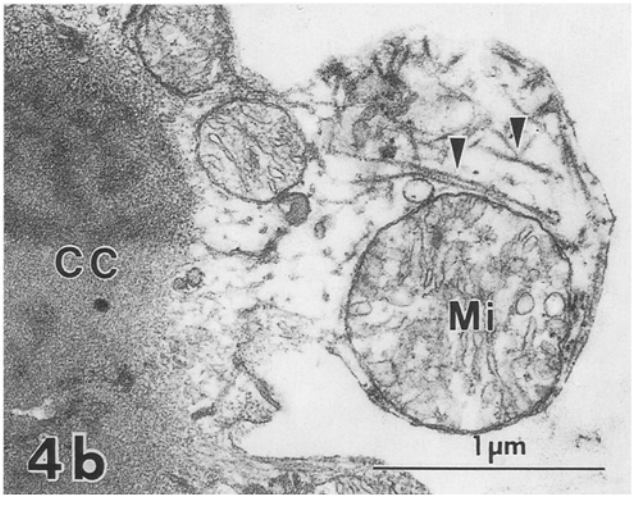



**Figs. 2–4.** Micrographs of freshly isolated myocytes collected and prepared *in situ* on Nucleopore filters for SEM and TEM studies. Cells designated for SEM studies (Figs. 2a, 3a and 4a) were fixed at 20° C, dehydrated, and then transferred to absolute acetone and critical point dried from $CO_2$. For TEM studies cells were double fixed in glutaraldehyde and $OsO_4$ at 20° C. For some experiments tannic acid was added to fixatives. **Fig. 2a–b.** SEM micrograph (**a**) showing the surface morphology of a rod shaped cell and a round cell, and TEM micrograph (**b**) of a cell fixed with the addition of tannic acid showing microtubular profiles (*arrowheads*) between nucleus (*N*) and a myofibril (*MF*). **Fig. 3a–b.** SEM micrograph (**a**) showing mitochondrial containing "blebs" (*asterisks*) on the sarcolemmal surface of a round cell with intervening thin fibres (*arrowheads*), and TEM micrograph (**b**) showing the separation of a rod shaped myocyte into two main domains during round cell formation, i.e. a central core (*CC*) of contractile filaments surrounded by small (*asterisk*) and large (*double asterisk*) subsarcolemmal blebs containing numerous expelled mitochondria. **Fig. 4a–b.** SEM micrograph (**a**) at a large magnification of expelled mitochondria (*Mi*) inside a subsarcolemmal bleb of a round cell and the network of thin fibres some of which appear to terminate on the mitochondrial surface (*arrowheads*), and TEM micrograph (**b**) showing the central core (*CC*) of a round cell at left and detail of a small mitochondrial (*Mi*) containing bleb at right with the appearance of thin fibres (*arrowheads*) in close proximity to the mitochondria

up in 0.01 *M* PBS for 30 min. Sections were then exposed for 60 min at 20° C to monoclonal mouse anti-β-tubulin (code N 357; Amersham, Amersham Int., UK), diluted 1:100–1:500 with 1% swine serum and 0.8% BSA made up in 0.01 *M* PBS. After rinsing with 0.8% BSA in 0.01 *M* PBS, sections were incubated for 2 h at 20° C in FITC-conjugated rabbit anti-mouse IgG (Dakopatts, Copenhagen, DK) diluted 1:80 with 1% swine serum and 0.8% BSA made up in 0.01 *M* PBS. After rinsing, the sections were finally embedded in a *p*-phenylen-diamine/glycerol solution. Controls included staining with omission of the primary antibody.

