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THE ROLE OF THE CYTOSKELETON IN THE REGULATION OF STEROIDOGENESIS

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Summary—The slow step in steroid synthesis involves the transport of cholesterol from lipid droplets in the cytoplasm to the first enzyme in the pathway—the cytochrome *P*450 that converts cholesterol to pregnenolone (*P*450scc) which is located in the inner mitochondrial membrane. ACTH stimulates this intracellular transport of cholesterol in adrenal cells (Y-1 mouse adrenal tumour cells and cultured bovine fasciculata cells) and this effect of the trophic hormone is inhibited by cytochalasins, by anti-actin antibodies and DNase I suggesting that the response to ACTH requires a pool of monomeric (G-) actin that can be polymerized to F-actin. Recent studies have shown that lipid droplets and mitochondria of adrenal cells are both attached to intermediate filaments. Moreover ACTH reorganizes the cytoskeleton and changes the shape of the cell. These observations suggest a mechanism for transport of cholesterol that involves reorganization and contraction of actin microfilaments which may, in turn, cause movement of droplets and mitochondria together through their common attachment to intermediate filaments.

In attempting to understand the mechanism by which ACTH and LH stimulate steroid synthesis in their respective target organs, we began by considering which step(s) in the pathway is (are) stimulated by these hormones. When ACTH acts on Y-1 mouse adrenal tumour cells, the slowest step in the pathway is the conversion of cholesterol to pregnenolone (called side-chain cleavage) [1] which takes place in mitochondria. However isolated mitochondria from cells stimulated by ACTH do not show an increase in the production of pregnenolone in spite of a great increase in steroid synthesis produced by ACTH in the cells from which the mitochondria are prepared [1]. This suggested that the slow step specifically stimulated by ACTH is before side-chain cleavage. The only step before this reaction when stores of cholesterol are available is the transport of cholesterol from lipid droplets, where it is stored in part as ester, to the inner mitochondrial membrane where the side-chain cleavage enzyme (cytochrome *P*450scc) is located. A similar conclusion was reached by Garren *et al.* [2] using a different approach. The response of Leydig cells to LH showed similar characteristics [3]. Certainly it is a strange situation for an enzyme to acquire its substrate from a distant location—in this case lipid droplets outside the mitochondrion. In considering the nature of this step of intracellular transport it seemed likely that it involved the cytoskeleton and in particular microfilaments which are potentially contractile and subject to rapid reorganization. Moreover the response to ACTH in Y-1 cells involves an intense rounding of the cells which must involve a radical rearrangement of the cytoskeleton [4]. Other evidence for these views and further studies of the transport of steroidogenic cholesterol are presented by Jefcoate *et al.* [5] elsewhere in this volume.

To test the possible involvement of microfilaments in the response to ACTH we used various cytochalasins and found that these agents inhibit the steroidogenic responses to ACTH and cyclic AMP [Fig. 1(A)] [6, 7]. Although numerous control experiments supported the idea that the effect of the inhibitor was specific and although inhibition was rapidly and completely reversed by removing cytochalasin, more direct evidence for involvement of actin was necessary. When well characterized polyclonal antibodies to actin were injected into Y-1 cells by means of liposomes, the response to ACTH was inhibited and once again inhibition was exerted specifically at the step of cholesterol transport [Fig. 1(B)] [8]. These findings showed that inhibition was specific for actin but they left open the question of how the antibody acts

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and how much anti-actin was required to cause inhibition. These questions were answered by injecting DNase I into Y-1 cells by way of red cell ghosts [9]. The distribution of DNase and the amount injected were determined by labelling the enzyme with FITC (fluorescence microscopy) or with ^{125}I. These studies showed that DNase I also inhibits the effect of ACTH on cholesterol transport [Fig. 1(C)], that the effect is extranuclear i.e. not the result of an effect on DNA and that 50% inhibition was obtained by injecting 10^7 molecules of DNase I per cell. Evidently from the well known mechanism by which DNase I binds monomeric or G-actin and prevents polymerization to F or filamentous actin, it would appear that the effect of ACTH on cholesterol transport requires a pool of G-actin—capable of polymerizing to F-actin [9].

