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Abstract: The mitochondria are critical for steroidogenesis since the ability of cholesterol to move into mitochondria to be available for cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1) determines the efficacy of steroid production. Different enzymes are localized in the mitochondria vs. the endoplasmic reticulum to produce the final steroid hormone, thus, suggesting that mitochondrial dynamics (a balance between fusion/fission events) might be relevant for this process. Several proteins, such as PKA, MEK and extracellular signal-regulated kinases, which are essential to complete steroidogenesis, form a mitochondria-associated complex. The physical protein-protein interactions between protein kinases and key factors during the transport of cholesterol takes place in the contact sites between the two mitochondrial membranes; however, no mitochondrial targeting sequence has been described for these protein kinases.
In addition, the steroids molecules are lipids that have to move from the mitochondria to the endoplasmic reticulum, and back to the mitochondria to finally be secreted. They do not have any binding protein within the steroidogenic cells nor do they accumulate in lipids droplets, therefore the question that arises as to how the protein kinases move to the mitochondria and how the steroid precursors transit between different organelles avoiding any contact with the cytoplasm? In the last few years accumulating evidence suggest that the specificity in the hormonal signal can be achieve through the spatial and temporal regulation of the response.
In the present manuscript we present data showing that it was possible to demonstrate that an individual cell's response could be initiated and localized to the site of LH-receptor complex formation. Using single cells and limiting stimulation to a "patch" of membrane help in understanding a membrane signal transduction at the single cell level and the role of compartmentalized responses in the mechanism of peptide hormone action in steroid synthesis.

We also present data showing that the hormonal stimulation triggers mitochondrial fusion and that mitochondrial fusion does not only correlate-with but also is an essential step in steroid production, both events being dependent on PKA activity. We also show that the hormone-stimulated relocalization of ERK1/2 in the mitochondrion, a critical step during steroidogenesis, depends on

mitochondrial fusion. Strikingly, we found that mitofusin 2 expression, a central protein for mitochondrial fusion, is upregulated immediately after hormone stimulation. Moreover, mitofusin 2 knockdown is sufficient to impair steroid biosynthesis. Together, our findings unveil an essential role for mitochondrial dynamics during steroidogenesis. These discoveries highlight the importance of the reorganization of organelles in specialized cells, prompting the exploration of the impact that organelle dynamics has on biological processes that include, but are not limited to, steroid synthesis.

Cover Letter

Buenos Aires, 13th September, 2012

Molecular and Cellular Endocrinology

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Dear Dr Schimmer,

Please find enclosed a copy of the manuscript entitled “The spatial and temporal regulation of the hormonal signal. Role of mitochondria in the formation of a protein complex required for the activation of cholesterol transport and steroids synthesis." by Poderoso C. et al.

I hope that you will find this manuscript conform to the journal policy and acceptable for publication.

Sincerely yours,

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The spatial and temporal regulation of the hormonal signal. Role of mitochondria in the formation of a protein complex required for the activation of cholesterol transport and steroids synthesis.

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Abstract

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In addition, the steroids molecules are lipids that have to move from the mitochondria to the endoplasmic reticulum, and back to the mitochondria to finally be secreted. They do not have any binding protein within the steroidogenic cells nor do they accumulate in lipids droplets, therefore the question that arises as to how the protein kinases move to the mitochondria and how the steroid precursors transit between different organelles avoiding any contact with the cytoplasm? In the last few years accumulating evidence suggest that the specificity in the hormonal signal can be achieve through the spatial and temporal regulation of the response.

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Keywords: Mitochondria, fusion, mitofusin, steroid secretion, mitochondrial re-arrangement, cAMP, F1CRhR, stimulation at the single cell level.

Abbreviations

CYP11A1:cytochrome P450, family 11, subfamily A, polypeptide 1
PKA: cyclic-AMP-dependent protein kinase
ACTH: adrenocorticotropin hormone
LH: Luteinizing hormone
IMM. Inner mitochondrial membrane
CYP11A1: Cytochrome P450 cholesterol side chain cleavage enzyme
P5: Pregnenolone
ER: The endoplasmic reticulum
OMM: Outer mitochondrial membrane
ERK1/2: Extracellular signal-regulated kinases
AKAps: The A-kinase anchor proteins
mt-YFP: Mitochondria-targeted YFP
hCG: Human chorionic gonadotropin
cAMP: 8Br-cAMP
Mito-GFP: Mitochondria-targeted GFP
P4: Progesterone
EGF: Epidermal growth factor
Mfn: Mitofusin
OPA1: Optic atrophy
Drp1: Dynamin related protein 1
MAM: Mitochondria associated membrane
Acsl4: Acyl-CoA sinthetase-4
PTP: Protein tyrosine phosphatase
StAR: Steroidogenic acute regulatory
AII: Angiotensin II
K^+. Potasium
TSPO: Translocator protein VDAC: Voltage dependent anion channel

Article Outline

1. Introduction

It is well recognized that the interaction of a peptide hormone with its receptors on the surface of a cell and the initiation of signal transduction are the most important steps in its mechanism of action. There has been a great amount of data describing the kinetics of the hormone-receptor interaction as well as the activation of second messenger formation. However, several important questions regarding the mechanism of peptide hormone action still remain elusive.

The concept of "compartmentalization" has been utilized to explain some inconsistencies in second-messenger action (Greengard, 1978). One example is the fact that resting intracellular cyclic-AMP concentration are three orders of magnitude higher

than the measurable K_a of cyclic-AMP-dependent protein kinase (PKA), which is, nevertheless, not fully activated under these conditions (Rubin and Rosen, 1975). Is the PKA compartamentalized or "protected" from resting cyclic-AMP levels and thus only responds to *de novo* synthesis of cyclic-AMP localized to the vicinity of the receptor-activated cyclase?

Steroid hormones are synthesized in steroidogenic cells of the adrenal gland, ovary, testis, placenta, and brain and are required for normal reproductive function and body homeostasis. Steroid synthesis is regulated by trophic hormones, specifically, adrenocorticotropin hormone (ACTH) in adrenocortical cells and luteinizing hormone (LH) in testicular Leydig and ovarian cells, respectively. These hormones activate G protein-coupled receptors resulting in the activation of adenylyl cyclase and an increase in intracellular cyclic-AMP levels (Dufau, Baukal and Catt, 1980). This increase promotes the activation of PKA, protein synthesis and protein phosphorylation (Dufau, Tsuruhara, Horner et al., 1977) (Podesta, Milani, Steffen et al., 1979,Podesta, Milani, Steffen et al., 1979,Rae, Gutmann, Tsao et al., 1979). All these processes contribute to the delivery of cholesterol from the outer to the inner mitochondrial membrane, the rate-limiting step in steroid production.

Steroid synthesis is initiated at the inner mitochondrial membrane (IMM), where the cytochrome P450 cholesterol side chain cleavage enzyme (CYP11A1) catalyzes the conversion of cholesterol to pregnenolone (P5) (Hall, 1985), Then P5 enters the endoplasmic reticulum (ER) where further enzymatic reactions occur. Afterwards, the steroid formed returns to the mitochondrion to produce the final steroid hormone. Remarkably, it is widely accepted that the translocation of cholesterol from the outer mitochondrial membrane (OMM) to the IMM is the rate-limiting step in the production of all steroids (Simpson, 1979,Jefcoate, McNamara, Artemenko et al., 1992). Therefore,

the mitochondria are critical for steroidogenesis since the ability of cholesterol to move into mitochondria so as to be available for CYP11A1 determines the efficacy of steroid production.

Several proteins, such as PKA, MEK and extracellular signal-regulated kinases (ERK1/2) (Rone, Fan and Papadopoulos, 2009,Poderoso, Converso, Maloberti et al., 2008), which are essential to complete steroidogenesis form a mitochondria-associated complex. The physical protein-protein interactions between protein kinases and key factors during the transport of cholesterol takes place in the contact sites between the two mitochondrial membranes (Rone et al., 2009), however, no mitochondrial targeting sequence has been described for these protein kinases.

In addition, the steroid molecules are lipids that have to move from the mitochondria to the ER, and back to the mitochondria to finally be secreted. Steroids do not have any binding protein within steroidogenic cells nor do they accumulate in lipids droplets, therefore how do the protein kinases move to the mitochondria and how do the steroid precursors transit between different organelles avoiding contact with the cytoplasm?

In the last few years evidence has been presented suggesting that the specificity of the hormonal signal can be achieved through the spatial and temporal regulation of the response. For example, the phosphorylation of proteins in the different intracellular compartments of the cell which are induces by PKA and the MAP-kinase family is critical for the regulation of multiple functions. This activation of kinases in different intracellular compartments changes according to the specific stimulus. In the steroidogenic system that use cyclic-AMP as the second messenger the signal originating in the plasma membrane produces the translocation of PKA and ERK1/2 to the mitochondria (Poderoso et al., 2008). This translocation is essential for the normal regulation of steroid synthesis (Poderoso et al., 2008). This phenomenon can be

observed also in other physiological systems. In the case of epidermal growth factor stimulus, for example, ERK is also translocated to the nucleus. The striking features are that neither PKA nor ERK have any peptide signal that directs them to either the mitochondria or the nucleus. Neither do transport proteins for these kinases exist in these cells.

During the last few years the majority of studies about the location of kinases in the different compartments of cells were directed at analyzing the mechanism of retention of these kinases in the different organelles; such as the studies of the different the A-kinase anchor proteins (AKAps) for PKA (Feliciello, Gottesman and Avvedimento, 2005,Carlucci, Lignitto and Feliciello, 2008). However, no studies have attempted to understand how proteins without specific signal peptides are able to travel to their respective sites of action. In this manuscript we will present a series of experiments to answer these important questions regarding the mechanisms of hormone action.

2. Hormone stimulation at the single cell level.

It is known that certain responses generated by cyclic-AMP are much more efficient when the messenger is produced through receptor activation, suggesting a compartmentalization between the site of cyclic-AMP production and the cyclic-AMP-dependent protein kinase system. Unfortunately, direct evidence for such compartmentalization has been scarce.

The regulation of steroid synthesis is one of the systems where the compartmentalization of the hormonal signal may be important for the correct functioning of the steroidogenic cells. Using single cells and limiting stimulation to a "patch" of membrane has made possible understanding how one form of signal transduction proceeds, *i.e.* the mechanism by which neurotransmitter occupied-receptors

lead to the gating of individual ion channels (Siegelbaum and Tsien, 1980,Camardo, Shuster, Siegelbaum et al., 1983).

