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Experimental Production of "Septa" and Apparent Subdivision of Muscle Mitochondria

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Abstract

The rapid formation (in less than 45 min) of internal septa and the apparent subdivision *in situ* of mitochondria from cardiac and skeletal muscle are described following a variety of experimental treatments. For example, the ionophore A23187, caffeine, DNP, ruthenium red, and the insecticide lindane have been applied to intact, glycerinated, and chemically skinned skeletal muscle fibers and to cardiac muscle strips from both amphibians and mammals. In some mitochondria, the two compartments are in the same configuration; in others they are different. The significance of these mitochondrial septa is discussed, and it is suggested that the findings are consistent with the hypothesis that a variety of experimental procedures can promote rapid mitochondrial division.

Introduction

There are now a number of accounts of mitochondria seen in electron-micrographs which possess transverse septa, apparently dividing them into two separate organelles. In particular, partitioned mitochondria have been described in detail in cardiac muscle cells of canaries and a variety of mammals [1] and in liver, adrenal cortex, and fat cells [2], while mention of the phenomenon is reported in a number of reports (see review, Tandler and Hoppel [1]). The proportion of mitochondria showing transverse septa is markedly increased following certain experimental treatments. Mice maintained for 6–8 weeks on a riboflavin-free diet had greatly enlarged hepatic mitochondria, but these were restored to normal dimensions within 3 days by injections of riboflavin, the giant organelles apparently undergoing division characterized by the development of a membranous partition [3]. The

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frequency of occurrence of septate mitochondria also rises markedly in hepatocytes [2] and cardiac muscle cells [1] from cuprizone-fed mice.

However, we have shown that apparent subdivision of mitochondria can be induced very much more rapidly by appropriate experimental treatment [4]. Exposure of mouse diaphragm muscle to the divalent cation ionophore A23187 *in vitro* probably causes the release of Ca^{2+} from the sarcoplasmic reticulum (S.R.), and the muscle mitochondria swell and undergo configurational changes which may be associated with Ca^{2+}-uptake. When maximally swollen, after exposure to A23187 for some 30 min, internal septa are apparently developed in a high proportion of the mitochondria. The purpose of the present communication is to show that similar apparent subdivisions can be produced experimentally in mitochondria from both skeletal and cardiac muscle equally rapidly by other treatments. One example is the exposure of frog skeletal muscle to the insecticide lindane (hexachlorocyclohexane, γ-isomer). We believe that such a finding is of interest, not only for the light that it may throw on the problems of division and fusion of mitochondria, but also on the toxicological effects that this insecticide (which is still in widespread use) may have at the ultrastructural level. Secondly, mitochondrial septa can be produced rapidly in both skinned and glycerinated mammalian skeletal muscle fibres. Finally, we show that a number of experimental procedures can produce the same effect in isolated strips of frog and mouse cardiac muscle as that described in skeletal muscle. Treatments that cause the development of internal septa in muscle mitochondria frequently, but not invariably, also cause severe and extensive damage to the myofilaments.

Materials and Methods

Exposure to Lindane

Cutaneous pectoris muscles of the frog *Rana temporaria* were removed and pinned out in Ringers solution at 10°C. They were then transferred to a beaker containing 20 ml Ringers solution plus 5×10^{-5} M lindane for 40 min at 22.5°C ($\pm$ 0.5°C). The conditions were chosen to be comparable with parallel studies on the pharmacology of lindane at the presynaptic terminals of the frog neuromuscular junction [5]. Exposure of normal, isolated mouse diaphragm muscle to test agents was carried out as described previously [4, 6].

Glycerination of Diaphragm Muscle

Bal/b mice (12–20 g) were killed by cervical dislocation and their diaphragms were quickly removed, pinned out on dental wax, and placed in

20% glycerol (v/v) on ice. Small pieces of diaphragm were removed at intervals, washed in 0.1 M KCl, and tested in contracting solution at 22°C which was composed of (mM): KCl 100, Na_2ATP 10, $CaCl_2$ 1, $MgCl_2$ 1; contraction was monitored under the microscope.

Preparation of Skinned Skeletal Muscle Fibers

Mouse diaphragm was isolated and pinned out as above and placed in skinning solution at 0°C (after Wood et al. [7]) which contained (mM): EGTA 5, K-propionate 170, Mg-acetate 2, Na_2ATP 2, imidazole 5, pH 7.0. Small pieces of diaphragm were removed at intervals, washed in 170 mM K-propionate, and tested for contraction at 22°C. Contracting solution contained (mM): K-propionate 170, Na_2ATP 10, $CaCl_2$ 1, $MgCl_2$ 2, pH 7.0. Contractions were normally seen after some 3 hr.

Mouse Cardiac Muscle Strips

The mouse was killed as above and the heart was quickly dissected out and washed in Krebs mammalian saline (22°C). The auricle and ventricle were separated and then sliced into small strips which were transferred to the oxygenated (95% O_2, 5% CO_2) test solution (8 ml) of Krebs saline containing the test agent at 37°C. Frog (*Rana temporaria*) cardiac muscle strips were prepared similarly in Ringers solution at 22°C.