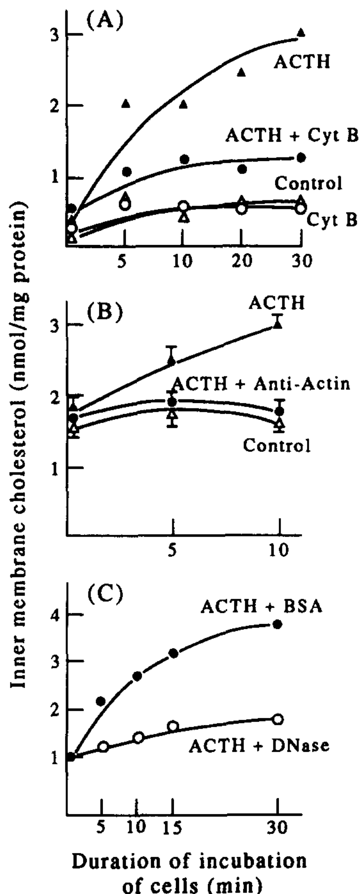

Fig. 1. The influence of actin on stimulation of cholesterol transport by ACTH. Y-1 adrenal cells were incubated with aminoglutethamide (0.76 mm) with and without ACTH (2 pm) and cytochalasin D (10^{-6} M), anti-actin antibodies entrapped in liposomes or DNase I in red cell ghosts. Following incubation mitochondria were isolated and the concentration of cholesterol in the inner mitochondrial membrane was measured relative to protein. Values are means of duplicate determinations.

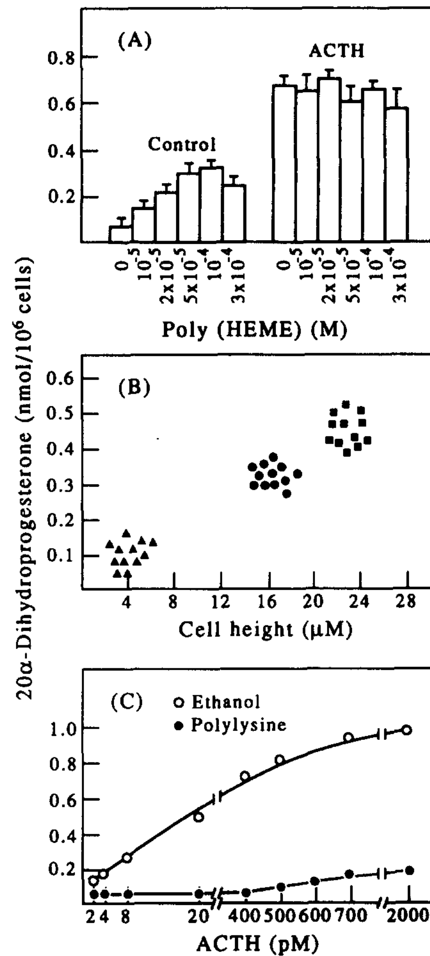

Fig. 2. The effects of poly (HEMA) and polylysine surfaces on the production of steroids by Y-1 cells. (A and B) Cells were grown on plastic dishes treated with ethanol or the concentrations of poly (HEMA) shown. The production of 20α-dihydroprogesterone by the cells was measured in the absence and presence of ACTH (A). (B) shows correlation between steroid production and cell height. (C) Cells were grown on dishes treated with ethanol or poly-D-lysine with and without ACTH. The production of 20α-dihydroprogeserone was measured. Values are means of duplicate determinations.

Because of the involvement of the cytoskeleton in this response it was important to determine whether or not calmodulin influenced the response to ACTH. Trifluoperazine was shown to inhibit the acceleration of cholesterol transport produced by ACTH and injection of calmodulin into Y-1 cells by liposomes stimulates this transport in the absence of ACTH [10].

