Adult Rat Cardiomyocytes Cultured in Creatine-deficient Medium Display Large Mitochondria with Paracrystalline Inclusions, Enriched for Creatine Kinase

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Abstract. In adult regenerating cardiomyocytes in culture, in contrast to fetal cells, mitochondrial creatine kinase (Mi-CK) was expressed. In the same cell, two populations of mitochondria, differing in shape, in distribution within the cell and in content of Mi-CK, could be distinguished. Immunofluorescence studies using antibodies against Mi-CK revealed a characteristic staining pattern for the two types of mitochondria: giant, mostly cylindrically shaped, and, as shown by confocal laser light microscopy, randomly distributed mitochondria exhibited a strong signal for Mi-CK, whereas small, "normal" mitochondria, localized in rows between myofibrils, gave a much weaker signal. Transmission EM of the giant mitochondria demonstrated paracrystalline inclusions located between cristae membranes. Immunogold labeling with anti-Mi-CK antibodies revealed a specific decoration of these inclusions for Mi-CK. Addition of 20 mM creatine, the substrate of Mi-CK, to the essentially creatine-free culture medium caused the disappearance of the giant cylindrically shaped mitochondria as well as of the paracrystalline inclusions, accompanied by an increase of the intracellular level of total creatine. Replacement of creatine in the medium by the creatine analogue and competitor β-guanidinopropionic acid caused the reappearance of the enlarged mitochondria. It is believed that the accumulation of Mi-CK within the paracrystalline inclusions, similar to those observed in certain myopathies, represents a compensatory effect of the cardiomyocytes to cope with a metabolic stress situation caused by low intracellular total creatine levels.

It was shown in preceding publications (3, 5, 9, 11, 17, 29) that adult rat cardiomyocytes (ARCs)[1] in culture represent a suitable in vitro system for the investigation of cardiac differentiation. There it was demonstrated that ARCs in long term cultures in many ways repeat embryonic and fetal differentiation steps. Fully differentiated heart cells express mitochondrial creatine kinase (Mi-CK) in conjunction with the cytosolic creatine kinase isoforms MM-, MB-, or BB-CK, with a significant fraction of MM-CK being specifically associated subcellularly in a compartmented fashion at intracellular sites of high energy turnover, e.g., at the myofibrillar M-band, the sarcoplasmic reticulum, etc. (39, 59), which has led us to the postulation of the phosphocreatine-circuit model (60, 61). Expression and appearance of Mi-CK in rat heart in vivo, however, occurs only during postnatal development paralleling more or less the appearance of MM-CK within the M-line structure of myofibrils which does not occur before 2 wk after birth (35). Concomitantly, fetal cardiomyocytes in culture do not express and accumulate Mi-CK. ARCs in culture though accumulate Mi-CK in their mitochondria, a feature characteristic for adult heart tissue and freshly isolated rod-shaped ARC. This is converse to contractile structures in cultured polymorphic ARC, which, at least to some extent, express a pattern similar to fetal cardiomyocytes (9, 11, 12, 29, 30).

Mi-CK is localized at the outer face of the inner mitochondrial membrane (40, 45) where it has been reported to be concentrated also at contact sites between outer and inner membranes (1, 20). Mi-CK exists in two oligomeric forms, dimers and octamers, and we have suggested recently that the latter may form functionally coupled multienzyme "energy channeling" complexes together with ATP/ADP translocators and outer mitochondrial pores (41) at inner and outer mitochondrial membrane contacts (20, 61).

The ARCs, when cultivated in creatine-free medium, are shown here to contain two distinct populations of mitochondria in one and the same cell: small, round mitochondria arranged along myofibrils showing only weak fluorescent staining and irregularly distributed cylindrically shaped, elongated mitochondria which show a very strong staining for Mi-CK and also show paracrystalline mitochondrial inclusions. Even though an extensive structural characterization of these inclusions by EM has been performed (13, 53), the

1. *Abbreviations used in this paper*: ARC, adult rat cardiomyocyte; Mi-CK, mitochondrial creatine kinase.

structures have evaded a detailed analysis of their chemical composition. Here, we present evidence by immunogold labeling that these inclusions are highly enriched for Mi-CK representing one of the major components of these structures.

As it was possible to cause the disappearance of the cylindrically shaped mitochondria as well as of their paracrystalline inclusions by addition of creatine to the medium, cultured adult cardiomyocytes are an in vitro model system to study the influence of culture conditions on mitochondria and specifically on mitochondrial creatine kinase. Some of the data presented here have been reported at the 34th annual meeting of the American Biophysical Society (Riesinger, I., M. Eppenberger-Eberhardt, P. Schwarb, M. Messerli, H. M. Eppenberger, and T. Wallimann. 1990. *Biophys. J.* 57:551a).

Materials and Methods

Isolation and Culture of Cells

Ventricular cardiac muscle cells of adult 2-mo-old Sprague-Dawley-Javonas rats were isolated as described by Claycomb and Palazzo (5) with some modifications as described in detail by Eppenberger et al. (11). The culture medium consisted of conditioned medium according to Claycomb and Lanson (4). The medium was changed first after 6 d. The fresh growth medium consisted of Medium 199 (Amimed AG, Basel, Switzerland) with 20% preselected FCS (Readysystem AG Labor, Zurzach, Switzerland) and 1% penicillin-streptomycin (Gibco Laboratories, Grand Island, NY). To inhibit fibroblast overgrowth, cytosine arabinoside (10 μM) was added throughout the culture period. Creatine (Sigma Chemical Co., St. Louis) was added to the culture medium at a concentration of 20 mM and β-guanidinopropionic acid (β-GPA; Sigma Chemical Co.) at a concentration of 10 mM. Fetal heart ventricle cells (19 d old) were dissociated in 0.2% trypsin according to Eppenberger et al. (8) and grown in the same medium as the adult cells. Cells for subsequent electron microscopic investigations and for confocal microscopy were grown on carbon-coated glass coverslips. Freshly isolated rod-shaped cells were centrifuged onto glass support directly after isolation.

Intracellular Creatine Determination

In the ARCs, which were scraped off the culture dishes, the intracellular creatine was estimated colorimetrically according to Eggleton et al. (7). Protein was measured according to Lowry et al. (23).

Antibodies and Fluorescence Reagents

mAbs against chicken heart Mi-CK (46) and polyclonal antichicken heart Mi-CK (41) and antichicken heart C-protein antibodies (2) were prepared in our laboratory. Rabbit antipig mitochondrial aspartate amino transferase (mAAT) antibodies were kindly provided by Dr. Heinz Gehring, University of Zürich. As second antibodies, fluoroisothiocyanate-conjugated goat anti-rabbit IgG (FITC; Cappel) and tetramethyl rhodamine isothiocyanate-conjugated goat anti-mouse IgG (Rhodamine, Cappel) were used, and for confocal microscopy donkey antimouse Texas red (Dianova, Hamburg, FRG) was used. The second antibody for immunogold labeling was goat anti-rabbit IgG coupled to 10-nm gold particles (Janssen, Belgium). Living cells were labeled with Rhodamine 123 (Rho 123; Sigma Chemical Co.).

Immunoblotting Experiments

Tissue samples of adult and fetal heart ventricles and of freshly isolated and cultured adult and fetal cardiomyocytes were separated on 10% SDS-polyacrylamide gels (22), and further processed as described in a previous publication (11). Gels were then transferred to nitrocellulose papers (57) and incubated with undiluted mAb against Mi-CK in order to visualize the Mi-CK.

Immunocytochemistry and Standard Fluorescence Microscopy

For double immunofluorescence, the cells were incubated with mAb against Mi-CK together with polyclonal antiheart C-protein or together with anti-mAAT antibodies.

The cells were washed with PBS, fixed for 20 min in 3% paraformaldehyde/PBS, and washed again in PBS. Membranes of cells and of mitochondria were permeabilized by 0.5% Triton/PBS for 5 min. Further washing with PBS was followed by incubation for 30 min with the primary antibodies. After washing with PBS, the cells were incubated with the respective second antibodies. After another rinsing with PBS the cells were mounted in 50% glycerol buffered with 0.1 M glycine, pH 9, or, in order to prevent the preparations from bleaching during the use of the confocal microscope, in 70% glycerol buffered with 0.1 M Tris-HCl, pH 9.5, to which 0.5 g/100 ml n-propyl-gallate (Sigma Chemical Co.) had been added.