Electron Microscopy

Muscle strips were prepared by replacing the bathing solution with fixative [8] at 21°C for 30 min. Fixation was completed by transfer to a fresh change of the same solution at 21°C for 2.5 hr. Specimens were washed in 0.1 M sodium cacodylate with 2.5 mM $CaCl_2$, pH 7.2, for 30 min (two changes). The tissue was then cut into smaller pieces and postfixed in OsO_4 for 2 hr at room temperature. After this, the pieces of muscle were cut into small blocks for subsequent washing in cacodylate buffer (two changes), dehydration through the graded alcohol series, and embedding in resin [9]. Sections were cut at 60–90 nm, stained with uranyl acetate and lead citrate [10], and examined on an AEI Corinth 500.

Reagents

All inorganic salts and ethanol were AnalaR grade. Lindane (γ-isomer), ATP, EGTA, ruthenium red, caffeine, 2,4-dinitrophenol (DNP), and imidazole were obtained from Sigma Chemical Co., St. Louis, USA. A23187 was a gift of Lilly Research Centre, Windlesham, Surrey. The stock solutions of A23187 and lindane were dissolved in ethanol; the final concentrations for experiments were A23187 6.25 μg ml^{-1}, ethanol 6.25 μl ml^{-1}, lindane 5 $\times$

10^{-5} M, and ethanol 0.5%. Control solutions contained a comparable concentration of ethanol.

Results

A typical sequence of events of the changes in mitochondrial ultrastructure following exposure of frog skeletal muscle to lindane (5×10^{-5} M) is as follows. There is a progressive loss of cristal organization and ultimately the organelle assumes a vacuolated appearance [5]. However, many of the mitochondria apparently become subdivided by the development of septa; Figures 1 and 2 illustrate a characteristic aspect in which the septa appear to be developing within the mitochondria and two septa appear to be developing simultaneously in one of the organelles illustrated in Fig. 2. Figures 3–5 show mitochondria with apparently complete subdivision. Concomitantly, the muscle fibers show extensive subcellular damage [5]. These ultrastructural changes in the mitochondria produced by exposure to lindane were not observed when Ca^{2+} was omitted from the extracellular medium.

Fig. 1. Mitochondria in frog skeletal muscle that has been treated for 40 min with lindane (5×10^{-5} M) at 22.5°C. Apparent development of internal septum. Bar = 250 nm.

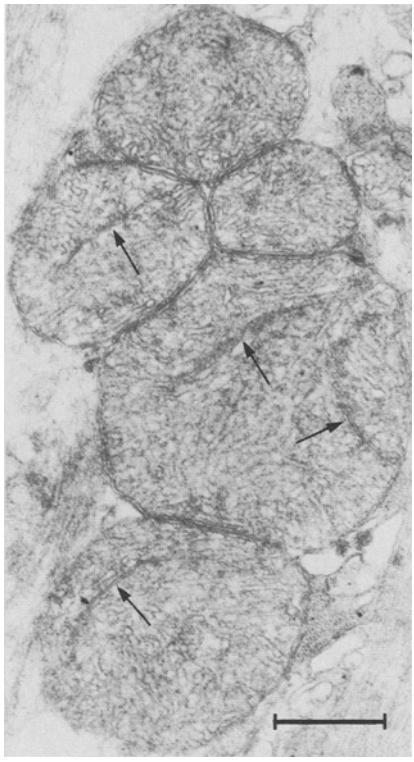

Fig. 2. Same as in Fig. 1. One mitochondrion appears to be developing two internal septa. Bar = 500 nm.

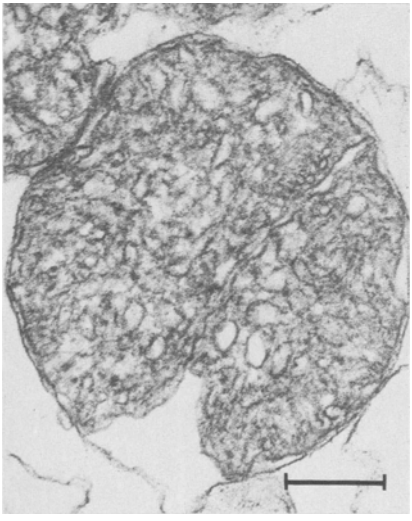

Fig. 3. Same as in Fig. 1. Mitochondria completely subdivided. Bar = 250 nm.

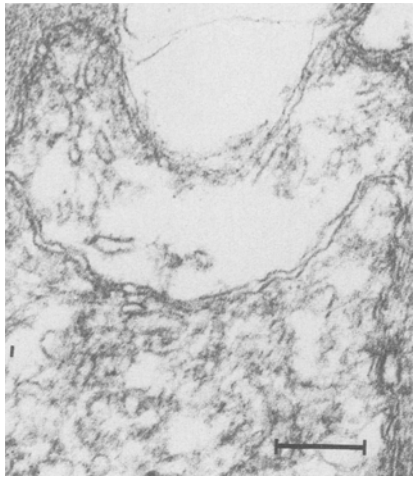

Fig. 4. Same as in Fig. 3. Bar = 250 nm.