To gain further insight into the role of the cytoskeleton in steroid synthesis, we grew Y-1 cells on poly (HEMA) and on poly-D-lysine. Poly (HEMA) is a polymer of methacrolate that can be used to cover positive charges on plastic culture dishes. This has the effect of limiting attachment of cells to the dish and hence

causing the cells to grow in rounded form. Poly-D-lysine causes the opposite effect i.e. cells become tightly bound to the plastic and are unusually flat. The degree of rounding of cells can be measured by determining cell height with a radiofrequency transducer attached to the stage of the microscope. Cells grown on poly (HEMA) show enhanced steroid synthesis without addition of ACTH [Fig. 2(A)] and this increase in production of steroids is closely correlated ($r = 0.92$) with the degree of rounding [Fig. 2(A and B)] [11]. Polylysine inhibits steroid synthesis and the response to ACTH [Fig. 2(C)] [11]. The effect of poly (HEMA) is not seen by adding the compound to the incubation medium; cells must be grown on a surface coated with this agent and the effect is exerted on the step involving cholesterol transport [11]. Evidently the shape of the cell influences steroid synthesis; presumably this influence is exerted by way of the cytoskeleton [11]. At this stage it was necessary to turn to microscopy to explore these events in greater detail.

Thin sections of Y-1 cells embedded in epon and examined by transmission electron-microscopy (TEM) show conspicuous lipid droplets 0.2 to 2.0 μm in diameter (Fig. 3). The fact that droplets remain intact through the severe procedures required for TEM is surprising and suggested that the droplets may be protected by a covering or capsule around each droplet. In view of the involvement of the cytoskeleton in cholesterol transport described above, we decided to determine whether the droplets are associated with the cytoskeleton. When Y-1 cells are extracted *in situ* i.e. on gold grids, with Triton X-100 1% for 10 min,

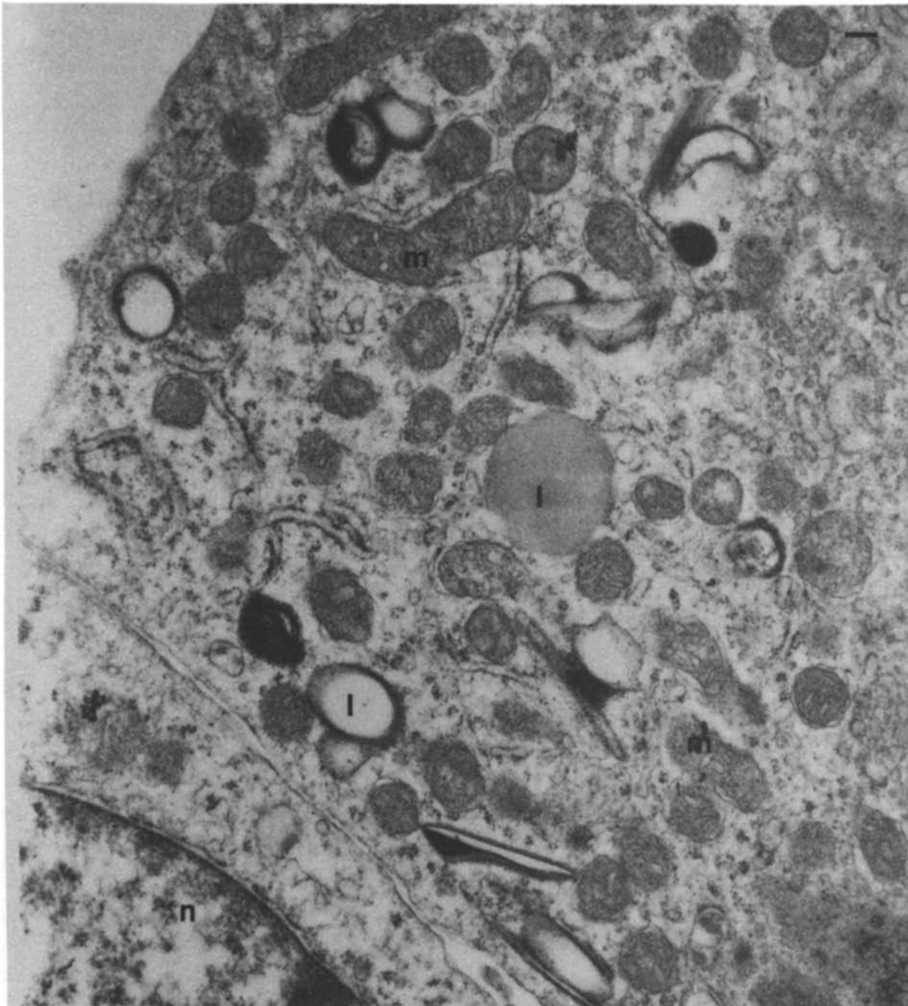

Fig. 3. TEM of Y-1 adrenal cell. Thin section *en face* was prepared from cells cultured as a monolayer. 1: lipid droplet; m: mitochondrion; n: nucleus. Bar: 0.25 μm.