Freshly isolated rod-shaped cells were incubated in medium containing Rho 123 at a concentration of 10 μg/ml for 10 min. After changing the medium, fluorescence staining of the mitochondria of the living cells was immediately observed and again after 6 d in culture. Cell preparations were observed under a Zeiss Standard Model 18 microscope (C.F. Zeiss, Oberkochen, West Germany) equipped with epifluorescence optics.

Confocal Microscopy

The confocal microscope system used for this study consists of a Zeiss Axioplan, a Bio-Rad MRC-600 confocal scanner, and a Silicon Graphics Personal Iris computer. The improved axial resolution of the confocal microscope as compared to the conventional microscope allows us to take images at different focal planes within the specimen showing only information originating in that very plane. The two channels (Texas red and fluorescein) were acquired simultaneously and digitized with a dynamic range of 8 bit. To produce the color composite, the Texas-red channel image is shown on the red gun and the fluorescein image on the green gun of the cathode raytube, respectively. No further processing was applied to the images produced.

To show the position and the thickness of the mitochondria, the Mi-CK channel data were further processed to introduce artificial shadows (58). This algorithm simulates the optical process of fluorescence in the following way. The data are lit with parallel rays (source at infinity) at an oblique angle. This light is then absorbed by the structures in the data set according to their measured intensity (i.e., bright structures absorb a large amount of light, dark ones absorb only little). The calculated fluorescent emission of the lit structures is observed at an orthogonal view. To allow shadows to be formed, a layer of constant intensity is added at the bottom of the stack. The simulated absorption and thus also the emission are linearly related to the measured fluorescence intensities.

EM

Chemical Fixation: Embedding and Polymerization. Cultured ARCs (9 d) grown on glass coverslips were fixed for 1 h in 0.1 M sodium cacodylate with 3% glutaraldehyde, washed twice in 0.1 M sodium cacodylate, post-fixed for 1 h in 1% OsO_4 in 0.1 M sodium cacodylate, washed twice in distilled H_2O, stained en bloc overnight with 0.5% aqueous uranyl acetate, dehydrated in an ascending series of ethanol/H_2O mixtures, and infiltrated in Epon/Araldite (24). Gelatin capsules filled with fresh 100% resin were placed on top of the coverslips and the samples were heat-polymerized at 60°C for 24 h. Thin sections were poststained with uranyl acetate and lead citrate (36).

Cryofixation: Embedding and Polymerization. High-pressure freezing. Cultured ARC (9 d) grown on carbon-coated glass coverslips and rod-shaped cardiomyocytes centrifuged onto the glass support directly after isolation were dipped in hexadecen (55) and placed between two aluminum holders. The sandwich was transferred to the commercial high pressure freezer HPM 010 (Balzers Union, Balzers, FL) and immediately frozen. *Freeze-substitution.* After cryoimmobilization, the sandwiches were opened under liquid nitrogen and the saphirglasses were quickly transferred to Eppendorf tubes filled with 0.5% uranyl acetate in −90°C cold ethanol. The samples were then substituted in a Balzers FSU 010 equipment for 9 h at −90°C, 6 h at −60°C, and 2 h at −30°C, followed by washing with anhydrous acetone at −30°C.

Embedding. The samples were infiltrated in a 30% solution of Epon/Araldite in acetone for 2 h at a temperature of −30°C. After another 2 h of infiltration at the same temperature with a concentration of ~60% Epon/

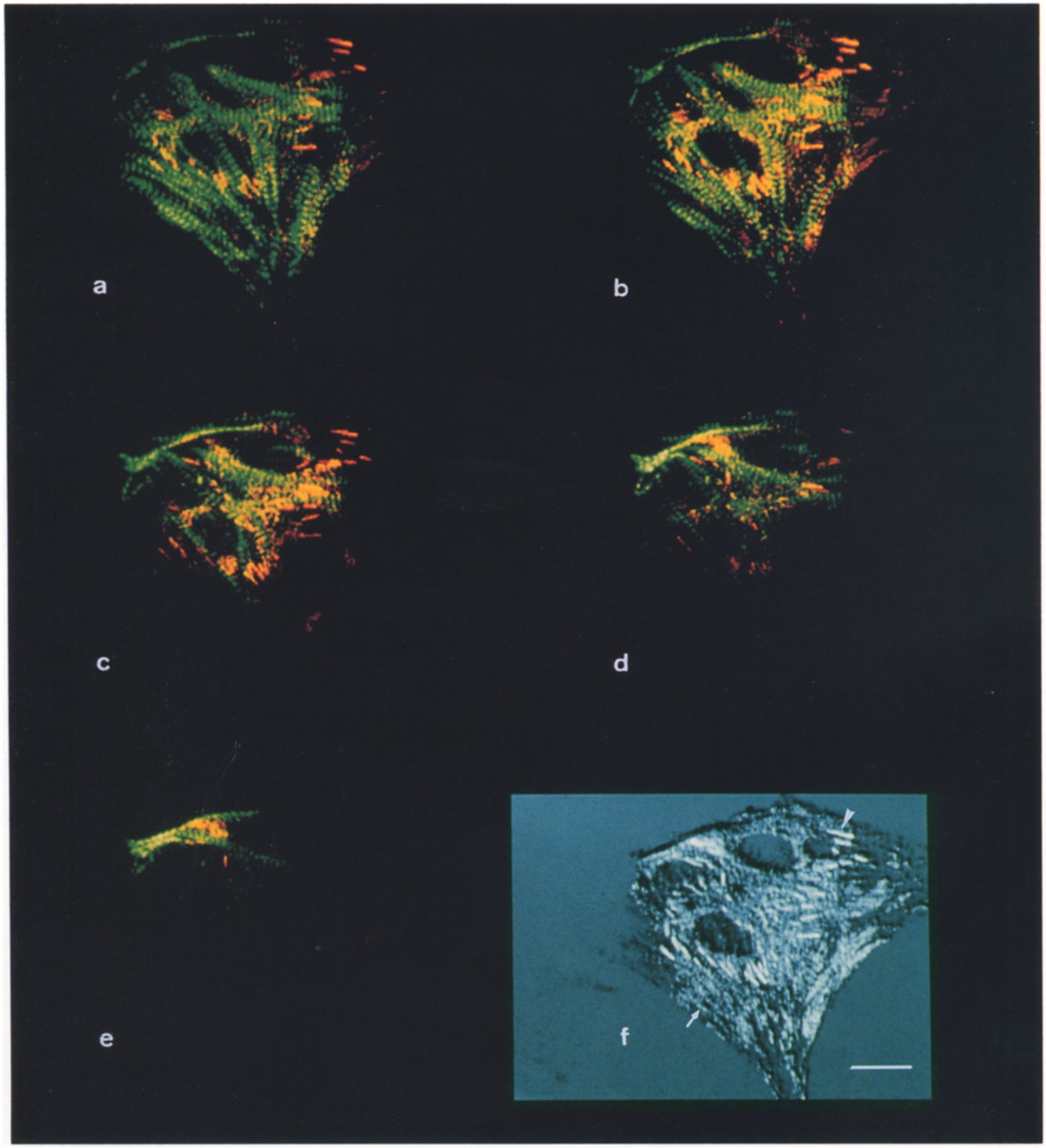

Figure 1. Confocal micrograph. A series of five images taken at consecutive focal planes are shown from the bottom of the cell (*a*) through the top of the cell (*e*). ARCs cultured in creatine-free medium for 9 d are double stained with antibodies against cardiac C-protein (green, FITC-conjugated second antibody) and Mi-CK (red, Texas-red conjugated second antibody). To clarify the position and the thickness of the mitochondria, an artificially shadowed picture of the Mi-CK channel is shown in *f* (for details see Materials and Methods). (*arrow*) Row of small, round mitochondria; (*arrowhead*) giant cylindrically shaped mitochondria. Bar, 15 μm.

Araldite, the samples were warmed up to RT and transferred to a 100% solution of Epon/Araldite for 2 h. The last step was repeated with freshly prepared Epon/Araldite, whereupon heat polymerization followed at 60°C for 24 h and thin sections were prepared according to the protocol described for chemical fixation.