Using this same approach should help in understanding another form of membrane signal transduction at the single cell level and the role of compartmentalized responses in the mechanism of peptide hormone action in steroid synthesis.

2.1 Compartmentalization of structural changes

In order to determine if it is possible to study the mechanism of LH action at the single cell level, changes in the ultrastrutural morphology of cultured MA-10 Leydig cells were examined in cells stimulated in either a general or in a limited area of their plasma membrane. In figure 1A we show a single cell under control conditions exhibiting a flattened appearance and which is almost devoid of microvilli on its surface. In contrast, after perfusion with a low concentration (2 ng/ml) of LH, a completely round shape with numerous microvilli on the cell surface is observed (Figure 1B). Limited stimulation of the cell by puffing with an electrode containing LH (20 ng/ml) shows numerous microvilli in the area of stimulation with partial "rounding up" in which the flattened appearance is less pronounced (Figure 1C). This is in agreement with the observation in cultured granulose cells that treatment with gonadotropin or cyclic-AMP produced totally or partially rounded up cells with numerous microvilli on their surface (Amsterdam and Rotmensch, 1987) and with the observation that changes in cell shape due to hormone stimulation have been described *in vivo* and *in vitro* (Amsterdam, Knecht and Catt, 1981,Schimmer, 1980,Schimmer, 1979).

We have taken advantage of the patch-clamp technique in orders to stimulate a Leydig cell in a defined portion of its plasma membrane and to determine if its response is likewise localized. In this way the hormone can interact with a limited number of its

receptors, since the tight seal prevents leakage of the peptide in or out of the patch (Neher, Sakmann and Steinbach, 1978,Podesta, Solano and Lemos, 1991). The Leydig cell response can be observed by a phenomenon already described for LH/hCG in Leydig cells and for ACTH in adrenal cells: the alteration in cell shape called “round up”.

When the cells are stimulated for 15 minutes via a section of membrane tightly sealed to an electrode containing LH (20 ng/ml) in every case (n=5) a very localized area, corresponding to the patch of membrane which was in the electrode, exhibited a morphological change (Figure 2). This compartmentalized response was clearly a partial “rounding up” (large arrow), visible as such when focusing up and down. Furthermore, associated with this limited morphological response there was a localized “extracellular bubble” (see small arrow), observable due to the different in viscosity of the cell’s secretion and the incubation buffer. Note that the morphological changes are maintained after the loss of the seal and removal of the stimulating electrode. Thus, localized stimulation of the plasma membrane may also produce localized secretion. The possibility that this could be simply a result of mechanical artifacts due to touching the cell can be ruled out since bath application of hormone could also produce “extracellular bubbles” (data not shown). The “extracellular bubbles” suggest localized secretion, because its production is always correlated with stimulation. We have never observed any “extracellular bubbles” in the absence of either localized or individual cellular “rounding up” (n=7) and it does not develop until well (30 min) after touching the cell. This observation again argues against the possibility that the bubble simply reflects a localized loss of membrane due to mechanical contact artifacts.

2.2 Compartmentalization of cAMP

A fluorescent indicator for the cAMP signalling pathway has been developed (Adams, 1999) by labeling the catalytic subunit (C) and the regulatory subunits (R^{I} & R^{II}) of cAMP-dependent protein kinase, so that in the labeled holoenzyme (termed FlCRhR), where the subunits recombine, the dyes are close enough for resonance energy transfer (Adams, 1999). cAMP liberates the C subunits and, therefore, changes the ratio of emission amplitudes at the two relevant In Fig. 3 non-confluent MA-10 cells in culture were individually injected, using pressure through a micropipette (Adams, 1999), with 56 µM FlCRhR in its standard buffer (see Methods). Injection volumes were approximately 2-10% of cell volumes and gave estimated final concentrations (0.2 - 2 µM) approximating or exceeding that of the intrinsic holoenzyme. Digital images of the fluorescence were obtained as previously described (Adams, 1999). Ratios are averages over the entire cell. Fig. 3 shows the time courses of labeled kinase emission ratio (500-530/570+ nm; broad range collection) reflecting the free cAMP concentrations (0.01 - 1 µM) in single MA-10 cells. Before stimulation (panel A) resting [cAMP] is <0.1 µM, in agreement with basal levels of cAMP in population studies on MA-10 cells (0.05 to 0.1 µM). 14 s after puffing with an electrode filled with 20 ng/ml of LH (panel B) there is an augmentation in that part of the MA-10 cell nearest the application. In panel C, 44 s after the intial puff, the whole cell has higher levels of cAMP, reflected by the labeled kinase emission ration, and the levels in the neighboring cell have also increased. The elevated [cAMP] continues 214 s after the initial puff of LH (panel E) but the third and fourth cell (to the right) have not responded. In order to determine if these last cells are capable of responding, we puffed LH directly onto the third right-most cell and this resulted in a 'localized' increase in [cAMP] 14 s later (panel F). Again the elevated [cAMP] persisted 84 s later (panel H). Thus, video imaging using FlCRhR makes possible a kinetic analysis of the cAMP responses (Adams et al 1999).

As shown in Fig. 4 the cAMP rise in cell 1 nearest the puff application is rapid (observable within 14 s) and shows a difference between the left (o) and right (triangle) portion of the cell. The elevation in cAMP in the left portion of the cell is maintained while the right side declines more rapidly. This is probably due to continued occupancy of the receptors on the side nearest the puff by the hormone. Cell 2 responded 53 s later and to a lesser extent. This could be due either to the intercellular transfer of the second messenger or the later arrival of the diluted puff to this cell. Since this cell's response is maintained, compared to the nearest portion of cell 1, this indicates that this cell is probably responding directly to LH. As can be observed in Fig. 3 the response to a puff of LH on cell 3 gave a more localized elevation of cAMP, again within 14 s. This would be the fastest (10-30 secs) known response for a peptide hormone for LH and another peptide action.

2.3 Compartmentalization of steroid release

In addition, individual cell secretion can be observed after localized LH stimulation by the use of the reverse-hemolytic plaque assay (Neill and Frawley, 1983). In this assay the cells are co-incubated with rabbit red blood cells which are coupled via protein-A with an anti-progesterone antibody. After LH stimulation, the culture is incubated in the presence of complement. This incubation produces the formation of red cell ghosts due to the destruction of the red blood cell after the interaction of the antibody and the progesterone secreted after cell stimulation in the presence of complement. Figure 5A show a single cell under control conditions exhibiting intact red cells surrounding a Leydig cell. In contrast, after general perfusion with a low concentration (2ng/ml) of LH, a completely dark area around the Leydig cell can be observed as a consequence of red cell disruption and ghost formation (Figure 5B). Limited stimulation of the cell by

an electrode containing LH (20ng/ml) shows only limited red cell disruption and ghost formation (Figure 5C) in the area of puffing.

These studies demonstrated the stimulation by a peptide hormone of an individual steroidogenic cell in a prescribed region of the plasma membrane. This localized response was achieved using the patch-clamp technique, thus limiting the stimulation to those receptors in the patch of membrane within the tip of the microelectrode. In this way the hormone can interact only with a limited number of receptors, since the GigaOhm seal prevents "leakage" of hormone in or out of the patch area (Neher et al., 1978).

It was possible to, thus, demonstrate that an individual cell's response could be initiated and localized to the site of LH-receptor complex formation.

In contrast, patching did not elicit rounding up in any of the control studies. Moreover, in some cases the same cell when used as a control did not produce any morphological change if there were saline in the electrode, but did when there was hormone in the electrode. The time- and hormone-dependence of the response strongly argues against its being solely the results of mechanical contact artifacts.

In conclusion, these results indicate that a cell can give a partial or localized response which is limited and proportional to the area of stimulated receptors. We have shown that stimulated LH receptors, which are coupled to adenylate cyclase, lead to an internal response which remains localized to the site of stimulation.

The parameter to monitor the response was the morphological change cellular "rounding up", which is mediated by cAMP stimulation of a cAMP-dependent protein kinase (Rae et al., 1979). As mentioned before, this rounding up phenomena has been observed at the ultra-structural level using electron microscopy on groups of cells in culture (Soto, Kliman, Strauss et al., 1986). In this study the time course for the

sterodogenic response of cells to the hormones LH and human chorionic gonadotropin (hCG) and the morphological rounding up of cells parallel each other. Such changes in cell shape may include the repositioning within the cell of organelles involved in steroidogenesis, one of them being mitochondria.

Changes in cell shape and size due to gonadotropin stimulation have been described *in vivo* and *in vitro* (Amsterdam et al., 1981). The mechanism of cell shape change presumably involves alteration of the cytoskeleton. Electron microscopy and histochemical studies in a monolayer of cultured granulosa cells show that flattened, unstimulated cells often possess thick actin filaments and are almost devoid of microvilli on the cell surface. After treatment with gonadotropin or cAMP the cells became highly aggregated and "round up" with increased numbers of microvilli on the cell surface (Amsterdam and Rotmensch, 1987). Microvilli are rich in thin filaments (actin), and accumulation of microfilaments and microtubules beneath aggregates of receptor-bound gonadotropin has been demonstrated (Amsterdam and Rotmensch, 1987) An interesting consequence of the changes in cell shape and cytokeletal consequence are the centripetal movements of widely dispersed organelles after exposure to gonadotropin or cAMP (Soto et al., 1986). Mitochondria, which are involved in steroid biosynthesis, concentrate in the perinuclear region. Clustering of these organelles in the perinuclear region, concurrent with hormone-induced shape change, might facilitate movements of substrate among organelles and thus enhance steroid synthesis. It has also establish that this phenomenon of "rounding up" is mediated by cAMP stimulating a cAMP-dependent protein kinase (Rae et al., 1979,Amsterdam et al., 1981)

3. Mitochondrial shape and steroid synthesis.

In an effort to identify the mitochondrial changes produced by hormonal stimulation, we investigated the effect of elevating cAMP levels on mitochondrial shape. Following cAMP stimulation it can be observed by electronmicoscopy that there is a reordering of mitochondria after treatment of MA10 Leydig cells with cAMP for one hour (Figure 4).

In basal conditions (Figure 6A), mitochondria have an orthodox structure with narrow cisternae and a more spherical shape. In hormone-stimulated cells (Figure 6B), mitochondria are larger in diameter and appeared elongated and tubular as compared to the round mitochondria found in control cells. Interestingly, EMs showed that large portions of filamentous ER appeared in close proximity to enlarged mitochondria.

