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cAMP-Induced Histones H3 Dephosphorylation Is Independent of PKA and MAP Kinase Activations and Correlates With mTOR Inactivation

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ABSTRACT

cAMP is a second messenger well documented to be involved in the phosphorylation of PKA, MAP kinase, and histone H3 (H3). Early, we reported that cAMP also induced H3 dephosphorylation in a variety of proliferating cell lines. Herein, it is shown that cAMP elicits a biphasic H3 dephosphorylation independent of PKA activation in cycling cells. H89, a potent inhibitor of PKA catalytic sub-unite, could not abolish this effect. Additionally, H89 induces a rapid and biphasic H3 serine 10 dephosphorylation, while a decline in the basal phosphorylation of CREB/ATF-1 is observed. Rp-cAMPS, an analog of cAMP and specific inhibitor of PKA, is unable to suppress cAMP-mediated H3 dephosphorylation, whereas Rp-cAMPS effectively blocks CREB/ATF-1 hyper-phosphorylation by cAMP and its inducers. Interestingly, cAMP exerts a rapid and profound H3 dephosphorylation at much lower concentration (50-fold lower, 0.125 mM) than the concentration required for maximal CREB/ATF-1 phosphorylation (5 mM). Much higher cAMP concentration is required to fully induce CREB/ATF-1 gain in phosphate (5 mM), which correlates with the inhibition of H3 dephosphorylation. Also, the dephosphorylation of H3 does not overlap at onset of MAP kinase phosphorylation pathways, p38 and ERK. Surprisingly, rapamycin (an mTOR inhibitor), cAMP, and its natural inducer isoproterenol, elicit identical dephosphorylation kinetics on both S6K1 ribosomal kinase (a downstream mTOR target) and H3. Finally, cAMP-induced H3 dephosphorylation is PP1/2-dependent. The results suggest that a pathway, requiring much lower cAMP concentration to that required for CREB/ATF-1 hyper-phosphorylation, is responsible for histone H3 dephosphorylation and may be linked to mTOR down regulation. J. Cell. Biochem. 117: 741–750, 2016.

KEY WORDS: HISTONE H3 PHOSPHORYLATION; MAMMALIAN TARGET OF RAPAMYCIN (mTOR); PROTEIN KINASE A (PKA); cAMP-DEPENDENT RESPONSE ELEMENT (CREB) AND CREB ASSOCIATED TRANSCRIPTIONAL FACTOR 1 (ATF-1); MITOGEN-ACTIVATED PROTEIN KINASES (MAPK, ERK, p38); 3′-5′-CYCLIC ADENOSINE MONOPHOSPHATE (cAMP); RIBOSOMAL S6 KINASE 1 (S6K1)

3′-5′-cyclic adenosine monophosphate (cAMP) is a ubiquitous second messenger that activates a variety of signaling cascades. Among them, the most studied is the protein kinase A (PKA). PKA is a serine/threonine specific protein kinase hetero-tetramer that consists of two homodimer catalytic subunits (C) and two homodimer inhibitory subunits [Kim et al., 2005; Sands and Palmer, 2008]. Binding of cAMP to the inhibitory subunits releases the active C subunits, which translocates into the nucleus, targeting

Abbreviations: cAMP, 3′,5′-cyclic adenosine monophosphate; PKA, protein kinase A; C, PKA catalytic subunits; CREB, cAMP-dependent response element; ATF-1, CREB associated transcriptional factor 1; pCREB/ATF-1, phosphorylated CREB/ATF-1; Epac or GEF, small GTPase protein directly activated by cAMP; H3, histone H3; MAPK, mitogen-activated protein kinases; MEK/ERK, extra cellular signal-regulated kinase; p38, MAPK stress signal; SOS, Son of sevenless; RAS, GTPase super family; PP1/2, protein phosphatase 1 and 2; S6K1, ribosomal S6 kinase 1; phosS6K1, phosphorylated S6K1; Rp-cAMPS, Rp-Adenosine 3′,5′-cyclic monophosphorothioate analog of cAMP inhibiting PKA; H89, N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide inhibitor of PKA; HRP, horse radish peroxidase-conjugated anti-rabbit secondary antibodies; H3S10ph, histone H3 phosphorylated at Ser 10; H3K9, 14ac, histone H3 acetylated at Lys 9 and 14; H3K4me2, histone H3 dimethylated at lysine 4; Msk1, a kinase down stream of ERK and p38, which phosphorylates histone H3; pCREB/ATF-1, phosphorylated CREB/ATF-1; p21 (CIP1/WAF1) and p27 (Kip1), cyclin-dependent kinase D inhibitors; OA, Okadaic acid.

The authors declare no conflict of interest.

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specific transcription factor substrates such as cAMP-dependent response element (CREB) and CREB associated transcriptional factor 1 (ATF-1) [Meinkoth et al., 1993]; concomitant with histone H3 phosphorylation [DeManno et al., 1999; Salvador et al., 2001]. Interestingly, histone H1.4 variant is phosphorylated by PKA, inducing its dissociation from mitotic chromosome [Chu et al., 2011]. The exchange RAS small GTPase protein directly activated by cAMP (Epac also known as GEF which belongs to the RAS GTPase super family and activates MAPK) is another example of signaling pathway activated by cAMP [de Rooji et al., 1998; Kawasaki et al., 1998]. MAPK/MEK/ERK (mitogen-activated protein kinases and extra cellular signal-regulated kinase) and MAPK p38 stress pathways are also common targets of cAMP activity, in a PKA-dependent and independent way [de Rooji et al., 1998; Houslay and Kolch, 2000; Zheng et al., 2000; Richards, 2001; Stork and Schmitt, 2002; Waltereit and Weller, 2003; Chio et al., 2004]. On the other hand, for example, the mechanism by which cAMP activates RAS or ERK in B16 melanoma and normal human melanocytes is not known, but it does not involve PKA, Epac or the classical RAS activator, Son of sevenless (SOS) [Busca et al., 2000]. Another set of cAMP effectors is the cAMP-gated membrane ion channels, which regulate the influx of cations into the cytosol in response to cAMP [Kaupp and Seifert, 2002; Puljung et al., 2014].