### *Pre-embedding immunoelectron microscopy*

Cells were chemically stabilized, quick-frozen, processed for cryoultramicrotomy and transferred in the same manner as described above for immunofluorescence microscopy. Sections at a thickness of 100 nm were collected on the teflon bottom of Heraeus Petriperm (Heraeus, Hanau, FRG) dishes, immersed in 1% newborn calf serum (Gibco) in 0.01 *M* PBS for 30 min, then in 0.2 *M* glycine in stabilization buffer for 16 min, and thereafter in 5% goat serum and 0.8% BSA made up in 0.01 *M* PBS for 30 min. Sections were then exposed for 60 min at 20° C to monoclonal mouse anti-β-tubulin (code N 357; Amersham), diluted 1:100 with 1% goat serum and 0.8% BSA made up in 0.01 *M* PBS. After rinsing with 0.8% BSA in 0.01 *M* PBS, sections were treated for 4 h at 20° C with the Ultra Gold Goat-anti-Mouse $IgGG_5$-complex (EM grade; Penninsula Laboratories Europe Ltd; St. Helens) diluted 1:5–1:10 with 1% goat serum and 0.8% BSA made up in 0.01 *M* PBS. After extensive washing the immunolabelled sections were postfixed with 2% glutaraldehyde in 0.1 *M* cacodylate buffer for 2 h (pH 7.2), postfixed in 2% $OsO_4$, dehydrated with ethanol, embedded in Epon and then cut and stained for electron microscopic examination. Controls included staining with omission of the primary antibody.