In this regard, mitochondrial shape could be a determinant in the hormonally-stimulated differential mitochondrial distribution.

To investigate changes in mitochondrial shape, we evaluated mitochondrial reordering based on a described characterization of multiple mitochondrial shapes (Braschi and McBride, 2010). For that purpose we transiently transfected MA-10 Leydig cells with mitochondria-targeted YFP (mt-YFP) (Karbowski, Arnoult, Chen et al., 2004) and then stimulated them with hCG or with 8Br-cAMP (cAMP), a cell permeable analogue of the second messenger. We studied the distribution of the fluorescence under hormonal stimuli.

Mitochondria were symmetrically distributed surrounding and excluding the nucleus in control cells while an asymmetric distribution was observed in stimulated cells (see Z-stack of both stains in Figure 7 B upper panel).

In agreement with the rounded shape category observed in the electronmicroscopy study, when mitochondria were visualized by transfection with mt-YFP, punctuated shape was observed under control condition. Cellular stimulation with hCG or cAMP resulted in the conversion of punctiform or short mitochondria to highly elongated and

interconnected organelles (Figure 7 A). This change in mitochondrial shape (punctuated vs tubular) is associated with fusion of the mitochondria (Braschi and McBride, 2010).

Mitochondrial shape changes were quantified by image analysis when cells with different mitochondrial appearances were counted. This mitochondrial shape has been determinates with hCG and/or a membrane-permeable second messenger 8Br-cAMP. Mitochondrial elongation or fusion was readily apparent within the first hour after cellular stimulation. Moreover, fusion shaped-mitochondria with threads surrounded nuclei after stimulation (Figure 7C). Under experimental conditions, we clearly distinguished two of these categories: punctuated and tubular shapes (Figure 7A, right and left, respectively). Remarkably, a direct correlation between this shape of the mitochondria and progesterone (P4) production was observed in all cases (Duarte, Poderoso et al. PLoS ONE 2012 in press). Moreover, when we used a slower steroidogenic stimulus, epidermal growth factor (EGF), a delay in mitochondrial shape changes was observed (Duarte, Poderoso et al. PLoS ONE 2012 in press). Cells treated with H89 significantly reduced the mitochondrial fusion observed after hormone or cAMP stimulations. As expected, steroid synthesis under both stimuli was also diminished by H89 (Duarte, Poderoso et al. PLoS ONE 2012 in press).

4. Mitochondrial fusion

Changes in mitochondrial morphology are generally taken as evidence for a change in the balance between fission and fusion (Liesa, Palacin and Zorzano, 2009).

Mitochondrial fusion/fission events, a mechanism also referred as “mitochondrial dynamics” (Liesa et al., 2009), is important for maintaining the integrity of these organelles. Mitochondrial dynamics allows mitochondrial replication, repair of defective mitochondria, selective elimination of depolarized mitochondria via mitophagy and propagation of intra-mitochondrial calcium waves. It has been described

that mitochondrial plasticity facilitates the movement of these organelles within the cell (Chada and Hollenbeck, 2003), and that mitochondrial rearrangements are important for the normal function of the cell, and protection against ageing-related changes (Nakada, Inoue, Chen et al., 2001). In addition, it has been described that mitochondrial fission is related to metabolic disorders such as hyperglycemia (Yu, Robotham and Yoon, 2006).

The above-mentioned findings demonstrate that mitochondrial dynamics plays an important role in many cellular functions. Despite the importance of this process, the mechanistic details of the regulation of mitochondrial fission-fusion dynamics remains to be completely elucidated, and, in particular, is not well characterized in hormonal regulation of cellular functions.

Although the key role of mitochondria in steroid synthesis is well accepted, there are no studies exploring the relationship between mitochondrial dynamics and the regulation of the onset of steroidogenesis.

It has been proposed that two dynamin-like GTPases are involved in mitochondrial fusion. Mitofusin (Mfn) 1 and 2 are implicated in the modulation of mitochondria-mitochondria and ER-mitochondria interactions. Mfn 1 and 2 are located in the outer mitochondrial membrane mediating mitochondrial fusion in concert with another GTPase, OPA1 (optic atrophy 1), in the inner membrane. Mfn 1 and 2 are extensively expressed in tissues, as demonstrated in brain (mainly Mfn2), liver, adrenal glands and testis (Eura, Ishihara, Yokota et al., 2003). It has been proven that Mfn2 is enriched at contact sites between ER and mitochondria, regulates morphology of the former and directly tethers the two by means of trans-organelle homotypic and heterotypic interactions. Even when several lines of evidence support a regulation of Mnf2 by diverse metabolic conditions, such as type 2 diabetes and obesity (Bach, Pich, Soriano et al., 2003), modulation of Mnf2 levels has not been demonstrated under hormonal

regulation of different cellular functions. Mitochondrial fission requires dynamin related protein 1 (Drp1), a cytosolic protein, that is recruited to the outer mitochondrial membrane by a poorly characterized multiprotein complex. Drp1 is subjected to post-translational modification by ubiquitylation, sumoylation, nitrosylation and phosphorylation (Chan, Lin, McNally et al., 1987,Cereghetti, Stangherlin, Martins de Brito et al., 2008,Wasiak, Zunino and McBride, 2007,Cho, Nakamura, Fang et al., 2009). Recent works have shown that PKA recruitment to the mitochondria resulted in mitochondrial elongation by Drp1 phosphorylation and inactivation in neurons (Merrill, Dagda, Dickey et al., 2011).

The enzyme acyl-CoA synthetase-4Acsl4 is an essential enzyme during steroid synthesis (Maloberti, Lozano, Mele et al., 2002,Maloberti, Castilla, Castillo et al., 2005,Cornejo Maciel, Maloberti, Neuman et al., 2005), which is located in a specialized compartment of the ER known as the mitochondria associated membrane (MAM). Since steroid synthesis requires both mitochondria and Acsl4, we thought that an increased interaction between both components might enhance steroidogenesis. To determine the effect of cAMP stimulation on Acsl4 distribution, we evaluated the co-localization of this MAM enzyme with the mitochondrial one (mt-YFP). We observed that cAMP increased the co-localization between Acsl4 and mt-YFP. Interestingly, inhibition of mitochondrial fusion was sufficient to diminish the co-localization after the cAMP stimulus (Duarte, Poderoso et al. PLoS ONE in press). Since hormone stimulation increases Acsl4 levels, we followed the effect of fusion inhibition on Acsl4 levels in total extracts. We observed no effect on total protein levels, indicating that the increased co-localization with mt-YFP is not a result of an increase in Acsl4 levels. In support of these experiments, we observed that Acsl4 levels are increased in isolated mitochondria upon cAMP stimulation, an effect that was not observed under fusion

inhibition (Duarte, Poderoso et al. PLoS ONE in press). Therefore, increased MAM-Acsl4 interaction with mitochondria correlated with mitochondrial fusion and steroid synthesis.

Together, these data strongly suggest that mitochondrial fusion is necessary to increase the association of mitochondria with MAM, a probably central event for an efficient transport of steroidogenesis intermediates.

Using primary cultures of adrenocortical and interstitial testicular Leydig cells as well as the established cell lines MA-10 and Y1 of murine Leydig and adrenal tumors, respectively, we have demonstrated that protein tyrosine phosphatase (PTP) inhibitors reduced hormone- or cAMP-induced stimulation of steroid biosynthesis (Maciel, Poderoso, Gorostizaga et al., 2001,Paz, Cornejo Maciel, Maloberti et al., 2002,Poderoso, Maciel, Gorostizaga et al., 2002). Apart from their inhibitory effect on steroid synthesis, PTP inhibitors affect both hormone-induced steroidogenic acute regulatory (StAR) protein and mRNA synthesis (Paz et al., 2002). Collectively, our studies demonstrated that PTPs serve as common intermediaries in the mechanism of action of steroidogenic hormones independently of the signal transduction pathway evoked by ACTH, LH, Angiotensin II (AII) and Potasium (K^+) (Mele, Duarte, Paz et al., 2012). Our studies also indicate that PTP activity is involved in StAR induction as well.

We have recently identified SHP2 as a PTP necessary for ACSl4 expression and steroid synthesis (Cooke, Orlando, Maloberti et al., 2011). To further determine whether SHP2 is also involved in the changes observed in mitochondrial morphology, we performed knockdown experiments using two different and specific short hairpin RNAs (shRNAs) directed against SHP2 protein. We found that knockdown of SHP2, by means of shRNAs, decreased steroid synthesis and mitochondrial fusion both by cAMP and hCG

stimulation, thus suggesting that mitochondrial fusion depends, at least in part, on SHP2 activity (Duarte, Poderoso et al. PLoS ONE).

4.1 Steroidogenesis requires Mitofusin 2

Mfn2 protein has been proposed as an important mitochondrial protein involved in the fusion formation of the mitochondrial network (Hoppins, Lackner and Nunnari, 2007) The machines that divide and fuse mitochondria (Huang, Yu and Yoon, 2007) (Zorzano, Liesa and Palacin, 2009,Zorzano, Sebastian, Segales et al., 2009).

To study the role of Mfn2 in steroid synthesis, we generated two specific shRNAs directed against Mfn2. First, we verified the correct knockdown of Mfn2 using both shRNA constructs. Both shRNA-Mfn2 significantly decreased the levels of this protein (Duarte, Poderoso et al., PLoS ONE 2012 in press). Next, we evaluated the effect of Mfn2 knockdown on mitochondrial fusion and steroid synthesis. We observed that knockdown of Mfn2 reduced a mitochondrial fusion and significantly decreased steroid production by cAMP stimulation. We, thus, concluded that Mfn2 participates in steroid synthesis through mitochondrial fusion.

4.2 Mitofusin 2 expression is regulated by cAMP and hCG in MA-10 cells

Although several lines of evidence support different types of regulation of Mfn2, hormonal regulation of Mfn2 levels in cellular differentiation has not been demonstrated. Thus, we analyzed the effects of cAMP and hCG on Mfn2 expression. We observed that both treatments induced Mfn2 mRNA levels. Remarkably there is a rapid mRNA induction at 30 min with cAMP and hCG. A longer time of stimulation did not show significant differences compared to control cells. We further analyzed protein expression levels under hCG and cAMP stimulation. The same stimulation pattern was observed in protein expression (Duarte, Poderoso et al. PLoS ONE in press). Together

these results demonstrate for the first time that Mfn2 expression is hormonally regulated and that mitochondrial fusion is stimulated and required for steroidogenesis.