While much attention has been centered on the cAMP-induced activation by phosphorylation of signaling cascades, an interesting aspect, yet, less well documented of cAMP signaling is its ability to regulate protein phosphatases by various mechanisms [Scott and Lawrence, 1998; Ahn et al., 2007; Stipanovich et al., 2008; Rodriguez-Collazo et al., 2008b]. cAMP antagonizes phosphatases. In this respect, it has been published that some protein phosphatase 1 (PP1) proto-inhibitors are activated by PKA, upon specific phosphorylation of specific amino acids, after which the inhibitors gain an exquisite and potent specificity for PP1 catalytic subunit (PP1c). For example, NIPP1, a nuclear protein, binds and inactivate PP1c ($Ki = 0.2$ nM) upon its phosphorylation by cAMP- and cGMP-dependent protein kinases [Trinkle-Mulcahy et al., 1999; Herzig and Neumann, 2000; Cohen, 2002]. After activation of the dopamine D1 receptor, in neurons, DARPP-32 is phosphorylated by PKA at Thr34 and converted into a potent inhibitor of PP1 [Hemmings et al., 1984]. Additionally, cAMP-dependent activation of protein phosphatase-2A and dephosphorylation of DARPP-32 at Ser 97 inhibits its nuclear export. The nuclear accumulation of DARPP-32 phosphorylated at Thr34 enhancers the phosphorylation of histone H3 at serine 10 [Stipanovich et al., 2008].

Less clear, however, is the agonistic role of cAMP in the activation of phosphatases. Some reports show that PKA activation by cAMP leads to PP2A activation by phosphorylating its B56∂ subunit [Usui et al., 1998; Ahn et al., 2007]. Although mechanistic details are unknown, cAMP-mediated ribosomal S6K1 dephosphorylation, in a PKA-independent way, has been documented [Graves et al., 1995; Monfar et al., 1995; Lin and Lawrence, 1996; Scott et al., 1996]. These kinases are downstream effectors of mammalian target of rapamycin (mTOR), a key kinase controlling protein synthesis, histone acetylation [Rohde and Cardenas, 2003], cell growth and G1 transition to S-phase [Crespo and Hall, 2002]. Interestingly, mTOR down-regulates phosphatase activity [Scott and Lawrence, 1998; Crespo and Hall, 2002] and evidences show that cAMP may inactivate this kinase by unknown mechanism [Graves et al., 1995; Scott and Lawrence, 1998].

We reported that cAMP and its inducers dephosphorylate histone H3 in dividing cell lines, whereas in quiescent cells, cAMP transiently induced histone H3 hyperphosphorylation [Rodriguez-Collazo et al., 2008b]. We demonstrate that cAMP-induced H3 dephosphorylation was independent of ERK and p38 MAPK and Epac pathways [Rodriguez-Collazo et al., 2008a,b]. Less clear, however, was the role of PKA activation in histone dephosphorylation [Rodriguez-Collazo et al., 2008a] and the possible phosphatases involved. In this paper, we therefore address the role of PKA in histone H3 dephosphorylation and the possibility that cAMP-mediated dephosphorylation activity may be associated to mTOR signaling. Additionally, we investigate the possible phosphatases responsible for cAMP-induced Ser10 dephosphorylation on H3.

Herein, pharmacologically is documented that potent and specific inhibitors of PKA could not abolish cAMP-mediated histone H3 dephosphorylation. H89 and Rp-cAMPS (Rp-Adenosine 3′,5′-cyclic monophosphorothioate, an analog of cAMP), which specifically inhibits PKA, did not prevent H3 dephosphorylation by cAMP or forskolin, while Rp-cAMPS and H89 induced a decline in the basal or induced phosphorylation of CREB/ATF-1. Interestingly, cAMP exerts its dephosphorylation activity on H3 at much lower concentration (50-fold lower) than the concentration required for CREB/ATF-1 maximal phosphorylation, canonical PKA effectors. Conversely, much higher cAMP concentration potently abrogates histone H3 dephosphorylation, which correlates with the hyperphosphorylation of CREB/ATF-1. Moreover, cAMP-induced H3 dephosphorylation does not overlap either with the onset of phosphorylation of the MAP kinase signaling members, p38 and ERK and the transcription factors CREB/ATF-1. Surprisingly, rapamycin (an mTOR specific inhibitor), cAMP and its inducers elicit identical dephosphorylation kinetics on S6K1 ribosomal kinase (a downstream target of mTOR) and H3. Finally, cAMP-induced H3 dephosphorylation is PP1 dependent.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Unless otherwise specified the reagents for cell treatment and antibodies used were purchased from Cell Signaling Technology and Upstate (NY) and used as manufacturer recommended. The appropriate secondary conjugated horseradish peroxidase-conjugated anti-rabbit antibodies (HRP, Jackson Immunology Research) were used 1/5000 to detect the primary antibodies.

Cell line 1470.2 [Rodriguez-Collazo et al., 2008b], were maintained in high-glucose DMEM, containing 10% fetal bovine serum (Atlanta Biologicals), 2 mM pyruvate, 4 mM glutamine and 10 μg/ml of ampicillin and streptomycin. Exponentially growing cells were seeded at 20% confluence in 30 or 100 mm dishes (Falcon) and grown for 14–16 h prior to treatment with various reagents at concentrations and times indicated in the figures. 8-Br-cAMP and H89 (N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide) were purchased from Sigma. Okadaic acid (OA) was purchased

from Calbiochem. Rp-cAMPS was obtained from Santa Cruz Biotechnology.

GEL ELECTROPHORESIS, PROTEIN TRANSFER, AND IMMUNOBLOTTING

For all immunoblotting experiments, cells were rapidly washed two times with pre-warmed (37°C) DMEM (without serum) after treatment to minimize cell disturbance [Rodriguez-Collazo et al., 2008b]. Total cell lysates were generated by the direct addition of 2× reducing sample buffer (60 mM Tris–HCl, pH 7, 120 mM DDT, and 0.6% SDS) or by using 0.2 M HCl for histone extraction after sulfopropyl-Sepharose purification and concentration [Rodriguez-Collazo et al., 2009, 2014].

Cellular extracts were resolved by electrophoresis in sodium dodecylsulfate (SDS)–polyacrylamide gels electrophoresis (PAGE), using a broad range of polyacrylamide concentration as indicated in the figure legends. Proteins were visualized by staining of gels with either GelCode Blue Stain Reagent (Pierce; vendor Fisher, cat. no. PI-24590), or Coomassie Blue R-250; or staining of membranes with Ponceau S (Sigma–Aldrich, cat. No. P3504), after western transfer of proteins.

