



The Roles of Microfilaments and Intermediate Filaments in the Regulation of Steroid Synthesis

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Much of the cholesterol used in steroid synthesis is stored in lipid droplets in the cytoplasm of steroid-forming cells. The cholesterol ester in these droplets is transported to the inner mitochondrial membrane where it enters the pathway to steroid hormones as free cholesterol—the substrate for the first enzyme, namely $P450_{\text{sec}}$. It has been shown that this transport process governs the rate of steroid synthesis and is specifically stimulated by ACTH and its second messenger. The stimulating influence of ACTH on cholesterol transport is inhibited by cytochalasins, by monospecific anti-actin and by DNase I demonstrating that the steroidogenic cell must possess a pool of monomeric actin available for polymerization to F actin if it is to respond to ACTH and cyclic AMP. It has been shown that the two structures involved in cholesterol transport (droplets and mitochondria) are both bound to vimentin intermediate filaments in adrenal and Leydig cells. In addition these filaments are closely associated with the circumferential actomyosin ring in which they are crosslinked by actin microfilaments. In permeabilized adrenal cells Ca^{2+} /calmodulin phosphorylates vimentin and this change is known to disrupt intermediate filaments and to cause contraction of actomyosin by phosphorylating myosin light chain kinase. Ca^{2+} /calmodulin stimulated cholesterol transport and steroid synthesis and causes rounding of the responding cells by contraction of the actomyosin, if ATP is also added at the same time. Other agents that disrupt intermediate filaments include anti-vimentin plus ATP in permeabilized cells which also results in rounding of the cell. Acrylamide exerts a similar effect in intact adrenal cells and in addition causes rounding of the cells and increase in steroid synthesis without increase in cyclic AMP. It is also known that if adrenal cells are grown on surfaces treated with poly(HEMA), the cells grow in rounded form and steroid synthesis is increased in proportion to the degree of rounding ($r = 0.92$). This response does not involve increase in cellular levels of cyclic AMP. It is proposed that *in vivo* where the cell is always round and cannot show more than strictly limited change in shape, ACTH activates Ca^{2+} /calmodulin possibly by redistributing cellular Ca^{2+} . Ca^{2+} /calmodulin in turn promotes phosphorylation of vimentin and myosin light chain. The first of these phosphorylations shortens intermediate filaments and the second promotes contraction of the actomyosin ring with internal shortening and approximation of lipid droplets and mitochondria. Details of the earlier events (activation of Ca^{2+} /calmodulin) and later changes (transfer of cholesterol to the inner membrane) remain to be elucidated. It is clear however that the action of ACTH requires increase in cellular cyclic AMP. These experimental responses bypass this step in the response to ACTH.

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INTRODUCTION

When it became apparent that the stimulating influence of ACTH and its second messenger on the rate of steroid synthesis was exerted at some step(s) in the

pathway before the conversion of cholesterol to pregnenolone in mitochondria, the possibility that such stimulation involves increased transport of cholesterol to mitochondria, was considered [1a]. This idea was finally established by the direct measurement of the transport process *in vitro* [1b, 2] and later confirmed *in vivo* [3]. It is now clear that this step is made up of several steps, i.e. transport to mitochondria, transport

within mitochondria (possibly from outer to inner membrane) and movement within the inner membrane [4]. It was further found that transport to mitochondria requires actin microfilaments [1b, 2], while transport within the mitochondrion requires synthesis of new protein [5, 6]. How actin microfilaments produce such transport was at first unclear. However it is known that ACTH and cyclic AMP cause adrenal cells to undergo extreme rounding and this is presumed to result from a contractile event involving the cytoskeleton.

It has been known for some time that steroidogenic cholesterol is stored in conspicuous lipid droplets as cholesterol ester [7]. The cholesterol appears in the inner mitochondrial membrane as the free sterol where it is converted to pregnenolone [8]. Although the exact mechanism of the transport of cholesterol is not understood, it has been suggested that under the influence of ACTH, lipid droplets move to and touch mitochondria (so-called docking) [9]. The need for an enzyme to acquire supplies of substrate from outside the organelle in which it is located, remains one of the remarkable features of the steroidogenic pathway. In the meantime it is considered that in the adrenal cortex, cholesterol ester serves as a reliable marker for lipid droplets [10]. In some steroidogenic cells cholesterol is made from acetate and is not stored in droplets to more than a limited extent. The mechanism by which this newly synthesized cholesterol is moved to mitochondria is not known.