Similar effects are frequently found in mammalian skeletal fibers that have had their plasmalemma removed, and Figs. 6–10 give typical examples in different experimental conditions. Mitochondria from glycerinated fibers incubated in 0.1 M KCl with the ionophore A23187 (6.25 μg ml^{-1}, 37°C, 40 min) are shown in Fig. 6, where the arrow indicates electron-dense material extending in from the outer boundary. Mitochondria from such fibers incubated in 0.1 M KCl plus 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM ATP (30 min; 21°C) are shown in Fig. 7; the large organelle is seen to be subdivided by

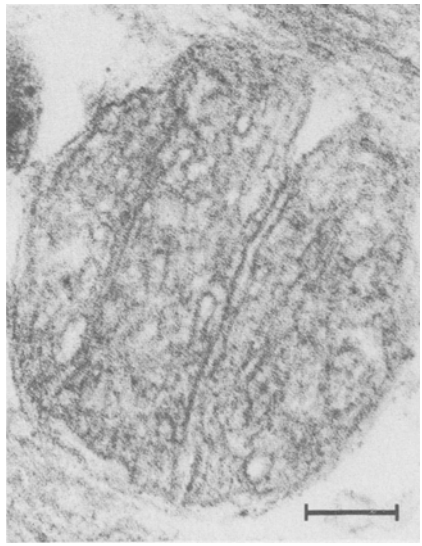

Fig. 5. Same as in Fig. 3. Bar = 125 nm.

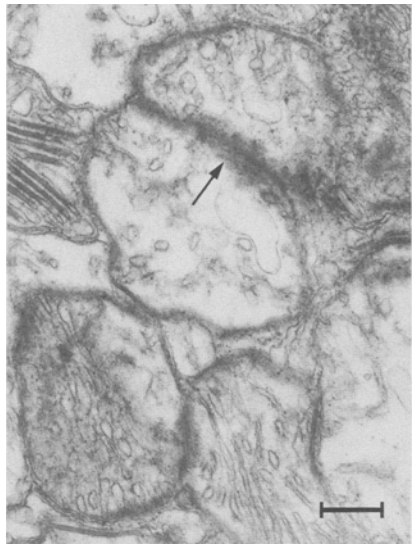

Fig. 6. Mitochondria in glycerinated mammalian skeletal muscle fibers after exposure to A23187 (6.25 μg ml^{-1}) for 40 min. A number of developing septa can be seen; the arrow indicates electron-dense material extending in from the outer boundary; bar = 250 nm.

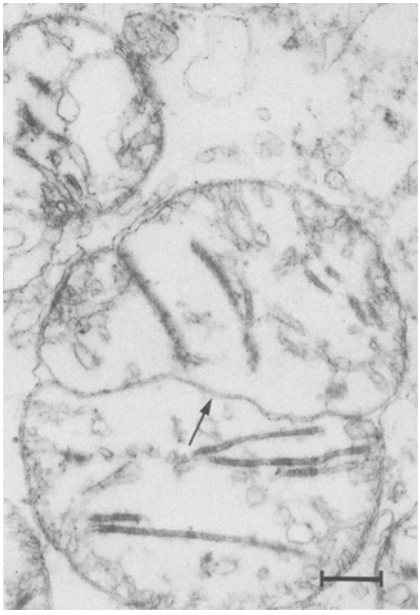

Fig. 7. Mitochondria in glycerinated mammalian skeletal muscle fibers after 30-min incubation in 1 mM Ca^{2+} plus 10 mM ATP (21°C); complete transverse septum. Bar = 250 nm.

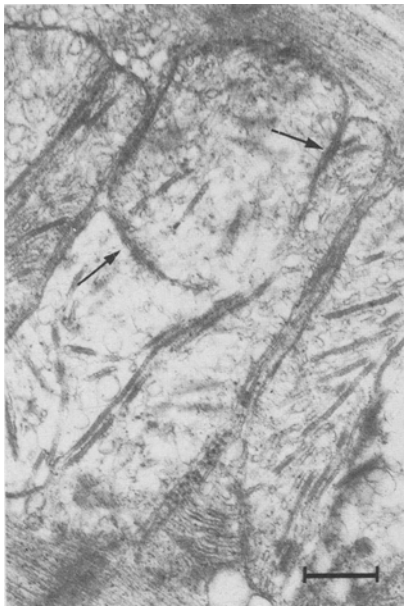

Fig. 8. Mitochondria in glycerinated mammalian skeletal muscle fibers after incubation for 30 min in 1 mM Ca^{2+} (37°C); arrows indicate where septa appear to be developing by infoldings from lateral margins; the mitochondria at upper left and lower right may be divided into two areas of different configurational states. Bar = 125 nm.

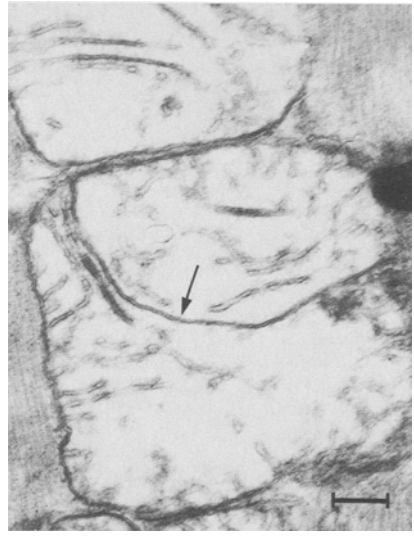

Fig. 9. Mitochondria in chemically skinned mammalian skeletal muscle after incubation in A23187 (6.25 μg ml^{-1}) for 50 min (37°C); arrow indicates a complete septum. Bar = 250 nm.