Western transfer of proteins onto 0.1 mm pore size nitrocellulose membrane (Whatman, Protean, BA79, Superior Nitrocellulose Membrane; vendor Fisher, cat. No. 09-301-120) was carried out in a Hoefer Semi-dry transfer unite 77 (GE Healthcare, cat no 80-6211-86) for 1 h at 9mA per cm^2 of membrane in 1× Tris-Glycine running buffer containing 0.05% SDS and 7% methanol. After incubation with primary antibodies (described in figure legends) the membranes were incubated with HRP secondary antibodies and detected using a chemiluminescence assay (Pierce; vendor, Fisher, cat. no. PI-32106), after exposure to X-ray films.

RESULTS AND DISCUSSION

cAMP-INDUCED A BIPHASIC HISTONE H3 DEPHOSPHORYLATION FOLLOWED BY HISTONE H3 DEACETYLATION AND CELL CYCLE ARREST

Despite the fact that cAMP-mediated histone phosphorylation by activation of PKA [Chu et al.; DeManno et al., 1999; Salvador et al., 2001] or by inhibiting nuclear PP1 [Stipanovich et al., 2008]; the phosphatase holoenzymes, second messengers, and signaling controlling histone dephosphorylation are poorly characterized. Although the signaling pathway involved was not elucidated, PP2 phosphatase catalytic subunit is involved in global histone dephosphorylation during heat shock of Drosophila [Nowak et al., 2003]. Ionizing radiation promotes an ATM-dependent activation of nuclear PP1c with the subsequent dephosphorylation of histones [Guo et al., 2002]. We reported that cAMP signaling dephosphorylated histone H3 in proliferating cell lines by unclear mechanisms [Rodriguez-Collazo et al., 2008a,b]; whereas, in quiescent cells, cAMP stimulus promoted a transient histone H3 hyperphosphorylation [Rodriguez-Collazo et al., 2008a].

It has been documented that treatment of breast cancer cells MDA-MB-157 with cAMP induces a PKA-independent biphasic increasing of p21 (CIP1/WAF1) and p27 (Kip1) proteins (two cyclin-dependent kinase D inhibitors, thereby blocking cell growth and proliferation at G1). p21 and p27 stabilization was independent of their mRNA synthesis, which raised only after 2 h, suggesting rather an effect in the stabilization of protein syntheses [Rao et al., 1999]. Curiously, cellular down regulation of mTOR by histidine depletion or by its specific inhibitor rapamycin have similar effects [Leung-Pineda et al., 2004]. Interestingly, results in Figure 1A show a biphasic induction of Ser10 dephosphorylation by cAMP with very similar kinetics to that reported for p21 and p27 oscillations. Our findings show that 90% of H3 dephosphorylation occurred within 5 min of cell treatment (Fig. 1A, lane 5 min) and recovers back at 2 h. At 8 h of treatment, Ser10 undergoes a second wave of dephosphorylation concomitant with a drastic and persistent H3 deacetylation of Lys9/14 (H3K9,14ac) (Fig. 1A, upper and middle panels, respectively). This may imply that cAMP simultaneously influence two disparate events: chromatin remodeling (Fig. 1A) and protein synthesis [Rao et al., 1999] both maybe acting synergistically in reshaping cellular architecture (Fig. 1C). After histone H3 dephosphorylation and deacetylation, cell proliferation arrests (Fig. 1C). This effect was independent of increasing expressions of associated-chromatin alpha-PP1 (Fig. 1B1), a major phosphatase responsible for mitotic histone H3 dephosphorylation [Qian et al., 2011], and PP1c (Fig. 1B2), as jugged by immunoblotting. Altogether, suggest that cAMP-induced dephosphorylation of H3 operates in a signal transduction manner and is an earlier event, whereas H3 deacetylation occurs much later and follows H3 dephosphorylation. Relevant to this finding is the fact that histone deacetylases (HDAC1 and HDAC2) have been found in multi-protein complexes, containing PP1 and its regulatory subunit (PNUT), or in complexes with PP2A and its regulatory subunit PP2R5A [Canettieri et al., 2003]. Additionally, in the nucleus HDAC1 targets PP1 to specific promotors with the subsequent dephosphorylation of phosphorylated CREB and histone H4, during the attenuation phase, after CREB phosphorylation by forskolin [Canettieri et al., 2003]. Altogether may suggest that cAMP stimulus of specific phosphatases, targeting H3 dephosphorylation, may recruit HDACs to the site of dephosphorylation, leading to a latter H3 deacetylation.

cAMP-INDUCED H3 DEPHOSPHORYLATION IS NOT PREVENTED BY PKA INHIBITORS

We next addressed if inhibitors of PKA antagonizes cAMP-induced histone H3 dephosphorylation. PKA is a canonical cAMP effector that can be specifically inhibited by a cAMP analog Rp-cAMPS [Dostmann, 1995] and by a more broad kinase inhibitor H89 [Lochner and Moolman, 2006]. The two compounds have different chemical structures. While H89 is an analog of ATP, thus it competes with ATP for binding to PKA free catalytic subunits (IC_{50} reported is in the range between 48 and a 135 nM [Chijiwa et al., 1990; Davies et al., 2000; Lochner and Moolman, 2006]); Rp-cAMPS is an analog of cAMP, which binds to the PKA inhibitory subunits and keeps PKA in a locked conformation. From the Rp-cAMPS-PKA complex, cAMP is unable to release PKA catalytic subunits [Dostmann, 1995]. Thus, Rp-cAMPS appears to be only specific for PKA inhibition, acting one step earlier than H89, and it is recommended to corroborate the results obtained with H89 [Lochner and Moolman, 2006].