a cytoskeleton remains and can be examined as a whole mount by freeze drying without sectioning. Such whole mounts retain lipid droplets of the same size as those seen in TEM [Fig. 4(A)] [12]. Moreover, if the cytoskeletal whole mounts are treated with ammonium sulphate to produce intermediate filaments by removing microfilaments and microtubules, lipid droplets remain bound to these filaments [Fig. 4(B)]. Tilting the sample (−18 to +18°) shows that the droplets are bound directly to the filaments [12]. In view of the extensive extraction and washing procedure used in such preparations, we can conclude that lipid droplets are very tightly bound to intermediate filaments. The filaments can be identified by measurement of diameter (10 nm) and by immunoelectron microscopy using anti-vimentin followed by protein A conjugated to gold particles [12]. The co-localization of lipid droplets and intermediate filaments was confirmed by fluorescence microscopy using nile red to stain lipid droplets and anti-vimentin with second antibody conjugated to FITC to stain intermediate filaments [12]. In addition fluorescence microscopy reveals a complete capsule containing vimentin surrounding each droplet [12]. To exclude artefacts arising from the processes

Table 1. Cholesterol ester in fractions of adrenal cells

	Cholesterol ester (nmol/10^6 cells)
Whole cells	6.2 ± 0.3
Extract 1	1.9 ± 0.5
Residue 1 (cytoskeleton)	3.8 ± 0.9
Extract 2	1.8 ± 0.5
Residue 2 (IF)	2.0 ± 0.8
Extract 3 (ethanol)	1.7 ± 0.3
Residue 3	0.2 ± 0.1

Y-1 cells were extracted with Triton X-100 to give extract 1 and cytoskeleton which was treated with $(NH_4)_2SO_4$ to give extract 2 and intermediate filaments (IF). The filaments were in turn extracted with ethanol to give extract 3 and extracted filaments. Values are means ± SE where $n = 6$.

of extraction and preparation for electron microscopy, we have examined unextracted and mildly extracted adrenal cells by electron microscopy and immunofluorescence. These studies confirmed the work discussed above with whole mounts [13].

We have found two ways of removing lipid droplets from intermediate filaments namely complete extraction by means of ethanol (which allows direct measurement of cholesterol and cholesterol ester in the extract) and by 6 M urea which removes intact droplets [12]. The extracted filaments remain as naked fibres from which various proteins have been stripped by extraction [Fig. 4(C)]. It is, generally agreed