Immunolabeling. About 50-nm-thick Epon/Araldite sections of the freeze-substituted cultured or rod-shaped cardiomyocytes were blocked with Tris-buffered gelatin (0.5% BSA and 0.2% gelatin in TBS, pH 7.6) for 30 min, washed with TBS, and incubated for 2 h with affinity-purified polyclonal rabbit antibodies raised against chicken cardiac Mi-CK (41). After

thoroughly washing the sections with TBS, the second antibody (goat anti-rabbit IgG coupled to 10-nm gold particles, from Janssen was applied for 45 min. Both antibodies were diluted with Tris-buffered gelatin 1:10 and 1:20, respectively. As a control, preimmunserum was used in the same way. Several washing steps with TBS and finally ultrapure H_2O followed. The sections were double stained with uranyl acetate (5 min) and Reynolds lead citrate (2 min) and examined in a JEM 100-C transmission electron microscope at 100 kV. It was not necessary to etch the surface of the Epon/Araldite sections before postembedding labeling, for pretreatment with 1% NaOH in 50% ethanol, as recommended by Kuhlmann and Peschke (21), did not improve the labeling intensity.

Results

Two Populations of Mitochondria Differing in Shape and Staining Intensity with mAbs against Mi-CK

When ARCs were kept in culture for 9 d, at a stage when they generally have assumed a polymorphic shape, they showed a well-developed myofibrillar apparatus and had made new cell-to-cell contacts by rebuilding intercalated discs (10, 11). By indirect immunofluorescence staining with monoclonal anti-Mi-CK antibodies (46), followed by observation by confocal laser scanning microscopy, two different populations of mitochondria were revealed. A sequence of confocal sections (Fig. 1, *a–e*) showed the distribution of mitochondria and myofibrils from bottom to top of the ARC: myofibrils stained with an antibody against cardiac C-protein followed by an FITC-conjugated second antibody appear green, whereas mitochondria stained with anti-Mi-CK mAb followed by a Texas red–conjugated second antibody appear red. Most of the giant mitochondria are located in an irregular fashion around the nuclei within the intermediate focal sections of the cells. The whole stack of optical sections of the channel exclusively with the Mi-CK signal was digitally processed using a shadow projection algorithm (58) and was displayed in Fig. 1 *f*. Here, a clear difference in the intensity of the fluorescent signal between the cylindrical and the normal round-shaped mitochondria, which were packed between adjacent myofibrils, was observed. The ratio, estimated by measuring the relative staining intensities for Mi-CK as obtained from a linear analogue/digital converter scheme in the confocal microscope, was in the order of 5:1 in favor of the cylindrically shaped mitochondria.

To see whether Mi-CK was selectively accumulated within the latter population of mitochondria, double immunofluorescence was performed with monoclonal anti-Mi-CK antibodies and polyclonal anti-mAAT, (kindly provided by Dr. H. Gehring and Prof. P. Christen, University of Zürich). While a strong fluorescence for Mi-CK was again observed in the cylindrically shaped mitochondria, only faint or no staining at all for mAAT, which is a matrix protein, was evident in these mitochondria. However, the round-shaped, small mitochondria appeared evenly stained for mAAT (Fig. 2).

Cylindrically Shaped Mitochondria with Paracrystalline Inclusions Highly Enriched in Mi-CK

ARCs were cultured for 9 d on carbon-coated glass coverslips and either chemically fixed with glutaraldehyde (Fig. 3 *b*) or cryofixed and cryosubstituted (Fig. 3, *a* and *c*), and then thin sections were prepared for electron microscopic analysis. Highly ordered paracrystalline inclusions could be observed preferentially in the elongated and giant mitochondria. The inclusions appeared as parallel straight lines (Fig. 3 *b*) or as sheet-like structures (Fig. 3, *a* and *c*) depending on the angle of sectioning. Often, especially in slightly oblique sections, a periodicity could be seen within these structures (Fig. 3 *c*). When 50-nm-thick epon sections of high pressure frozen and freeze-substituted cells were incubated with specific, affinity-purified polyclonal anti-Mi-CK antibodies followed by a second antibody coupled with 10-nm colloidal gold particles, a heavy gold labeling predominantly at the paracrystalline inclusions of the particular mitochondria could be demonstrated, whereas mitochondria of the same cell without such paracrystalline inclusions

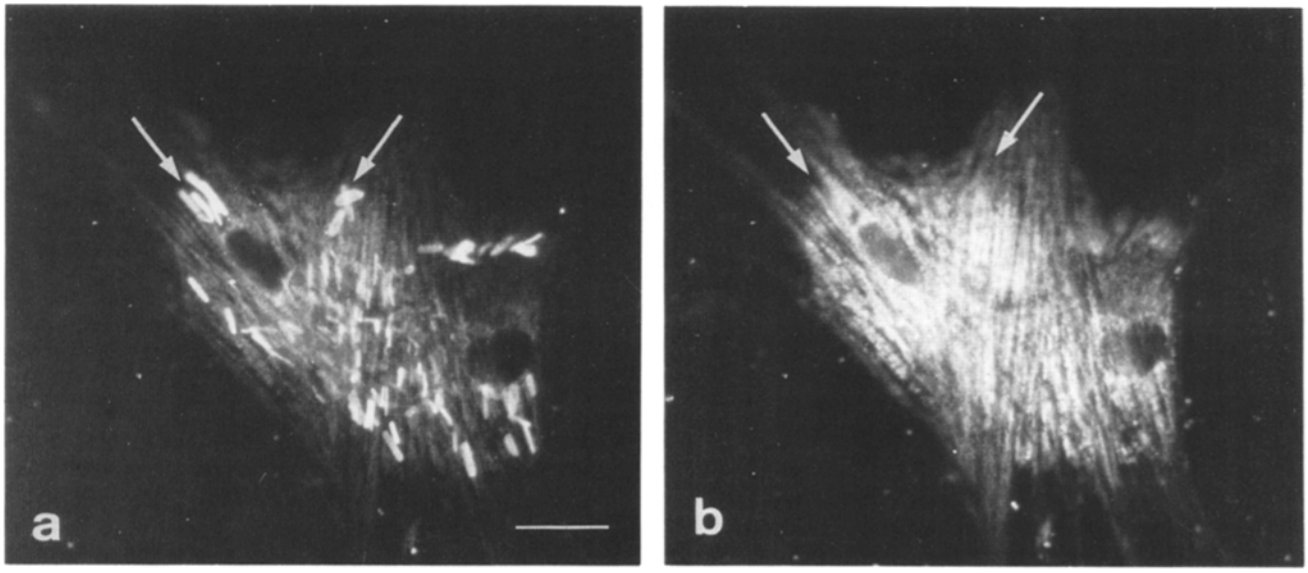

Figure 2. Double immunofluorescence labeling for Mi-CK (Rho) (*a*) and mitochondrial aspartate amino transferase (FITC) (*b*) of ARC cultured for 9 d in medium without creatine. The Mi-CK–positive cylindrically shaped mitochondria (*a*, *arrows*) show very little or no staining for mitochondrial aspartate amino transferase (*b*, *arrows*). Bar, 30 μm.