## Results

### *General cellular morphology*

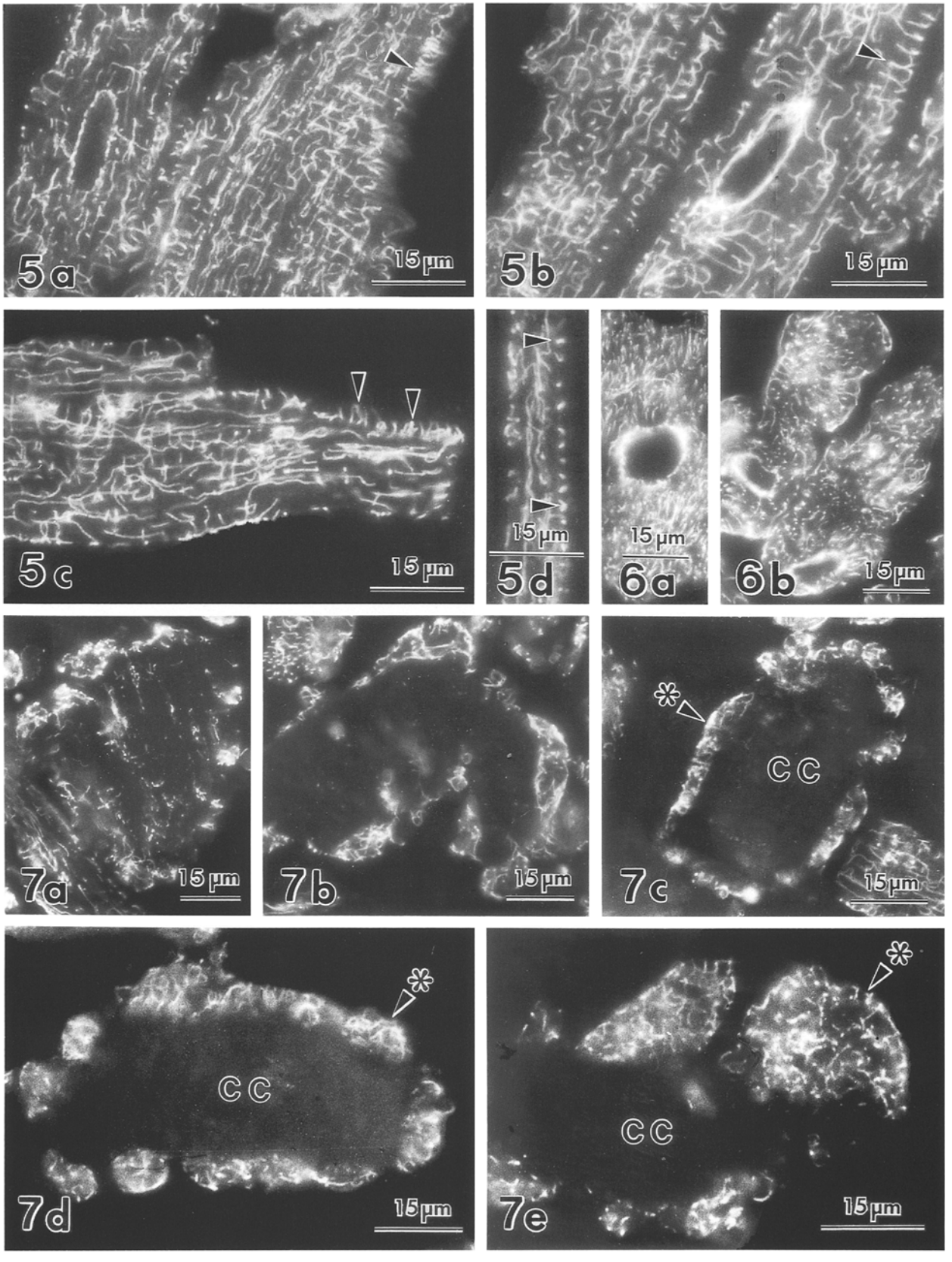
*TEM and SEM preparations.* The freshly isolated myocytes consisted of approximately 85% elongated rod shaped cells (Fig. 2a and b) and 15% square shaped and round cells (Figs. 2a, 3a and b). The rod cells were cylindrical in shape and exhibited a relaxed striated appearance. The general ultrastructure of the rod cells showed no visible structural injuries with the exception of the torn nexuses. Thin sections of rod cells fixed in a glutaraldehyde/tannic acid solution at room temperature revealed profiles of apparently well preserved microtubules that were particularly evident in the perinuclear area (Fig. 2b). During hypercontraction the rod cells were drastically shortened until the cell acquired a squared shape. These square cells were finally converted into round cells (Figs. 2a and 3a–4b). During this process the cells developed subsarcolemmal blebs filled with numerous expelled mitochondria, membrane vesicles, and fibres. The blebs surrounded a central core of contractile filaments (Fig. 3a and b). When examining the subsarcolemmal blebs of round cells in SEM and TEM preparations, a network of thin fibres appeared in the intermitochondrial spaces. These fibres were localized in close proximity to the mitochondria and some of them appeared to terminate on the mitochondrial surface (Fig. 4a and b).