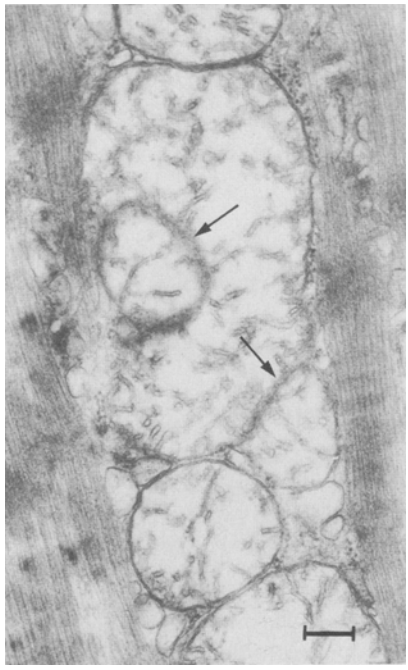

Fig. 10. Mitochondria in chemically skinned mammalian skeletal muscle after incubation in 1 mM Ca^{2+} (15 min, 37°C); arrows indicate developing septum. Bar = 250 nm.

a transverse septum but is enclosed in a complete unit membrane. Figure 8 shows closely apposed mitochondria from fibers incubated in 0.1 M KCl plus 1 mM $CaCl_2$ (30 min; 37°C), and septa (arrows) can be seen, apparently developing by infoldings from the lateral margins. Some organelles appear to be subdivided into two areas of different configurational state.

Mitochondria found in chemically skinned muscle fibers are shown in Figs. 9 and 10; in Fig. 9 the muscle strip was incubated in 170 mM K-propionate plus A23187 for 50 min at 37°C (pH 7.0), and a septum apparently subdividing the organelle can be seen. The muscle strip shown in Fig. 10 was incubated for 15 min after skinning in 170 mM K-propionate plus 1 mM Ca^{2+} (pH 8.5; 37°C), and apparent early formative stages of internal septa (arrows) can be seen. The picture is strikingly similar to that described previously in intact mammalian muscle following A23187 treatment [4] and shown in Fig. 22.

The results from the experiments concerning the effect of A23187 on mammalian cardiac muscle strips are of interest in that, under the conditions studied, the ionophore does not cause myofilament damage, unlike its action on amphibian [5, 11, 12] and mammalian [6] skeletal muscle. Nevertheless, the mitochondria swell and undergo the same ultrastructural changes seen in mammalian skeletal muscle [4]. Furthermore, many of the electron micro-

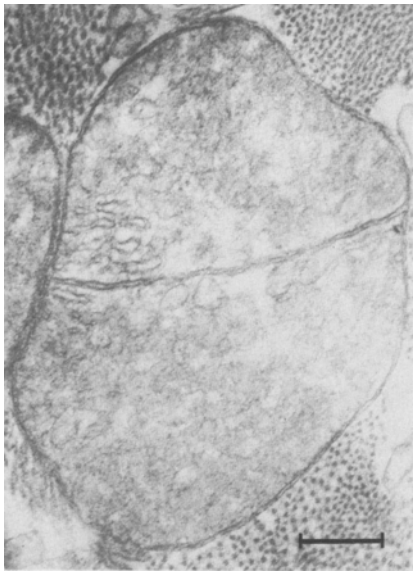

Fig. 11. Mitochondria from mammalian cardiac muscle strips following exposure to A23187 (6.25 μg ml^{-1}) for 45 min at 37°C. Septum complete; bar = 250 nm.

graphs show mitochondria with internal septa (Figs. 11–15). In some, the septum is complete, gently curving, and divides the mitochondrion into halves each of which is in the same energetic configuration (Figs. 11 and 12); in others the two sections are in clearly different configurational states (Figs. 13 and 14). Some mitochondria show septa that are apparently developing, either by lateral inpushings (Fig. 15) or by the internal development of electron-dense material (Figs. 13 and 15).

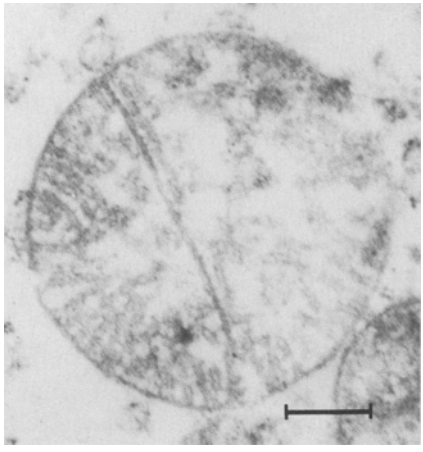

Fig. 12. Same as in Fig. 11; bar = 250 nm.

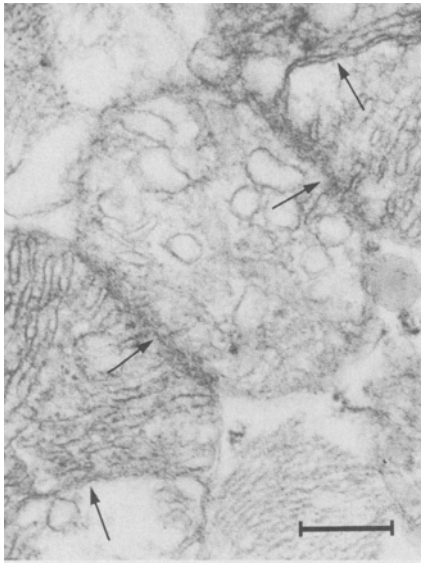

Fig. 13. Same as in Figs. 11 and 12. Arrows indicate developing or complete septa; mitochondrion in lower left has two sections in different configurational states. Bar = 250 nm.

