UNIVERSITY OF NAPLES "FEDERICO II"

PhD Program

Molecular Pathology and Physiopathology

School of Molecular Medicine

XXVIII Cycle

Control of PKA signaling by the Ubiquitin proteasome system (UPS)

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Academic year 2014-2015

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ORIGINAL PUBBLICATIONS

1. Lignitto L, Arcella A, Sepe M, **Rinaldi L**, Delle Donne R, Gallo A, Stefan E, Bachmann VA, Oliva MA, Tiziana Storlazzi C, L'Abbate A, Brunetti A, Gargiulo S, Gramanzini M, Insabato L, Garbi C, Gottesman ME, Feliciello A. (2013) Proteolysis of MOB1 by the ubiquitin ligase praja2 attenuates Hippo signalling and supports glioblastoma growth. *Nature Communication.*2013, 4:1822. Doi:10.1038/ncomms2791.

2. Sepe M, Lignitto L, Porpora M, Delle Donne R, **Rinaldi L**, Belgianni G, Colucci G, Cuomo O, Viggiano D, Scorziello A, Garbi C, Annunziato L, Feliciello A.(2014) Proteolytic control of neurite outgrowth inhibitor NOGO-A by the cAMP/PKA pathway. *Proc. Natl. Acad. Sci.* 2014, 111(44):15729-15734.

3. **Rinaldi L**, Sepe M, Donne RD, Feliciello A.(2015) A dynamic interface between ubiquitylation and cAMP signaling. *Front. Pharmacol.* 2015, 6:177.

4. **Rinaldi L.***, Delle Donne R.*, Sepe M., Porpora M., Garbi C., Chiuso F., Gallo A., Parisi S., Russo L., Bachmann V., Huber R. G., Stefan E., Russo T. and Feliciello A.(2016) praja2 regulates KSR1 stability and mitogenic signaling . *Submitted to Cell Death and Desease.*

5. **Rinaldi L.**, Delle Donne R., Sepe M., Porpora M., Sauchella S., Chiuso F., Nisticò R., Piccinin S., Stefan E., Feliciello A. Control of PKA signaling by the Ubiquitin proteasome system (2016). *Manuscript in preparation.*

LIST OF ABBREVIATION

PDE Phosphodiesterase

AC Adenylyl cyclase

GPCR G protein coupled receptor

PKA Protein kinase A

UPS Ubiquitin Proteasome System

CHIP C-terminus of Hsc70 Interacting protein

cAMP cyclic AMP

PKA-C Catalytic subunit of protein kinase A

FSK Forskolin

HSPI Hsp70 Inhibitor

ABSTRACT

Stimulation of G protein coupled receptors(GPCRs) causes the increase of cAMP intracellular levels. The main effector of cAMP signaling is Protein kinase A (PKA), which, in its inactive form, is constituted by two catalytic (Cs) and two regulatory (Rs) subunits. The binding of cAMP to the Rs causes the disassembly of the holoenzyme and the release of the Cs in the cytoplasm, with the conseguent phosphorylation of a wide array of cellular substrates. The duration and the amplitude of the PKA signaling is dependent on the amount of free C subunits in the cell. Here I contributed to identify a novel mechanism of PKA signaling attenuation, based on the ubiquitination and degradation of the C subunit of PKA (PKA-C). Stimulation of GPCRs induced poly-ubiquitination and degradation of PKA-C , causing the decrease of PKA substrates activation. I identified the complex HSP70/CHIP as responsible for this ubiquitination. Interfering with CHIP expression or inhibiting the HSP70 activity impeded PKA-C ubiquitination and sustained PKA signaling.

Thus the HSP70/CHIP complex regulates the total concentration of C subunits, tuning the strength and duration of PKA signaling in response to cAMP.

1.INTRODUCTION

1.1 The cAMP-dependent signal transduction pathway

Extracellular signals, such as hormones and growth factors, regulate multiple cellular activities. Intracellular transduction systems receive the signals and transmit them to elicit specific biological responses. Since living cells are exposed to a wide variety of simultaneous stimuli, the tight regulation of the intracellular transduction pathways is crucial for the appropriate response.

Cell surface signal transducing systems are the most common way for information to flow into the cell. In these systems, plasma membrane receptors recognize extracellular signals and, in turn, activate intracellular messengers, also known as second messengers.

The first second messenger to be identified was cyclic adenosine 3′,5′-monophosphate (cAMP) (1). Since its discovery, all the elements of the cAMP transduction pathway have been intensively studied, generating a complex and finely regulated network of scaffolding and regulatory proteins (2) . The intracellular levels of cAMP are controlled by the balance between two classes of proteins: the Adenylyl Cyclases (ACs), enzymes that convert ATP in cAMP, and the Phosphodiesterases (PDEs), enzymes that hydrolyze the 3'-5' phosphodiester bond in cAMP, producing 5' AMP (3).

G protein coupled receptors (GPCRs) belong to a large family of proteins characterized by seven trans-membrane helices (7 TMs). GPCRs transduce signals from the extracellular microenvironment to inside cell (4). GPCRs include receptors for sensory signal mediators, for biogenic amines (dopamine, epinephrine, isoproterenol), and for peptide hormones (FSH, GH).

The binding of extracellular ligands to GPCR at cell membrane causes a conformational change in the receptor, allowing the binding to the α subunit of the G proteins (Gαs). The activated Gα exchange the GDP with GTP triggering the dissociation of the α subunit from the Gβ and Gγ subunits. The dissociated Gα activates the Adenylate Cyclase (AC), which generates cAMP at discrete points of the plasma membrane (5) .

cAMP acts through three distinct classes of effectors: cAMP-dependent protein kinase (PKA), RAP exchange proteins (EPACs), and cAMP gated ion channels (cNGC) (6).

Following the stimulation of GPCRs, PKA translocates into the nucleus. Once in the nucleus it stimulates the transcription of several factors, through phosphorylation of the cyclic AMP-responsive element binding protein (CREB) at Ser 133 (7) . Phosphorylated CREB binds to cAMP response elements (CREs) of gene promoters and recruits histone acetyl transferases CREB binding protein (CBP) and p300 to activate the transcription of target genes (8).

Continuous or repeated pulses of hormone stimulation down-regulate GPCR activation. This phenomenon is called receptor desensitization (9). Desensitization can be homologous or heterologous, the first one consists in a loss of response to agonists that act to a particular subtype of GPCRs, whereas the heterologous desensitization refers to a more general effect involving the simultaneous loss of agonists responsiveness at multiple GPCRs (10). The deregulation of cAMP/PKA signaling, or genetic mutation of its components are associated with pathological conditions, such as cardiac dysfunctions (11), familial breast cancer (12) and schizophrenia (13).