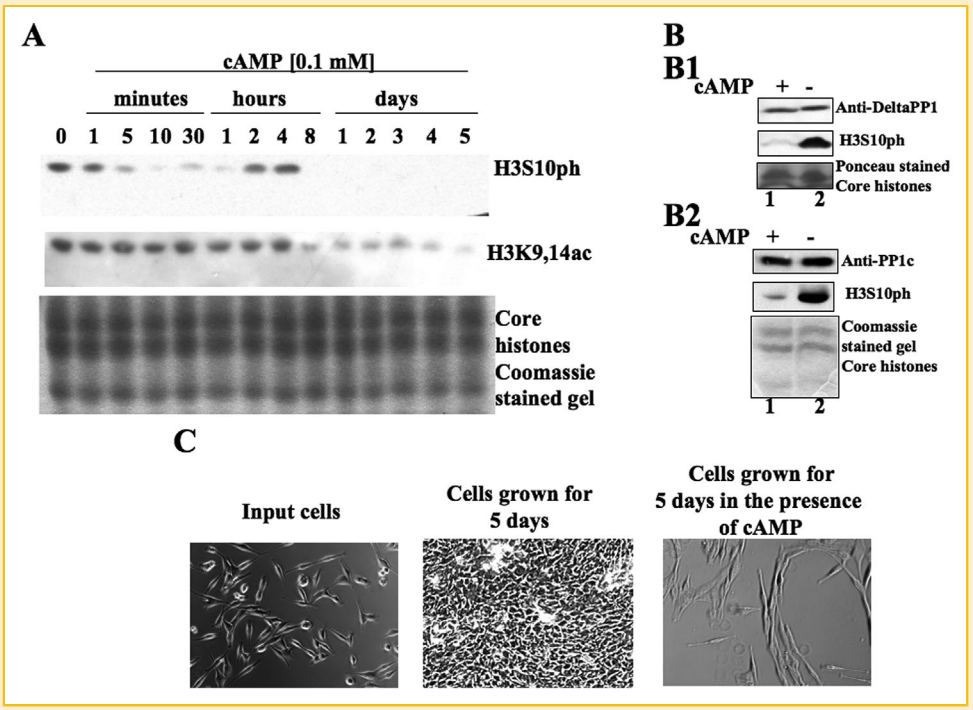

Fig. 1. cAMP-induced a rapid and biphasic histone H3 dephosphorylation followed by its slower deacetylation and cell cycle arrest. (A) cAMP (0.1 mM) induces a rapid and biphasic histone H3S10ph dephosphorylation followed by its deacetylation at lysines 9 and 14. (B) The dephosphorylation process is independent of PP1 expression. (C) Proliferating cancer cells become quiescent concomitant with persistent H3 dephosphorylation and deacetylation upon cAMP treatment. 1470.2 breast carcinoma cells (20% confluence in DMEM/10% BFS) were treated with 0.1 mM cAMP or vehicle alone (10 μl H_2O/10 ml medium) at increasing time (indicated at the top of A). Kinetics of phosphorylated and acetylated H3 were followed by immunoblotting with specific antibodies for each modified residues, extracted from whole cell with 2× SDS-reducing sample buffer. Phosphorylated Ser10 on H3 (H3S10ph, A, upper panel) and acetylated Lys9/14 on H3 (H3K9, 14ac, A, middle panel) are indicated at the right. Equal protein loading control, using core histones, was verified by Coomassie staining of a parallel run gel (A, bottom panel). The expression of delta-PP1, a chromatin-associated phosphatase, and phosphorylated H3 were verified with specific antibodies in cAMP-treated and untreated cells for 1 h (upper panel B1, indicated at the top and right, respectively). Middle panel in B1 is the immunoblotting for phosphorylated Ser10 on H3 (H3S10ph, indicated at the right). At the bottom in B1 is indicated the protein loading control, using Ponceau staining of histones. Note that in B1, bottom panel, the samples were separated by 10.5%-SDS–polyacrylamide gel electrophoresis (PAGE), thus the four core histones could not be fully resolved. Expression of the catalytic subunit of PP1 (PP1c) and histone H3 dephosphorylation, separated by 16%-SDS-PAGE (B2), were followed with specific antibodies for the blotted proteins, extracted as in A, treated or untreated with cAMP (indicated at the top panel in B2). The histone protein loading control is at the bottom of B2. Phase contrast photography of input logarithmically proliferating cells, after 5 days untreated proliferating cells, and after 5-days cAMP-treated and proliferation-arrested cells is indicated at the top of C.

The best-characterized effectors of activated PKA are cAMP-dependent response element (CREB) and CREB associated transcriptional factor 1 (ATF-1) [Servillo et al., 2002]. Activation of CREB/ATF-1 can be monitored using specific antibodies against the respective phospho-proteins, which in the case of CREB is serine 133. Treatment of cycling 1470.2 cells with either Rp-cAMPS or H89 did not prevent forskolin- and cAMP-induced H3 dephosphorylation, while basal phosphorylation of CREB/ATF-1 is clearly inhibited (Fig. 2; compare lanes 1, 2, 3, and 4; and lanes 5 with 6 and 7). Interestingly, H89 alone was able to provoke a drastic dephosphorylation of both H3S10ph and pCREB/ATF-1 (lane 9, upper and middle panel, respectively); whereas Rp-cAMPS alone did not down regulate basal levels of H3S10ph (lane 8, upper panel). However, noticeable is that Rp-cAMPS alone effectively down regulated the phosphorylation of CREB/ATF-1 (lane 8, middle panel) and that this compound effectively blocks forskolin-induced CREB/ATF-1 hyper-phosphorylation, but not histone H3 dephosphorylation (lane 3). Thus, it may be concluded that cAMP-mediated Ser10 dephosphorylation may be PKA-independent. Unlike to Rp-cAMPS, which specifically inhibits PKA activation [Dostmann, 1995; Lochner and Moolman, 2006]; H89 inhibits other kinases [Lochner and Moolman, 2006], for example, in the MAP kinase pathway it inhibits Msk1, which are also responsible for Ser10 phosphorylation on H3 [Thomson et al., 1999]. The inhibition of a broad spectrum of kinases by H89 may be responsible for the drastic H3 dephosphorylation observed here and may act synergistically with cAMP (lane 7, upper panel).

In vitro, we tested the possibility that cAMP-induced histone H3 dephosphorylation may be due to the inhibition of Msk1 by cAMP, a downstream MAP kinase target that directly phosphorylated H3, and the result showed that cAMP inhibited this kinase at millimolar concentrations (data not shown), suggesting that cAMP is not a direct inhibitor of such kinase.