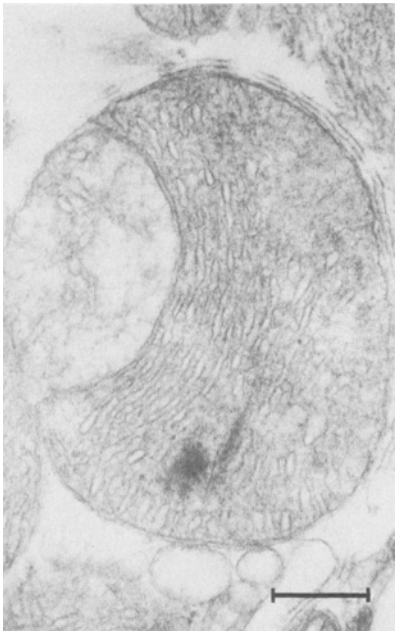

Fig. 14. Same as in Fig. 11. Septum complete with two sections in different configurational states. Bar = 250 nm.

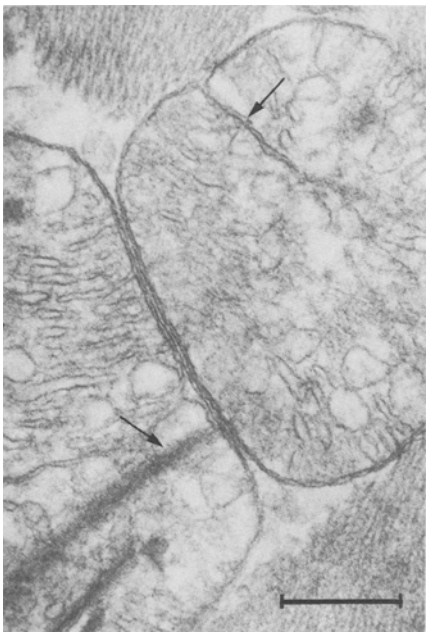

Fig. 15. Same as in Fig. 11. Arrows indicate septa developing, one by lateral inpushing, the other by the internal development of electron-dense material; bar = 500 nm.

However, treatment of mouse cardiac muscle strips with DNP (10^{-4} M, 25 min, 37°C) causes extensive myofilament damage, comparable with that described in mammalian skeletal muscle after A23187 treatment [6], and, again, the mitochondria show apparent subdivision with septa (Fig. 23), either by lateral inpushings (Fig. 16) or by the internal development of electron-dense material (Fig. 17). Treatment of the isolated mouse diaphragm with 10^{-4} M DNP for 30 min at 37°C also causes mitochondrial swelling in the skeletal muscle fibers, and there appears to be an increase in the numbers of these organelles and the formation of internal septa (Fig. 25).

Treatment of frog skeletal muscle with A23187 does not cause the development of mitochondrial septa [11]; furthermore, we have found that simple exposure of frog cardiac muscle strips to A23187 does not cause the myofilament destruction found in frog skeletal muscle, a feature in common with mammalian cardiac muscle. Nevertheless, myofilament degradation, accompanied by the formation of mitochondrial septa, can be produced in frog cardiac muscle, although the latter phenomenon is found less commonly than in mammalian skeletal muscle. Figure 18 shows a mitochondrion (which may be completely subdivided) from frog cardiac muscle exposed to A23187 in Ca^{2+}-free saline for 15 min which was then transferred for 5 min to a depolarizing saline solution containing Ca^{2+} and A23187 and in which $[K^+]$ was raised to 20 mM. Under these conditions, the depolarization augments

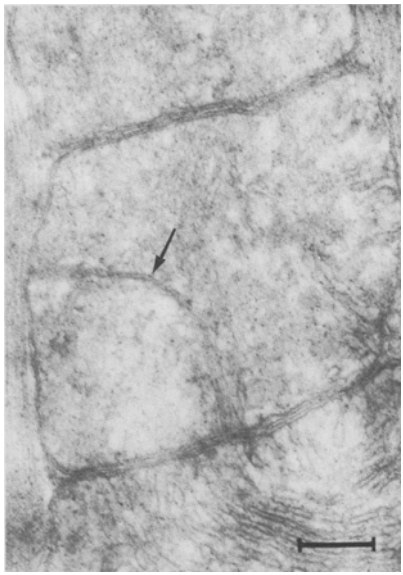

Fig. 16. Mitochondria from strips of mouse ventricle treated with 10^{-4} M DNP for 25 min at 37°C. The photograph shows a septum, apparently developing by a lateral inpushing, which appears to cut off one corner of the organelle, a condition that is found quite frequently. Bar = 250 nm.

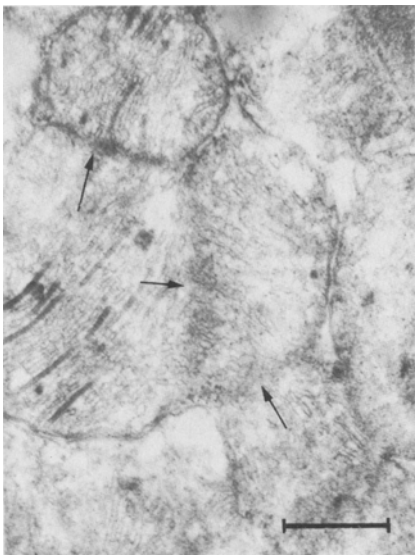

Fig. 17. Same as in Fig. 16; bar = 250 nm. The photograph shows the internal development of electron-dense material in a number of positions.