5. Conclusions

Herein we have shown that using a combination of fluorescence imaging, patch-clamp, and reverse-hemolytic plaque methods we were able to describe the kinetics of an individual cell's response to hormone stimulation. Furthermore we have been able to correlate compartmentalized second-messenger responses with localized release of steroids. We have also shown that following cAMP stimulation it can be observed by electronmicoscopy that there is a reordering of mitochondria after treatment of MA10 Leydig cells with cAMP. In addition mitochondrial fusion induced after hormone treatment is able to regulate specialized cellular functions, such as steroid synthesis. We found that mitochondrial fusion precedes and is required for steroid biosynthesis. We proposed a novel mechanism in which mitochondrial fusion is determined by a cross-talk between serine/threonine phosphorylation and tyrosine dephosphorylation events triggered by hormonal stimulation. This mechanism involves PKA and the tyrosine phosphatase SHP2 activity in the mitochondrial fusion changes. Furthermore, we demonstrated that mitochondrial fusion allows the localization of key proteins for steroid synthesis such as ERK1/2 and Acsl4 in mitochondria to achieve steroidogenesis. We also showed for the first time that Mfn2 expression is hormonally regulated and is needed for steroidogenesis. Together, we provide compelling evidence demonstrating not only the requirement but also the molecular basis for linking mitochondrial fusion with the proper activation of full steroidogenesis.

It is known that phospho/dephosphorylation events are obligatory in cholesterol transport to the inner mitochondrial membrane, and that PKA is one of the proteins

required for this process. We determined that mitochondrial fusion depends on PKA activity. Furthermore, it has been recently proposed that PKA increases mitochondrial fusion events and cell survival (Merrill et al., 2011). Also, PTPs activity is required for steroid synthesis. We have recently identified SHP2 as a PTP that participates in the cAMP-dependent activation of steroidogenesis (Cooke et al., 2011) Its activity is modulated by PKA phosphorylation after ACTH challenge in adrenocortical cells . Our data show that PTPs are involved in the mitochondrial switch to the fusion shape and establish that SHP2 is the PTP that participates in the mitochondrial fusion mechanism.

Steroidogenic hormones promote a notable change in cell shape, implicating reorganization of the actin cytoskeleton, preceding steroid biosynthesis and secretion. Thus, it is conceivable that SHP2 activity modulates the actin cytoskeleton with a direct relationship with mitochondrial sucellular distribution and fusion.

Reorganization of organelles and contact between membranes can be an important process in steroid production and secretion through the plasma membrane. The localization of several enzymes is critical to ensure appropriate steroidogenesis rates. In steroidogenic cells, Acsl4 is induced after hormonal stimulation (Cornejo Maciel et al., 2005) and is localized in the ER, particularly in the MAMs (Mikkola, Saris, Grigoriev et al., 1999,Lewin, Van Horn, Krisans et al., 2002). Interestingly, we detected that Acsl4 is not associated with mitochondria if mitochondrial fusion is inhibited. Restored mitochondrial fusion re-localized this protein in the mitochondrial context. On the other hand, it has been proposed that Acsl4 could have a role in MAMs enhancing the mitochondrial and ER membrane fluidity (Knudsen, Jensen, Hansen et al., 1999).

These data suggest that the increased contacts between Acsl4 and mitochondria during mitochondrial fusion could allow steroid movement between these two organelles. In addition, we demonstrated that ERK1/2 locates to the mitochondria after hCG/8Br-

cAMP. Furthermore, ERK1/2 mobilization is dependent on SHP2-mediated mitochondrial fusion.

Mitochondrial fusion is carried out by mitofusins, among which Mfn2 is mainly involved. We added new evidence on the correlation between mitochondrial fusion and steroid production. Our results indicate that fusion *per se* is obligatory for steroid biosynthesis and rule out any other unspecific effect of mitochondrial fusion inhibition on steroidogenesis.

We showed for the first time that Mfn2 mRNA and protein levels are modulated by hCG and 8Br-cAMP in this cellular type. This suggests that mitochondrial fusion could transfer an increase in cellular energy when abrupt changes, such as steroid hormone synthesis, are required.

In summary, we have provided a large body of evidence about how the compartmentalized cAMP response and mitochondrial fusion operates in steroidogenic cells. More significantly, our results provide a framework for understanding how liposoluble steroid hormones can shift between mitochondria and the MAMs, by non-vesicular trafficking, to reach the plasma membrane without moving through the cytoplasmic hydrophilic milieu.

Very recently it has been demonstrated that upon hormonal stimulation a multiprotein mitochondrial complex is formed (Papadopuolos et al., Molecular Endocrinology, in press). This 800-kDa mitochondrial protein complex containing the OMM components of the transduceosome translocator protein (TSPO), Voltage dependent anion channel (VDAC), the matrix protein ATAD3A, and the IMM protein CYP11A1 that is required for steroidogenesis allows this complex to be capable of binding and segregating cholesterol from structural OMM lipids and has the ability to synthesize steroids.

Therefore, upon hormonal stimulation, this multiprotein mitochondrial complex could function in the pooling of cholesterol at the OMM, an activity that would then allow StAR to mobilize the cholesterol for transfer into the adjacent IMM and its subsequent conversion to pregnenolone. The fusion of the mitochondria may play a role in the formation of this complex.

In conclusion we propose that after hormone treatment there is an induction of Mitofusin 2 (Figure 6) that will allows mitochondrial fusion and this step may trigger the association of the ER with the mitochondria and the formation of a multiprotein complex that triggers the transport of cholesterol from the OMM to the IMM, a rate limiting step in steroid synthesis.

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Figures legends

Figure 1. Ultrastrutural morphology of cultured MA-10 Leydig cells examined in cells stimulated in either a general or in a limited area of their plasma membrane.

All are SEMicrographs of MA-10 cells in culture. A) MA-10 cell in control saline. B) similar ce;; after [puffing with broken (R<1 MΩ) electrode containing 20 ng/ml oLH. C) similar cell after puffing with unbroken electrode (R=10 MΩ). Scale bar = 10 µm. MA-10 cell cultures were immediately fixed with 2% glutaraldehyde in 100 mM Phosphate buffered saline pH 7.2, for 30 min. All steps of Scanning Electron Microscopy (SEM) preparation were performed at room temperature. Cultures were washed 3 x 10 min in the same buffer and post-fixed in buffered 1% Osmium tetraoxide for 30-60 min. After 2 washes for 10 min in distilled water, the cultures were dehydrated in ethanol. After ethanol dehydratation the culture was rinsed in acetone, critical point dried in CO_2 and. The culture were then examined using an AMR 1000A SEM at 10-20kV.

Figure 2. Stimulation of a single MA-10 Leydig cell, in a defined portion of its plasma membrane using the patch-clamp technique.

All panels are Hoffmann-modulated contrast micrographs of MA-10 cells in normal medium. Left) Electrode (R=15 MΩ) filled with 20 ng/Ml oLH positioned 1-2 µm above central Leydig tumor cell in field and max postive pressure applied for 3 s. (right) 15 mins after puffing and after removing pipette there is a large, general effect in the central cell (bottom arrow). Also, note "extracellular bubble" (top arrow). Bar = 30 µm.

Fire-polished hard-glass electrodes with resistances of 10-20 MegaOhms (MΩ) were used to achieve seal resistances of >10 GigaOhms (GΩ). Seals were most easily achieved during the first hour after removal of culture medium. The size of the pipet tip opening were calculated to be between 0.5-1 μm^2, giving the minimum size of the patches of plasma membrane sealed off. In fact, the area of the patches is considerably (~10X) bigger and most probably correspond to the size to the localized responses

observed with LH in the pipet. Standard patch-clamp techniques were used to achieve cell-attached patches.

Figure 3: **Fluorescent imaging of free [cAMP] in MA-10 cells.**

Pseudocolors, as calibrated in the right hand color scale. Pseudocolor brightness reflects the mean of two emission intensities, represent ratios of fluorescein to rhodamine emissions from FlCRhRII microinjected into four cells. Low ratios (0.85 relative to a fluorescein-rhodamine standard mixture) are coded in blue and indicate low cAMP. Progressively higher ratios and cAMP concentrations are displayed as green, yellow, orange, red, and magenta, the latter corresponding to a ratio of 1.60 relative to the dye standard. Images have been spatially smoothed by convolution with a 5x5 pixel kernel. **A**: Before stimulation, cAMP is very low, <100 nM. **B**: 14 sec after a puff of LH from a pipet filled with a 20 ng/ml solution, delivered where indicated by the arrow.. [cAMP] first rises only in the cell closest to the site of delivery. **C**: 44 s after the LH puff, the immediately adjacent cell begins to show a significant increase in [cAMP]. **D**: 114 s after the LH puff, the 2nd cell has now reached its peak [cAMP]. **E**: 214 s after the LH puff, [cAMP] is still unchanged in cells 3 and 4. **F**: 14 s after a second puff of LH delivered near the boundary of cells 3 and 4, [cAMP] is increasing locally closest to the site of application. **G**: 24 s after the 2nd puff of LH, the increase in [cAMP] has spread throughout cell 3. **H**: 84 s after the 2nd puff of LH, cell 3 is yet higher in [cAMP], and the rise has finally spread to cell 4. Scale bar indicates 50 µm.(also see (Adams, 1999)

Fig. 4: **Kinetics of FlChR response to hormone and second messenger**.

Time courses of labeled kinase emission ratio (500-530/570+ nm) reflecting free cAMP concentrations (0.8 - 2 µM) in individual MA-10 cells. Kinetics of cAMP changes from Fig. 3. Cell 1 shows a difference between the left (o) and right (triangle) portion of the

cell . Puff was applied to cell 3 (light square; ⌂) after 214 s and the response in cell 4 is indicated by dark squares (■).

Figure 5 . Individual MA-10 Leydig cell secretion, after localized LH stimulation, by the use of the reverse-hemolytic plaque assay.

MA-10 Leydig cells are co incubated with rabbit red blood cells which are coupled via protein A with an anti-progesterone antibody.