*Immunofluorescence studies.* Indirect immunofluorescence in 0.1-, 1- and 2-μm-thick cryosections of quick-frozen rod cells, showed the characteristic, specific β-tubulin anti-body staining (Figs. 5a–d and 6a and b). The microtubules as revealed by monoclonal anti-β-tubulin were found to be brightly and distinctly stained. An abundant label was observed in the perinuclear area (Figs. 5a and b, 6a and b). Also, the remaining parts of the cytoplasm in rod cells were generally characterized by a densely developed network of tortuous running microtubules. The complexity of this network however varied among the myocytes. The overall orientation of these fibres was in parallel with the longitudinal axis of the cell. In addition, a striking feature of most cell profiles was the presence of transversely oriented fibres that sometimes were localized in the central parts of the myocyte, but most frequently resided in the subsarcolemmal areas. These fibres appeared to encircle the myocyte in the subsarcolemmal position (Fig. 5a–d).

In hypercontracting square cells this pattern of β-tubulin label became less distinct. Thus, at early stages of square cell formation, bands and small areas of a weak β-tubulin staining persisted in the central parts of cell (Fig. 7a). At more advanced stages of square cell formation the anti-β-tubulin had completely vanished from the central parts (Fig. 7b). At both stages however, a bright β-tubulin staining reaction still appeared in the outer bleb containing domain. Finally, in round cells anti-β-tubulin staining appearing in curved bands, loops, and spots, was localized solely in the outer domain (Fig. 7c, d and e). In such cells staining was totally absent from the central filamentous core. No specific staining, and very little background was observed in controls.