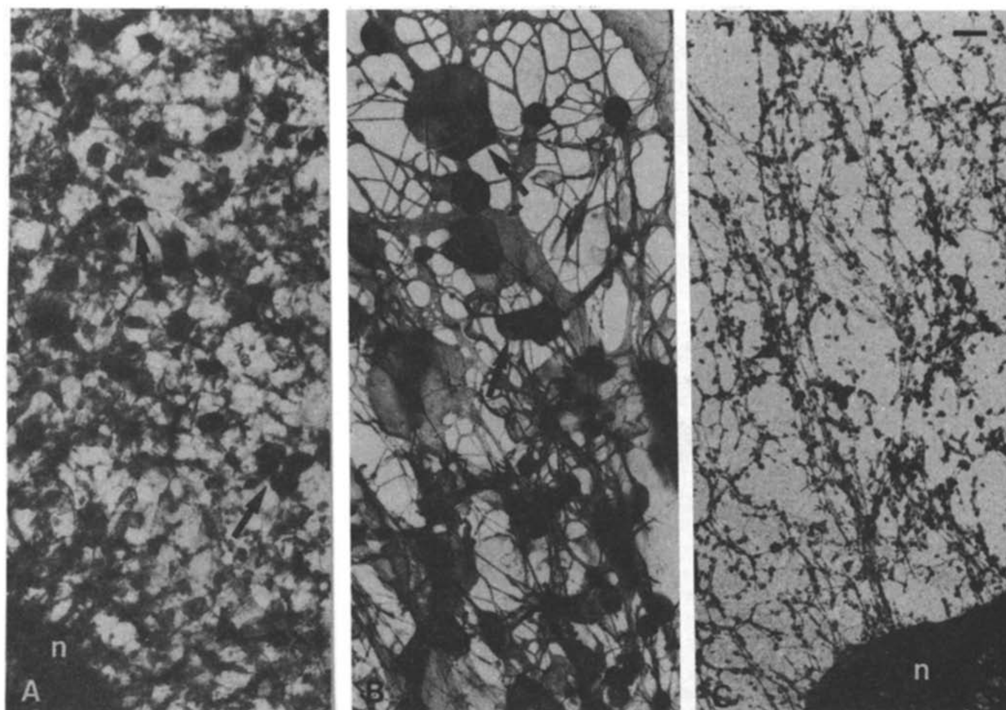

Fig. 4. TEM of adrenal cell cytoskeleton. Y-1 cells were grown on gold grids and whole mounts of cytoskeletons and intermediate filaments were prepared. (A) cytoskeleton, (B) intermediate filaments and (C) intermediate filaments extracted with ethanol. n: nucleus; large arrows: lipid droplets. Bar: 0.35 μm.

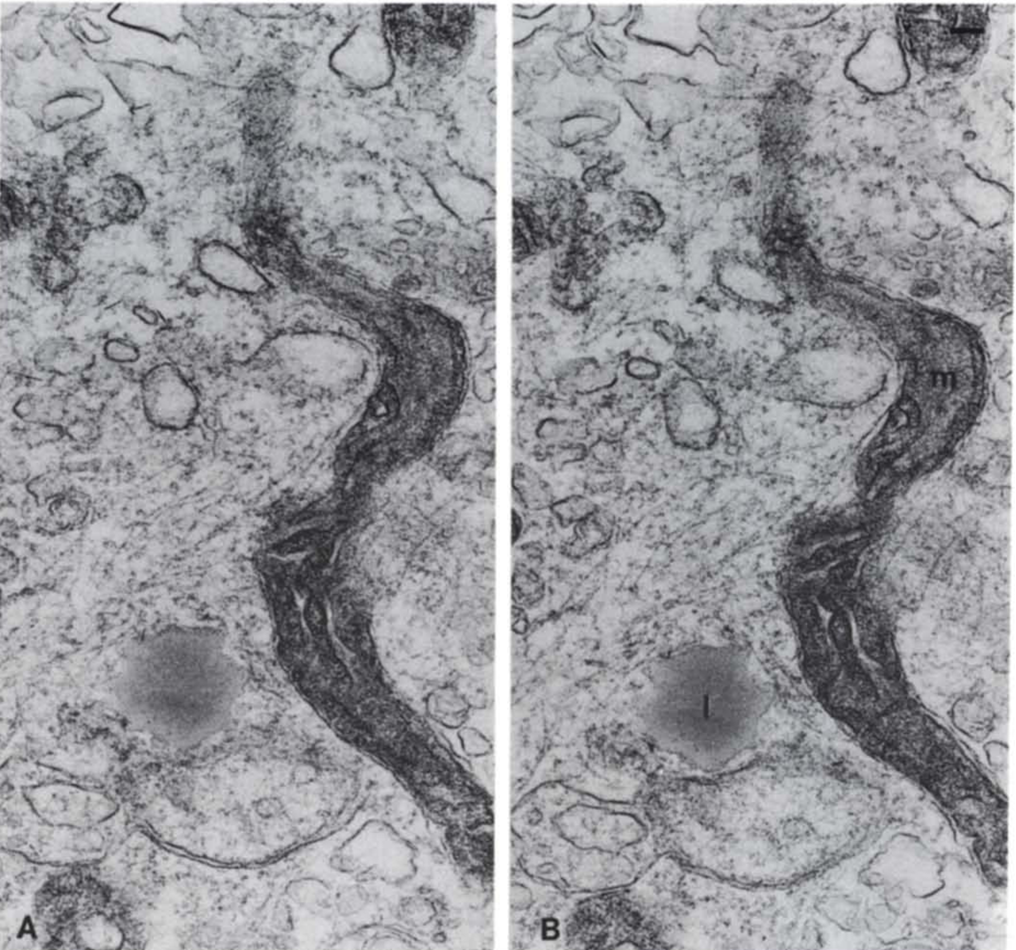

Fig. 5. TEM of adrenal cells subjected to lysis. Bovine fasciculata cells were subjected to osmotic lysis on culture dishes. The broken cells were fixed and prepared for electron microscopy. The figure shows stereo pairs A = +12°, B = −12°. l: lipid droplet; m: mitochondrion. Bar: 0.11 μm.