Left Panel: Cells are incubated for one h in the absence of LH. Middle Panel: Cells are treated during one h with LH (general perfusion). Right Panel: Cells are incubated with LH (puffing application: see arrow). After LH stimulation the cultures are incubated in the presence of complement. This incubation produces the formation of red cell ghosts due to the destruction of the red blood cell after the interaction of the antibody and the progesterone secreted after cell stimulation.

Figure 6. Representative electron micrographs of mitochondria in MA-10 cells. Cells were stimulated with (right panel) or without 8Br-cAMP (1mM) for 1 h (left panel). Scale bars, 0.2 μm. m indicate mitochondria and ER endoplasmic reticulum.

Figure 7 . Confocal images of MA-10 Leydig cell

A. Representative confocal 3D images of MA-10 Leydig cell transfected with mtYFP (mito, Green) and stimulated after 24 hour with (Left panel) or without hCG (right panel) for 1 h. Scale bar, 10 μm.

B. Z-axis stacks of confocal mtYFP images with or without 1 hour of 8Br-cAMP (Upper panel). The lower panel shows the added intensity for all stacks in the region indicated by the white line in the cell. Fluorescence was measured with LSM image Browser software. Scale bar, 10 μm.

C. Representative confocal 3D images of MA-10 Leydig cell transfected with mtYFP (mito, Green) and stimulated after 24 hour with (Left panel) or without 8Br-cAMP (1

mM) (right panel) for 1 h. Scale bar, 10 µm (upper panel). The lower panel shows the intensity for the region indicated in the 3D image by the white line in the cell. Scale bar, 10 µm.

Figure 8 . Schematic representation of the association of ER and Mitochondria of a steroidogenic cell after the induction of Mitofusin 2.

Left panel represents a non-stimulated steroidogenic cell where the mitochondria, the proteins MEK1/2, ERK1/2, PKA, PAP7, DBI and ER are separated without any physical interaction. Right panel represents the reorganization of the mitochondria after Mfn2-induced-mitochondrial fusion allowing the association of the different proteins with the mitochondria and the ER.

References

[1] Greengard, P., 1978. Phosphorylated proteins as physiological effectors, Science. 199, 146-52.

[2] Rubin, C.S. and Rosen, O.M., 1975. Protein phosphorylation, Annu Rev Biochem. 44, 831-87.

[3] Dufau, M.L., Baukal, A.J. and Catt, K.J., 1980. Hormone-induced guanyl nucleotide binding and activation of adenylate cyclase in the Leydig cell, Proc Natl Acad Sci U S A. 77, 5837-41.

[4] Dufau, M.L., Tsuruhara, T., Horner, K.A., Podesta, E. and Catt, K.J., 1977. Intermediate role of adenosine 3':5'-cyclic monophosphate and protein kinase during gonadotropin-induced steroidogenesis in testicular interstitial cells, Proc Natl Acad Sci U S A. 74, 3419-23.

[5] Podesta, E.J., Milani, A., Steffen, H. and Neher, R., 1979. Steroidogenesis in isolated adrenocortical cells. Correlation with receptor-bound adenosine e 3':5'-cyclic monophosphate, Biochem J. 180, 355-63.

[6] Podesta, E.J., Milani, A., Steffen, H. and Neher, R., 1979. Adrenocorticotropin (ACTH) induces phosphorylation of a cytoplasmic protein in intact isolated adrenocortical cells, Proc Natl Acad Sci U S A. 76, 5187-91.

[7] Rae, P.A., Gutmann, N.S., Tsao, J. and Schimmer, B.P., 1979. Mutations in cyclic AMP-dependent protein kinase and corticotropin (ACTH)-sensitive adenylate cyclase affect adrenal steroidogenesis, Proc Natl Acad Sci U S A. 76, 1896-900.

[8] Hall, P.F., 1985. Role of cytochromes P-450 in the biosynthesis of steroid hormones, Vitam Horm. 42, 315-68.

[9] Simpson, E.R., 1979. Cholesterol side-chain cleavage, cytochrome P450, and the control of steroidogenesis, Mol Cell Endocrinol. 13, 213-27.

[10] Jefcoate, C.R., McNamara, B.C., Artemenko, I. and Yamazaki, T., 1992. Regulation of cholesterol movement to mitochondrial cytochrome P450scc in steroid hormone synthesis, J Steroid Biochem Mol Biol. 43, 751-67.
[11] Rone, M.B., Fan, J. and Papadopoulos, V., 2009. Cholesterol transport in steroid biosynthesis: role of protein-protein interactions and implications in disease states, Biochim Biophys Acta. 1791, 646-58.
[12] Poderoso, C., Converso, D.P., Maloberti, P., Duarte, A., Neuman, I., Galli, S., Maciel, F.C., Paz, C., Carreras, M.C., Poderoso, J.J. and Podesta, E.J., 2008. A mitochondrial kinase complex is essential to mediate an ERK1/2-dependent phosphorylation of a key regulatory protein in steroid biosynthesis, PLoS One. 3, e1443.
[13] Feliciello, A., Gottesman, M.E. and Avvedimento, E.V., 2005. cAMP-PKA signaling to the mitochondria: protein scaffolds, mRNA and phosphatases, Cell Signal. 17, 279-87.
[14] Carlucci, A., Lignitto, L. and Feliciello, A., 2008. Control of mitochondria dynamics and oxidative metabolism by cAMP, AKAPs and the proteasome, Trends Cell Biol. 18, 604-13.
[15] Siegelbaum, S.A. and Tsien, R.W., 1980. Calcium-activated transient outward current in calf cardiac Purkinje fibres, J Physiol. 299, 485-506.
[16] Camardo, J.S., Shuster, M.J., Siegelbaum, S.A. and Kandel, E.R., 1983. Modulation of a specific potassium channel in sensory neurons of Aplysia by serotonin and cAMP-dependent protein phosphorylation, Cold Spring Harb Symp Quant Biol. 48 Pt 1, 213-20.
[17] Amsterdam, A. and Rotmensch, S., 1987. Structure-function relationships during granulosa cell differentiation, Endocr Rev. 8, 309-37.
[18] Amsterdam, A., Knecht, M. and Catt, K.J., 1981. Hormonal regulation of cytodifferentiation and intercellular communication in cultured granulosa cells, Proc Natl Acad Sci U S A. 78, 3000-4.
[19] Schimmer, B.P., 1980. Cyclic nucleotides in hormonal regulation of adrenocortical function, Adv Cyclic Nucleotide Res. 13, 181-214.
[20] Schimmer, B.P., 1979. Adrenocortical Y1 cells, Methods Enzymol. 58, 570-4.
[21] Neher, E., Sakmann, B. and Steinbach, J.H., 1978. The extracellular patch clamp: a method for resolving currents through individual open channels in biological membranes, Pflugers Arch. 375, 219-28.
[22] Podesta, E.J., Solano, A.R. and Lemos, J.R., 1991. Stimulation of an individual cell with peptide hormone in a prescribed region of its plasma membrane results in a compartmentalized cyclic AMP-dependent protein kinase response, J Mol Endocrinol. 6, 269-79.
[23] Adams, S., Bacskai, B., Taylor, S. & Tsien, R.Y. , 1999. In Fluorescent and Luminescent Probes for Biological Activity: A Practical Guide to Technology for Quantitative Real-Time Analysis, WT Mason, ed; Elsevier Science. 10:133-150
[24] Neill, J.D. and Frawley, L.S., 1983. Detection of hormone release from individual cells in mixed populations using a reverse hemolytic plaque assay, Endocrinology. 112, 1135-7.
[25] Soto, E.A., Kliman, H.J., Strauss, J.F., 3rd and Paavola, L.G., 1986. Gonadotropins and cyclic adenosine 3',5'-monophosphate (cAMP) alter the morphology of cultured human granulosa cells, Biol Reprod. 34, 559-69.

[26] Braschi, E. and McBride, H.M., 2010. Mitochondria and the culture of the Borg: understanding the integration of mitochondrial function within the reticulum, the cell, and the organism, Bioessays. 32, 958-66.
[27] Karbowski, M., Arnoult, D., Chen, H., Chan, D.C., Smith, C.L. and Youle, R.J., 2004. Quantitation of mitochondrial dynamics by photolabeling of individual organelles shows that mitochondrial fusion is blocked during the Bax activation phase of apoptosis, J Cell Biol. 164, 493-9.
[28] Liesa, M., Palacin, M. and Zorzano, A., 2009. Mitochondrial dynamics in mammalian health and disease, Physiol Rev. 89, 799-845.
[29] Chada, S.R. and Hollenbeck, P.J., 2003. Mitochondrial movement and positioning in axons: the role of growth factor signaling, J Exp Biol. 206, 1985-92.
[30] Nakada, K., Inoue, K., Chen, C.S., Nonaka, I., Goto, Y., Ogura, A. and Hayashi, J.I., 2001. Correlation of functional and ultrastructural abnormalities of mitochondria in mouse heart carrying a pathogenic mutant mtDNA with a 4696-bp deletion, Biochem Biophys Res Commun. 288, 901-7.
[31] Yu, T., Robotham, J.L. and Yoon, Y., 2006. Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology, Proc Natl Acad Sci U S A. 103, 2653-8.
[32] Eura, Y., Ishihara, N., Yokota, S. and Mihara, K., 2003. Two mitofusin proteins, mammalian homologues of FZO, with distinct functions are both required for mitochondrial fusion, J Biochem. 134, 333-44.
[33] Bach, D., Pich, S., Soriano, F.X., Vega, N., Baumgartner, B., Oriola, J., Daugaard, J.R., Lloberas, J., Camps, M., Zierath, J.R., Rabasa-Lhoret, R., Wallberg-Henriksson, H., Laville, M., Palacin, M., Vidal, H., Rivera, F., Brand, M. and Zorzano, A., 2003. Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism. A novel regulatory mechanism altered in obesity, J Biol Chem. 278, 17190-7.
[34] Chan, Y.L., Lin, A., McNally, J., Peleg, D., Meyuhas, O. and Wool, I.G., 1987. The primary structure of rat ribosomal protein L19. A determination from the sequence of nucleotides in a cDNA and from the sequence of amino acids in the protein, J Biol Chem. 262, 1111-5.
[35] Cereghetti, G.M., Stangherlin, A., Martins de Brito, O., Chang, C.R., Blackstone, C., Bernardi, P. and Scorrano, L., 2008. Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria, Proc Natl Acad Sci U S A. 105, 15803-8.
[36] Wasiak, S., Zunino, R. and McBride, H.M., 2007. Bax/Bak promote sumoylation of DRP1 and its stable association with mitochondria during apoptotic cell death, J Cell Biol. 177, 439-50.
[37] Cho, D.H., Nakamura, T., Fang, J., Cieplak, P., Godzik, A., Gu, Z. and Lipton, S.A., 2009. S-nitrosylation of Drp1 mediates beta-amyloid-related mitochondrial fission and neuronal injury, Science. 324, 102-5.
[38] Merrill, R.A., Dagda, R.K., Dickey, A.S., Cribbs, J.T., Green, S.H., Usachev, Y.M. and Strack, S., 2011. Mechanism of neuroprotective mitochondrial remodeling by PKA/AKAP1, PLoS Biol. 9, e1000612.
[39] Maloberti, P., Lozano, R.C., Mele, P.G., Cano, F., Colonna, C., Mendez, C.F., Paz, C. and Podesta, E.J., 2002. Concerted regulation of free arachidonic acid and hormone-induced steroid synthesis by acyl-CoA thioesterases and acyl-CoA synthetases in adrenal cells, Eur J Biochem. 269, 5599-607.