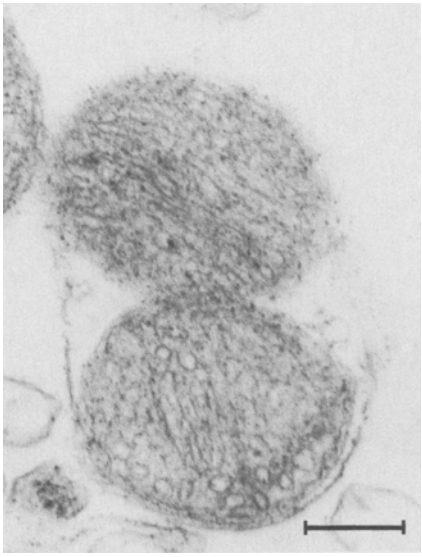

Fig. 18. Mitochondrion from a strip of frog ventricle exposed to A23187 (6.25 μg ml^{-1}) for 15 min in Ca^{2+}-free solution which was then transferred for 5 min to a solution containing 1.8 mM Ca^{2+}, 20 mM K^{+}, and A23187 (6.25 μg/ml). 22°C. Bar = 250 nm.

the action of the ionophore, and extensive damage to the cardiac muscle was found. Figure 19 shows mitochondria with internal septa from frog cardiac muscle exposed to 30 μM ruthenium red for 40 min.

Exposure of frog skeletal muscle to caffeine causes extensive myofilament damage [12]. Our present studies show that, even at 10 mM, caffeine

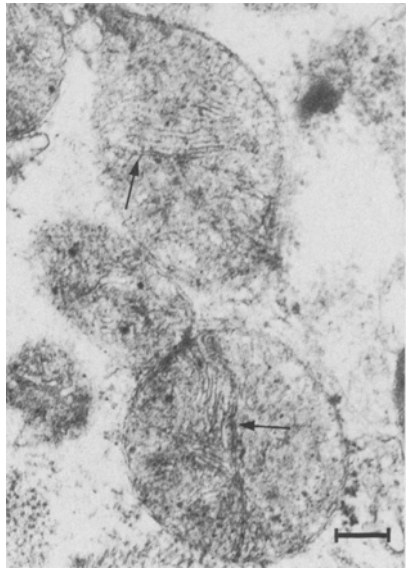

Fig. 19. Mitochondria from a strip of frog ventricle exposed to 30 μM ruthenium red for 40 min at 22°C. The organelles are apparently subdivided into two sections by septa (arrows). Bar = 250 nm.

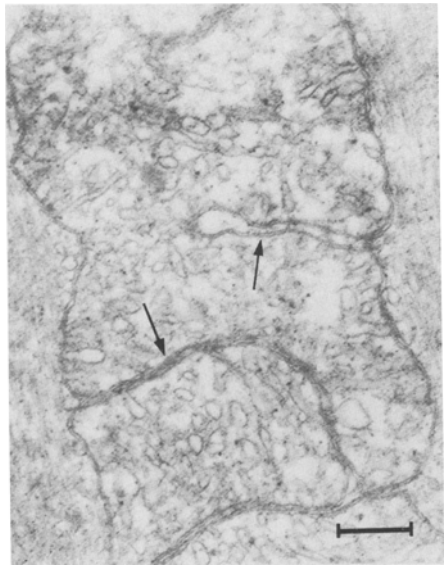

Fig. 20. Mitochondrion from mouse diaphragm muscle which was electrically stimulated for 8 min (1 stimulus per sec) during a 40-min exposure to 10 mM caffeine at 37°C. Arrows indicate an apparent lateral infolding and also where a septum separates one corner of the mitochondrion. Bar = 250 nm.

does not always have this effect on mammalian skeletal muscle. However, if the mouse diaphragm preparation is electrically stimulated during exposure to 10 mM caffeine (8 min stimulation during a total exposure to caffeine of 40 min; 37°C) or is depolarized by raising $[K^+]_o$ to 20 mM, both myofilament degradation and mitochondrial septa can be found (see Fig. 20). In certain muscles, caffeine treatment (10 mM) alone does cause muscle contraction

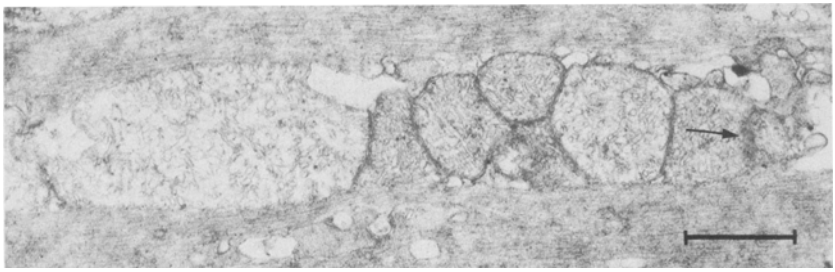

Fig. 21. Mitochondria from frog skeletal muscle exposed to 5×10^{-5} M lindane for 40 min (22.5°C). Arrow shows electron-dense material developed within one mitochondrion. Bar = 1 μm.

