LIPID DROPLETS IN CALCIUM-INDUCED STRUCTURE INTERACTIONS IN ADRENOCORTICOCYTES

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Information about prof. E.A. Lukyanetz is described on p. 167.

Introduction

Recently it has been shown that, similarly to other secretory cells, intracellular calcium signaling is the main mechanism which is activated in response to the external stimulus and provides a steroidogenesis in adrenocortical cells (Aptel et al., 1996; Brandenburger et al., 1996; Cherradi et al., 1998; Rossier, 1997). However, in comparison to adrenocorticocytes from zona glomerulosa (ZG), in the cells from zona fasciculata (FZ), the role of Ca^{2+} entering through Ca^{2+} channels in steroidogenesis is unclear. Thus, it was shown that cAMP cascade rather than Ca^{2+} signaling is involved in steroid hormone release in FZ cells (Cote et al., 2001; Coyne et al., 1996), despite the expression of voltage-gated calcium channels (T- and L-type) in these cells (Barbara and Takeda, 1995; Schrier et al., 2001).

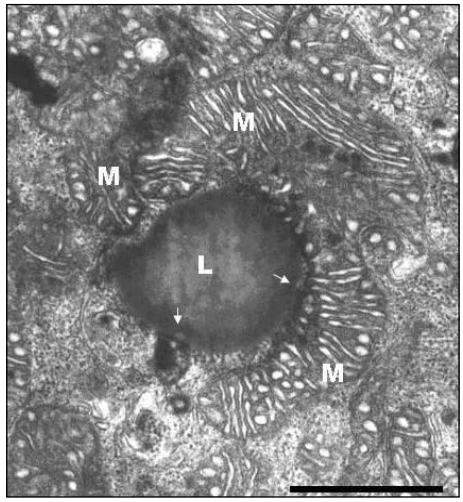
A very important feature of adrenocortical cells is the fact that enzymes participating in synthesis of steroid hormones are located in different compartments of the cell (Ishimura and Fujita, 1997). Thus, conversion of cholesterol to pregnenolone and the final stages of corticosteroid synthesis occur in mitochondria and involve cytochromes P450 (among them P450scc cholesterol side-chain cleavage cytochrome, product of the CYP11A gene) located on the internal surface of mitochondrial membrane (Ishimura and Fujita, 1997), whereas the intermediate stages resulting in synthesis of deoxycortisol or deoxycorticosterone from pregnenolone occur in the membranes of endoplasmic reticulum (ER) (Kim et al., 1997). Recently the data appeared that other organoid members may participate in this process. in addition to these two main structures.

The purpose of our experiments was to establish the role of Ca^{2+} signaling in morphological ultrastructural changes connected with steroidogenesis of organelles in adrenocortical cells from zona fasciculata/reticularis.

Methods

Cell culturing and electron microscopy. A 4-day culture of adrenocortical cells from bovine adrenals was used for electron microscopy researches. The detailed description of feeding such culture was described previously in details (Koval et al., 2000; Koval et al., 2001). Cells were grown on polyornithine-covered acklar plates and then were fixed by 2.5 % glutaraldehyde in 0.1 M phosphate buffer. After triple washing, they cells were treated with 1 % OsO_4 solution for 1 h. In separate experiments, the cells were loaded by Ca^{2+} via depolarization by 2 s exposure to 100mM KCl or pretreatment with 10 μM A23187 (calcium ionophore) in Ca^{2+}-free solution followed by 2 s incubation in Ca^{2+}-containing medium, and then were post-fixed. The ultrastructure of cells was examined on ultrathin sections (50÷60 nm thickness) contrasted by uranylacetate under an electronic microscope JEM-100 CX (JEOL, Japan). Preparations were analyzed at ×6,700÷×40,000 magnification. The morphological analysis was carried out with using "KSlite" (Kontron Electronik, Germany) software. The values are given as mean ± S.E.M. Statistical significance analysis of differences was performed using ANOVA analysis.