[40] Maloberti, P., Castilla, R., Castillo, F., Maciel, F.C., Mendez, C.F., Paz, C. and Podesta, E.J., 2005. Silencing the expression of mitochondrial acyl-CoA thioesterase I and acyl-CoA synthetase 4 inhibits hormone-induced steroidogenesis, Febs J. 272, 1804-14.
[41] Cornejo Maciel, F., Maloberti, P., Neuman, I., Cano, F., Castilla, R., Castillo, F., Paz, C. and Podesta, E.J., 2005. An arachidonic acid-preferring acyl-CoA synthetase is a hormone-dependent and obligatory protein in the signal transduction pathway of steroidogenic hormones, J Mol Endocrinol. 34, 655-66.
[42] Maciel, F.C., Poderoso, C., Gorostizaga, A., Paz, C. and Podesta, E.J., 2001. LH/chorionic gonadotropin signaling pathway involves protein tyrosine phosphatase activity downstream of protein kinase A activation: evidence of an obligatory step in steroid production by Leydig cells, J Endocrinol. 170, 403-11.
[43] Paz, C., Cornejo Maciel, F., Maloberti, P., Walsh, L.P., Stocco, D.M. and Podesta, E.J., 2002. Protein tyrosine phosphatases are involved in LH/chorionic gonadotropin and 8Br-cAMP regulation of steroidogenesis and StAR protein levels in MA-10 Leydig cells, J Endocrinol. 175, 793-801.
[44] Poderoso, C., Maciel, F.C., Gorostizaga, A., Bey, P., Paz, C. and Podesta, E.J., 2002. The obligatory action of protein tyrosine phosphatases in ACTH-stimulated steroidogenesis is exerted at the level of StAR protein, Endocr Res. 28, 413-7.
[45] Mele, P.G., Duarte, A., Paz, C., Capponi, A. and Podesta, E.J., 2012. Role of intramitochondrial arachidonic acid and acyl-CoA synthetase 4 in angiotensin II-regulated aldosterone synthesis in NCI-H295R adrenocortical cell line, Endocrinology. 153, 3284-94.
[46] Cooke, M., Orlando, U., Maloberti, P., Podesta, E.J. and Maciel, F.C., 2011. Tyrosine phosphatase SHP2 regulates the expression of acyl-CoA synthetase ACSL4, J Lipid Res. 52, 1936-48.
[47] Hoppins, S., Lackner, L. and Nunnari, J., 2007. The machines that divide and fuse mitochondria, Annu Rev Biochem. 76, 751-80.
[48] Huang, P., Yu, T. and Yoon, Y., 2007. Mitochondrial clustering induced by overexpression of the mitochondrial fusion protein Mfn2 causes mitochondrial dysfunction and cell death, Eur J Cell Biol. 86, 289-302.
[49] Zorzano, A., Liesa, M. and Palacin, M., 2009. Role of mitochondrial dynamics proteins in the pathophysiology of obesity and type 2 diabetes, Int J Biochem Cell Biol. 41, 1846-54.
[50] Zorzano, A., Sebastian, D., Segales, J. and Palacin, M., 2009. The molecular machinery of mitochondrial fusion and fission: An opportunity for drug discovery?, Curr Opin Drug Discov Devel. 12, 597-606.
[51] Mikkola, R., Saris, N.E., Grigoriev, P.A., Andersson, M.A. and Salkinoja-Salonen, M.S., 1999. Ionophoretic properties and mitochondrial effects of cereulide: the emetic toxin of B. cereus, Eur J Biochem. 263, 112-7.
[52] Lewin, T.M., Van Horn, C.G., Krisans, S.K. and Coleman, R.A., 2002. Rat liver acyl-CoA synthetase 4 is a peripheral-membrane protein located in two distinct subcellular organelles, peroxisomes, and mitochondrial-associated membrane, Arch Biochem Biophys. 404, 263-70.
[53] Knudsen, J., Jensen, M.V., Hansen, J.K., Faergeman, N.J., Neergaard, T.B. and Gaigg, B., 1999. Role of acylCoA binding protein in acylCoA transport, metabolism and cell signaling, Mol Cell Biochem. 192, 95-103.

***Highlights (for review)**

In the present manuscript we present data showing that it was possible to demonstrate that an individual cell's response could be initiated and localized to the site of LH-receptor complex formation. Using single cells and limiting stimulation to a "patch" of membrane help in understanding a membrane signal transduction at the single cell level and the role of compartmentalized responses in the mechanism of peptide hormone action in steroid synthesis.

We also present data showing that the hormonal stimulation triggers mitochondrial fusion and that mitochondrial fusion does not only correlate-with but also is an essential step in steroid production, both events being dependent on PKA activity. We also show that the hormone-stimulated relocalization of ERK1/2 in the mitochondrion, a critical step during steroidogenesis, depends on mitochondrial fusion. Strikingly, we found that mitofusin 2 expression, a central protein for mitochondrial fusion, is upregulated immediately after hormone stimulation. Moreover, mitofusin 2 knockdown is sufficient to impair steroid biosynthesis. Together, our findings unveil an essential role for mitochondrial dynamics during steroidogenesis. These discoveries highlight the importance of the reorganization of organelles in specialized cells, prompting the exploration of the impact that organelle dynamics has on biological processes that include, but are not limited to, steroid synthesis.

Figure 1

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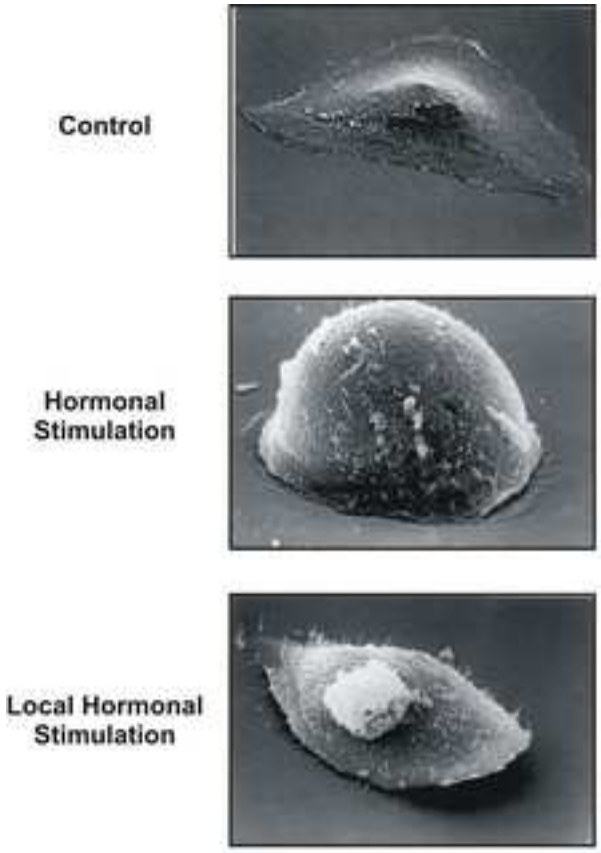

Figure 2
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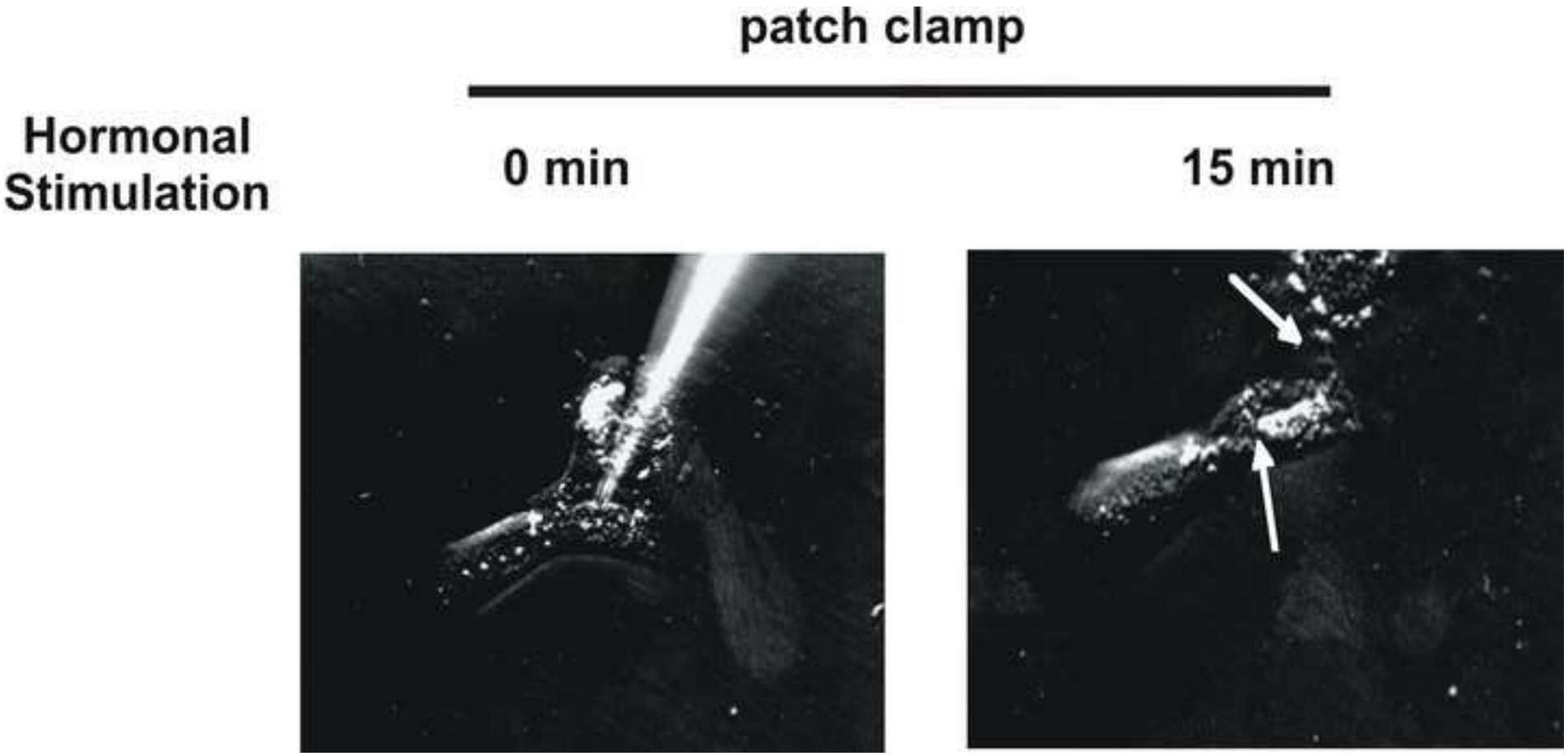