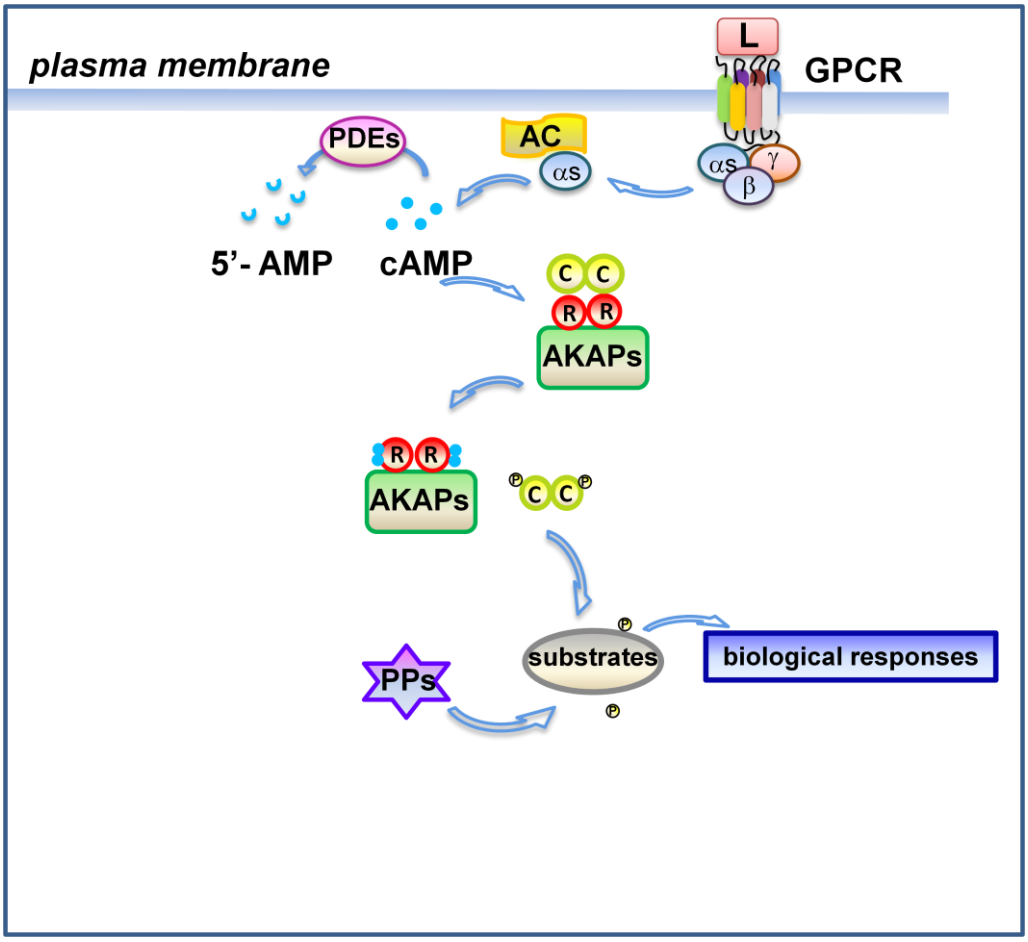

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Figure 1. cAMP signaling The binding of a ligand to the GPCR causes the dissociation of heterotrimeric G proteins and the cosequent activation of AC by Gα subunit. AC converts ATP into cAMP; cAMP binds the regulatory subunits of PKA, eliciting the dissociation from the catalytic subunits. The free catalytic subunits phosphorylate a wide array of substrates, evoking plenty of biological responses. cAMP levels are regulated by PDEs that converts the cAMP in 5' AMP.

1.2 Protein Kinase A (PKA)

In eukaryotes most of the effects elicited by cAMP depend on the activation of PKA. PKA is an holoenzyme constituted by two regulatory (Rs) and two catalytic (Cs) subunits. Rs exist in two major forms: RI and RII, each form have two different subtypes, alpha and beta, and every isotype is encoded by a different gene. Depending on type of R subunits (RI or RII) present in the holoenzyme, PKA is classified as type 1 and type 2, with different sensitivity to cAMP activation and different localization (14).

Catalytic subunits exist in three different subtypes, alpha, beta and gamma. Cα and Cβ subunits are highly homologous (93%), exibit similar substrate specificity and are broadly expressed in all tissues (Ulher M.D., et al. 1986) (15). The Cγ subunit is more tissue-specific and less sensitive to PKA specific inhibitors, compared to Cα and Cβ (16).

The activity of PKA is modulated by a family of proteins called PKA inhibitors (PKI). These proteins are characterized by a heptapeptide sequence (LLRASLG) called Kemptide motif. This motif acts as pseudo-substrate, it binds to and inhibits the C subunit (17).

The binding of two molecules of cAMP to each R subunit causes the dissociation of the holoenzyme and release of the two active C subunits. Free C subunits catalyze the transfer of the terminal phosphate of ATP to a Serine/Threonine residue located at position 4 of the PKA consensus site (Arg-Arg-X-Ser/Thr) of the target protein. By phosphorylating intracellular substrates, PKA regulates essential aspects of cell physiology (18).

PKA C subunit was the first kinase whose structure has been resolved by crystallography. This kinase is composed by two lobes, the upper

lobe (residues 25-120) and the lower lobe (128-310), and the catalytic site is positioned between the two lobes. The key residues for kinase activity are the Asparagine-166, which serves as catalytic base, the Lysine-168 that neutralizes the negative charge of the ATP phosphate group during its transfer to the substrate and the Arginine-184 that chelates the Mg^{2+} ions (19), (20).

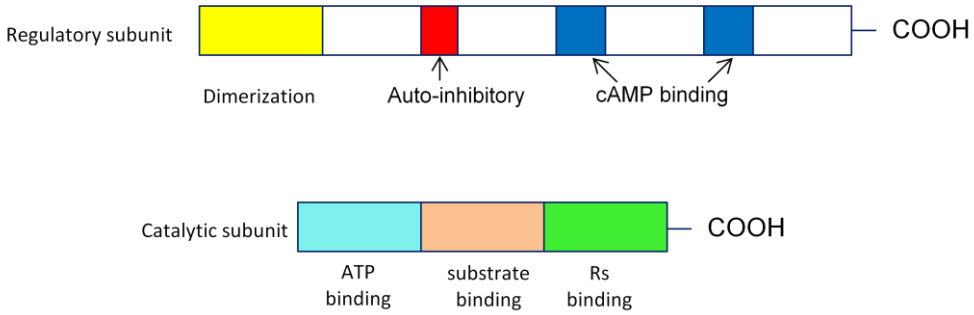

Figure 2. Schematic structure of PKA subunits Regulatory subunit of PKA is characterized by a dimerization domain at the N-terminal end of the protein, an auto-inhibitory domain and the two cAMP binding domains.

The catalytic subunit has an ATP binding domain located at its the N-terminus, a substrate binding domain in the middle region and one R-binding domain at its C-terminus.

The functional and the biochemical features of the PKA holoenzymes are mostly determined by the relative abundance of the intracellular Rs. The localization of PKA within the cell is mediated by scaffold proteins, called A-Kinase Anchor Proteins (AKAPs). AKAPs belong to a group of proteins that share the common feature to target PKA in proximity of its substrates (21). Each AKAP contains a PKA-binding motif that binds the R subunit of PKA and a targeting domain that directs the kinase to specific subcellular compartments (22). Moreover, AKAPs can organize multiplex signalling complexes at the precise site where the stimulus is generated, through the binding with other components of the cAMP cascade, as GPCRs, phosphodiesterase (PDEs), ACs, protein phosphatases (14),(23).

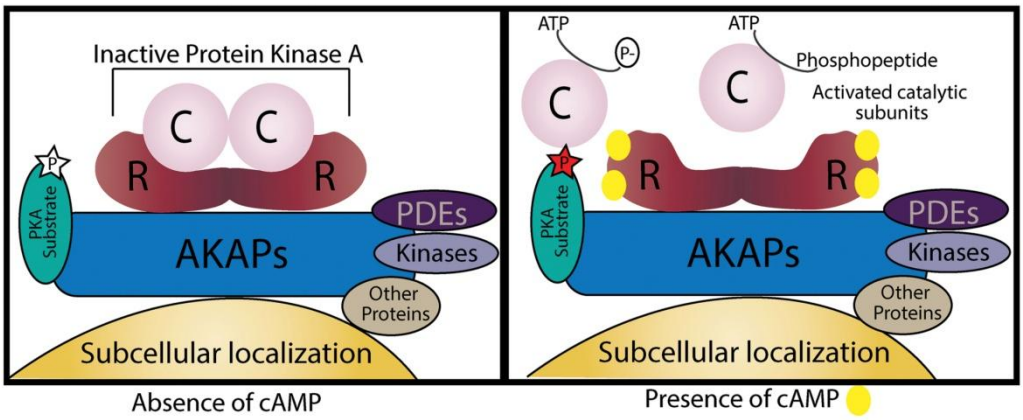

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Figura 3. AKAP transduceosome AKAPs role is to target PKA and its interactors to specific subcellular localizations. They can also function as signaling scaffolds for other signaling enzymes, this particular phenomenon compartimentalizes the signal making it faster and more efficient.

Following cAMP stimulation, PKA holoenzyme undergoes to phosphorylation at Ser 95 of RII subunits. As a consequence of this auto-phosphorylation, the affinity between the catalityc subunits and the regulatory subunits decreases (24). The other two phosphorylation events occur at two sites of the catalytic subunit: Ser338 and Thr197. Phosphorylation of Ser338 is an essential step for correct folding of the kinase and for its activity (25).

Activation of PKA is followed by a refractory phase caused by different mechanisms: 1. activation of Ser/Thr phosphatases (26); 2. activation of PDEs (27) ; 3. Downregulation of GPCRs (28); 5. inhibition of ACs (29) 4. transcriptional regulation of components of the cAMP network (30) ; 5. ratio between R and C subunits (31).

Dis-regulation of PKA is associated with a variety of pathological conditions. Thus, germline inactivating mutations or large deletions of the gene encoding for the RIα subunit (*PRKAR1A)* cause the Carney complex syndrome, a rare autosomal dominant syndrome, characterized by multiple endocrine tumors (32). *PRKAR1A* inactivation and PKA dysregulation have been implicated also in various types of adrenocortical pathologies associated with ACTH-independent Cushing syndrome (AICS). More recently, somatic mutations of PKA Cα subunit have been associated with the pathogenesis of cortisol-producing adrenocortical tumors. These mutations abolish the interaction between the R and C subunits, causing a long-lasting cAMP-independent activation of the PKA (33) .