DOSE-RESPONSE AND TIME-COURSE OF cAMP-INDUCED HISTONE H3 DEPHOSPHORYLATION IS INDEPENDENT OF PKA AND MAP KINASE ACTIVATION

PKA activation requires larger over-saturating concentration of cAMP in order to be fully dissociated from its inhibitory sub-unities

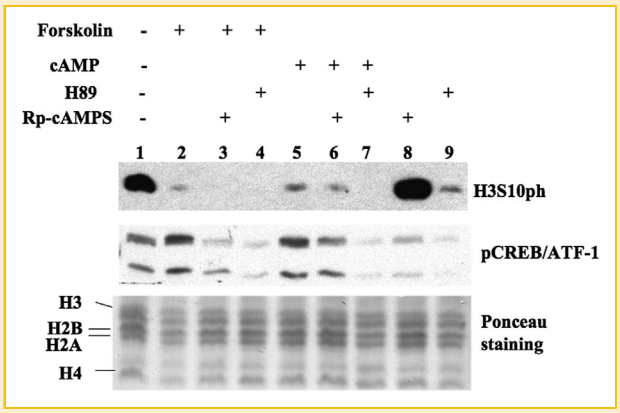

Fig. 2. cAMP-induced H3 dephosphorylation is not prevented by PKA inhibitors. 1470.2 cells were treated for 30 min with either Rp-cAMPS (2 mM, a specific PKA inhibitor), or H89 (20 μM, an ATP competitive inhibitor of PKA ATP-binding catalytic subunit). After that, cAMP (0.1 mM), forskolin (10 μM, an agonist of adenylate cyclase), or H_2O vehicle (10 μl, tame 0, line 1) were added for 1 h, as indicated at the top of the figure. Total cell extracts were subjected to SDS–PAGE and immunoblotting analysis. Immunoblotting was carried out with an antibody against phosphorylated CREB (1B6, mouse mAb, Cell Signaling Technology), which detects endogenous levels of CREB only when is phosphorylated at serine 133 (pCREB/ATF-1, indicated at the right). This antibody also detects the phosphorylated form of the CREB-related protein, ATF-1. Phosphorylated H3 Ser10 (H3S10ph, indicated at the right) was detected with rabbit polyclonal antibodies. Note that H89 alone induced H3 dephosphorylation (lane 9), whereas Rp-cAMPS did not (lane 8). Equal protein loading was verified using as reference the blotted histones stained with Ponceau S.

for its maximal activation [Kopperud et al., 2003]. This has been documented by showing that larger excess of cAMP over catalytic-inhibitory PKA-subunit complexes poorly activate PKA [Kopperud et al., 2002]. Since we had evidences that cAMP-induced dephosphorylation of H3 is most likely PKA-independent (Fig. 2), we wonder if much lower cAMP concentration would be required to induce H3 dephosphorylation rather than stimulation of PKA activity [Kopperud et al., 2002]. If so, this would be an additional evidence supporting the results obtained in Figure 2. Induction of CREB/ATF-1 phosphorylation by cAMP in a PKA- or MAPK-dependent way has been widely accepted [Johannessen and Moens, 2007; Sands and Palmer, 2008]. Additionally, it is known that ERK and p38 can be activated by cAMP in a PKA-dependent or -independent way [Kawasaki et al., 1998; Pham et al., 2000; Zheng et al., 2000; Stork and Schmitt, 2002; Waltereit and Weller, 2003; Chio et al., 2004]. Therefore, we reasoned that following the dose-response and time-course of cAMP-induced CREB/ATF-1, ERK, and p38 phosphorylation, by immunoblotting with specific antibodies, would be informative on whether their phosphorylations overlap and are linked to histone H3 dephosphorylation. After treatment, cycling mammary adenocarcinoma cells were lysed with 2× SDS-reducing sample buffer for immunoblotting. Importantly, all of the analyzed phospho-proteins were carried out on the same blotting membrane and at the same time to avoid possible conflict with respect to time exposure of antibody signals to the X-ray film (Fig. 3).

Increasing concentrations of cAMP, from 0.125 up to 5 mM, provoke a sustained increase in CREB/ATF-1 phosphorylation in dose and time-dependent manners (Fig. 3, pCREB/ATF-1). Additionally, at lower cAMP concentrations a sharp H3 dephosphorylation is induced in a time-dependent fashion (Fig. 3, compare the dose-response kinetics of dephosphorylation of Ser10 (H3S10ph) and stimulation of CREB/ATF-1 phosphorylation throughout the process). Much lower cAMP concentrations are required for a much more effective H3-dephosphorylation, within a shorter time (compare phosphorylated H3 at 0.125 mM and at 5 mM of cAMP concentration). Whereas, when higher concentration of cAMP was used, a histone dephosphorylation delay was observed, and the recover of phosphorylation of H3 occurred earlier (Fig. 3, H3S10ph), most likely attributable to the activation of kinases, targeting H3

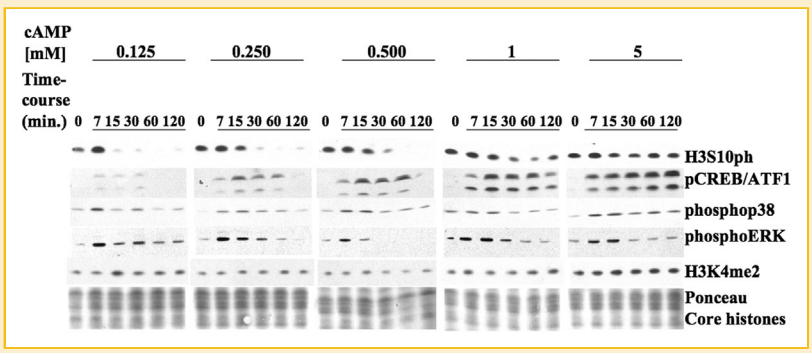

Fig. 3. Dose-response and time-course of cAMP-induced histone H3 dephosphorylation is independent of PKA and MAP kinase activation. After treatment with cAMP, total cell extracts were separated by 15% SDS–PAGE and electro-blotted onto nitrocellulose membranes. The blotted proteins were stained with Ponceau S and the core histones are shown as a loading control (bottom). Blotted proteins, on the same membrane, were sequentially incubated, overnight at 4°C, with specific antibodies for phosphorylated Ser10 on H3 (H3S10ph), phosphorylated CREB/ATF-1 (pCREB/ATF1), phosphorylated p38 at Thr180/Tyr182 (phosphop38), and phosphorylated ERK at Thr202/Tyr204 (phosphoERK) are indicated at the right. Note that the immunoblotting was carried out on the same membrane for H3S10ph, pCREB/ATF-1, phosphop38, and phosphoERK, thereby the time of exposure to the X-ray film for all phosphoproteins was identical for all cAMP concentrations and recorded kinetics. Dimethylated Lys 4 on H3 (H3K4me2), from the same experiment, was independently run and tested in an independent membrane. An anti-rabbit conjugated HRP (Jackson Immunology Research) 1/5000, was used to detect the primary antibodies. Concentration of cAMP and time of treatment are indicated at the top of the figure.