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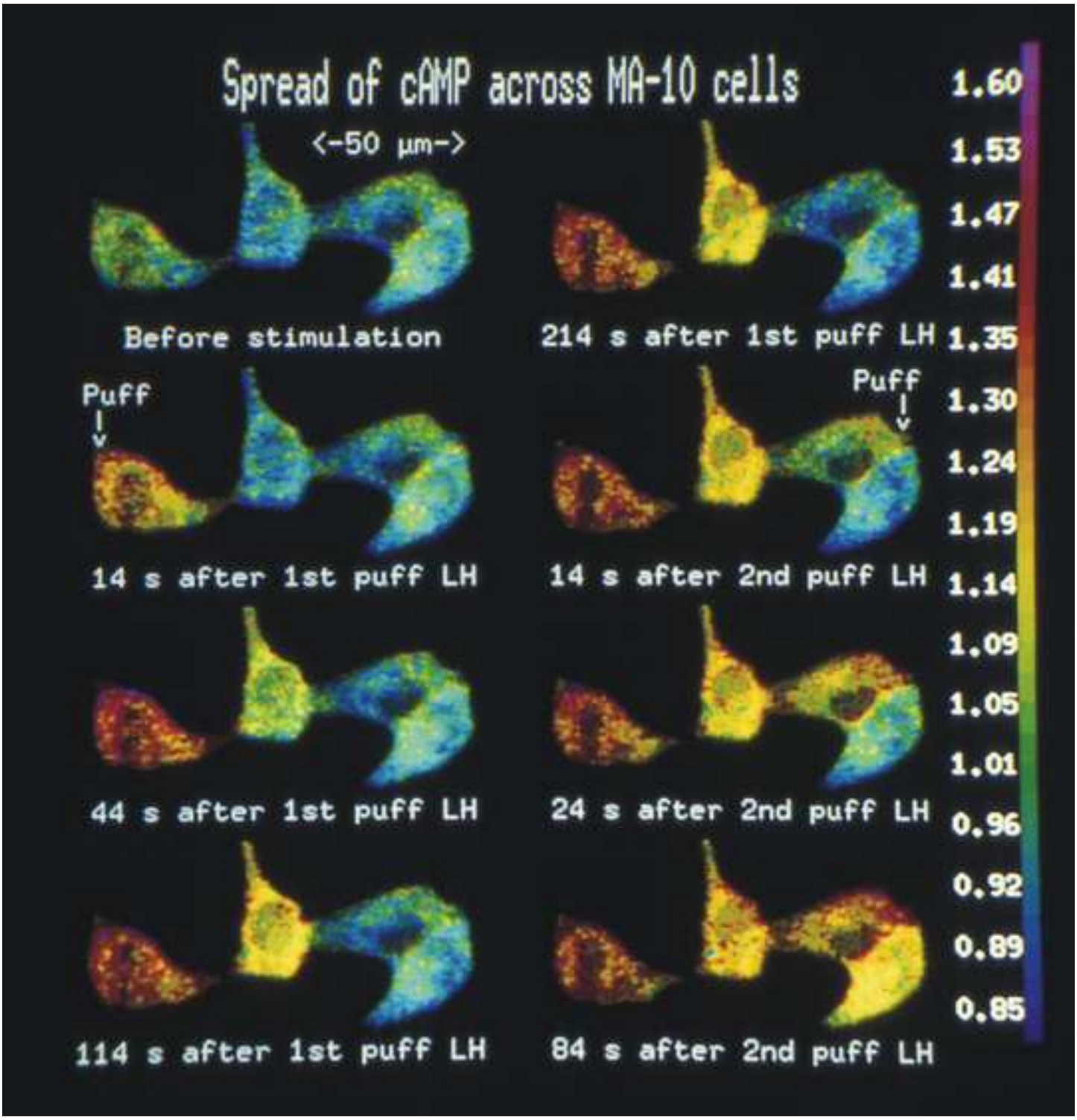

Figure 4

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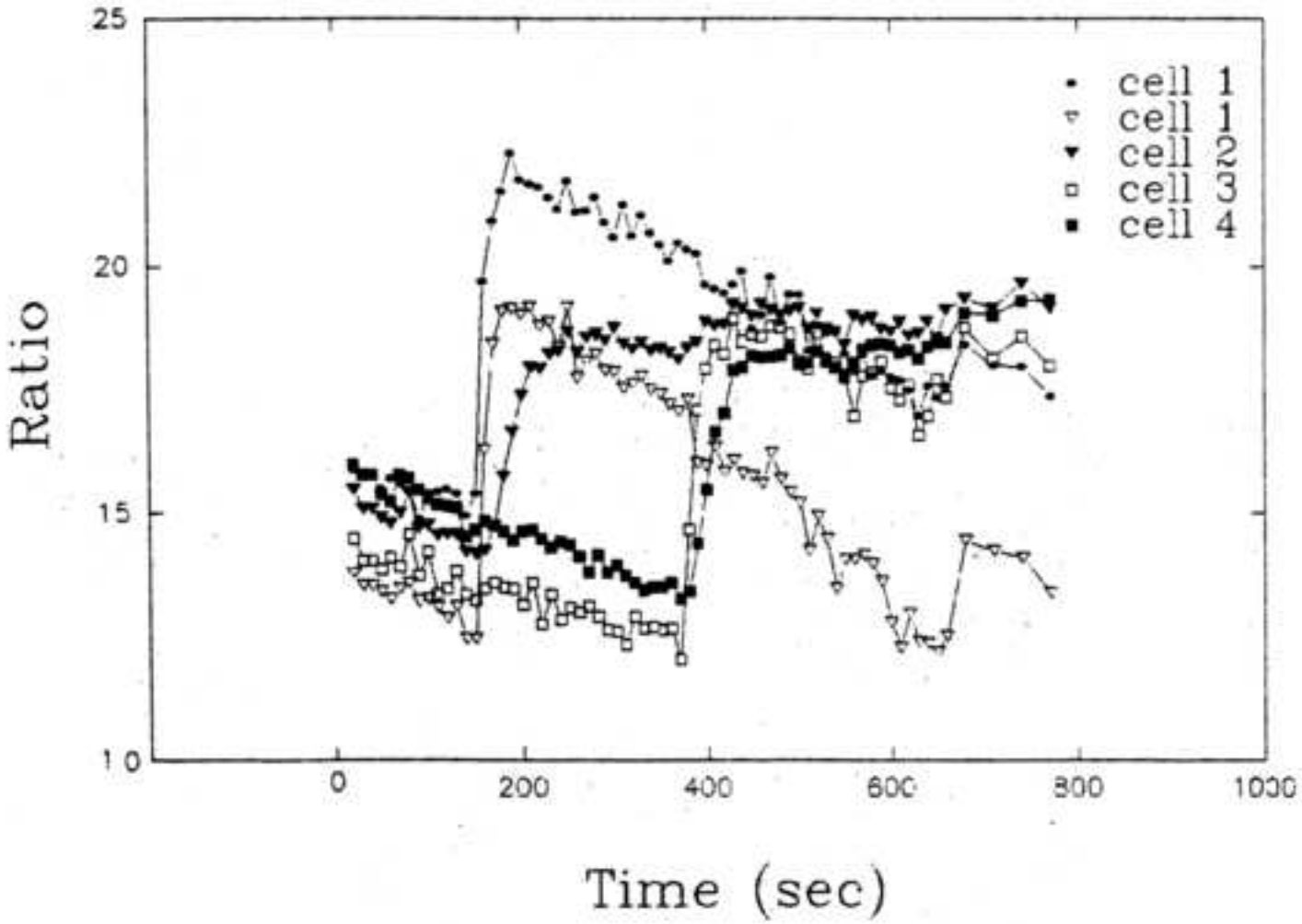