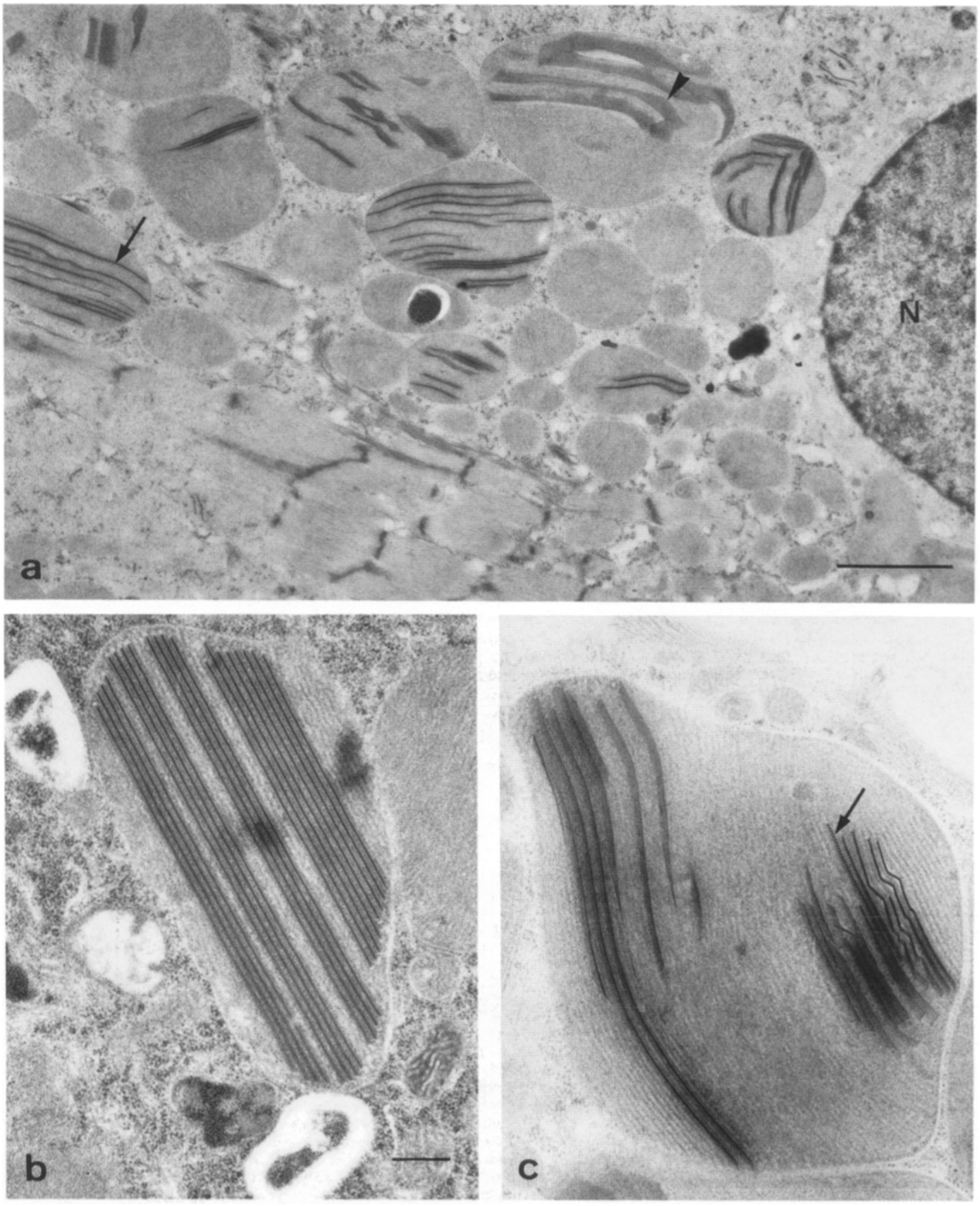

Figure 3. Electron micrographs of ARC, 9 d cultured without creatine. The sections were cut parallel to the support of the culture dish. Cells were either chemically fixed (*b*) or freeze substituted (*a* and *c*) before embedding in Epon/Araldite. (*a*) Note the two populations of mitochondria: giant mitochondria with electron-dense inclusions appearing as parallel straight lines (*arrow*) or sheet-like structures (*arrowhead*) besides normal ones. The mitochondrion in the chemically fixed cell displays straighter parallel lines (*b*) than in the freeze-substituted cell (*c*). Note the periodicity within these structures (*arrow*, *c*). Bars: (*a*) 3 μm; (*b* and *c*) 450 nm.

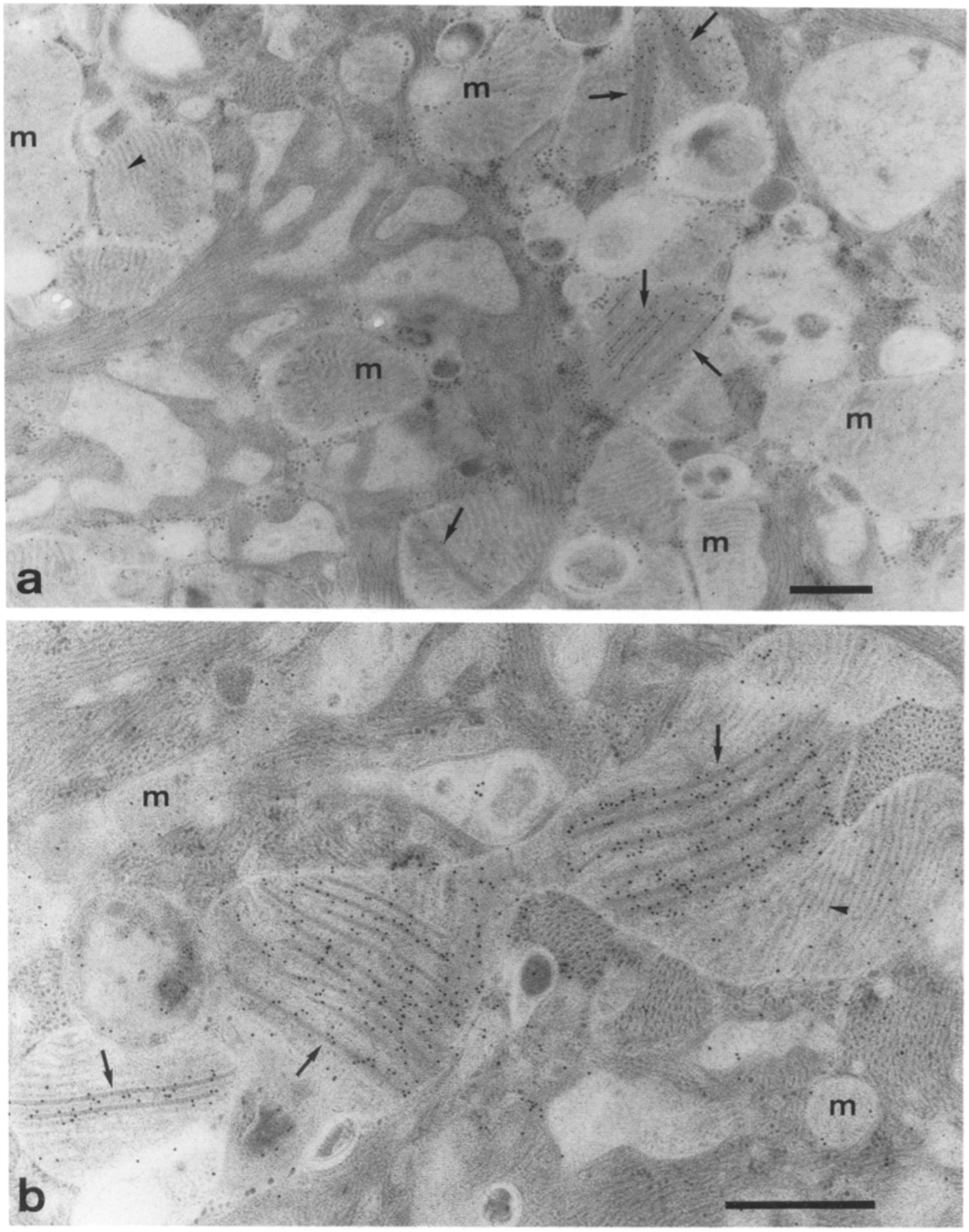

Figure 4. (*a*) Survey of freeze-substituted ARC labeled with affinity-purified anti-Mi-CK antibodies. The gold grains are localized predominantly at the inclusions in the giant mitochondria (*arrows*), whereas mitochondria without such special structures show only few grains distributed over the whole mitochondrial area (*m*). (*b*) Detailed micrograph of the same cell. (*m*) Mitochondria; (*arrows*) inclusions labeled by gold grains; (*arrowheads*) cristae membranes. Bar, 0.5 μm.