1.3 The ubiquitin proteasome system (UPS)

The ubiquitin-proteasome system is an important mechanism of protein degradation that is involved in the homeostasis of numerous cell functions, such as cell growth, differentiation, survival and metabolism.

Proteolysis via UPS involves the modification of the substrate proteins by the covalent attachment of multiple ubiquitin molecules (poly-ubiquitination). The ubiquitin tagged protein is eventually targeted to proteasomal degradation (34).

The ubiquitynation is a process mediated by the attachment of ubiquitin to the amino group of lysine residues of a target protein. This process requires a series of ATP dependent enzymatic steps catalyzed by E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme) and E3 (ubiquitin ligating enzyme). The E3 ubiquitin ligase enzyme, which tags the substrate with the ubiquitin molecules, determines the target specificity of the process (35).

E3 ligases belong to four different families: HECT (Homologous to the E6-AP Carboxyl Terminus), RING (Really Interesting New Gene), U-box, and PHD (plant homeodomain). The HECT is the only E3 ubiquitin ligases that directly ubiquitinate the substrates. They function similarly to E1 and E2 enzymes, as they contain a central cystein residue that acts as an acceptor for ubiquitin.

The RING E3 ligases constitute the largest family characterized by a cystein-rich zinc binding domain. They do not have a direct role in protein ubiquitination, instead they act as scaffold, helping the substrate proteins and the E2 enzymes to interact (36).

The U-box is a 74 amminoacid domain, structurally related to the RING finger domain. Like RING finger E3s, they facilitate the E2 interaction with the substrate (37).

All the modifications described can involve the attachment of both a single ubiquitin molecule (mono-ubiquitination) and a multiple ubiquitin moieties (poly-ubiquitination) (38). E3 ubiquitin ligases can catalyze either mono- and poly-ubiquitination.

The destiny of a polyubiquitinated protein depends of which lysine residue of the ubiquitin molecules is attached to the substrate. Ubiquitin molecules linked through the lysine at position 48 (K48) target proteins to the proteasome. However, all the lysines of the ubiquitin molecule can be involved in the covalent binding to the next ubiquitin molecule (K6, K11, K27, K29, K48, and K63-) *(39)*

Poly-ubiquitination of a protein is mostly related to protein degradation through the proteasome. Alternatively, polyubiquitinated proteins can also follow a non-degradative pathway (40). In particular, this mechanism can control the intracellular trafficking of the target protein or its activity (41). In this case, de-ubiquitinating enzymes (DUBs) can remove the ubiquitin chain and restore the localization/activity of the modified proteins (40).

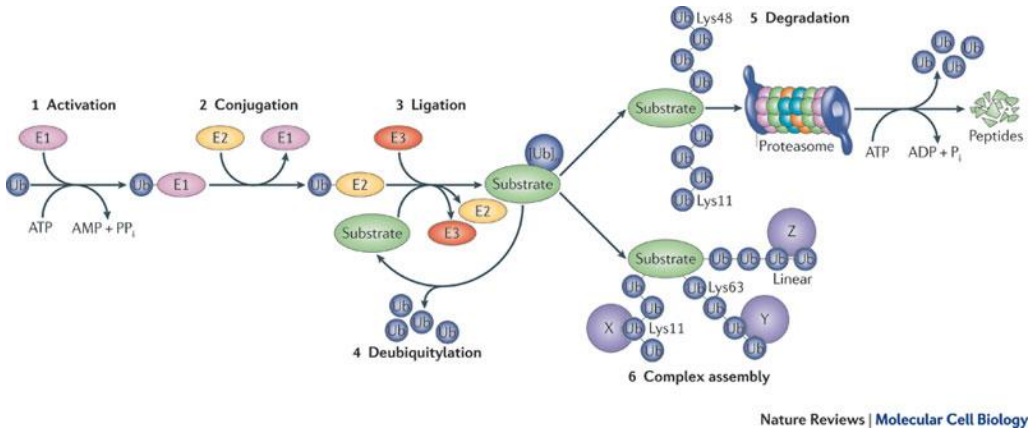

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Figure 4. Schematic representation of Ubiquitin system The conjugation of ubiquitin molecules to substrates requires coordinated action of three enzymes: the ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme E2 and the E3 ubiquitin ligase, that links the ubiquitin molecules to substrates. Once ubiquitinated, the proteins can be degraded by the proteasome or de-ubiquitinated by specific DUB enzymes.

1.4 Carboxy-terminus of HSP70 interacting protein (CHIP)

CHIP (C-terminus of Hsc70 Interacting protein), also known as STIP1 homology and U-Box containing protein 1 (STUB1), is a very conserved protein, ubiquitously expressed, that belongs to the family of U-box E3 ubiquitin ligase. It binds to the molecular chaperone Hsc70, controlling both the ubiquitination and the turnover of the Hsc-70 bound clients (42).

Heat shock cognate 70 (Hsc70) is a costitutively expressed member of the molecular chaperones Hsp70 family. It is an important member of the protein quality control system, and works together with a list of co-chaperones to help the refolding of proteins, and to degrade the misfolded proteins (43, 44).

The concerted activity of Hsc70 and CHIP is fundamental to reduce, through the ubiquitin-proteasome system, the levels of disease-associated and misfolded proteins, like the microtubule-binding protein tau, or the cystic fibrosis transmembrane conductance regulator (45, 46). CHIP also modifies Hsc70 by adding canonical and non canonical poly-ubiquitin chains (47), increasing Hsc70 activity and enhancing its turn-over (48, 49).

CHIP interacts with Hsc70 through a C-terminus located motif , composed of 8 residues (GPTIEEVD) (50). A similar motif is present also in the Hsp90 molecular chaperone, allowing CHIP to ubiquitinate Hsc70- and Hsp90-bound substrates (51). The function of CHIP strictly depends on its capability to bind to Hsc70 or Hsp90. A tetratricopeptide repeat (TPR) domain located at the N-terminus of CHIP mediates the binding to HSP70/90. The TPR consist of 3 to 16 degenerate 34 amminoacid motifs constituted by anti-parallel α-helical hairpins (52).

A U-box domain present at the C-terminus of CHIP displays ubiquitin ligase activity. CHIP provides a direct link between the protein quality control system directed by chaperones and the proteasome system, and it is deputed to regulate the cellular balance between folding and degradation, playing a key role in the maintenance of cellular homeostasis.

Inactivating mutations in STUB1 gene have been recently linked with different form of ataxia and hypogonadism. In particular mutations in this gene cause spinocerebellar ataxia, autosomal recessive 16 (SCAR16). Loss of CHIP function in mice results in behavioral and reproductive impairment, miming the human ataxic phenotipe (53, 54).

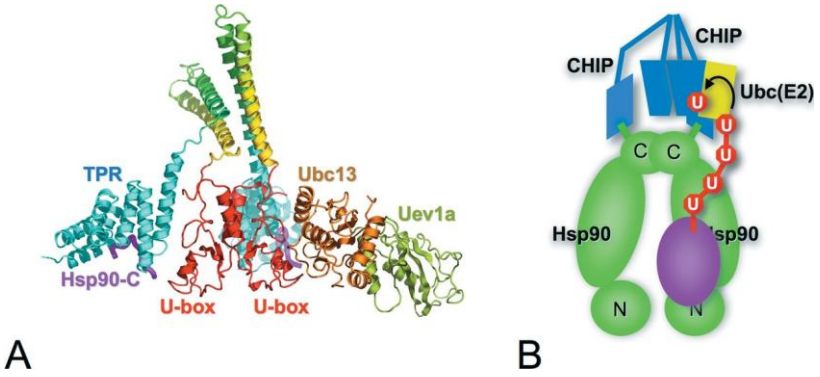


Figure 5. Chaperone-directed E3-ubiquitin ligase CHIP (**A**) Model of CHIP E3–E2 complex. The model is a composite of crystal structures of the full-length CHIP dimer bound to the C-terminal peptide of Hsp90 (magenta), and the crystal structure of a complex of the CHIP U-box dimer bound to the heterodimeric Lys^{63}-specific E2 ubiquitin-conjugating enzyme Ubc13–Uev1a. The unusual asymmetric structure of CHIP allows it to bind to both C-terminus of an Hsp90 dimer, but to recruit only one E2 system. (**B**) Schematic figure showing how a client protein bound to Hsp90 could be polyubiquitinated by CHIP as a necessary prelude to degradation by the proteasome. U, ubiquitin

2.AIM OF THE THESIS

2.1 AIM OF THE THESIS

The laboratory where I completed my PhD program is interested in the mechanisms underlying cAMP signaling in mammalian cells. In the last years, our attention was focused on the regulation of cAMP signaling by the UPS. In this context, it is emerging a reciprocal regulation between cAMP-PKA and the UPS at different levels, giving rise to a complex regulatory network that controls key biological activities. We have demonstrated that PKA stability and activity can be regulated by the UPS. Thus, the RING-H2 ligase praja2 once activated by PKA, primes R subunits to the UPS. By lowering the levels of inhibitory R subunits, praja2 increases PKA-dependent phosphorylation and downstream signaling(55) .