Figure 5

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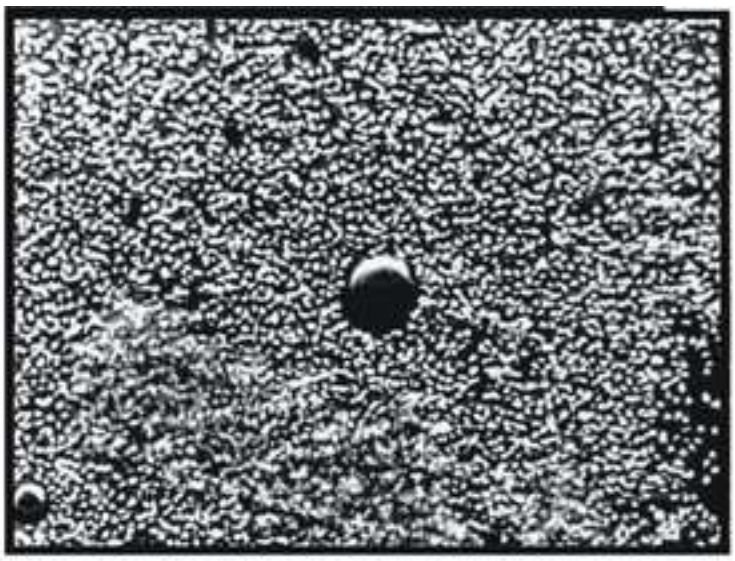
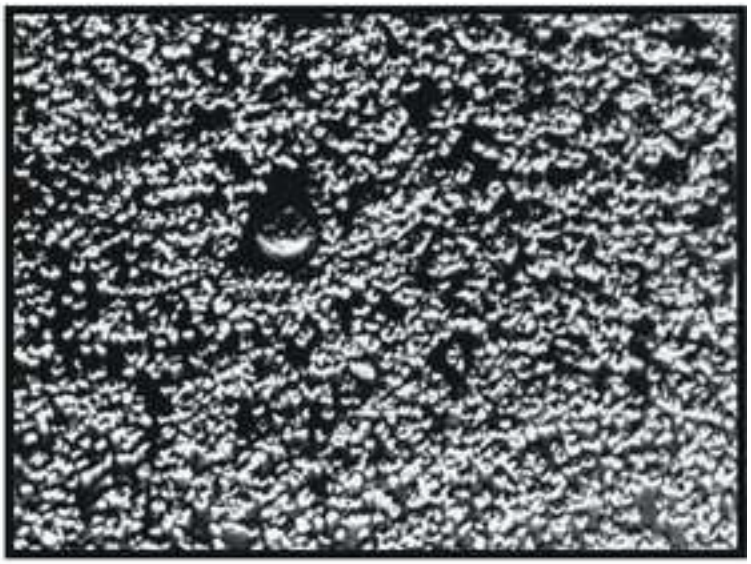
Control



General Stimulation



Local Stimulation

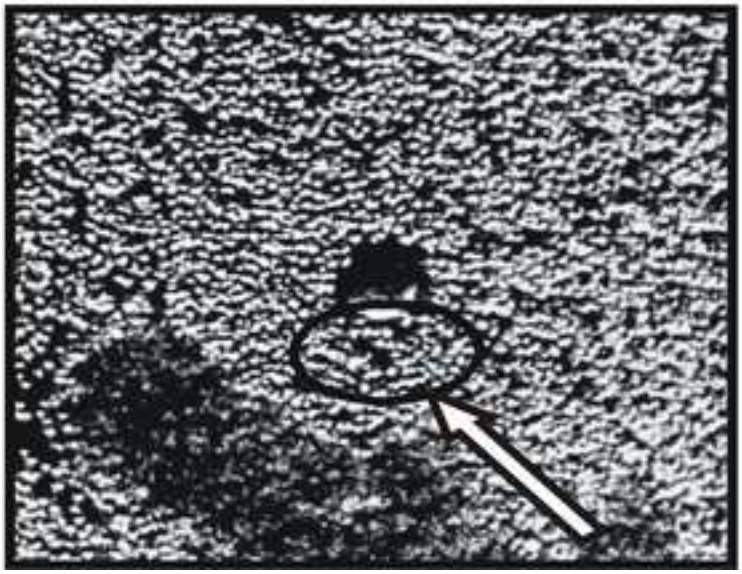


Figure 6

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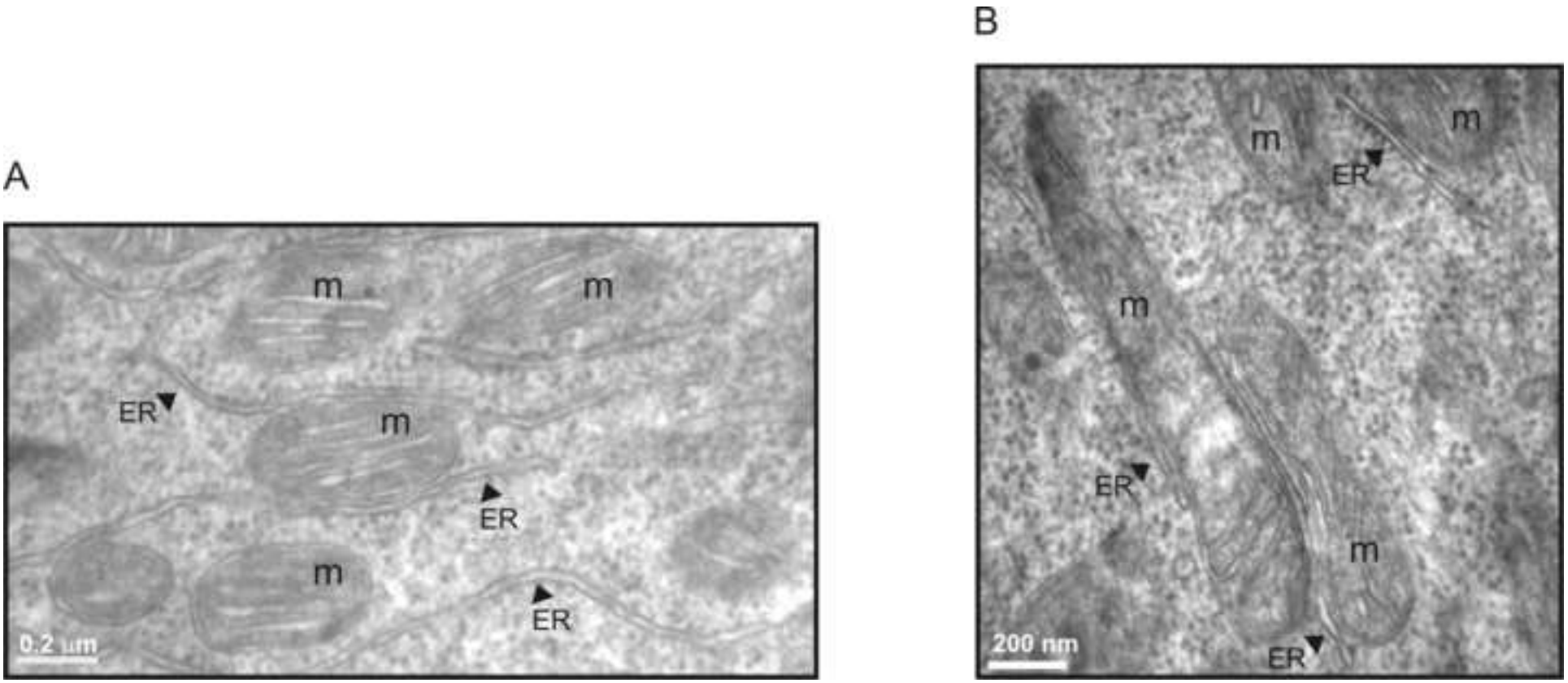

Figure 7

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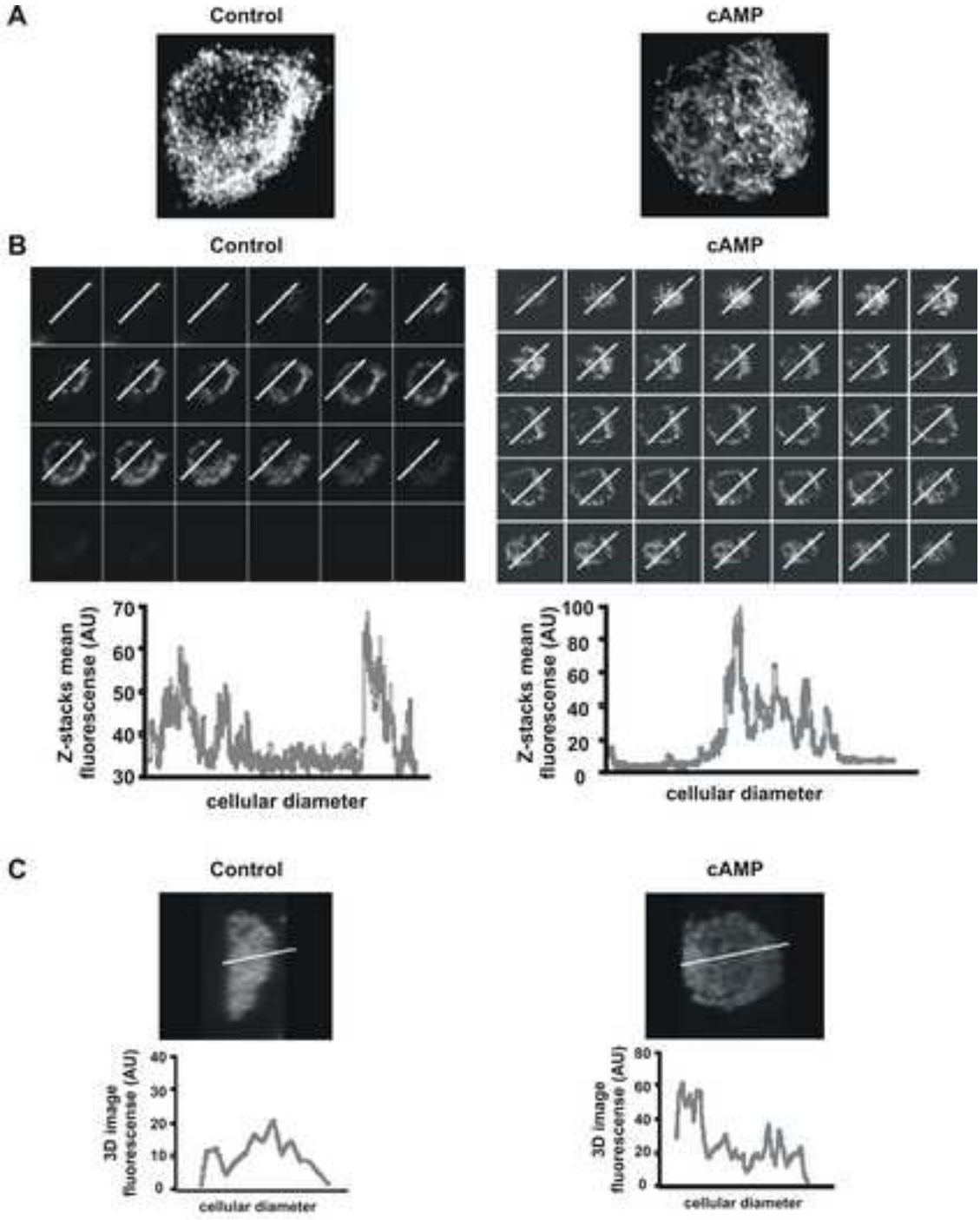

Figure 8

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