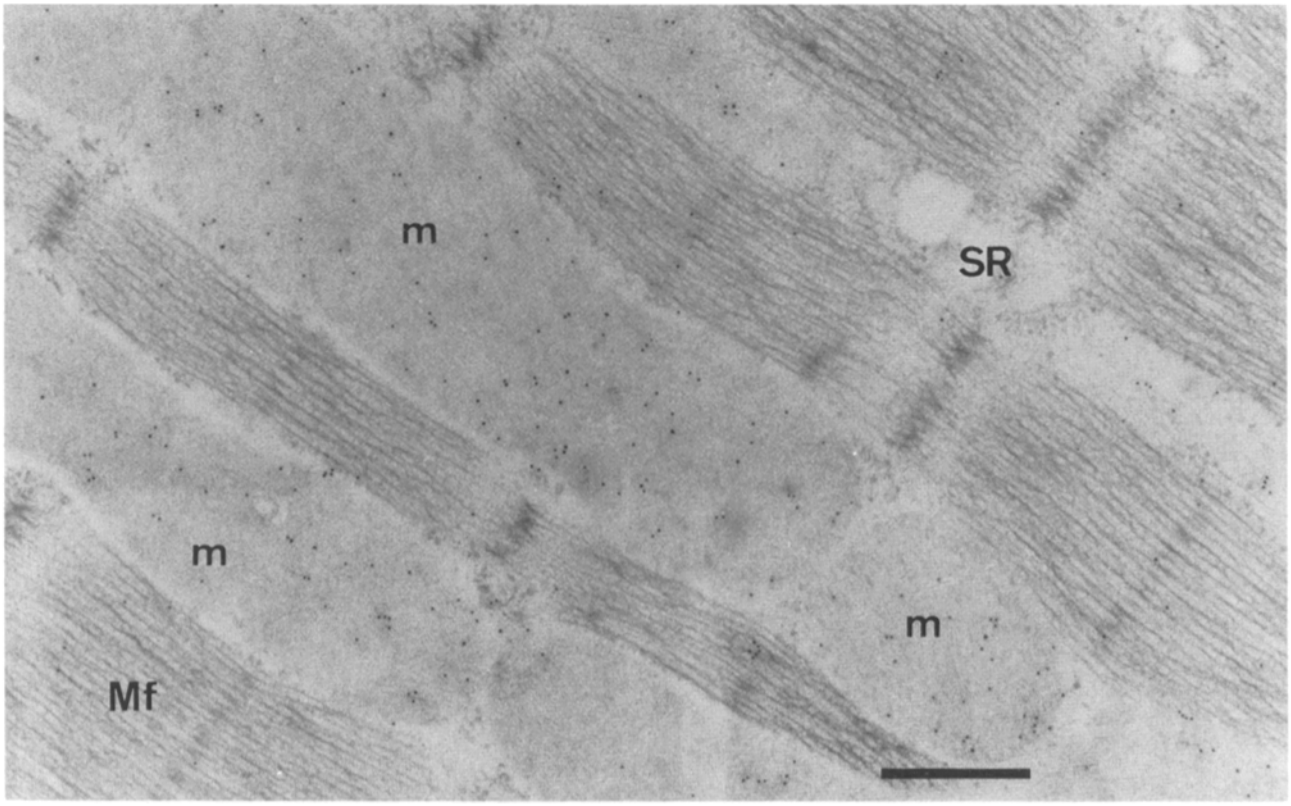

Figure 5. Freeze-substituted, rod-shaped cell immunolabeled with affinity-purified anti-Mi-CK antibody. Goat antirabbit-gold (10 nm) was used as second antibody. No inclusions are found within mitochondria of the freshly isolated rod-shaped cardiomyocytes. (*SR*) sarcoplasmatic reticulum; (*m*) mitochondria; (*Mf*) myofibrils. Bar, 0.5 μm.

showed fewer grains, which were distributed over the whole mitochondrial area (Fig. 4, *a* and *b*).

The Appearance of These Special Cylindrically Shaped Mitochondria with Mi-CK-containing Inclusions Depends on the Concentration of Creatine in the Culture Medium

A dependence of the formation of these Mi-CK-containing inclusions on the cell and the culture conditions became evident when freshly dissociated rod-shaped cardiomyocytes, which had not yet been exposed to culture medium, were centrifuged on a glass support and subsequently prepared for electron microscopic analysis. No paracrystals were present within the mitochondria of these cells and immunogold labeling for Mi-CK revealed a more or less random distribution of gold grains over the mitochondria which were regularly stacked between the myofibrils (Fig. 5).

Since the ARCs were kept in an essentially creatine-free culture medium, whereas cardiac cells in vivo are provided with creatine from the blood plasma (14, 49), the total creatine concentration within the cultured cells was determined as low as 29 ± 9 nmol/mg protein and, correspondingly, also the level of phosphocreatine synthesized by cardiac CK isoenzymes must have been low. Addition of 20 mM creatine to the culture medium enhanced the cellular concentration of total creatine approximately eight times to 202 ± 72 nmol/mg protein and, to our surprise, this higher level of creatine also had a dramatic effect on the morphology of the mitochondria in the cells. ARC as shown in Fig. 6, when kept in culture medium substituted with 20 mM creatine for 9 d, contained rows of only normally shaped mitochondria between the myofibrils showing rather strong signals for Mi-CK (Fig. 6 *f*). By contrast, in ARC cultured in creatine-free medium, clearly the two described populations of mitochondria appeared (Fig. 6 *c*).

When creatine was added throughout the culture period, the appearance of cylindrically shaped mitochondria in ARC could be prevented. But the addition of creatine was necessary only during certain culture time periods. Cells cultured for the first 5 d in medium supplemented with 20 mM creatine contained only normally shaped mitochondria as shown in Fig. 6 *f*; when the same cells were transferred to creatine-free medium for an additional 5 d, they still did not display any cylindrically shaped mitochondria. Moreover, in cells already displaying two populations of mitochondria after 7 d in creatine-free medium, an additional 3 d of creatine substitution was enough to cause the disappearance of the cylindrically shaped mitochondria. These experiments prove that the appearance of the special mitochondria described with the peculiar accumulation of Mi-CK therein is related to low intracellular creatine levels, but is reversible by adding high creatine concentrations to the culture medium. The influence of creatine on the morphology of the mitochondria and the disappearance of paracrystalline inclusions was confirmed by EM on thin sections of ARC that had been cultured in the presence of 20 mM creatine for 9 d: in those cells no inclusions were present at all and the mitochondria were small, round, and always normal looking (Fig. 7 as compared to

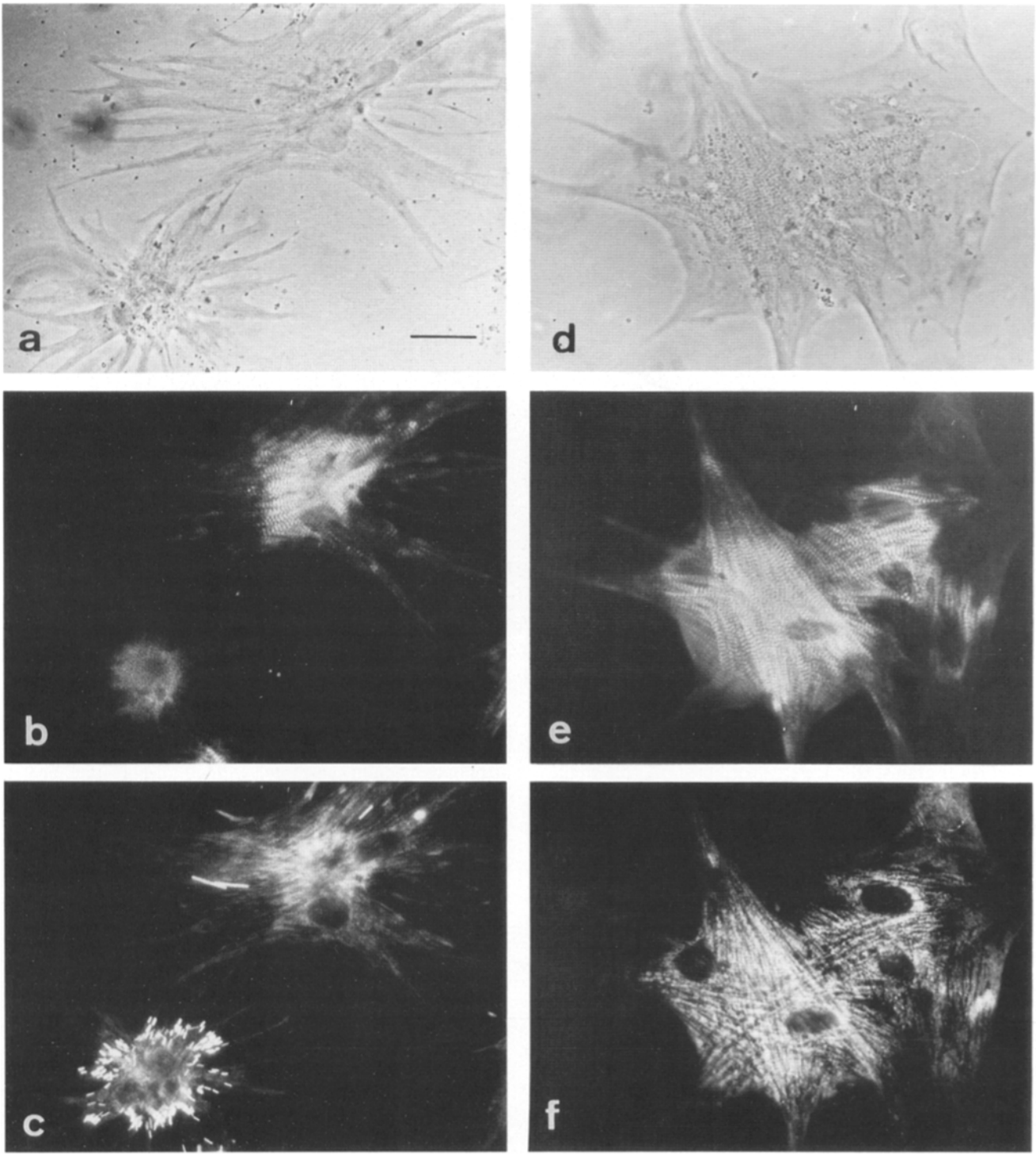

Figure 6. Double immunofluorescence labeling for cardiac C-protein (FITC, *b* and *e*) and for Mi-CK (Rhod, *c* and *f*) in ARC kept in culture during 9 d without creatine (*a–c*) or supplemented with 20 mM creatine (*d–f*). When creatine was added to the medium, the cylindrically shaped, partly giant and strongly Mi-CK-positive mitochondria displayed in Fig. 7 *c*, were replaced by rows of normally shaped, evenly but less stained Mi-CK-positive mitochondria localized between myofibrils in Fig. 7 *f*. Bar, 30 μm.