The aim of my PhD project was to find a connection between the UPS activity and the stability of PKA catalytic subunit (PKA-C), identifying the E3 ubiquitin ligase responsible for PKA-C degradation and evaluating the physiological consequences of PKA-C regulation by the UPS.

More specifically, my attention was focused on the following points:

- Understand if PKA-C is a target of the UPS
- Identify the E3 ubiquitin ligase that controls PKA-C stability
- Analyze the complex formed by PKA-C and the E3 ligase.
- Define the functional relevance of the PKA-C modulation by the UPS.

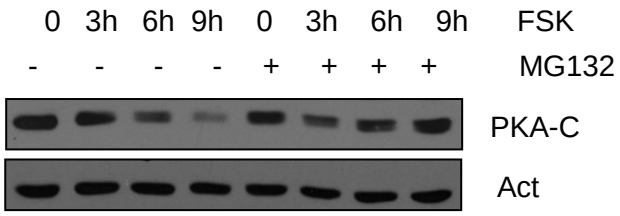
3.RESULTS

3.1 The UPS is responsible for PKA-C degradation.

Given this role of cAMP and the proteasome in the regulation of the PKA regulatory subunits stability, I asked if PKA-C is also degraded by the UPS.

To this aim, I treated HEK293 cells with Forskolin (FSK), a diterpene that activates the transmembrane adenylate cyclase, and analyzed the levels of PKA-C subunit in cell lysates. The data shown in **Fig 6a and 6b** revealed that prolonged FSK treatments (for 6 and 9 hours) caused a severe decrease of PKA-C levels. To understand was involved in PKA-C degradation, the experiment was replicated by pre-treating the cells with MG132, a proteasome inhibitor. As shown in **Fig 6a and 6b**, the treatment with MG132 was sufficient to abrogate the PKA-C degradation induced by FSK.

a)



b)

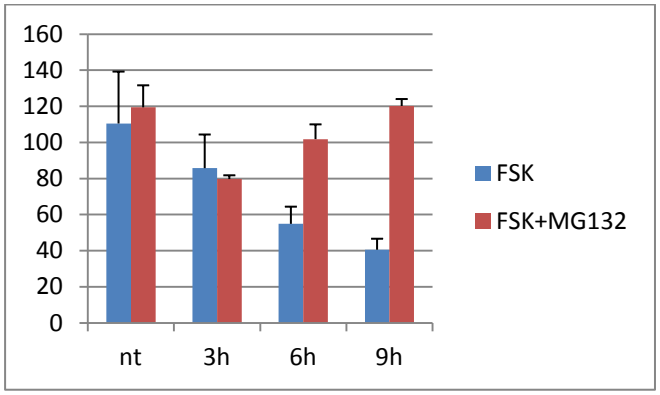


Fig.6. MG132 reverses cAMP dependent PKA-C degradation. a) HEK293 were treated with Forskolin (FSK, 40 uM) for the indicated times and harvested. Total lysates (20 ug) were immunoblotted with anti-PKA-C polyclonal antibody. Where indicated the cell were pretreated with MG131. **b**) Quantitative analysis of the experiment.

3.2 PKA-C is ubiquitinated in response to cAMP stimulus.

Given the role of the proteasome in PKA-C degradation, the next step was to understand if PKA-C was ubiquitinated in response to cAMP stimulation. To test this hypothesis, I performed *in vivo* ubiquitination assays. HEK293 cells were transfected with hemaglutinin (HA)-tagged ubiquitin, treated with FSK and harvested after 5' to 60' minutes of treatment. The lysates were immunoprecipitated with anti-PKA-C antibody, and the precipitates were immunoblotted with anti-HA antibody. **Fig. 7** shows that FSK induces the accumulation of poly-ubiquitinated form of PKA-C.

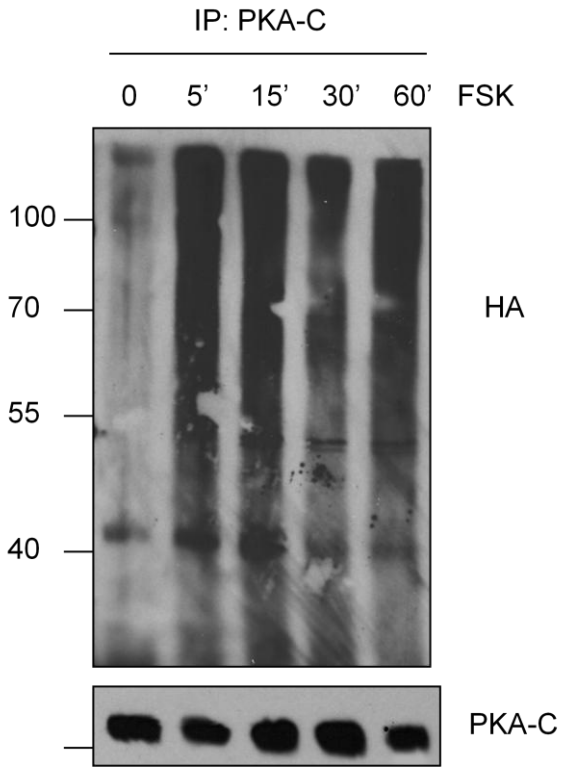


Fig 7. HEK293 were transfected with HA-ubiquitin. 24 hours after transfection cells, cells were treated with FSK (40uM) and harvested at the indicated time points. Lysates (1 mg) were immunoprecipited with anti-PKA-C antibody. The precipitates were immunoblotted with anti-HA and anti-PKA-C antibodies.

3.3 PKA-C interacts with the E3 ligase CHIP and the molecular chaperone HSP70.

Since PKA-C is efficiently ubiquitinated under cAMP stimulus, it was necessary to identify the E3 ligase responsible for this ubiquitination. By mass spectrometry analysis, I isolated several interactors of PKA-C. Among these, I found the E3 ligase CHIP and its molecular chaperone HSP70 as potential PKA-C interactors. These two proteins are known to form a dimeric complex, where HSP70 recognizes the target protein and the co-assembled CHIP ubiquitinates it. Therefore, I tested if HSP70, CHIP and PKA-C constitute a trimeric complex in cells. First, I performed a co-immunoprecipitation assay using lysates from cells transfected with the CHIP and HSP70 target. Where indicated, cells where stimulated with FSK. As shown in **fig. 8**, PKA-C is bound to HSP70, both under basal conditions or following FSK stimulation. CHIP was present in the HSP70 immunoprecipitates. In contrast, a CHIP mutant lacking the HSP70 binding activity (CHIP-K30A) was not recruited within the HSP70/PKA-C complex, indicating that HSP70 was necessary for CHIP binding to PKA-C.

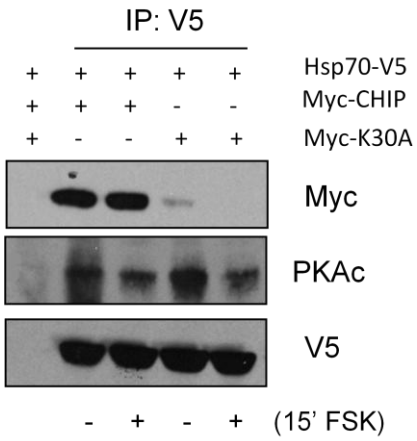


Figure 8. HEK293 cell were transfected with V5-HSP70 and Myc-CHIP (wt or mutant K30A). 24 hours after transfection, cells were treated with FSK for 15 minutes and harvested. Lysates were immunoprecipited with anti-V5 antibody and the precipitates were immunoblotted with the described antibodies.

To understand if the recruitment of CHIP on the PKAc/HSP70 complex was regulated, I performed co-immunoprecipitation assays using antibodies against PKA-C. As shown by **figure 9**, FSK treatment increases the levels of CHIP bound to PKA-C/HSP70, suggesting that the cAMP rise was required for the constitution of the trimeric complex. CHIP mutant (K30A) was used as negative control.

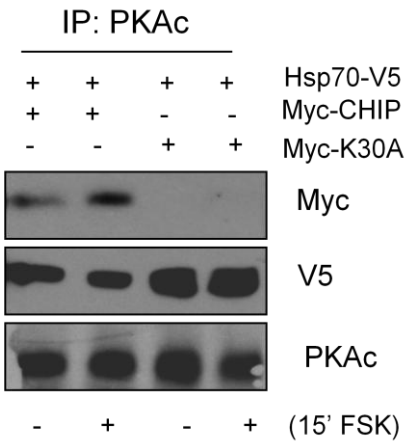


Figure 9. HEK293 cells were transfected with V5-HSP70 and Myc-CHIP (wt or mutant K30A). 24 hours after transfection, cells were treated with FSK for 15 minutes and harvested. Lysates were immunoprecipited with anti-PKAc antibody and the precipitates were immunoblotted with the described antibodies.