ATTACHMENT OF LIPID DROPLETS TO INTERMEDIATE FILAMENTS

If microfilaments are necessary for transport of cholesterol to mitochondria, it seemed appropriate to look for an association between droplets and the cytoskeleton. Bovine fasciculata cells were cultured on gold grids and extracted with Triton X-100 to yield a whole mount of the cytoskeleton (i.e. the full three dimensional thickness of the cytoskeleton) which was examined with transmission electron microscopy. Lipid droplets were present in large numbers [11]. Since in some of these preparations microtubules were not preserved, it was concluded that droplets were attached either to microfilaments or to intermediate filaments or to both. When the cytoskeletons were treated with ammonium sulphate to give a preparation of intermediate filaments [12], actin and any remaining tubulin were removed [11] leaving a nuclear cytoskeleton together with intermediate filaments. In these whole mounts, lipid droplets were still present in large numbers [11]. Clearly at least some of the lipid droplets are tightly associated with intermediate filaments and direct physical contact between droplets and filaments was demonstrated by tilting thin sections and viewing stereopairs [11, 13]. The major protein in the intermediate filaments was identified as vimentin by immunoelectron microscopy [11].

Immunofluorescence using Nile red to stain the droplets and anti-vimentin in indirect immunofluorescence, demonstrated the co-localization of droplets and vimentin intermediate filaments. Moreover, it became clear that all the droplets present were closely associated with filaments [13, 14]. In addition, immunofluorescence revealed that the droplets are surrounded by a capsule containing vimentin. It is likely that this capsule protects the lipid contents of the droplets from the various agents used in making these preparations. Moreover, the close association between droplets and filaments was confirmed by thin section electron microscopy in which the samples were not extracted, thereby excluding the unlikely possibility that this association results from an artefact of extraction [13].

To investigate the recovery of the droplets through these procedures, it was found that these structures can be removed quantitatively from the filaments by means of ethanol or urea. Using cholesterol ester as a marker for lipid droplets, it was found that approximately one third of these droplets were removed from the cell by extraction with Triton X-100 (1%, 5 min), one third was removed by conversion of the resulting cytoskeleton to intermediate filaments by ammonium sulphate and one third was extracted from the intermediate filaments by ethanol [11]. It would appear that one third of the droplets is either free or loosely attached to some cellular structure, one third is either associated with microfilaments or is loosely associated with intermediate filaments and one third is tightly associated with intermediate filaments.

ATTACHMENT OF MITOCHONDRIA TO INTERMEDIATE FILAMENTS

If lipid droplets or their contents are transported to mitochondria and if lipid droplets are tightly attached to intermediate filaments it was clearly important to learn how mitochondria are related to the cytoskeleton. Since these organelles are so much more fragile than intermediate filaments it is possible that they are not seen in preparations of cytoskeleton and intermediate filaments because they are destroyed by the Triton used during the procedures described above, not because they are free within the cytoplasm.

Clearly more gentle methods of disrupting the cell were required if this problem were to be approached. Three methods were used for this purpose namely, osmotic lysis, homogenization and mild extraction with Triton. Following these procedures cell residues were pelleted for the preparation of thin sections and examination by transmission electron microscopy. These procedures removed sufficient cell membranes and other opaque structures to permit penetration of the electron beam thereby revealing association of mitochondria with intermediate filaments [14]. Direct

physical contact between these structures was demonstrated by tilting the samples and stereopairs [14].

Furthermore indirect immunofluorescence with anti-cytochrome oxidase and anti-vimentin revealed co-localization of cytochrome oxidase and vimentin [14]. In this study a method of double indirect immunofluorescence was used which was called 4:3 (four antibodies, three species) [15]. This method allowed two antigens to be examined with a single light filter which facilitates demonstration of co-localization. In the present case the method not only revealed co-localization of mitochondria and intermediate filaments but also revealed that all mitochondria were associated with filaments—where there were no filaments there were no mitochondria and vice-versa. Again, stereopairs revealed direct physical contact between these structures. Clearly the association of droplets and mitochondria with intermediate filaments is too intimate and too frequent to be the result of chance. Finally, transmission electron microscopy showed numerous examples of intermediate filaments which were in direct contact with both lipid droplets and mitochondria [16]. These findings were highly significant for understanding the mechanism by which droplets are transported to mitochondria. Although ideas of guide wires and train tracks would be premature, nevertheless intermediate filaments could provide direction for a process in which transport occurs from droplets to mitochondria.