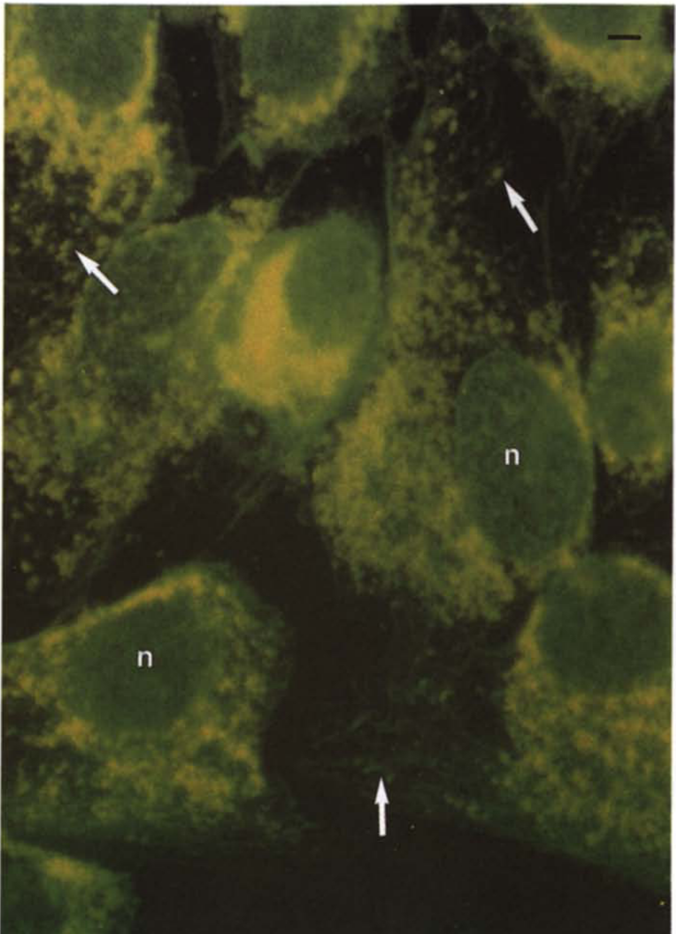

Fig. 6. FITC of Y-1 cells. Cells were subjected to Triton extraction 0.5% for 5 min, fixed and processed for double indirect immunofluorescence (see text). Arrows show co-localization of mitochondria with intermediate filaments. n: nucleus. Bar: 2.5 μm.

that cholesterol ester serves as a reliable marker for lipid droplets in the adrenal cortex [13]. Cholesterol ester was measured in Y-1 cells at each stage of the extraction procedure described above (Table 1). It will be seen that approximately one-third of the droplets are removed during the Triton extraction (extract 1) so that the cytoskeleton (residue 1) contains about two-thirds of the droplets tightly bound. These droplets are almost equally divided between the intermediate filaments (residue 2) and the ammonium sulphate extract (extract 2). Ethanol (extract 3) removes most of the remaining droplets (Table 1). Evidently there are at least three fractions of droplets: free or unbound droplets loosely bound in the cell and removed by Triton, droplets bound to the cytoskeleton and finally droplets remaining tightly bound to intermediate filaments. It appears that about half of the cytoskeletal droplets are either bound to cytoskeletal structures other than intermediate filaments or are bound more loosely to the filaments than the fraction that remains with the filaments after treatment with ammonium sulphate. It should be noted that, as expected, values for residue 1 are approximately equal to values for residue 2 plus extract 2 and that values for residue 2 are approximately equal to those for extract 3 plus residue 3.

The use of whole mounts proved helpful in these studies since these preparations yield three dimensional information. However it is possible, although very unlikely, that the binding of lipid droplets to intermediate filaments is artefictitious. To eliminate this possibility we have made thin sections of cultured adrenal cells broken by osmotic lysis (Fig. 5). This procedure produces minimal disruption of the cytoskeleton and preserves the *in vivo* distribution of organelles. Intermediate filaments can be seen attached to droplets in TEM (Fig. 5) [13]. Clearly this attachment cannot be attributed to artefact [13].