(i.e., by MAPK-Msk1/2, PKA, etc.). Because activation of MAPK signaling did not change much at either lower or higher concentration of cAMP (compare 0.125 mM cAMP with 5 mM and see further discussion bellow); one may tentatively favor the idea that hyperphosphorylation of CREB/ATF-1, at higher cAMP concentration, may, perhaps, be attributable to hyperactivation of PKA. A maximal CREB/ATF-1 phosphorylation is detected at 5 mM cAMP, reaching a peak at 2 h. At this concentration, H3 dephosphorylation is almost abrogated. Over-activation of PKA may be antagonizing H3 dephosphorylation in this type of cycling cells, perhaps due to the fact that H3 is a direct substrate of PKA [DeManno et al., 1999; Salvador et al., 2001]. Curiously, it has been published that a variety of PP1 inhibitors are activated by PKA, upon specific phosphorylation of certain amino acids, after which the inhibitors gain an exquisite and potent specificity for PP1c. For example, NIPP1, a nuclear protein, binds and inactivate PP1c upon its phosphorylation induced by cAMP- and cGMP-dependent protein kinases (Ki = 0.2 nM) [Trinkle-Mulcahy et al., 1999; Herzig and Neumann, 2000; Cohen, 2002]. These findings may be additional explanations of why higher concentration of cAMP prevents histone H3 dephosphorylation (Fig. 3). Because cAMP signaling also activates MAP kinase pathways [de Rooji et al., 1998; Kawasaki et al., 1998; Pham et al., 2000; Pak et al., 2002], and activation of MAP kinase can lead to H3 hyperphosphorylation [Mahadevan et al., 1991; Thomson et al., 1999; Dunn and Davie, 2005], the phosphorylated ERK and p38 were also followed with specific anti-phospho antibodies for each protein. It is clear that the onset of phosphorylation of ERK and p38 does not overlap with the onset of H3 dephosphorylation at any cAMP concentration (Fig. 3, H3S10ph, phosphop38, and phosphoERK, see 7 min of treatment for all cAMP concentrations). As it was discussed above, it should be noted, that the levels of hyper-phosphorylation of ERK and p38 by cAMP remain relatively similar at either lower or higher cAMP concentration during the treatment. This may support the idea that MAPK is not involved in the over-phosphorylation of CREB/ATF-1 at higher cAMP concentration and that either MAPK pathway is not responsible for the increasing blockade of loss in phosphate from H3 observed from 0.5 up to 5 mM cAMP, which, therefore, may be hypothesized to be attributable to PKA activation. However, by simply following CREB/ATF-1 phosphorylation on Ser133 is difficult to predict that PKA over-activation is responsible for the inhibition of H3 dephosphorylation. Firstly, although not necessarily induced by cAMP, Ser133 on CREB can be phosphorylated by multiple kinases [Johannessen and Moens, 2007]. Secondly, cAMP, directly or indirectly, activates multiple pathways that can phosphorylate CREB [Sands and Palmer, 2008]. Thirdly, we did not directly measure the activation of PKA by cAMP during this experiment. Thus, it cannot be excluded that the progressive inhibition of H3 dephosphorylation, concomitant with CREB/ATF-1 hyperphosphorylation, at increasing cAMP concentration, may be due to the activation of unknown signaling pathway(s). Consequently, this is an important aspect to be investigated in the future.

Note that within this period of cAMP treatment, neither dimethylated Lys 4 H3 (Fig. 3, H3K4me2) nor acetylated H3 (Fig. 1) substantially changed, suggesting that cAMP activity is specific towards histone H3 dephosphorylation.

To summarize: What did we learn from the results in Fig. 3?

- Cycling cells are hypersensitive to low-doses of cAMP leading to a much more effective H3 dephosphorylation than at higher cAMP concentration. Higher cAMP concentrations antagonize H3 dephosphorylation.
- cAMP-mediated H3 dephosphorylation is independent of MAP-kinase activation.
- Even when lower doses of cAMP activate MAP kinase pathways, the dephosphorylation of H3 prevailed over its hyperphosphorylation by such pathways.
- Sustained activation of CREB/ATF-1, by higher cAMP concentration, correlated with the inhibition of H3 dephosphorylation.
- A minimum dose of cAMP might be recommended for cell-proliferation arrest of this type of carcinoma cell line. Indeed, 0.1 mM cAMP was successfully used by us, in experiment 1C to arrest cell proliferation.

H89, A POTENT INHIBITOR OF PKA, INDUCES WITH SIMILAR KINETICS TO THAT OF cAMP A RAPID HISTONE H3 DEPHOSPHORYLATION

H89 is an isoquinoline sulfonamide derivative without the cyclic phosphate moiety present in cAMP, which compete for the binding of ATP to the activated catalytic subunit of PKA (IC_{50} between 48 and 135 nM) [Chijiwa et al., 1990; Davies et al., 2000]; thereby, effectively preventing PKA catalytic subunit to phosphorylate its specific targets. In vitro, H89 is also a potent inhibitor for Msk1 (IC_{50} ~120 nM [Lochner and Moolman, 2006], a MAPK enzyme directly phosphorylating H3 [Thomson et al., 1999; Dunn and Davie, 2005]), less potent for MAPKAP-K1b (IC_{50} ~2.8 μM) and PKBα (IC_{50} ~2.6 μM) [Davies et al., 2000]. Thereby, H89 blocks the mitogenic signal transduced from MAP kinase and PKA pathways to H3 [Davies et al., 2000]. The IC_{50} for the mitotic kinase Aurora A, in vitro, is ~103 μM and is expected to be higher for Aurora B [Vankayalapati et al., 2003]. Both kinases are involved in mitotic histone phosphorylation [Hsu et al., 2000; Zeitlin et al., 2001], thus H89 is excluded from inhibiting these kinases since, herein, it was used at 10 μM for cell treatment.

We also noticed that in Figure 2 lane 9, 10 μM H89 drastically reduced both Ser10 phosphorylation on H3 and CREB/ATF-1 as well. Therefore, we were motivated to study the kinetic of dephosphorylation of H3 by H89 and its effect on CREB/ATF-1 phosphorylation. Surprisingly, and similarly to 0.1 mM cAMP (Fig. 1A), yet 10-fold less concentrated (10 μM), H89 induces a rapid and potent dephosphorylation of H3 (Fig. 4, lane 5 min, upper panel) lasting for at least 2 h (lane 120 min) and recovering back at 240 min (lane 240 min.). Note that, conversely to cAMP (Fig. 3), CREP/ATF-1 are not hyperphosphorylated during H89 treatment, rather their basal phosphorylation is down-regulated, in a time-dependent manner, as one would expect (Fig. 4 middle panel, lanes 30 to 280 min). This finding supports the results in Figures 2 and 3 that activation of PKA and MAP kinases are not the pathways involved in H3 dephosphorylation by cAMP.