3.4 CHIP ubiquitinates PKA-C

The data above indicate that PKA-C, CHIP and HSP70 constitute a trimeric complex in cell extracts. Next, I asked if CHIP ubiquitinates PKA-C. To this aim, I transfected HEK293 cells with either CHIP (wt or mutant K30A) and HA-ubiquitin, and tested the accumulation of ubiquitinated PKA-C under growth conditions. The data shown in **fig. 10** indicates that CHIP promoted polyubiquitination of PKA-C. As expected, CHIP mutant (K30A) failed to ubiquitinate PKA-C.

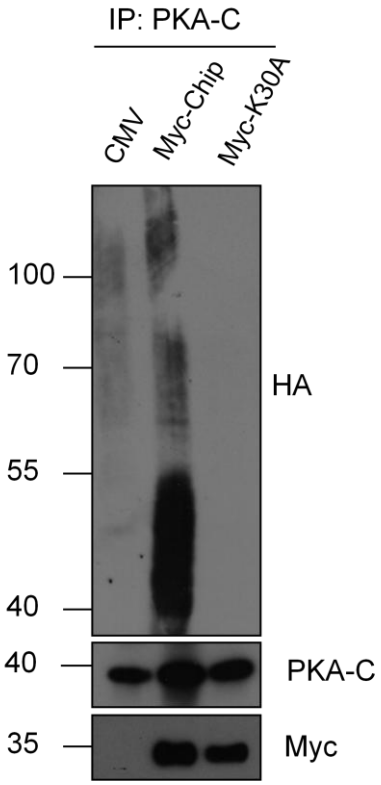


Fig.10. HEK293 cells were co-transfected with anti HA-ubiquitin and CHIP (wt or mutant K30A). 24 hours after transfection, cells were harvested. Lysates (1 mg) were immunoprecipited with anti-PKA-C antibody and immunoblotted with the indicated antibodies.

Since cAMP promotes PKA-C degradation through the UPS, I asked if CHIP ubiquitinates PKA-C following cAMP stimulation. To this aim, I performed the ubiquitination assay in cells expressing the CHIP mutant (K30A) and treated with the β-agonist Isoproterenol, a potent cAMP agonist. As expected, the K30A mutant reversed the isoproterenol-induced ubiquitination of PKA-C (**Fig 11**).

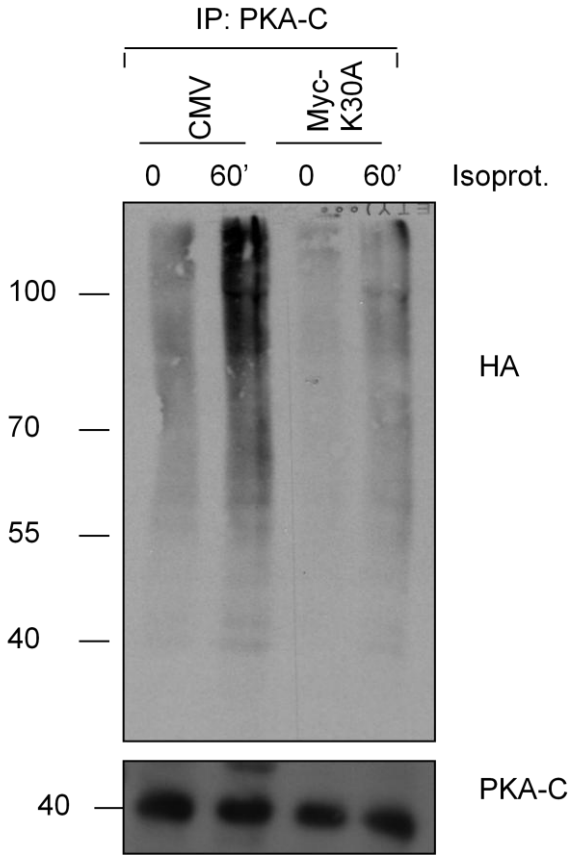
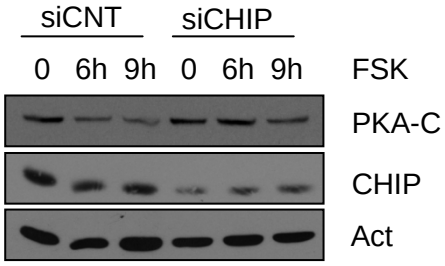


Fig 11. HEK293 cells were co-transfected with vectors encoding for HA-ubiquitin and mutant K30A. 24 hours after transfection, cells were treated with Isoproterenol and harvested 60 minutes later. Lysates (1 mg) were immunoprecipitated with anti-PKA-C antibody and immunoblotted with the indicated antibodie

3.5 CHIP degrades PKA-C.

The data described above indicate that CHIP constitutes a complex with PKA-C and HSP70, hence causing PKA-C poly-ubiquitination. Accordingly, I tested if CHIP was responsible of cAMP-induced degradation of PKA-C. To this aim, endogenous CHIP was down-regulated by RNAi. Cells were transfected were control siRNAs or siRNAs targeting endogenous CHIP. Tweenty-four hours from transfection, cells were left untreated or stimulated with FSK, to stimulate the PKA-C degradation. As shown in **Fig.12a** and **Fig.12b**, down-regulation of CHIP prevented PKA-C degradation induced by FSK treatment.

a)



b)

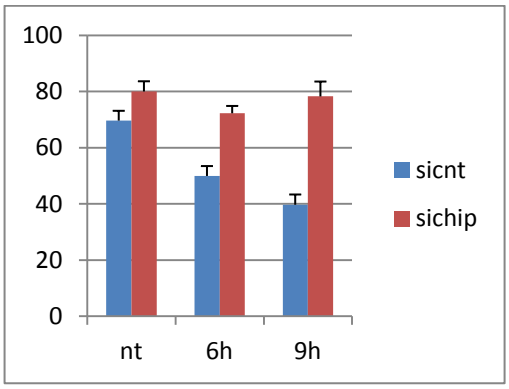


Fig 12. a) HEK293 cells were transfected with control siRNA or with siRNAs targeting CHIP. 48 hours after transfection, cells were treated with FSK for 6 and 9 hours. Lysates were immunoblotted with the indicated antibodies. b) Cumulative data of the experiment shown in a.

3.6 Inhibition of HSP70 inhibited PKA-C degradation by cAMP.

Published work from different laboratories demonstrate that CHIP requires HSP70 to ubiquitinate a given substrate (Feder- Hofmann, 1999; Imai, Yashiroda, 2003). Accordingly, I tested if pharmacological inhibition of HSP70 activity prevents PKA-C degradation. To this end I used an inhibitor (VER155008) that specifically targets HSP70, leaving working other HSPs. As shown in **fig.13**, treatment with VER155008 prevented the degradation of PKA-C induced by FSK.

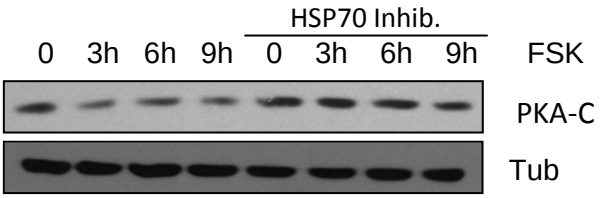


Fig.13 HEK293 cells were treated with the HSP70 inhibitor (VER155008, 40 uM), FSK (40 uM) or with both. Cells were harvested at the indicated times and lysates were immunoblotted with anti-PKA-C and anti-tubulin antibodies.

3.7 HSP70/CHIP complex regulates cAMP-dependent CREB phosphorylation.

Once activated, PKA-C translocates to the nucleus and phosphorylates the transcription factor CREB at Ser133, promoting phospho CREB/coactivator-dependent activation of gene transcription (Lonze et al. 2002). Given the role of HSP70/CHIP complex in the regulation of PKA-C stability, I asked if CREB phosphorylation at the PKA site was regulated by the complex. To this aim, I transfected HEK293 cells with the CHIP-K30A mutant and treated the cells with FSK for different times. As shown in **Fig 14**, expression of the K30A mutant enhanced CREB phosphorylation at Ser133, suggesting a role of CHIP in the regulation of CREB activity. To confirm that enhanced CREB phosphorylation in K30A expressing cells was, indeed, dependent on PKA, I treated the transfected cells with H89, a specific inhibitor of PKA. As predicted, the treatment with H89 reversed the effect elicited by K30A overexpression, causing a severe reduction of CREB phosphorylation in FSK-treated cells.