If the cytoskeleton plays an important role in the movement of lipid droplets to mitochondria and if some lipid droplets are tightly bound to intermediate filaments, it becomes necessary to understand the relation between mitochondria and cytoskeleton. By using mild extraction of adrenal cells (Triton 0.5% for 5 min) it is possible to extract enough cytoplasm and cell membranes to clear the cell and reveal the underlying cytoskeleton without removing mitochondria. Under these conditions it can be shown by electron microscopy that mitochondria are attached to intermediate filaments (Fig. 5). By using four antibodies, raised in three species, in immunofluorescence microscopy, the co-localization between mitochondria and intermediate filaments can be demonstrated in one photograph [14]. In Fig. 6 mitochondria are stained with rabbit anti-cytochrome oxidase and donkey anti-rabbit IgG (Texas red) and intermediate filaments by goat anti-vimentin and rabbit anti-goat IgG (FITC). Rabbit anti-goat IgG not only binds to goat anti-vimentin but it is also recognized by donkey anti-rabbit IgG. As a result both the mitochondria and intermediate filaments can be seen with the same filter. Co-localization is apparent on examination of individual mitochondria (Fig. 6). These studies have been accompanied by appropriate controls including normal IgG, antibodies pre-absorbed with antigen and no first antibody (not shown) [14].

To confirm these findings with fluorescence microscopy, electron microscopy of homogenized adrenal cells was performed (Fig. 7). It can be seen that intermediate filaments make direct contact with mitochondria [13]. Moreover we have recently shown that both lipid droplets and mitochondria are directly attached to intermediate filaments using stereo pairs with TEM [15].

All of the above findings have been made in both Y-1 adrenal cells and primary cultures of bovine fasciculata cells [12–14].

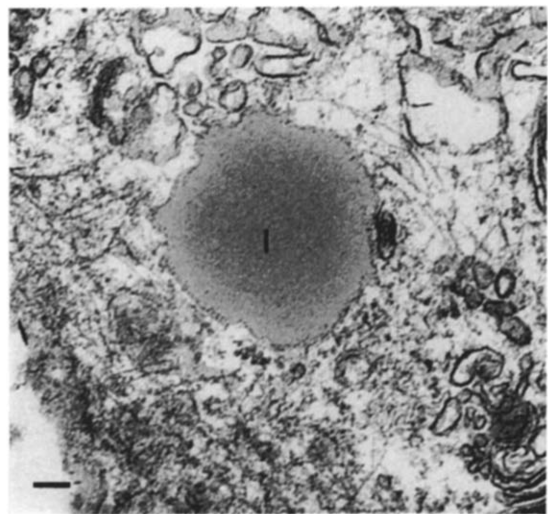

Fig. 7. TEM of adrenal cells subjected to osmotic lysis and processed for electron microscopy as a monolayer. Samples were photographed after tilting from +12 to −12. Bar: 0.125 μm.

DISCUSSION

The cytochrome *P*450 that catalyses the conversion of cholesterol to pregnenolone—a process called side-chain cleavage (scc) has been isolated by evolution in the inner mitochondrial membrane remote from the cytoplasmic stores of its substrate cholesterol which exists as cholesterol ester in cytoplasmic lipid droplets. The result of this separation is that an elaborate but regulated process of intracellular transport must bring substrate to enzyme. Not surprisingly perhaps the transport step is rate-limiting for the synthesis of steroids [1, 2]. It is clear that the cytoskeleton and in particular microfilaments and intermediate filaments, are involved in the transport.