RAPAMYCIN (A SPECIFIC INHIBITOR OF mTOR), ISOPROTERENOL, AND cAMP ELICIT, IN A SIGNAL TRANSDUCTION FASHION, HISTONE H3S10ph AND phosS6K1 DEPHOSPHORYLATION IN PROLIFERATING MAMMARY ADENOCARCINOMA CELLS

mTOR is an essential serine/threonine kinase member of the cellular phosphatidylinositol 3-kinase pathway, which regulates ribosome

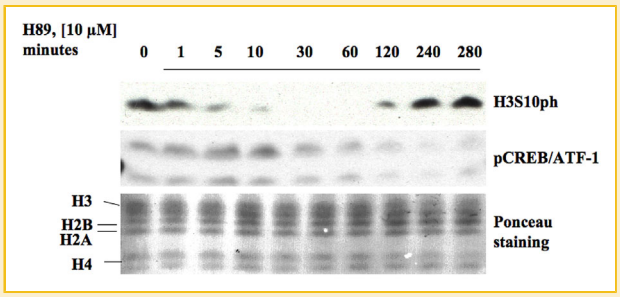

Fig. 4. H89 (10 μM), a potent inhibitor of PKA, induces with similar kinetics to that of cAMP, a rapid histone H3 dephosphorylation. 1470.2 cells were grown as described in Materials and Methods and Figure 1. Samples were prepared, resolved, and blotted as in Figure 1. Tame-course of H89 treatment are indicated at the top of the figure. Phosphorylated Ser10 H3 (H3S10ph, upper panel) and phosphorylated CREB/ATF-1 (pCREB/ATF-1, middle panel) were followed with specific anti-phospho-antibodies as described for other experiments. Protein loading control is indicated at the bottom, using core histones stained with Ponceau S as reference. Note that H89, as expected, is unable to stimulate CREB/ATF-1 phosphorylation. Moreover, at 30–280 min of incubation, a decline in phosphorylated CREB and ATF-1 is detected, suggesting upstream inhibition of specific kinases by H89.

biogenesis, translational, and transcriptional control [Crespo and Hall, 2002; Rohde and Cardenas, 2003; Tsang et al., 2003; Li et al., 2006; Shor et al., 2010]. mTOR localizes in both the cytoplasm and nuclei of cells [Kim and Chen, 2000; Zhang et al., 2002; Bachmann et al., 2006; Li et al., 2006; Shor et al., 2010] and has been linked to histone H4 deacetylation [Rohde and Cardenas, 2003] and to transcriptional control of ribosomal mRNA. Long-term mTOR inactivation by rapamycin, the mTOR specific inhibitor, leads to H4 deacetylation, ribosomal DNA chromatin condensation, Pol I delocalization, and ribosomal mRNA decay [Crespo and Hall, 2002; Rohde and Cardenas, 2003; Tsang et al., 2003].

Interestingly, cAMP is responsible for down regulation of mTOR and its downstream factors such as S6K1 and 4E-BP1/PHAS-1 [Graves et al., 1995; Scott et al., 1996; Scott and Lawrence, 1998]. However, the immediate response of H3 dephosphorylation induced by cAMP or rapamycin had not been investigated in the mentioned works. An interesting fact, although poorly understood, is that mTOR inactivation by rapamycin leads to PP2A-type phosphatase activation, which, in turn, dephosphorylates mTOR downstream targets, S6K1 and 4E-BP1/PHAS-1, implicated in translation [Peterson et al., 1999]. One could suggest a possible scenario in which mTOR, a pathway down-regulating phosphatase activity [Peterson et al., 1999], which is also down regulated by cAMP [Graves et al., 1995; Scott and Lawrence, 1998], could in turn regulate cAMP-mediated H3 dephosphorylation. Therefore, herein it is investigated whether cAMP-mediated mTOR inactivation could be a central element in both S6K1 and H3 dephosphorylation. Thus, we tested whether mTOR inhibition by rapamycin, cAMP and its inducers correlates with H3 dephosphorylation. First, we blocked mTOR activity with its specific inhibitor rapamycin and analyzed by immunoblotting the decay kinetics of phosphorylation of Thr389 on S6K1 and Ser10 on H3. As expected, rapamycin induces a potent and rapid dephosphorylation of S6K1, and surprisingly, concomitant with H3 dephosphorylation with similar kinetics (Fig. 5A). We next analyzed the effect of isoproterenol (a norepinephrine analog and agonist of adenylate cyclase) and cAMP on H3 and phosS6K1 dephosphorylation (Fig. 5B). Both compounds, fast and potently, stimulated with similar kinetics, both H3 and S6K1 dephosphorylation. This important finding suggested that mTOR may regulate gene activation through two well-established histone epigenetic markers, histone acetylation [Rohde and Cardenas, 2003] and phosphorylation. Of notice is the fact that the reported histone H4 deacetylation occurred after long-term mTOR inhibition by rapamycin [Rohde and Cardenas, 2003], whereas our results show that cAMP and rapamycin-induced H3S10ph dephosphorylation is an earlier and rapid event occurring in a signal transduction manner

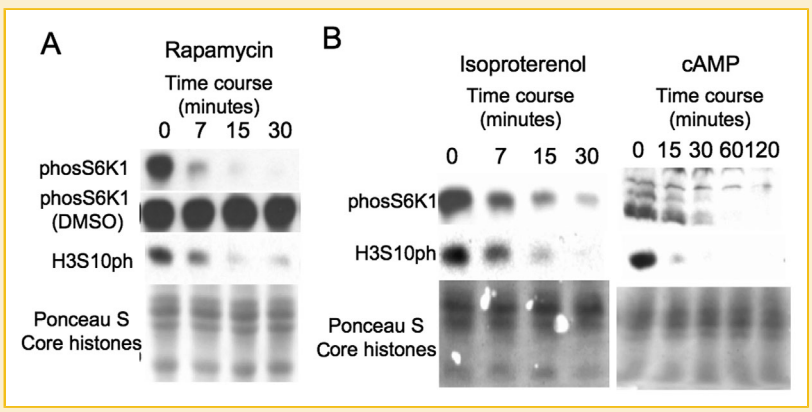

Fig. 5. Rapamycin, isoproterenol, and cAMP elicit a fast histone H3 and S6K1 dephosphorylation in proliferating mammary adenocarcinoma cells. (A) Cycling 1470.2 cells were treated either with 400 nM rapamycin or vehicle DMSO. (B) In parallel, cells were treated with isoproterenol (20 μM) or cAMP (0.1 mM) for the time indicated at the top of the figures. Untreated cells are indicated as time 0 at the top of the figures. Total cell extracts were subjected to immunoblotting. Dephosphorylation of H3 serine (H3S10ph) and Thr412 on S6K1 (phosS6K1) was followed by immunoblotting with specific anti-phospho-antibodies for each protein. Ponceau S staining of core histones, at the bottom, indicates equal protein loading.