Still the role of microfilaments in this process remained obscure. Since actin is capable of triggering myosin ATP-ase with consequent shortening and since adrenal cells contain abundant myosin, the possibility that the microfilaments cause shortening within the cell was considered. The well known rounding of adrenal cells may provide an example of such shortening. To study this possibility, a method of permeabilizing adrenal cells was developed in order to introduce various substances into the cells. This was achieved by extracting the cells with Triton X-100 (0.5% for 5 min). Such cells can be kept at 37°C for at least 3 h without changing shape. Indirect immunofluorescence in such cells shows that actin and myosin co-localise as a circumferential structure known as the actomyosin ring which is situated just inside the plasma membrane. Vimentin filaments, on the other hand show a radial distribution from the nucleus towards the periphery of the cell where they are joined by circumferential vimentin filaments which run just inside the plasma membrane but outside the actomyosin ring [17].

Transmission electron microscopy revealed three important facts about the distribution of actin, myosin and vimentin namely: (i) vimentin filaments penetrate the actomyosin ring to form a mixed network of actomyosin and vimentin; (ii) some intermediate filaments pass through the actomyosin ring and make direct contact with the plasma membrane; and (iii)

numerous microfilaments crosslink two or more intermediate filaments [17]. Evidently intermediate filaments are in intimate contact with the actomyosin ring and with the plasma membrane. Contraction of the ring would gather the contents of the cell in a spherical form bringing the attached vimentin filaments and the plasma membrane with it. Presumably this is what happens when the cell rounds.

These observations gave rise to a testable hypothesis, namely that the intermediate filaments keep the cultured cell in its extended form when it is unstimulated. Work in fibroblasts by Hollenback *et al.* [18] showed that permeabilized cells undergo an ATP-dependent and actin-dependent rounding accompanied by collapse of intermediate filaments. Presumably the ATP causes contraction of the actomyosin ring which in turn disrupts intermediate filaments.

We have used three agents to collapse intermediate filaments namely: (i) anti-vimentin; (ii) Ca^{2+} /calmodulin; and (iii) protein kinase C. In addition, Shiver *et al.* [19] used acrylamide for the same purpose.

(i) Permeabilized bovine fasciculata cells were treated with a monoclonal anti-vimentin directed towards the NH_2 terminus of the molecule. When ATP was added, the cells began to round up slowly after 60 min. When anti-vimentin alone is added no change in shape occurs but when both anti-vimentin and ATP are added together, the cells round up (beginning after 15 min) and the rounding becomes intense. Gawlitta *et al.* [20] and Klymkowski *et al.* [21] showed that in other cells anti-vimentin causes the entire intermediate filament population to collapse in a cap around the nucleus.

(ii) Ca^{2+} /calmodulin. It was pointed out above that Ca^{2+} /calmodulin stimulates cholesterol transport and steroid synthesis in adrenal [22] and Leydig cells [2]. When Ca^{2+} /calmodulin is added to permeabilized adrenal cells no change in shape is seen but if ATP is added at the same time, the cells rapidly become rounded and the rounding becomes intense. This rounding is prevented by trifluoperazine, EGTA and SMI (an inhibitor of myosin light chain kinase) [17]. When the permeabilized cells were incubated with [γ - ^{32}P]ATP followed by gel electrophoresis and autoradiography, it was found that Ca^{2+} /calmodulin increased phosphorylation of two proteins namely, vimentin and myosin light chain [17]. Earlier studies had shown that cytoskeletons of adrenal cells contain a tightly bound Ca^{2+} /calmodulin kinase 11 [23]. These findings suggest that the action of Ca^{2+} /calmodulin in promoting cholesterol transport and steroid synthesis involves the cytoskeleton in which phosphorylation of vimentin by the Ca^{2+} /calmodulin kinase would cause

disorganisation of intermediate filaments and phosphorylation of myosin light chain which would promote contraction of the actomyosin ring. These changes would in turn cause rounding of the cell. It has been shown that intermediate filaments are disrupted by phosphorylation with Ca^{2+} /calmodulin [24a].