*Immunogold electron microscopy.* The immunogold labelling in freshly isolated rod cells showed an extensive anti-β-tubulin labelling in the intermyofibrillar spaces

**Figs. 5–7.** Immunofluorescent localization of β-tubulin in frozen sections of freshly isolated cardiomyocytes. After chemical stabilization in a formaldehyde solution at 20° C, cells were shock-frozen at −150° C before cutting at −70° C in a cryoultramicrotome of 1-μm-thick sections that were later stained by indirect immunofluorescence. **Fig. 5a–d.** Localization of β-tubulin in frozen sections of rod shaped cells. Longitudinal orientation. The cell in **d** has been cut tangentially. Perinuclear β-tubulin label is shown in **a** and **b**. Also, note the presence of the dense network of microtubules in the cytoplasm outside the perinuclear area as well as subsarcolemmal β-tubulin positive fibres with an overall transverse orientation (*arrowheads in all panels*). **Fig. 6a–b.** Localization of β-tubulin in frozen sections of rod cells. Transverse-oblique orientations. Note perinuclear reaction as well as the high density of specific label throughout the remaining parts of the cytoplasm, appearing as spots or short strands of β-tubulin. **Fig. 7a–e.** Localization of β-tubulin in frozen sections of progressive stages of contracting square cells and round cells. During square cell formation (**a** and **b**), specific label is gradually vanishing from the central cytoplasmic areas. Finally, in round cells (**c**, **d** and **e**) specific label is totally absent from the central filamentous core (*CC*), while in both square and round cells an abundant label persists in the outer organelle containing domain (**a**, **b** and *asterisks* in **c**, **d** and **e**). High magnification in **e** of the interior of a subsarcolemmal bleb, shows specific label as punctate or strand-like elements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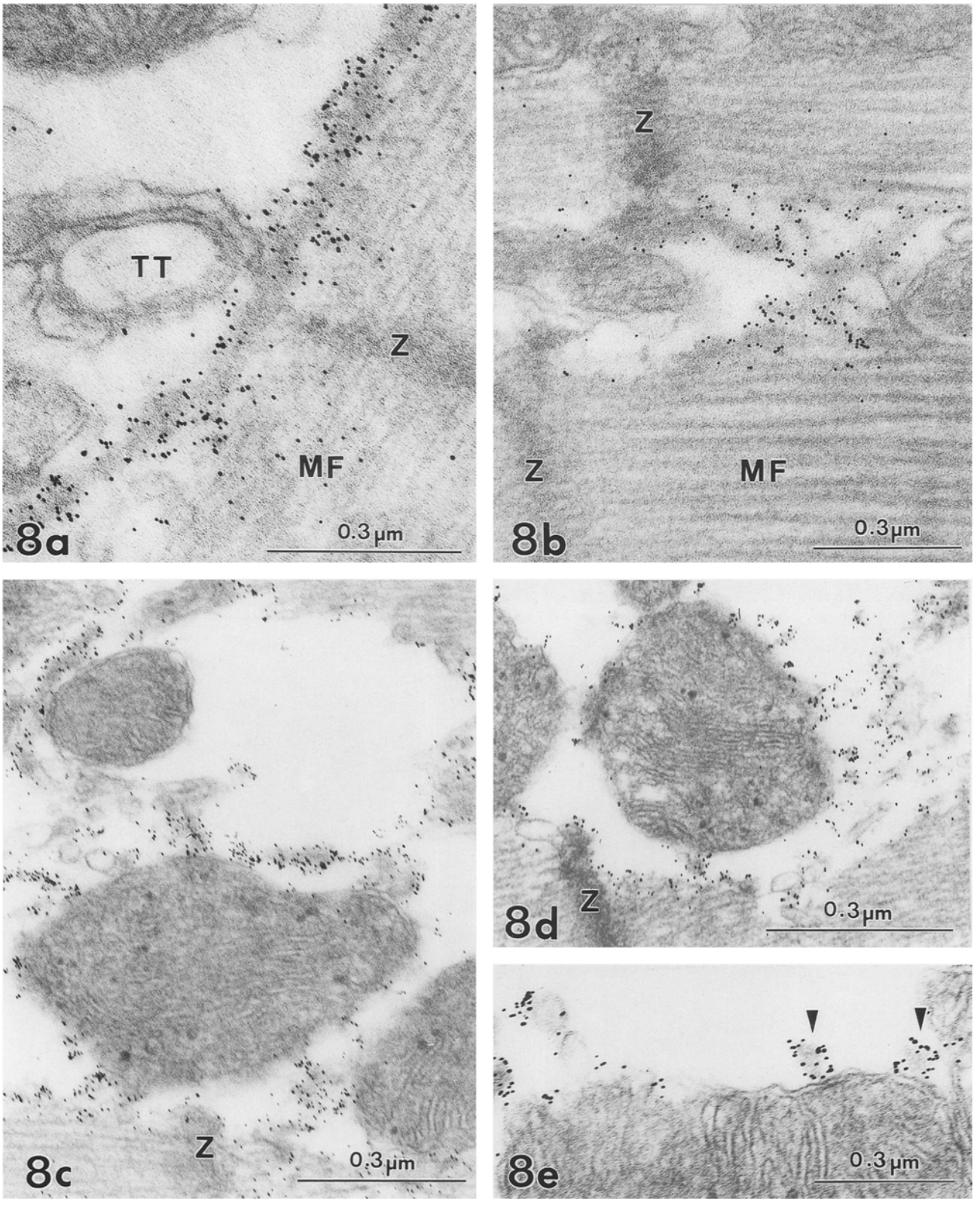