(Figs., 3, and 5 1), which is followed later on by H3K9,12ac deacetylation (Fig. 1). The results in Figure 5 suggest that protein translation and chromatin remodeling could be intrinsically entangled, implying that cAMP may simultaneously influence two disparate events, protein synthesis through S6K1 dephosphorylation, and chromatin remodeling through H3 dephosphorylation and deacetylation (Figs. 1 and 5, respectively); both acting, probably, synergistically in preventing cell cycle progression (Fig. 1C).

OKADAIC ACID, A POTENT AND SPECIFIC INHIBITOR OF PP1/2, BLOCKS cAMP-MEDIATED H3 Ser10 DEPHOSPHORYLATION IN A DOSE-RESPONSE MANNER

Here, we provide evidence that cAMP-induced H3 dephosphorylation is blocked by okadaic acid (OA) (Fig. 6); suggesting that cAMP is dephosphorylating H3 through PP1/2-type phosphatases.

It has been established that PP1/PP2 can down regulate histone phosphorylation under certain circumstances. For example, it is known that Aurora B kinase is likely to be responsible, at least in part, for H3 phosphorylation during prophase and metaphase and this kinase is in a complex with PP1, which renders aurora B inactive upon its dephosphorylation [Murnion et al., 2001; Bolton et al., 2002; Sugiyama et al., 2002]. Additionally, histone deacetylase (HDAC1 and HDAC2) have been found in a multi-protein complex containing PP1 and its regulatory subunit (PNUT) and PP2A regulatory subunit PP2R5 [Canettieri et al., 2003].

It has been published that PP1 and PP2-type phosphatases possess a remarkably differences in their sensitivity to okadaic acid (OA). PP2 is much more sensible to OA inhibition than PP1. In cultured cells, OA blocks PP2 at ~50 nM, whereas PP1 inhibition requires concentration around 1 μM [Honkanen and Golden, 2002]. Our finding that higher concentration of OA (500 nM) inhibits more effectively cAMP-mediated H3 dephosphorylation than lower concentration (100 mM), may be indicative of PP1ase being involved in cAMP-induced H3 dephosphorylation (Fig. 6, compare lanes 3, 100 nM with lane 5, 500 nM). Note that OA, at concentrations and time used here, does not have a significant effect on the basal level of phosphorylation of H3 in cells untreated with cAMP (Fig. 6, lanes 10–12). Neither vehicle alone (DMSO) has any effect (lanes 13–15).

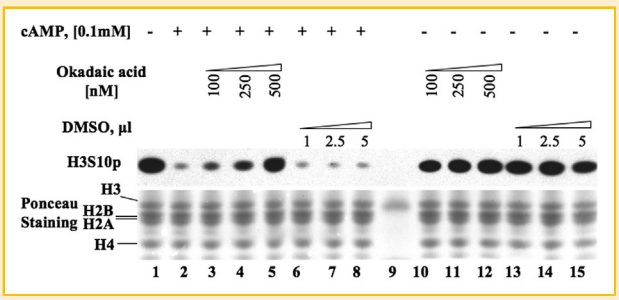

Fig. 6. Okadaic acid (OA) a potent and specific inhibitor of PP1/2 blocks cAMP-mediated H3 Ser10 dephosphorylation. Immunoblotting for phosphorylated Ser10 on H3 (H3S10ph, upper panel) was carried out for acidic extracted core histones from whole cells, treated or not with increasing concentration of OA in the presence or absence of cAMP. Before cAMP treatment, the cells were pre-treated with H_2O, OA, or DMSO for 10 min, and then for another additional 50 min, either in the presence of cAMP/OA mixture (lanes 3–5), cAMP alone (lane 2), no cAMP (H_2O vehicle, lane 1), cAMP/DMSO (OA vehicle, lanes 6–8), OA alone (lanes, 10–12) or DMSO alone (lanes 13–15). Lane 9 is a 14-kDa MW marker. Lower panel is the Ponceau S stained of histones as loading control. Note that 1-h treatment with OA, up to 500 nM OA, did not induce a hyperphosphorylation of H3 in control samples (lanes 10–12). The concentrations of OA and amounts of DMSO are indicated at the top of the figure.

CONCLUSIONS

cAMP is an ubiquitous second messenger with broad targets. Although decades have been needed to elucidate its most recent agonist (i.e., GEF/RAS/MAPK), it is shown here that cAMP dephosphorylates Ser10 on histone H3 independent of PKA and inversely correlates with CREB/ATF-1 hyperphosphorylation, canonical PKA targets. We published that cAMP-mediated H3S10ph dephosphorylation was independent of Epac (GEF) [Rodriguez-Collazo et al., 2008a]. Unable to directly show here the pathways involved in cAMP-induced H3 dephosphorylation, our results; however, suggest a link to a signaling rout, sensitive to low concentration of cAMP, which is PKA and MAPK-independent that may be canalized through down regulation of mTOR kinase. In fact, it is shown here that mTOR down regulation with rapamycin, cAMP and its agonist, elicited, with similar kinetics, both S6K1 and H3 dephosphorylation. The dose-response inhibition of cAMP-induced H3 dephosphorylation by okadaic acid suggests that PP1 may be responsible for cAMP-mediated H3 dephosphorylation.

As we earlier mentioned, it has been published that after long-term mTOR inhibition by rapamycin histone deacetylation occurred, affecting ribosomal mRNA transcription [Rohde and Cardenas, 2003; Tsang et al., 2003]. We hope that our findings will encourage the investigation on whether the rapid cAMP- and rapamycin-mediated histone H3S10ph dephosphorylation could be the leading processes provoking histone deacetylation, the mechanism involved in, and the connection, if any, to gene transcription.

AUTHORS' CONTRIBUTION

Pedro Rodriguez designed, executed, analyzed the experimental data, and contributed to write the paper. Juan Rojas contributed to write the paper.

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