(iii) Protein kinase C is tightly bound to adrenal cell cytoskeleton and when activated by phorbol esters, Ca^{2+} and phospholipid, this kinase causes rounding of the cytoskeletons [24b]. Moreover protein kinase C is specifically associated with intermediate filaments in other cells. This kinase phosphorylates vimentin and in microglial cells causes redistribution of vimentin and actin filaments [25]. In macrophages phorbol ester causes altered alignment of mitochondria and lysosomes along vimentin filaments [26]. In addition, the activity of protein kinase C is increased by ACTH and cyclic AMP [27, 28].

(iv) When acrylamide is added to Y-1 mouse adrenal tumor cells steroid synthesis is increased. This stimulating action is exerted at a step before side-chain cleavage of cholesterol [19]. Acrylamide is known to alter the distribution of intermediate filaments in Ptk1 cells [29]. Acrylamide also causes rounding of Y-1 cells [19].

These findings serve to bring together the two cytoskeletal elements that are clearly involved in the steroidogenic response to ACTH namely, microfilaments and vimentin intermediate filaments and to account for the role of Ca^{2+} /calmodulin in the regulation of cholesterol transport. It would seem that intermediate filaments promote the extended or relaxed state of the cell which is flat with low steroid output and relaxed actomyosin ring. Ca^{2+} /calmodulin can disrupt intermediate filaments and activate myosin, i.e. cause contraction of the actomyosin ring. These changes will cause the actomyosin ring to gather the cell in a more spherical form and through the association between the two systems of filaments, the intermediate filaments and the plasma membrane will be brought into the rounding process.

Simply growing cells in rounded form on dishes coated with poly(HEMA), is enough to stimulate steroid synthesis without increasing production of cyclic AMP [30]. It is noteworthy that acrylamide produces the same changes—again without increasing levels of cyclic AMP [19]. In this connection it is known that cytoskeletons of adrenal cells contain bound adenylate cyclase [31]. It is clear that more than one kinase can phosphorylate vimentin and hence cause disruption of vimentin filaments [21]. Anti-vimentin has the same effect [20, 21].

It is important to notice that the poly(HEMA) surface and acrylamide do not cause an increase in

cellular levels of cyclic AMP. Evidently these agents short-circuit the need for additional cyclic AMP. ACTH on the other hand must increase levels of the cyclic nucleotide to produce increased transport of cholesterol and rounding. Evidently cyclic AMP achieves, under physiological conditions, what these agents produce by unphysiological means. However poly(HEMA) and acrylamide enable the investigator to dissect those cellular changes which lead to the same end.

In some cells activation of actomyosin is enough to cause rounding, e.g. fibroblasts [18]. In other cases disruption of filaments alone is enough to cause rounding, e.g. Y-1 adrenal cells with acrylamide [19]. In yet other cases both factors appear to be required, e.g. Ca^{2+} /calmodulin in bovine fasciculata cells as reviewed above. These responses have not been fully clarified. Such factors as the extent and the rate of actomyosin contraction may prove important. In the meantime it should be pointed out that at least in the adrenal cell, if the response to any of the factors that cause rounding (ATP alone, anti-vimentin plus ATP, Ca^{2+} /calmodulin plus ATP) is allowed to progress to completion the entire cytoskeleton collapses around the nucleus [17].

CONDITIONS *IN VIVO*

We know of no condition in which anti-vimentin enters adrenal cells *in vivo* and acrylamide is purely an experimental agent. Permeabilized cells are at best mere shadows of intact cells. More important, the cells in an organ are not free to undergo drastic changes in shape. Indeed the flattened shape of the cultured cell is brought about by adjustment to a wholly unnatural surface. Nevertheless these experimental artefacts may reveal possible responses of the cell and its organelles to changes in intermediate and micro-filaments. What of the cell *in vivo*?

For the cultured cell to abandon the flattened shape in favour of the rounded form, intermediate filaments must undergo disruption or at least shortening so that the attached organelles would be expected to come closer together. The round cell on poly(HEMA) has never developed extended intermediate filaments which need to be shortened, so that the attached organelles may already be closer together than in the flattened cell. The adrenal cell *in situ* has a generally rounded shape like that of the cultured cell after treatment with acrylamide and that grown on poly(HEMA). We must conclude that in the adrenal cortex, ACTH brings droplets and mitochondria closer together by internal shortening without gross change in external shape.

In conclusion it should be pointed out that the changes described above are all seen in Leydig cells when these cells are stimulated by LH [16].

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