Fluorescent $[Ca^{2+}]_i$ measurements. Intracellular Ca^{2+} was monitored from individual cells with a Ca^{2+} photometric system (TILL Photonic, USA). This method is described elsewhere (Lukyanetz, 2001; Lukyanetz and Neher, 1999). Briefly, cells were loaded with 1÷2 μM fura-2 acetoxymethylester (fura-2AM; Molecular Probes, Eugene, OR, USA) for 30 min and subsequently washed for 30 min. Then they were transferred to a recording chamber where recordings at pair wavelenghts of 360 (F1) and 380nm (F2) were collected every 200 ms. The fluorescence signals were examined on an inverted microscope (Olympus IX70, Japan) using a dry ×40 objective (NA = 0.55), attached with photometric devices from TILL Photonics, USA. In all experiments, the signals were averaged through-



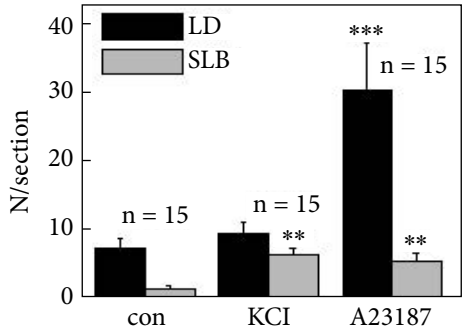


Fig. 2. Ca^{2+}-induced increase of steroidogenic activity in FZ/ZR cells. The mean values of the number of lipid droplets (black bars) and SLB (gray bars) per section in different experimental conditions are presented

Fig. 1. Ca^{2+}-induced interaction of lipid droplets with multiple mitochondria in adrenocortical cell from FZ/ZR zone. Cell was exposed to 100 mM KCl for 2 min before fixation. One can see a lipid droplet (L) located at the center of cell, enveloped by several mitochondria (M). White arrows show SLB in the places of contacts. Scale bar demonstrates 1 μm

out the cell. Data are presented as signals ratio (F1/F2). The excitation light waves were adjusted by monochromator Polychrome IV (TILL Photonics, USA) added with an amplifier EPC9 and software Pulse/XChart (HEKA Electronics, Germany).

Results

Previous studies of adrenocortical cells have shown that cells from different zones of adrenal cortex differ in their mitochondria, lipid droplets, endoplasmic reticulum (ER) and also in the presence of pigment lipofuscin granules (PLG). In our experiments the ultrastructural analysis of cultured cells from dissociated tissue of cortex of adrenal glands has shown two types of adrenocortical cells differing in mitochondrial cristae and in the presence of lipofuscin granules. One type of cells occurred much oftener and was characterized by large mitochondria with characteristic tubulo-vesicular crista (Fig. 1). According to these properties, such cells belong to the fascicular zone (FZ) (Bornstein et al., 1992), whereas the other type of cells was rarely observed in our experiments and was characterized by mitochondria with tubulo-lamellar cristae characteristic for the cells from the zona glomerulosa (ZG) (Belloni et al., 1987). Since in our experiments we observed mainly adrenocortocytes from FZ and ZR, the next data will concern only FZ/ZR cell types.

To show that Ca^{2+} can be involved in steroidogenesis in FZ/ZR cells, primarily we used microfluorescent method to measure intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by using Fura-2 dye. We compared the action of adrenocorticotropic hormone (ACTH) with activation of voltage-operated Ca^{2+} channels by KCl-in-

duced membrane depolarization. We found that short application of 100mM KCl evoked pronounced Ca^{2+} transient pointing that in this cell type activation of Ca^{2+} channels induces prominent Ca^{2+} influx into the cell. Application of 2 μM ACTH evoked a similar effect on the level of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in the same cell. These findings allowed us to suppose that Ca^{2+} ions are really involved in the steroidogenesis in this type of adrenocortical cells.

Morphological experiments showed that both depolarization of the cells by 100 mM KCl inducing Ca^{2+} influx into the cell via voltage-operated Ca^{2+} channels and Ca^{2+} ionophore A23187 (10μM) transferring Ca^{2+} into the cell induced morphological changes in the ultrastructure of adrenocortical cells. It was possible to observe droplets tightly contacting together and with other organels, Fig. 1. It has been proved that the lipid droplets contacted together frequently via specialized structures which we called the "spiral-like bodies" (SLB). After exposing the cells to Ca^{2+} inductors, the tight interactions of lipid droplets with mitochondria, sometimes with two and more, were remarkable (Fig. 1). The contacts between mitochondria and droplets could be also observed in control. The droplets preserved their electron dark contents at the periphery while the center remained electron lighter. The lipid droplets contacted with mitochondria frequently via specialized SLB, located on the droplets (white arrows). The space expansion between mitochondrial cristae and rounding of mitochondrial vesicles were observed after excitation. In the place of their contact with droplets, the vesicular cristae concentrated and lined up along the contacts with similar distances between each other. The mitochondrial vesicles were oriented perpendicularly to the contacts, pointing on the steroidigenesis in process.