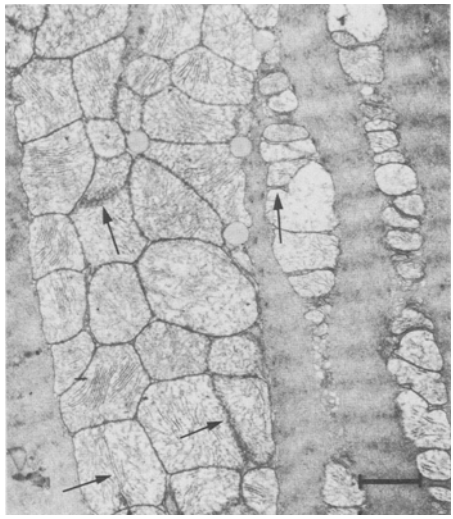

Fig. 22. Mouse diaphragm muscle exposed to A23187 (6.25 μg/ml) for 30 min at 37°C. Muscle fiber is heavily contracted and Z-line blurring can be seen, indicating the beginning of myofilament degradation. Note apparent chains of mitochondria believed to be formed by subdivision and also an area of swollen and packed mitochondria. A number of developing septa can be seen (arrows). Bar = 2 μm.

and myofilament damage and, at the same time, many of the mitochondria can be seen to have developed septa (Fig. 24).

Figures 21–25 show a series of electron-micrographs at lower power to illustrate the phenomena of mitochondrial swelling and the development of mitochondrial septa. As recorded above, the mitochondria of frog muscle do not swell dramatically and septa are much less commonly seen; Fig. 21 shows frog skeletal muscle treated with lindane, and the accompanying myofilament degradation can be detected. The most dramatic mitochondrial swelling is found in mammalian skeletal muscle, as shown in Figs. 22 and 25 where myofilament degradation can also be seen. These effects follow treatment with A23187 or DNP. The electron-micrographs show an apparent increase in the number of the mitochondria, perhaps by subdivision. For example, note apparent chains of mitochondria in Fig. 22, perhaps formed by folding or perhaps by subdivision of the organelles, and also the abnormal packing of the mitochondria in Figs. 22 and 25. Recently formed septa and the development of internal electron-dense material are indicated by arrows. Figure 24 shows muscle treated with caffeine in which initial division of many of the

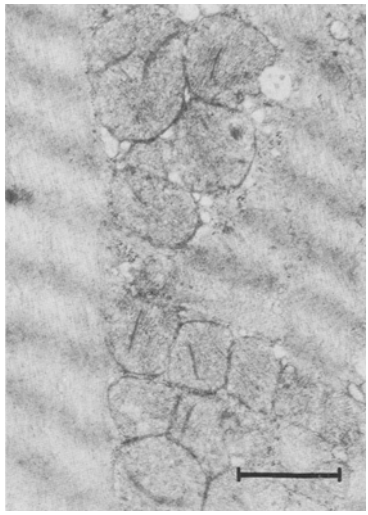

Fig. 23. Mouse ventricle muscle strips incubated at 37°C with 10^{-4} M DNP for 25 min and then for a further 25 min in 10^{-4} M DNP plus 6.25 μg/ml A23187. Note blurring of Z-lines, myofilament degradation, and the development of electron-dense material within some of the mitochondria. Bar = 2 μm.

mitochondria can be clearly seen; other parts of the muscle apparently show swollen chains of mitochondria similar to those in Fig. 22. It is possible, therefore, that Fig. 24 represents an early stage in the swelling and subdivision of the organelles which leads to the picture shown in Fig. 22. Cardiac muscle is shown in Fig. 23; the development of internal electron-dense material is shown in many of the mitochondria in the muscle treated with both DNP and A23187, and the myofilaments are heavily contracted.

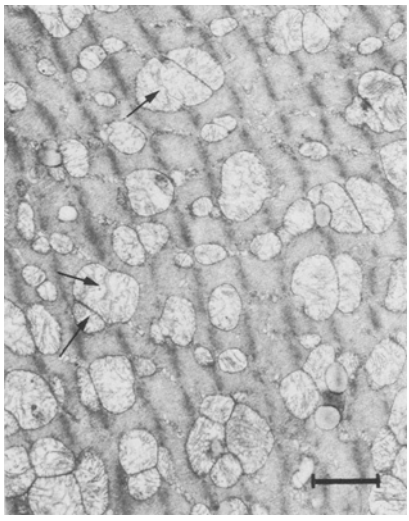

Fig. 24. Mouse diaphragm muscle exposed to 10 mM caffeine for 40 min at 37°C. Muscle fiber is heavily contracted with Z-line blurring. Septa can be detected in many of the mitochondria, and the arrows indicate two mitochondria which appear to be subdividing into three. Bar = 2 μm.