Fig. 3 *a*). As shown in Fig. 8, no obvious difference in the overall expression of Mi-CK was observed, when extracts of tissue and of cells cultured under various conditions (+/− creatine) had been investigated by immunoblotting. Primary cell cultures (2- and 6-d-old) of fetal cardiac cells, however, did not show any Mi-CK expression at all, confirming earlier data that this enzyme is accumulated only postnatally (35). In accordance with this, complementary cultures of fetal cardiomyocytes, double stained for Mi-CK and C-protein, did not reveal any positive signal for Mi-CK independently of whether the cells were grown in medium with or without creatine (not shown).

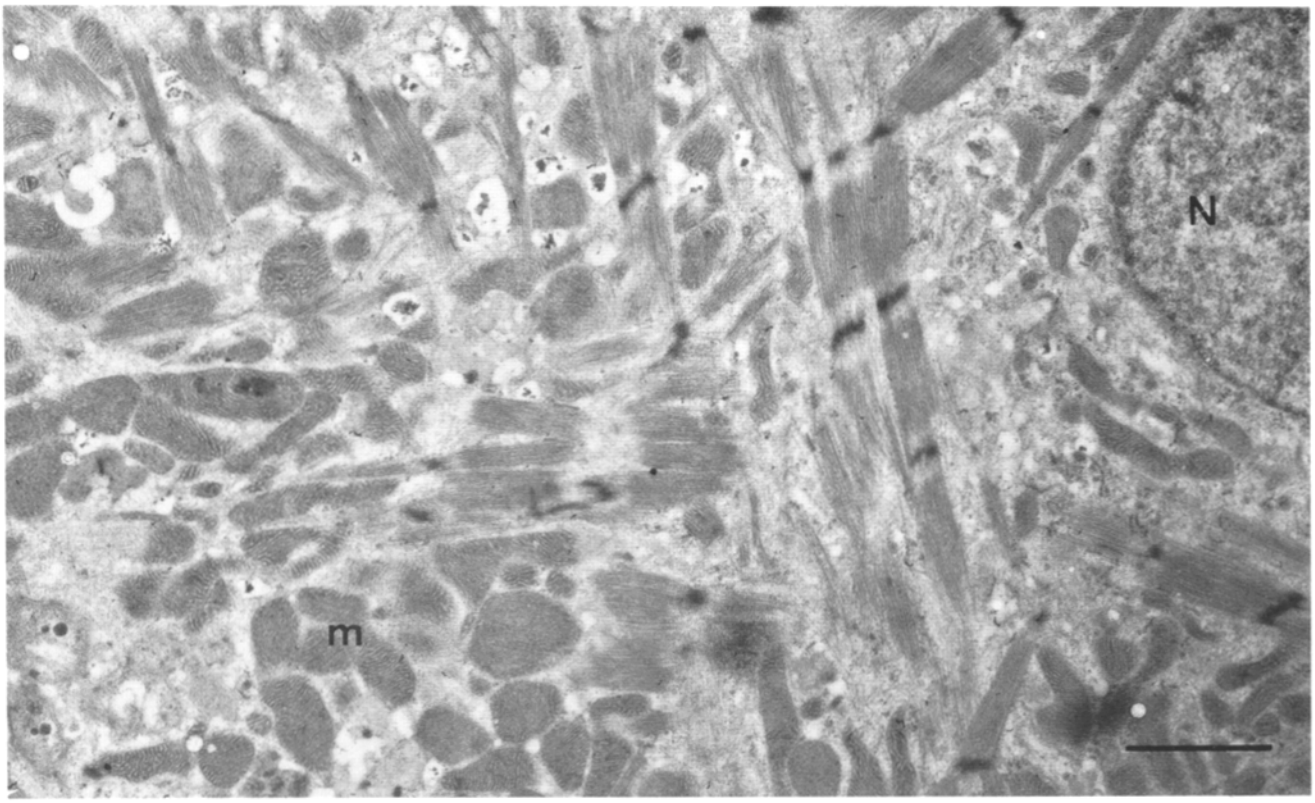

Figure 7. Low-magnification electron micrograph of a spread-out ARC maintained during 9 d in culture medium supplemented with 20 mM creatine. The cells were chemically fixed and embedded in Epon/Araldite. Note that all mitochondria are uniform and small. (*m*) Mitochondria; (*N*) nucleus. Bar, 3 μm.

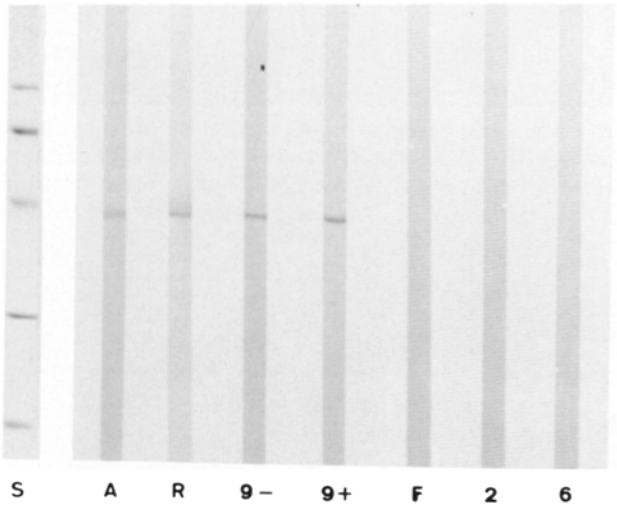

Figure 8. Nitrocellulose immunoblotting with mAb against Mi-CK of adult ventricular heart tissue (*A*), freshly isolated rod-shaped cells (*R*), ARC cultured for 9 d in medium without creatine (*9−*) or medium with 20 mM creatine (*9+*), fetal (19 d) rat heart tissue (*F*), and fetal cardiomyocytes cultured for 2(2) and 6(6) d in medium without creatine. Samples containing 165 μg protein were separated on 10% SDS-PAGE gels. SDS-PAGE low molecular weight marker by Bio-Rad was used as standard (*S*). No difference in overall expression of Mi-CK in adult cells and tissue is found, whereas fetal tissue and cells do not express Mi-CK.

The Creatine Effect Is Counteracted by the Creatine Analogue β-GPA

Intramitochondrial inclusions in skeletal muscle, but not in heart muscle, have been reported in vivo, where rats had been fed with the creatine analogue β-GPA, which is known to cause creatine and phosphocreatine depletion in muscle (31). It was then of interest to test if β-GPA could interfere with the creatine effect in mitochondria of ARC described above, and in some way could restore the standard "creatine-free" culture conditions. Double immunofluorescence for cardiac C-protein and Mi-CK was performed in 9-d-old ARC, which had been cultured for 6 d in a medium containing creatine in order to prevent the formation of the cylindrically shaped mitochondria, and to which 10 mM β-GPA had been added for the following 3 d (Fig. 9). In a number of β-GPA–treated cells the cylindrically shaped mitochondria clearly reappeared, even though many cells did not survive the treatment with β-GPA. Fetal cardiac cells, which do not express Mi-CK at all, do not seem to be very dependent on the availability of creatine, for they did not react to β-GPA (Fig. 10, *c* and *d*), that is, they survived the treatment and no Mi-CK-positive giant mitochondria appeared. This was also confirmed by data obtained from electron micrographs of fetal cardiomyocytes, which clearly demonstrated the absence of giant mitochondria with paracrystalline inclusions, when cells were kept in creatine-free medium (not shown).