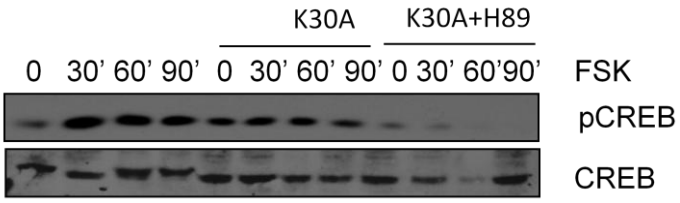
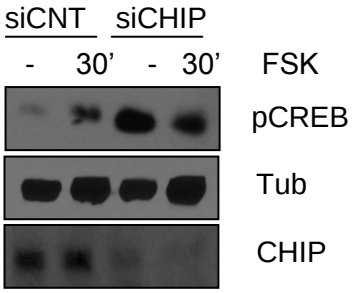


Fig 14. HEK293 cells were transfected with CHIP-K30A and treated FSK (40uM) for 30', 60' and 90'. Where indicated the cells were pretreated with with H89 (10uM). Lysates were immunoblotted with the indicated antibodies.

As shown in **Fig. 15a and Fig. 15b,** genetic silencing of endogenous CHIP confirmed the role of the ligase in the regulation of PKA-dependent phosphorylation of CREB.

a)



b)

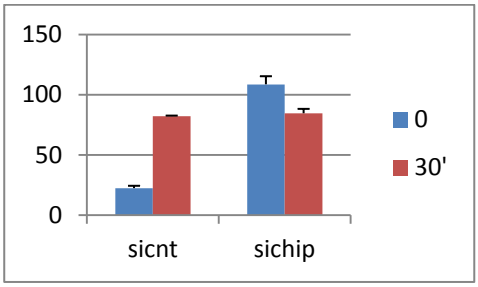


Fig. 15. a) HEK293 cells were transfected with control siRNA or with siRNA targeting endogenous CHIP. 48 hours after transfection, cells were left untreated or stimulated with FSK and harvested at the indicated times. Lysates were immunoblotted with the indicated anti-phospho CREB and anti-Tubulin antibodies. b) Quantitative analysis of experiment shown in a).

Next, I tested if HSP70 inhibition enhances CREB phosphorylation. To this aim, I treated the cells with HSP70 inhibitor and FSK, and evaluated CREB phosphorylation under basal and FSK stimulation. **Fig. 16** shows that HSP70 inhibition enhanced CREB phosphorylation, even

in the absence of FSK, and the levels of pohsphoCREB were insensitive to FSK treatment.

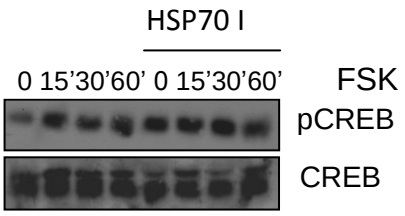


Figure 16. HEK293 cells were treated with FSK (40 uM). Where indicated, cells were pretreated with the HSP70 inhibitor (VER155008, 40 uM). Cells were harvested at the indicated times and lysates were immunoblotted with anti-phosphoCREB and anti-CREB antibodies.

3.8 HSP70/CHIP complex regulates c-fos transcription.

The binding of phosphoCREB to a cAMP responsive element (CRE) located within the *c*-fos gene promoter drives the transcription of the

downstream gene (56). Considering the role of HSP70/CHIP complex in the regulation of CREB phosphorylation, I tested if PKA-dependent *c*-fos transcription was regulated by CHIP. To this aim, I transfected HEK293 cells with control siRNA or with siRNA targeting CHIP. Cells where, then, treated with FSK and harvested at different time points from stimulation. By Real Time-PCR, I monitored the accumulation of *c*-fos mRNA under basal condition or following FSK stimulation, both in control and in CHIP-silenced cells. As shown in **fig.17**, activation of adenylate cyclase in control cells induced a robust increase of *c*-fos mRNA, at 30 and 60 minutes from treatment. In contrast, downregulation of CHIP enhanced basal transcription of *c*-fos by several folds over control values, without further increase of *c*-fos mRNA levels following FSK treatment.

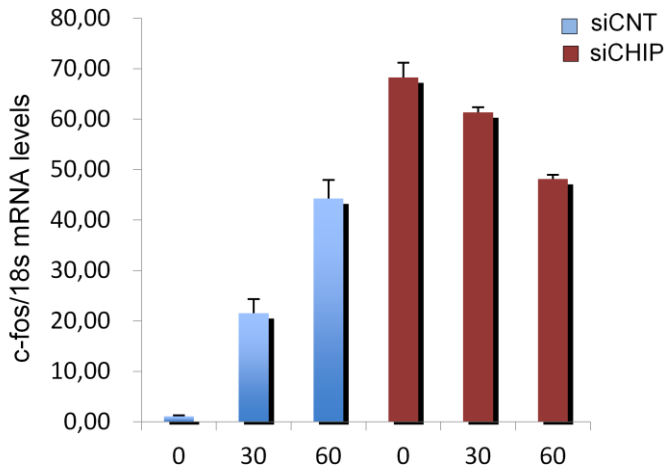


Figure 17 HEK293 cells were transiently transfected with control siRNA or with siRNA targeting endogenous CHIP. 48 hours after transfection,

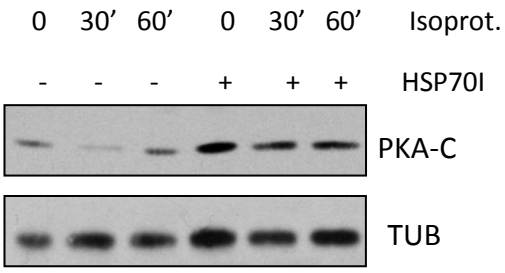
cells were deprived of serum for 18 h and then treated with FSK for 30 and 60 minutes. Total RNA was extracted and subjected to RT-qPCR analysis for *c*-fos mRNA.

3.9 HSP70 specific inhibitor prevent PKA-C degradation and promotes CREB phosphorylation in mouse brain.

Given the role of PKA in enhancing the neurotransmitter release at the synaptic connections (57), I tested if the regulatory mechanism of PKA action by CHIP was also operating in the mouse brain. To this aim, I performed an intracerebroventricular (icv) injection of the HSP70 specific inhibitor in C57B6 mice. 30 minutes later, the same brain area was perfused with isoproterenol. The mice were sacrified at different time points from stimulation and the hyppocampal tissues explanted. Tissue were homogenized and total lysates were immunoblotted for PKA-C and phosphoCREB. **Fig. 18** shows isoproterenol treatment reduced the levels of PKA-C in the hippocampal sections, compared to

basal values. As expected, pre-treatment with the HSP70 inhibitor increased the levels of PKA-C subunit in the hippocampal lysates, both in basal conditions as well as under isoproterenol treatment, by several folds over control values.

a)



b)

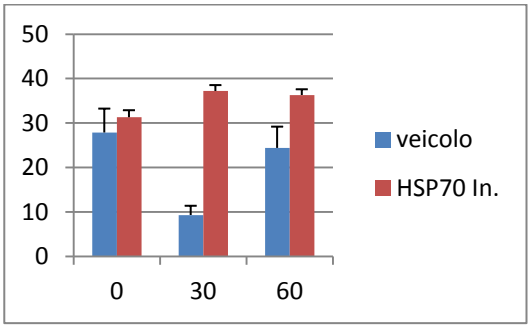


Figure 18. a) Hippocampal slices from mice treated with isoproterenol for 30 and 60 minutes or from mice pretreated with HSP70 inhibitor before the Isoproterenol treatment were homogenized and lysed. The lysates were immunoblotted with the indicated antibodies. b) Quantitative analysis of experiment shown in a).