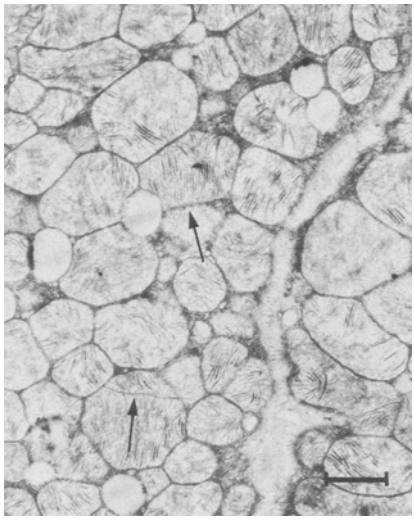

Fig. 25. Mouse diaphragm treated with 10^{-4} M DNP for 30 min at 37°C. Many of the mitochondria show developing septa. Bar = 1 μm.

Discussion

The presence of septa apparently subdividing mitochondria has been widely reported, particularly in muscle and liver cells (see reviews, Tandler and Hoppel [1, 13], and the introduction to this paper), and electron-micrographs have been presented which illustrate a possible sequence in the development of such septa, both by lateral infoldings of the mitochondrial membrane and by the apparent internal formation of a subdividing membrane [4]. However, the significance of such septa is still not clear. The possibility that they are fixation artifacts, dependent on such factors as differential rates of penetration of the fixative in different parts of the organelle, so resulting in one section of the mitochondrion being in the condensed and the other in the orthodox configuration, has been considered by Wakabayashi et al. [2]. The results of their study suggest that septa cannot be explained in this way, particularly in isolated mitochondria. Two possible processes that may be involved in the formation of apparently subdivided mitochondria are the fusion and fission of the organelles, and a number of suggestions have been made in this regard:

(1) In many partitioned mitochondria, the two sets of cristae on opposite sides of the septum were positioned at right angles [1].
(2) In mitochondrial fusion, the adjacent organelles are believed to become interlocked by protrusions which fit into recesses in their neighbors, whereas partitioned mitochondria have straight or gently curved septa and show no obvious interlocking [1].

(3) Suspensions of *isolated* mitochondria from beef heart showing septa have the inner membranes on one side of the partition in the condensed configuration and those on the other side in the orthodox configuration; when found *in situ,* however, the cristae are in the orthodox configuration on both sides of the septum [2].

Tandler and Hoppel [13] have reviewed the studies concerning mitochondrial biogenesis and they conclude that the evidence is consistent with the hypothesis that new mitochondria arise by steady growth and division of preexisting mitochondria.

The electron micrographs shown in the present study do not help to resolve the problem of whether mitochondria showing an apparent septum are dividing or are merely the result of fusion, consequent upon the swelling of the organelles. However, the results do show the following:

(1) Septa can be produced quite readily in mitochondria from skeletal and cardiac muscle *in situ* by a variety of experimental techniques.
(2) The septa develop very quickly (within 30–45 min), in contrast with previous studies where the mitochondria were found in tissues taken from animals maintained on various dietary regimes lasting several weeks.
(3) When A23187 is used to induce mitochondrial division *in situ* in cardiac muscle, both types of subdivided mitochondria can be found in the same cell; the two halves can be in either the same (Figs. 11 and 12) or different (Figs. 13 and 14) configurational states. Prior to these findings, the subdivision into two different energetic configurations has only been described in isolated mitochondria from liver, heart, brown fat, and adrenal cortex; the frequency of dividing mitochondria in such suspensions was 0.3–0.5% [2].
(4) Many of the mitochondria in these treated muscles show electron-dense inclusions, in some of which an organized membrane structure can be detected (Figs. 1, 2, 6, 7, 10, 13, 17, 22, and 23) and we have suggested that these structures may represent early stages of one method by which internal septa may form [4]. Similar paracrystalline and "rodlike" structures have been described in mitochondria from mammalian diaphragm muscle following postmortem aging [14] and in hypoxic organ culture [15]. Another mechanism of septum formation appears to be by the development of infoldings (Figs. 8, 15, 16, 20, and 22).

Our studies therefore do not demonstrate mitochondrial division *in situ* unequivocally, but the findings are consistent with the hypothesis that a variety of experimental techniques can probably rapidly induce division of these organelles in skeletal and cardiac muscle.

However, it is difficult from these studies to determine the precise trigger for the rapid development of mitochondrial septa. Only two features that the various treatments may have in common can be suggested: (1) an interference, either directly or indirectly, with normal mitochondrial functioning; (2) an alteration in free Ca^{2+} levels in the muscle cell. In some instances this is manifested by an accompanying contraction, degradation, and dissolution of the myofilaments [5, 6, 11, 12, 16]. Ca^{2+} may enter from outside the cell, be released from the S.R. or from the mitochondrion, or mitochondrial uptake may be impaired.

Thus, the divalent cation ionophore A23187 (Figs. 11–15, 22, 24) promotes a rise in muscle $[Ca^{2+}]_i$, as shown by the development of tension prior to myofilament dissolution, the Ca^{2+} being released from intracellular sites [11]. Caffeine (Fig. 20) probably promotes Ca^{2+} release from the S.R. [12]; the main action of lindane (Figs. 1–5) in promoting mitochondrial septation is probably in promoting Ca^{2+} entry since it is ineffective in the absence of extracellular Ca^{2+} [5]. DNP (Figs. 16, 17, 25) is a mitochondrial uncoupler while ruthenium red (Fig. 19) inhibits Ca^{2+}-uptake by the mitochondria. Postmortem aging [14], hypoxia [15], and degeneration in skeletal muscle [17–19] are all conditions where similar mitochondrial changes can be detected and where mitochondrial efficiency will be impaired.

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