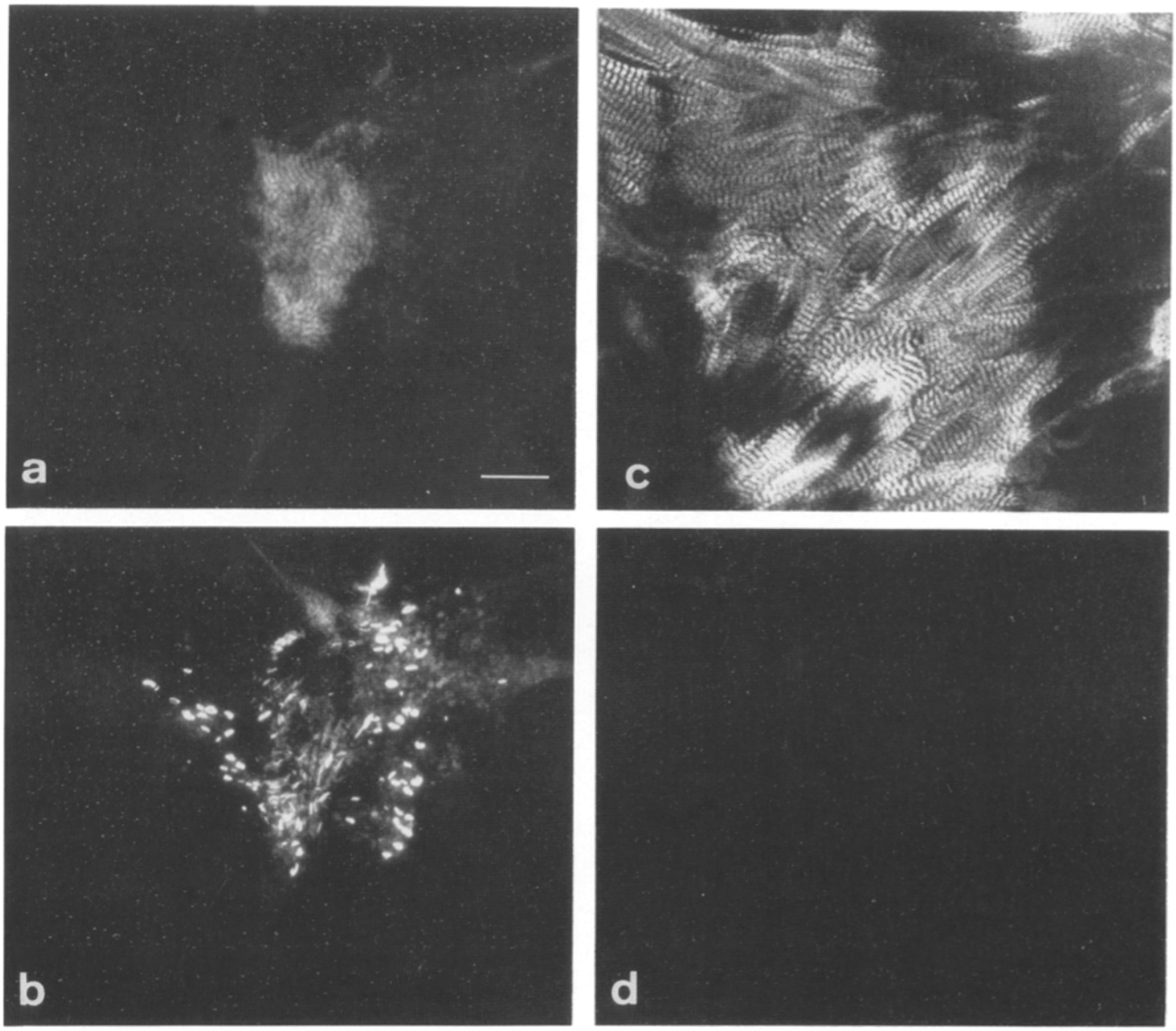

Figure 9. ARC cultured during 9 d, for the first 6 d in medium supplemented with 20 mM creatine and the last 3 d with 10 mM β-GPA (*a* and *b*). Fetal cardiomyocytes (*c* and *d*) cultured for 5 d in medium containing 10 mM β-GPA. Double immunostaining for (*a* and *c*) cardiac C-protein; (*b* and *d*) Mi-CK. In ARC, β-GPA caused a reappearance of cylindrically shaped, strongly Mi-CK–positive mitochondria (*b*), whereas the same drug had no effect in fetal cells (*d*). Bar, 20 μm.

To answer the question of whether the mitochondria present in ARC represent a population of mitochondria, which were newly formed by the redifferentiating ARC, or if all or most of the mitochondria were derived from the original rod-shaped cells dissociated from the adult heart, living freshly isolated cells were treated for 10 min with Rho 123. The mitochondria of these rod-shaped cells appeared in longitudinal rows between the myofibrils (Fig. 10 *b*). When the same cells were kept in culture, after 6 d, they still displayed a large number of Rho 123–positive mitochondria localized mainly in the center of the cell (Fig. 10 *d*). This suggested that the mitochondria initially present in newly isolated cells were mostly conserved and were reorganized and relocated during the redifferentiation of the cultured cardiomyocytes; thus, no obvious evidence for a generalized destruction of the preexisting mitochondria could be observed.

Discussion

Dedifferentiation and Redifferentiation of ARC in Culture: Persistence of Mi-CK

Unlike the contractile apparatus (9, 11), mitochondria of ARC do not seem to be involved in the process of dedifferentiation, for the same cells, when briefly labeled by Rho 123 right after dissociation, kept the marker for another 6 d inside their mitochondria indicating the preservation of the majority of the original mitochondria. Since these giant mitochondria have been reported to appear at an early stage of ischaemia (16) and in adult rat heart cells already 24 h after cell isolation (27), it could be that they originate by a process of fusion of smaller mitochondria as suggested for liver mitochondria (56). The reason why in ARC only part of the mi-