**Figs. 8–9.** Electron micrographs showing the presence of $\beta$-tubulin in freshly isolated cardiomyocytes based on pre-embedding immunogold localization in 100 nm-thick-cryosections. Cells were washed in stabilization buffer, fixed in a formaldehyde solution at room temperature and then shock-frozen at $-150°$ C before cutting in a cryoultramicrotome at kn $-90°$ C/sp $-70°$ C prior to immunostaining. All sections were subsequently osmicated, embedded in epon and finally processed for electron microscopic examination. **Fig. 8a–e.** Indirect immunogold labelling of $\beta$-tubulin in isolated rod shaped cells. Note that gold particles in **a** and **b** are specifically distributed between the myofibrils (*Mf*), and, in **c** and **d** in close proximity to the mitochondria. A particular close association between gold label and the outer mitochondrial membrane appears from panel **e** (*arrowheads*). *Z* in **a**, **b**, **c** and **d** points to Z-bands, and TT in **a** to a transverse tubule

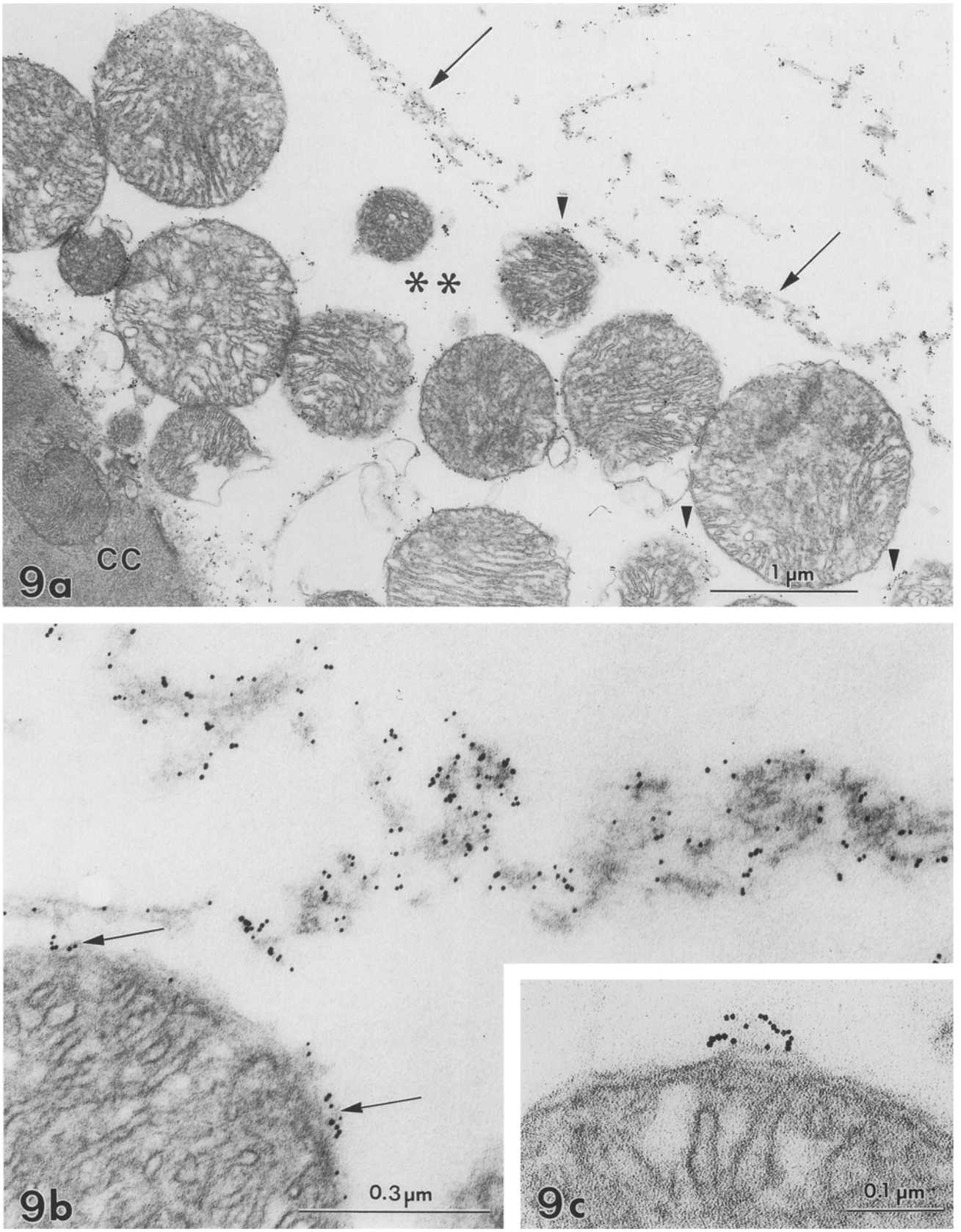


**Fig. 9a–c.** Indirect immunogold labelling of $\beta$-tubulin in round cells. While gold particles in **a** are completely absent from the central filamentous core (*CC*), a heavy label can be seen in the outer mitochondrial containing domain (*double asterisk*) covering fibres as well as the outer mitochondrial surface. Note specific labelling of fibres in **a** (*arrows*) and in upper right of micrograph in **b**. Also observe that gold particles are frequently distributed in close proximity to the outer mitochondrial membrane as indicated by *arrowheads* in **a** and by *arrows* in **b**. A point of close association between a profile of a gold labelled fibre and the outer mitochondrial membrane, appears at a high magnification in **c**. Note the slight bulging out of the mitochondrial membrane at the point of association

(Fig. 8a and b). An oblique to transverse orientation of microtubular labelling in relation to the longitudinal axis of the cell was particularly evident at the Z-discs and I-band levels. Moreover, an extensive pattern of anti-β-tubulin label did frequently occur in the cytoplasm between mitochondria (Fig. 8c, d and e). In such areas, close associations between the gold label and the outer mitochondrial membrane were observed (Fig. 8e). In round cells (Fig. 9a–c), the characteristic pattern of anti-β-tubulin reaction as observed in rod cells was completely absent from the central filamentous core (Fig. 9a). Thus, no gold label could be found in the core area, while a heavy label persisted in the outer domain. The latter emerged as rows of immunogold label that ran in various directions and covered fibres that extended between the mitochondria (Fig. 9a and b). Gold label appeared in all areas of the outer domain and was seen to cover fibres as well as the outer mitochondrial surface. Close associations between the labelled fibres and the outer mitochondrial membrane was demonstrated in this particular region of the cells (Fig. 9b and c). Specific immunogold labelling was absent in controls.