The same experiment was performed to analyze the levels of phosphoCREB. As shown in **Fig. 19a** and **Fig. 19a**, icv injection of VER155008 increased the basal levels of phosphorylated CREB, compared to the controls, and no further induction could be seen after isoproterenol treatment.

a)

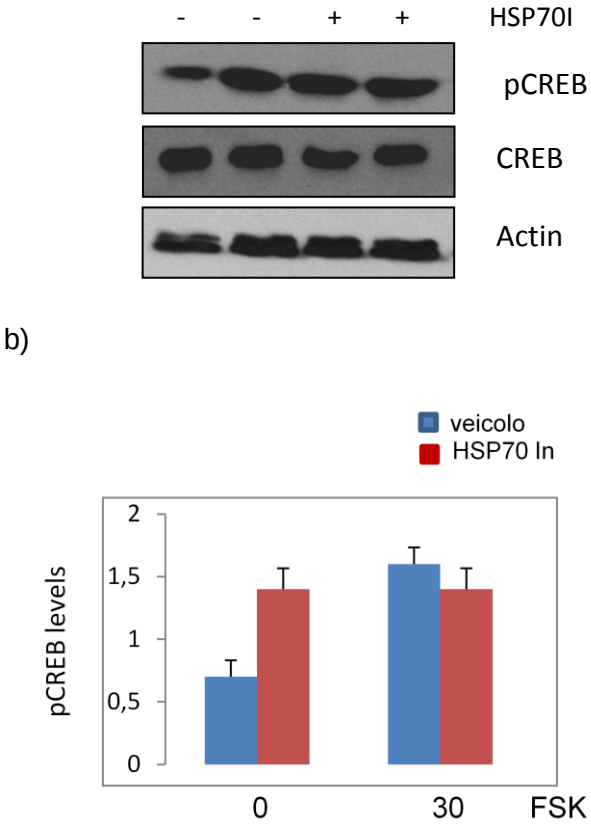


Figura 19. a) Hippocampal tissue explanted from control mice, treated with isoproterenol for 30 minutes, and from mice injected with HSP70 inhibitor before the isoproterenol treatment. The tissues were homogenized and the lysates were immunoblotted with the described antibodies. b) Quantitative analysis of experiment shown in a).

3.10 HSP70 inhibitor enhances L-LTP in mouse brain

Finally, I investigated the electrophysiological consequences of HSP70 inhibition on Long-Term Potentiation (LTP) at perforant pathway to granule cell synapses (PP-DG). We monitored the late phase of LTP (L-LTP) that is a typical a PKA-dependent form of memory (Malleret et al, 2010). Isolated brain slices were treated with FSK and Rolipram, an inhibitor of cAMP phosphodiesterases. We monitored the excitatory

post-synaptic potentials over a time period from stimulation. This protocol induced a potent L-LTP in control slices. As expected, inhibition of HSP70 prolonged the L-LTP compared to control slices, suggesting a role of HSP70/CHIP/PKA-C complex in cAMP-dependent events underlying to long-term memory in the brain (**fig 20**).

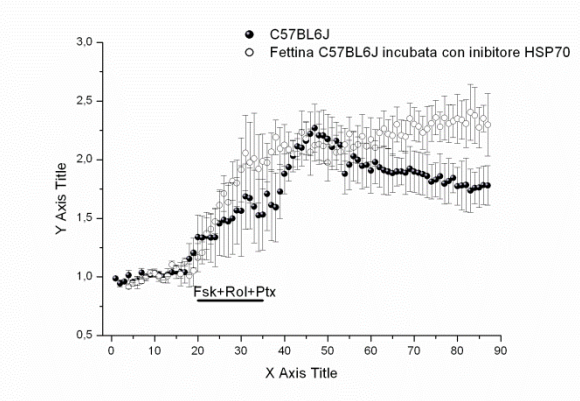


Figure 20. Time plot of field excitatory post-synaptic potential responses for group data, showing that the stimulation with FSK and Rolipram elicits normal LTP in controls, and prolonged LTP in VER155008-treated brain slices.

4. CONCLUSIONS AND DISCUSSION

4.1 Discussion

Activation of PKA is followed by a refractory phase in which the cell does not respond to a subsequent stimulus until it returns to its resting state. The attenuation of the cAMP response is controlled at different steps: desensitization of the membrane receptors, inhibition of adenylate cylase, activation of phosphodiesterases and of Ser/Thr phosphatases (Canettieri et al., 2003; Lamas and Sassone-Corsi, 1997)(31, 58). Work by Armstrong and colleagues showed that, in course of hormone stimulation, inhibition of PKA-C translation caused a reduction of PKA-C levels and hence attenuation of PKA pathway. This was considered as the main mechanism responsible of the refractory phase in hormone-responsive cells (Armstrong R et al., 1995) (30). Accordingly, PKA activity can be down regulated by decreasing the amount of free catalitic subunit or increasing the levels of the inhibitory regulatory subunits.

The UPS could contribute to the attenuation of the cAMP signaling at different steps. At cell membrane, ubiquitination and consequent proteolysis of receptors determines post-stimulus receptor desensitization (Bonifacino and Weissman, 1998) (41). Stimulatory Gas subunit and PDEs undergo to ubiquitination and proteasomal degradation, with significant consequences on the activated downstream signaling (Jenie et al., 2013; Huo et al., 2012)(59, 60). The UPS can also sustain the cAMP cascade. Thus, ubiquitin-directed proteolysis of R subunits by the RING ligase praja2 contributes to increase free PKA-C levels and to activate the downstream effectors of PKA (Lignitto et al., 2011) (55). Despite the wide range of biological activities controlled by PKA and the efforts to identify the regulatory mechanisms of PKA action, if and how the PKA-C subunit can be regulated by the UPS was largely unexplored.

Our work contributed to identify a novel mechanism of cAMP attenuation mostly mediated by the ubiquitination and proteolysis of PKA-C, creating a novel link between cAMP signaling and the UPS. The data shown in my thesis demonstrate that, following cAMP burst, PKA-C undergoes to proteolitic degradation. Interfering with the proteasome activity prevented the decline of PKA-C levels in course of GPCR stimulation. I have identified the HSP70/CHIP complex as responsible of PKA-C ubiquitination following cAMP stimulation. Interfering with the expression or activity of CHIP prevented PKA-C ubiquitination and proteolysis induced by cAMP. I found that HSP70 was in complex with PKA-C, even in the absence of external stimuli. Rise of cAMP levels induced recruitment of the E3 ligase CHIP within the complex, activating the negative-feedback regulatory loop. The molecular events controlled by HSP70/CHIP complex deeply impacted on PKA signaling. In particular, I observed that phosphorylation of CREB at the PKA site (Ser133) and the transcription of CREB-responsive genes, as *c*-fos, were up-regulated when CHIP was silenced.

HSP70 mediates the physical and functional interaction between CHIP and PKA-C. Thus, inhibition of HSP70, both in cells and in mouse brains, caused accumulation of PKA-C and consequent enhancement of CREB phosphorylation. The role of HSP70 in controlling PKA-C activity was confirmed by studying the electrophysiological events underlying to brain memory. Thus, treatment of hippocampal sections with the HSP70 inhibitor enhanced cAMP-induced form of LTP. Studies are in course to confirm these data by injecting the HSP70 inhibitor in the hippocampus of living mice and evaluating the electrophysiological events of such pharmacological treatment on the LTP. Furthermore, available CHIP KO mice will be analyzed to better understand the role of this ligase in the induction and maintenance of long term memory.

this is the first demonstration of attenuation of the cAMP signaling, based on the regulation of the stability of activated kinase (Fig. 21).

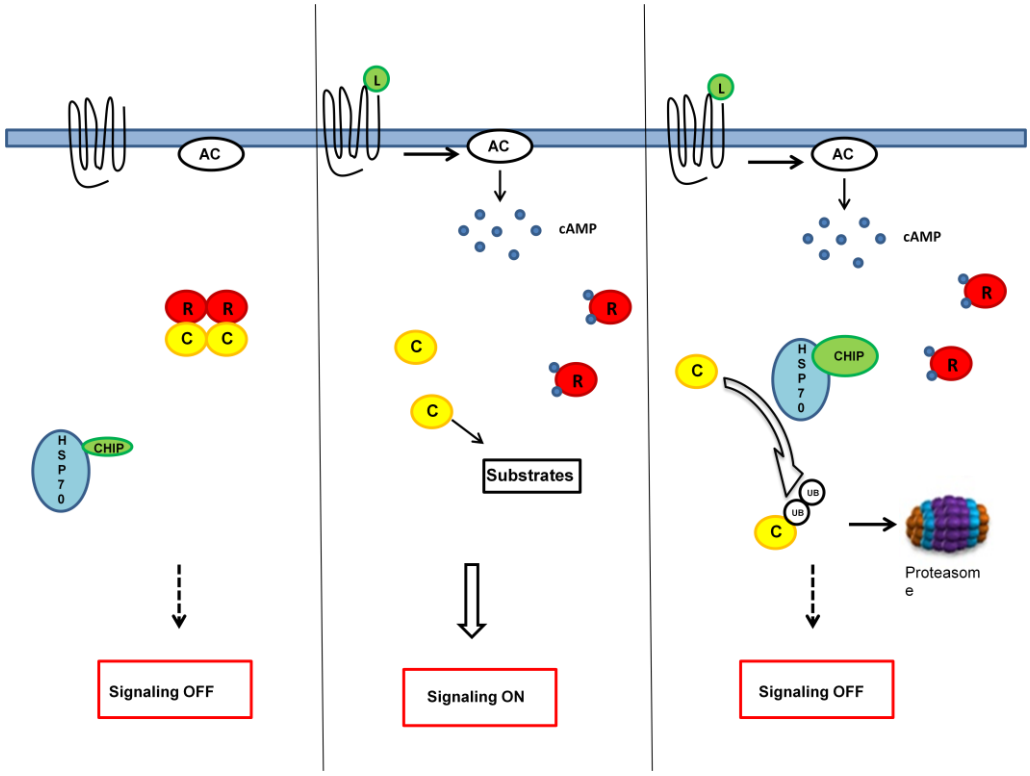


Figure 21. Proposed model. Under resting conditions, the tetrameric PKA holoenzyme is inactive. The binding of a given ligand to its cognate GPCR at plasma membrane turns ON the cAMP signaling, inducing dissociation of PKA holoenzyme and release of of free active PKA-C. Phosphorylation of a wide array of intracellular substrates by PKA regulates key biological functions. Following the burst phase, ubiquitination and proteolysis of PKA-C by CHIP-triggered UPS attenuates the PKA cascade.