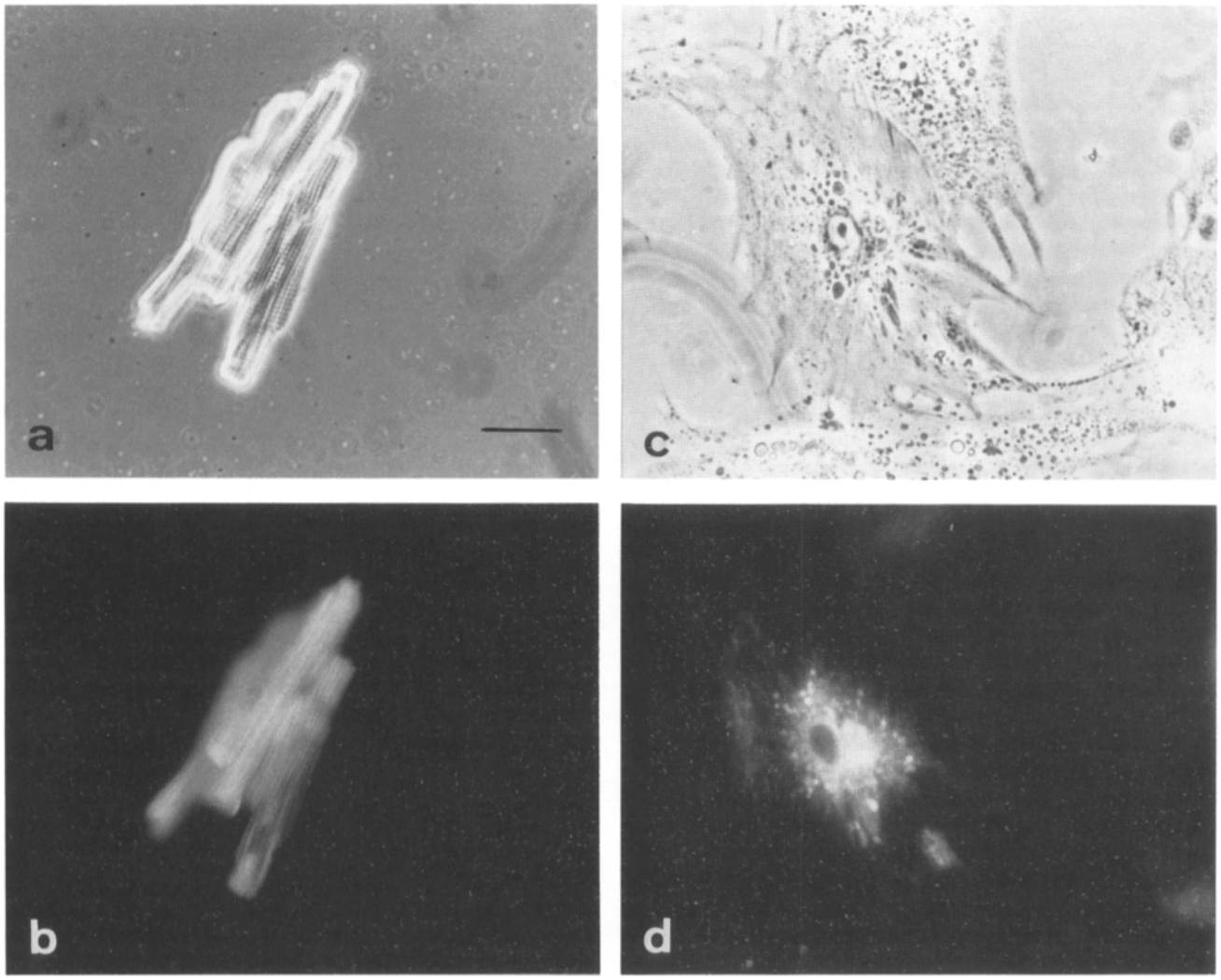

Figure 10. Living ARC treated with Rho 123. (*a* and *b*) Freshly isolated rod-shaped ARC after 10 min of treatment with Rho 123, mitochondria are organized in rows between the myofibrils. (*c* and *d*) Polymorphic ARC after 6 d in culture still containing Rho 123–positive mitochondria localized mainly in the perinuclear region. Bar, 30 μm.

tochondria are changed and form inclusions, may simply be that one population is more susceptible to the influences described. As a matter of fact, the existence of two anatomically distinct populations of cardiac mitochondria, intermyofibrillar and subsarcolemmal, has been known for a number of years (32, 33, 37, 54) and a substantially higher lability of the latter to both hypoxia and reoxygenation has been described (6). Also, inclusions have been seen mainly in mitochondria at the periphery of ischemic muscle fibers (16) and in mitochondrial myopathies within large subsarcolemmal clusters (25, 37). Thus, it is possible that the subsarcolemmal mitochondria in cultured ARC respond to the creatine deficiency, first with fusion into giant mitochondria and second with a compensatory accumulation of Mi-CK within the inclusions.

Significance of Formation of Paracrystalline Inclusions in Mitochondria in Myopathies, Which Show a Mitochondrial Involvement

The electron microscopic investigations did confirm the immunofluorescence results, demonstrating that the large fluorescently labeled mitochondria exhibit distinct inclusions in the intracristal space. An involvement of the cristae membrane in the structure of these inclusions is supported by a strong osmiophilic staining in conventionally processed tissue or by the absence of staining in cryosubstituted tissue (compare Fig. 3, *b* and *c*). The inclusions that have been reported in mitochondria of skeletal muscle subjected to ischaemia (16, 19, 37), in heart cells (47), in isolated mitochondria (15), as well as in many neuromuscular disorders and mitochondrial myopathies (18, 28, 34, 47, 48, 51, 52) look similar to the ones shown here. Inclusions in mitochondria have been described as early as 24 h after isolation of ARC, and it has been suggested that these inclusions are a consequence of severe mitochondrial damage due to the cell dissociation procedure (27), or that enzymes like CK, present in the mitochondrial intermembrance space, might be implicated in the formation of inclusions (16). The results presented here confirm this latter hypothesis.

Our findings that the appearance of mitochondrial inclusions correlates with a low intracellular total creatine and thus also a low phosphocreatine content, and that one of the prominent components of these inclusions is Mi-CK, are

certainly of special importance also from a clinical point of view (see below).

Mi-CK as a Main Constituent of the Mitochondrial Inclusions

The heavy immuno-gold labeling along the inclusions allowed the assumption that Mi-CK is one of their major components; since Mi-CK is located on the outer side of the inner mitochondrial membrane (40, 45), it is assumed that Mi-CK could be sandwiched as inclusions between cristae membranes, while in the cylindrical mitochondria the matrix protein mAAT, as shown by its absent or weak staining, is not involved in the paracrystals and its import into the matrix may even be inhibited.

The fact, that only very little or no gold labeling occurs in normally shaped mitochondria is in part due to the Epon postembedding labeling technique by which only mitochondrial structures with an abundance of Mi-CK antigen epitopes could be labeled. These results confirm that the cylindrically shaped and, in contrast to normal mitochondria, strongly Mi-CK-positive mitochondria found by immunofluorescence are identical with the mitochondria-containing paracrystalline inclusions. We cannot conclude, however, that Mi-CK is the only component of the inclusions, even though there is a good possibility that it is "stored" at this site in a paracrystalline or even crystalline form. Most important in this context is the observation made by Stadhouders, A. M., and colleagues at the University of Nijmegen (personal communication) that the two types of "intracristal inclusions" in mitochondria of human myopathic muscle (13) are also heavily labeled by our anti-Mi-CK antibodies. The appearance of these inclusions (13) seems similar to that of negatively stained flakes of pure Mi-CK protein crystals (42, 43) or to Mi-CK ribbons (44). This strongly suggests that the inclusions in mitochondria of ARC that appear in vitro under low creatine, stress-inducing conditions are of similar nature to those observed in vivo under pathological conditions in biopsies from patients suffering from occular myopathies (13) and also suggests once more that Mi-CK is a main component of these inclusions.

Effects of Creatine and of the Creatine Analogue β-GPA

The addition of creatine is mainly necessary during the first days in culture, representing the critical period, when the cells go through extreme changes and may still be leaky to some extent for smaller molecules. Although no indication exists as to how directly creatine brings about the effects on the mitochondria, its involvement is clearly demonstrated by experiments, where the creatine analogue β-GPA reversed the creatine effect by competition in ARC, previously grown in creatine-substituted medium. Ohira et al. (31) also had investigated the influence of β-GPA in vivo on the ultrastructure of mitochondria in skeletal muscle from rabbits previously fed with the drug and observed similar mitochondrial inclusions in skeletal but not in cardiac muscle of animals chronically treated with the drug. Other authors have demonstrated that in vivo feeding of β-GPA lowers the level of total creatine (creatine plus phosphocreatine) in muscle (26, 50), because it inhibits creatine entry into muscle from the blood plasma (14, 49). In our hands, ARC, supplemented with creatine, which had been treated afterwards with β-GPA, only partially survived, which is an indication of a "low-energy" state induced by the drug (26, 49, 50). Nevertheless, these results prove a complete reversibility of the phenomena initiated by a lack of creatine in the medium.

Functional Considerations of Mi-CK-rich Inclusions and Metabolic Adaptation of ARC

The cylindrically shaped large mitochondria with paracrystalline inclusions clearly appear as a consequence of a single factor, that is low external creatine levels. Concomitantly with low intracellular total creatine concentrations and thus also with low phosphocreatine energy stores, a heavy accumulation of Mi-CK within these inclusions is seen. The entire complex of events is reversed by addition of a single factor, namely chemically pure creatine. Since in creatine-deficient cells total Mi-CK does not seem to be overexpressed in general as judged semiquantitatively from the immunoblots (Fig. 8), one has to assume an accumulation of the enzyme specifically induced in the population of the cylindrically shaped mitochondria.

In a "metabolic adaptation hypothesis" one could postulate that the transphosphorylation of so called "energy-rich phosphate" from ATP to creatine, to yield phosphocreatine and to enable the transport of the latter out of the mitochondria, is made more efficient by an increased accumulation of Mi-CK in a highly ordered fashion along the cristae membranes. Thus, the low intracellular levels of creatine and creatine phosphate would be compensated by the metabolic adaptation via higher total amounts of Mi-CK within the cylindrically shaped large mitochondria, in other words, the cell would respond to its lower phosphocreatine energy status by increasing what we called the "transport function" of the phosphocreatine circuit (60, 61) via Mi-CK, representing an "energy chaneling" enzyme (40, 41). Since mitochondrial inclusions also occur in β-GPA–treated animals (31) where phosphocreatine levels in skeletal muscle as low as 10% of normal have been reported (26, 49, 50), such a metabolic adaptation to increase the energy transport capacity of the phosphocreatine circuit by Mi-CK (60, 61) deserves some merit.

Alternatively, one could argue that the giant cylindrically shaped mitochondria are merely representing degenerating pathological structures with some enzymes like Mi-CK, stored away at high concentrations, and with others like mAAT, present in lower amounts than normal or absent.

In our opinion, the highly ordered structure as well as the full reversibility of the mitochondrial inclusions in ARC by addition of creatine to the cell culture medium would make the "degenerative hypothesis" less likely.

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