## Discussion

In this study we have investigated the associations between β-tubulin and mitochondria in freshly isolated adult heart myocytes of the rat. Microtubules were first demonstrated in striated muscle in conventional electron microscopy (Page 1966; Hatt et al. 1970; Cartwright and Goldstein 1982). One of the technical problems involved in studies of freshly isolated adult cardiomyocytes is the poor penetration of antibodies even following permeabilization (Samuel et al. 1983; Zernig and Wiche 1985). Still another specific problem is the 15- to 35-μm diameter of the myocyte, since the out-of-focus fluorescence of the background produces a rather diffuse image (Rappaport and Samuel 1988). Here, we have used a technique for immunohistochemical labelling in 0.1 to 2.0-μm-thick frozen sections of the isolated myocytes that yields a high resolution image of the microtubular network (Sætersdal et al. 1989). For immunoelectron microscopy thin frozen sections were embedded in Epon after labelling and then sectioned, osmicated and examined in the electron microscope. This is a safe and stable technical procedure that allows for a precise localization of specific intracellular proteins in freshly isolated heart myocytes.

Our immunofluorescence studies demonstrate that freshly isolated, rod shaped cardiac myocytes exhibit a strong anti-β-tubulin staining in the perinuclear areas as well as in the remaining parts of the cytoplasm. Furthermore, it appears, from these studies as well as from our immunoelectron microscopic studies, that the microtubules of rod cells extend throughout the cytoplasm forming coiled structures that are primarily orientated in parallel with the longitudinal axis of the cell. These results agree with the conventional electron microscopic studies of microtubules in ventricular cells of various mammals (Goldstein and Entman 1979; Forbes and Sperelakis 1980), and with immunofluorescent and immunogold electron microscopic studies of tubulin in rat ventricular cells (Samuel et al. 1983; Watkins et al. 1987). Moreover, we report here that labelled fibres with an overall transverse orientation occur in addition to the axially oriented fibres. Thus, a consistent finding in most of the rod cells examined was the presence of transversely oriented fibres that encircled the cell in a subsarcolemmal position. Transversely oriented microtubules adjacent to the I band of heart muscle cells have been reported in the literature in a number of species (Ferrans and Roberts 1973; Goldstein and Entman 1979; Watkins et al. 1987), although no actual connections appear to exist between microtubules and desmin intermediate filaments (Bertier et al. 1984; Watkins et al. 1987; Rappaport and Samuel 1988). A somewhat unusual feature of our immunohistochemical preparations was that many myocytes revealed a dense microtubular network throughout the cytoplasm, i.e. also in areas outside the perinuclear region. These are areas in which microtubules of heart myocytes have been considered to be rather sparsely distributed (Rappaport and Samuel 1988). Yet, in a study of heart conduction cells, Thornell and Eriksson (1981) have suggested that microtubules might be more numerous in adult cardiac muscle than previously recognized. Here, the density of the network varied considerably from one myocyte to another, reflecting the dynamic instability of microtubules observed both *in vitro* and *in vivo*, in which they switch between a state of steady growth and one of relatively rapid shrinking (Bayley 1990; Bayley et al. 1990).

Evidence for the colocalization of mitochondria and microtubules in various types of cells has been presented in a number of reports (Smith et al. 1975; Heggeness et al. 1978; Nakamura and Ueda 1982; Cambray-Deakin et al. 1988). Our findings with immunofluorescence as well as in electron microscopic immunogold preparations of rod cells demonstrate that β-tubulin is confined to the perinuclear and interfibrillar spaces and, therefore, is largely colocalized with the cytoplasmic organelles. During severe contracture the myocytes are separated in two domains, an inner one consisting of irregularly oriented contractile fibres, and an outer one that almost exclusively contains expelled mitochondria and "fibres". Using monoclonal antibodies we have recently shown that at advanced stages of square cell formation, the 53 kDa intermediate filament protein desmin is entirely confined to the inner filamentous core, whereas no desmin label is found in the outer organelle-containing domain of the cell (Sætersdal et al. 1989).