Microfilaments could use three of their well known properties in the transport process namely: microfilaments are vectorial since they exhibit a defined direction; they have polarity because the two ends of the monomeric (G-actin) are distinct and must fit into the polymer (F-actin) always in the same direction so that the pointed ends are all aligned in the same direction and finally in the presence of myosin microfilaments are potentially contractile. Intermediate filaments on the other hand are not contractile and may provide the fundamental skeletal backbone of the cell. It would, however, be a mistake to think of intermediate filaments as fixed or immutable structures. They are capable of turnover, breakdown and reorganization although we do not yet understand the significance or the mechanism of such changes [17]. In any event the fact that the two chief players in these events (lipid droplets and mitochondria) are attached to the same structures is important. This organization may facilitate the coming together of these two structures although analogies to railway tracks etc are premature and dangerously seductive. Intermediate filaments whatever else they may be are not contractile. It is here that actin and microfilaments may come to the rescue by providing, with myosin, a contractile element to make use of the mutual attachment of droplets and mitochondria to a common cable and in this way bring droplets to mitochondria or perhaps vice-versa. The evidence is still incomplete but the possibilities are tantalizing. The cultured adrenal cell rounds up under the influence of ACTH. Clearly the cytoskeleton undergoes a major upheaval and peripheral cellular processes are retracted. Isometric tension in stress fibres is relaxed and actin depolymerizes to allow the cell to resume its primordial or rounded shape [18]. Meanwhile polymerization of actin monomers to give rise to new microfilaments must occur if steroidogenesis is to be accelerated. Forcing the cell to grow rounded stimulates this process while securing the cell in spread-out and flattened form prevents the steroidogenic pathway from expressing the stimulation normally produced by ACTH. In fact an essential step in the process of stimulation involves a gathering of organelles around the nucleus [19]—a process that occurs in other cells and is referred to as centralization e.g. in platelets [20]. This process of centralization requires microfilaments and brings organelles closer together. Such a process is triggered by ATP in fibroblasts where centralization was recently shown to involve radical reorganization of intermediate filaments under the influence of ATP [21]. In preliminary studies, we have found that injection of antibodies to smooth muscle light chain inhibits the steroidogenic responses to ACTH (unpublished). This finding is compatible with the involvement of actomyosin in these responses which could account for movement of droplets and other cellular structures in response to ACTH.

These processes have been presented in the adrenal cell and have all been repeated in Leydig cells where the findings are essentially identical. In Leydig cells the evidence for involvement of actin [22, 23] and Ca^{2+} in the response to LH has been published and it is known that lipid droplets are associated with intermediate filaments in several species [24]. The evidence is less complete because of technical problems but there is every reason to believe that the findings with adrenal cells apply to other steroidogenic tissues [25]. For example, studies in the ovary are consistent with the evidence presented here [26]. Again, we have recently established that direct contact between both droplets and mitochondria on the one hand and intermediate filaments on the other hand can be seen in stereo pairs of electron micrographs of Leydig cell cytoskeletons [27].

In considering the role of the cytoskeleton in steroidogenesis association between other components of the system may prove important e.g. microtubules and intermediate filaments [28]. In non-steroidogenic cells mitochondria have been shown to be associated with intermediate filaments [29].

All of these observations have been made *in vitro*. It is notoriously difficult to study the

cytoskeleton *in vivo*. Moreover, although rounding of cells in culture is instructive in revealing the need for the rounded form of the cell for effective cholesterol transport, the flattened shapes of cultured cells are abnormal and rounding up has no counterpart *in vivo* where the cells are already in a rounded form. The nearest approach to *in vivo* conditions is provided by electron microscopy of unextracted cells in which the fixation procedure is believed not to introduce artefacts. This method in which the cell is far from the *in vivo* state enabled earlier workers to make important observations about intermediate filaments and microfilaments in Leydig cells that are compatible with findings presented here [30, 31].

In conclusion, the complexities of cholesterol transport are far from completely resolved. For example, cholesterol may serve as a precursor of steroids without incorporation into droplets. A microvescular system is the most likely explanation for the transport of such cholesterol [32] and the cytoskeleton is likely to be required for the vectorial transport of such microvesicles. When low density lipoprotein serves to deliver cholesterol to steroidogenic cells it is de-esterified in lysosomes before it appears in lipid droplets en route to mitochondria [33]. This process involves re-esterification and a further de-esterification. Where the final release of free cholesterol occurs remains uncertain but lipid droplets contain free cholesterol [13]. In addition, carrier proteins are involved in cholesterol transport. For example endozepine is required for transport within mitochondria [34]. The exact function of other proteins that act in connection with transport of steroidogenic cholesterol e.g. sterol carrier protein 2 remains to be determined [35].

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