The SLB represented the electron light rounded structures with dark core located always on the surface of the droplets, and they looked like as electron-dense spiral body included in the electron-light pouch having a regular ovoid shape, Fig. 1 (white arrows). The mean diameter of the SLB equaled to 96 ± 8.3nm (n=16). The SLB could be observed in control conditions, however after increase of Ca^{2+} in cell cytoplasm their number was remarkably increased by about 5 times after KCl stimulation and by about 4.4 times after A23187 exposition, Fig. 2.

The bar graph in Fig. 2 represents the mean number of lipid droplets (black bars) and SLB (gray bars) calculated per one section. Totally, 15 sections from different cells were analyzed. It was proved that in control conditions the number of lipid droplets per section was 7.13 ± 1.5 (n = 15), in the case of KCl it was 9.33 ± ± 1.64 (n = 15) and 30.33 ± 6.8 (n = 15) after A23187 treatment. As it can be seen, that number of lipid droplets was significantly increased after A23187 treatment (by about 4 times) whereas increase of the droplet number after KCl stimulation was statistically insignificant (Fig. 2). It should be noted that not all droplets contained SLB and as rule every droplet contained single SLB. However, it is necessary to bear in mind, that an appearance of SLB in the section (50 nm thickness) could depend on SLB location respecting to the section plan.

Discussion

From our experiments we concluded, that tight ultrastructural interactions between various intracellular structures significantly increased after Ca^{2+}-induced priming of steroidogenesis in adrenocortical cells from zona fasciculata/reticularis. We observed the tight spatial contacts of lipid droplets with mitochondria and other organneles. The surface of lipid droplets contained the specialized morphological formations (SLB) in the contact sites with most of cases. We firstly describe these structures in steroidogenic cells. Some of the authors observed similar structures in lipid droplets, however they were mistakenly attributed to fixation artifacts. Our detailed inspection has shown that they really are the specialized structures. Besides, previously it was reported that similar formations were observed in lipid droplets from macrophage foam cells (McGookey and Anderson, 1983). These authors have reported whorls of bilayered membranes at specialized sites on the droplet surface, and provided evidence that these were the sites of core lipid hydrolysis by hormone-sensitive lipase (Brasaemle et al., 2000; Morimoto et al., 2000). This hydrolysis results in a surplus of envelope, which may then fold back upon itself (van Meer, 2001). Really, the fact of the increase of their number during cell excitation points on their role in steroidogenesis, since according to the existing data, Ca^{2+} can produce steroid hormone secretion in FZ/ZR cells (Capponi et al., 1988). Our statistical data have shown that the changes in the droplet's morphology were differentially influenced by KCl and the ionophore. Besides appearance of SLB was also significantly differed at two types of the way to increase an intracellular Ca^{2+}, being very pronounced at KCl depolarization. The latter can reflect an importance of the way by which Ca^{2+} enter into the cell. Really, an increase of lipid droplets diameters occurred only after stimulation of Ca^{2+} channels due to membrane depolarization. These our observations can be explained with data obtained recently in electrophysiological experiments on adrenal glomerulosa cells (Rossier et al., 1996a; Rossier et al., 1996b). These authors have shown the direct functional link between T-type calcium channel activity and steroidogenesis in ZG cells. They suggested a model in which calcium entering the cell through these channels can be further funneled, resulting in the confinement of intracellular calcium signaling (Rossier, 1997). Such way, Ca^{2+} can reach directly the intracellular places where steroidogenesis occurs.

Therefore, in the case of Ca^{2+} ionophore, when Ca^{2+} influx is undirected, Ca^{2+} ions can not reach the places associating with the lipid droplet enlargement (may be located inside of some of the organelles). We can suppose that the specialized spiral-like structures (SLB) observed in our experiments, that were formed only in the places of contact between lipid droplets with other organelles can be related with similar systems providing transport of proteins and lipids during steroidogenesis in adrenocortical cells.

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