According to the proposed model, the pathological conditions caused by inactivating mutations of CHIP, as it occurs in autosomal recessive spinocerebellar ataxia (SCAR16), could be a consequence of impaired functioning of the cAMP pathway. Therefore, restoring cAMP cascade in SCAR16 disease may have potential therapeutic effect on the progression of the disease. The mechanism regulated by CHIP can be also relevant in proliferative diseases, for example in those linked to tumor progression and metastasis. In this context, a recent work by Pattabiraman et al. shows that activation of PKA induces a mesenchymal-to-epithelial transition (MET) in metastatic mammary epithelial cell, preventing the growth and expansion of the metastatic mesenchimal tumor cells. Activation of PKA induces an epigenetic reprogramming of tumor initiating cells (TICs) which promotes their differentiation and loss of tumor-initiating properties (Pattabiraman et al., 2016)(61). In this context, it would be of interest to test the role of CHIP in MET of mesenchimal cancer cells and evaluate the consequence of CHIP inactivation or pharmacological inhibition of HSP70 in the growth and progression of metastatic epithelial lesions.

In conclusions, my work contributed to identify a novel mechanism of cAMP signal attenuation operating in mammalian cells that is required for post-stimulus recovery phase in hormone-stimulated cells. Exploring further the mechanism(s) regulating ubiquitination of PKA-C in course of hormone stimulation, and identifying other UPS targets in the cAMP cascade will provide important tools to dissect and manipulate this signaling network in human disorders.

5.METHODS

5.1 Cell lines.

Human embryonic kidney cell line (HEK293) were cultured in Dulbecco modified Eagle's medium, containing 10% fetal bovin serum in an atmosphere of 5% CO2.

5.2 Plasmids and transfection.

Vectors encoding the Myc-tagged CHIP and the Myc-tagged Chip K30A were provided by Dr. Francesca Carlomagno (University of Naples, Italy). V5-HSP70 was purchased from Addgene, HA-tagged ubiquitin was provided by Dr. Antonio Leonardi (University of Naples, Italy), HA-tagged PKA catalytic subunit type alpha was provided by Dr. Eduard Stefan (University of Innsbruck, Austria).PKA K310A and PKA K286A were generated by site-directed mutagenesis. siRNA targeting coding regions of CHIP were purchased from Life Technologies. The following is the siRNA sequence, targeting CHIP:

Sense sequence : UUACACCAACCGGGCCUUtt

Antisense sequence : CAAGGCCCGGUUGGUGUAAta

siRNA were transiently transfected using Lipofectamine 2000 (Invitrogen) at a final concentration of 100 pmol/ml of culture medium.

5.3 Antibodies and chemicals.

Rabbit polyclonal antibodies directed against PKA alpha catalytic subunit (immunoblot 1:4000), α-actin (immunoblot 1:2000) and CHIP (immunoblot 1:500) were bought from Santa Cruz; Myc epitope (immunoblot 1:2000), V5 epitope (immunoblot 1:2000) and α-tubulin (immunoblot 1:8000) from Sigma; CREB (immunoblot 1:1000) and pCREB (immunoblot 1:1000) from Millipore; hemaglutinin epitope

(immunoblot dilution 1:1000) (HA.11) from Covance. Forskolin was purchased from SIGMA and VER 155008 from Tocris Bioscience.

5.4 Western Blot Analysis.

Cells were washed twice with phosphate-buffered saline and lysed in saline buffer-1% Triton-X 100 (NaCl, 150 mM; Tris-HCl, 50 mM, pH8; EDTA, 5 mM) or, for immunoprecipitation assay, in saline buffer 0.5% NP40 (50mMTris-HCl, pH 7.4, 0.15MNaCl, 100 mM EDTA,0.5% NP40) containing aprotinin (5 µg/ml), leupeptin (10 µg/ml), pepstatin (2 µg/ml), 0.5 mm PMSF, 2 mmorthovanadate, and 10 mmNaF.

The lysates were cleared by centrifugation at 15,000 g for 10 min. An aliquote of whole cell lysate (WHL) (100 µg) were resolved on sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred on nitrocellulose membrane (Biorad, Milan, Italy) for 3 h. Filters were blocked for 1 h at room temperature in Tween-20 Phosphate buffer saline (TPBS) (PBS- Sigma, 0,1% Tween 20, pH 7.4) containing 5% non-fat dry milk. Blots were then incubated O/N with primary antibody. Blots were washed three times with TPBS buffer and then incubated for 1 h with secondary antibody (peroxidase-coupled antirabbit (GE-Healthcare) in TPBS. Reactive signals were revealed by enhanced ECL Western Blotting analysis system (Roche).

5.5 Immunoprecipitation and pull down assay.

Cells were homogenized in lysis buffer described above. Cell lysates (1.5 mg) were immunoprecipitated with the indicated antibodies. An aliquot of cell lysate (50 µg) or immunoprecipitates were resolved by SDS-PAGE gel and transferred to Protran membrane. The immunoblot analysis was performed as previously described. Pellets were washed four times in lysis buffer supplemented with NaCl (0.4 M final concentration) and eluted in Laemmli buffer. Eluted samples were

resolved on8%-SDS PAGE gel, transferred to nitrocellulose membranes and immunoblotted with the indicated antibody.

5.6 Protein extraction from murine brain tissue.

The tissue removed from the animal, was immediately placed in 5 volumes of Lysis buffer 1% Triton with DOC 0,5 %. The tissue was then homogenized on ice using an homogenizer. The sample was cleared by centrifugation for 10 minutes at 13000 RPM (4°C) and the supernatant was collected in 1,5 ml tube.

5.7 Electrophisiology.

All animal procedures were in compliance with the European Council Directive (86/609/EEC). Parasagittal hippocampal slices (400 μm) from mice were kept submerged at 30 °C and superfused (2–3 ml min^{-1}) with oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) containing (mM): 124 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 26 $NaHCO_3$ and 10 glucose. Slices were treated with VER 155008(40 uM)for 30 minutes before stimulation. Presynaptic stimulation was applied to the medial perforant pathway of the dentate gyrus using a bipolar insulated tungsten wire electrode, and field excitatory postsynaptic potentials were recorded at a control test frequency of 0.033 Hz from the middle one-third of the molecular layer of the dentate gyrus with a glass microelectrode. The transient E-LTP was induced by treatment with Forskolin (50 uM), whereas long-lasting L-LTP was induced with TBS consisting of nine bursts of four pulses at 100 Hz, 200 ms interburst interval, 5 min intertrain interval. All solutions contained 100 μM picrotoxin (Sigma) to block $GABA_A$ (γ-aminobutyric acid A)-mediated activity.

5.8 Animals.

C57 black mice (Charles River) were housed in diurnal lighting conditions (12 h darkness and 12 h light) and fasted overnight but allowed free access to water before the experiment. Experiments were carried out according to international guidelines for animal research and the experimental protocol was approved by the Animal Care Committee of the University of Naples.

5.9 Chemicals administration into the rat brain.

All mice, anaesthetized with chloral hydrate (400 mg kg^{-1}, intraperitoneally), were put on a stereotaxic frame. A 23 g stainless-steel guide cannula (Small Parts) was implanted into the right lateral ventricle, the third ventricle, using the stereotaxic coordinates of 0.5 mm caudal to bregma, 2 mm lateral and 2.5 mm below the dura. The cannula was fixed to the cranium using dental acrylic and small screws. VER 155008 (40uM) was administered 1 hour before isoproterenol stimulation. Rats were killed 30 min or 60 min later. Distinct brain regions (cortex, hippocampus and striatum) from the left and right hemispheres were isolated. Brain tissues were used for immunoblot analysis and RNA extraction.

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