The results presented here clearly show that β-tubulin is localized in the outer domain in advanced stages of square cells and in round cells. This implies that in a cell model with marked segregation of the contractile filaments and organelles, mitochondria are codistributed with microtubules in the total absence of desmin intermediate filaments. Moreover, these findings suggest that mitochondria are associated with microtubules rather than with desmin intermediate filaments (Ball and Singer 1982), and also that desmin and microtubules are prob-

ably not strongly connected in cardiac muscle cells (Watkins et al. 1987). Our immunoelectron microscopic studies of contracture development confirm these results and also the presence of anti-β-tubulin-positive fibres in the outer mitochondrial containing domain that might be identical with the type of fibre exhibited in our conventional TEM and SEM preparations. These latter fibres appeared to be specifically associated with the external surface of the mitochondria. Similar fibres in round cells that were reported to attach to the outer mitochondrial membrane have been demonstrated in conventional electron microscopy by Russo et al. (1981).

An association between microtubules and mitochondrial membranes was first observed in conventional electron microscopy (Smith et al. 1975; Nakamura and Ueda 1982; Raine et al. 1987), and was also suggested from immunoelectron microscopic studies of rat cardiac muscle (Watkins et al. 1987). Further, it recently has been demonstrated that tubulin is an integral part of the mitochondrial membranes (Bernier-Valentin et al. 1983; Hargreaves and Avila 1985), and that two major brain microtubule-associated proteins (MAP2 and tau) are able to bind to purified rat brain mitochondria (Jancsik et al. 1989; Linden et al. 1989). In conventional electron microscopy the distance between the microtubules and the outer mitochondrial membrane has been estimated to be 250 Å or less (Smith et al. 1975). Here, we have been able to demonstrate by a high resolution immunogold technique either a direct anti-β-tubulin labelling of the outer mitochondrial membrane or a labelling of fibres terminating in close contact with this latter membrane. We infer from these results the presence of a specific β-tubulin binding to the outer mitochondrial membrane that probably also involves microtubule based translocators and/or MAPs. Such contacts might be relatively few in number for any mitochondria-microtubule pair and, for this reason, may be difficult to detect in conventional thin section electron microscopy (Ball and Singer 1982).

The molecular composition and functional implications that might be involved in these contacts remain to be clarified. Intracellular organelle motility (Schnapp et al. 1985; Weiss 1987; Dabora and Sheetz 1988), in particular the motility of mitochondria (Forman et al. 1987; Raine et al. 1987), has been observed and studied in different cells. Although it is well established that intracellular motility in many cell types is closely associated with the microtubule-based translocators, kinesin (Vale et al. 1985), dynein (Paschal and Vallee 1987), and dynamin (Shpetner and Vallee 1989; Cleveland 1990), the molecular composition of the motors involved during *in vivo* conditions is less well known (Stebbings 1990). Recently, immunohistochemical based results have been presented that demonstrated kinesin molecules acting as ATP-dependent organelle transport motors by residing on the organelle surface and binding transiently to the microtubules (Pfister et al. 1989). An extension of the antibody technique as used in the present study is likely to be valuable in clarifying the substructure of the mitochondrial surface binding sites to β-tubulin in heart myocytes.

The results presented here suggest a state of dynamic instability of the cytoplasmic microtubules in isolated heart myocytes. Also, *in vivo* studies of microtubular turnover by microinjections of labelled tubulin subunits have shown that the majority of microtubules are labile whereas a minority are more stable (Farrel et al. 1987). The microtubular system, however, would probably require a substantial degree of stability in order to be suitable for organelle translocation (Bayley 1990). A multifunctional nature of a given microtubule might therefore be considered. Additional support for this position comes from recent studies which have demonstrated the presence of a number of β-tubulin isoforms in vertebrate cells, the most striking characteristic of which is the interspecies conservation of variable regions (Mac Rae and Langdon 1989). The differentiated expression of β-tubulin isoforms might imply that they are functionally discrete. This has often been interpreted as support for the multitubulin hypothesis, i.e. different isotypes have different functions within the cell (Fulton and Simpson 1976; Cleveland 1987). We suggest consistent with these latter studies that in rat cardiac muscle cells different β-tubulin isoforms coassamble into a given microtubule, and that its composition of isoforms would determine its multifunctional nature.

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