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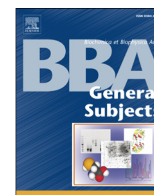

PI3K pathway is involved in ERK signaling cascade activation by histamine H2R agonist in HEK293T cells

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ABSTRACT

Background: Histamine, through histamine H2 receptor (H2R), modulates different biological processes, involving the modulation of PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways. Many evidences have demonstrated the existence and importance of the crossregulation between these two signaling pathways. The aim of the present work was to determine the molecular mechanisms leading to PI3K and ERK pathways modulation induced by the H2R agonist amthamine and to evaluate the possible interplay between them.
Methods: Phosphorylation levels of ERK and Akt were examined by Western blot in HEK293T cells expressing the human H2R, in the presence of H2R agonist and dominant negative mutants or pharmacological inhibitors of different proteins/pathways. Transcriptional activity assays were assessed to determine SRE activity. Amthamine-mediated cellular proliferation was investigated in MA-10A cells in the presence of PI3K inhibitor.
Results: H2R agonist inhibits PI3K/Akt/mTOR and stimulates Ras/MEK/ERK pathways. Moreover, PI3K/Akt/mTOR signaling inhibition is necessary to achieve H2R mediated ERK activation. In the presence of a constitutive active mutant of Akt, amthamine is not able to mediate ERK activation. This crosstalk affects classical ERK downstream targets such as Elk1 phosphorylation and the transcriptional activity of the SRE, classically associated to proliferation. We further demonstrate that amthamine-induced proliferation in Leydig MA-10 tumor cells, is enhanced by LY294002, a PI3K inhibitor.
Conclusions: These results describe a crosstalk between PI3K/AKT/mTOR and Ras/MEK/ERK pathways induced by H2R stimulation with implications in cell proliferation.
General significance: This work indicates that the modulation of PI3K/AKT/mTOR pathway by H2R in turn regulates Ras/MEK/ERK activation conditioning the proliferative capacity of the cells.
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1. Introduction

Histamine [2-(4-imidazolyl)-ethylamine] is an important mediator of many physiological and pathological processes, including inflammation, gastric acid secretion and neuromodulation. It is also associated with diverse biological responses which are related to tumor growth, including proliferation, angiogenesis, cell invasion, migration, differentiation, apoptosis and modulation of the immune response, indicating that may have a crucial role in cancer development and progression [28,29,41].

Its cellular effects are exerted by binding to four different G protein-coupled receptors (GPCRs), namely histamine receptors 1, 2, 3, and 4 (H1R, H2R, H3R, H4R) according to the order in which they were discovered. In most systems, H2R stimulation leads to adenylyl cyclase activation with subsequent increase in cAMP accumulation and PKA activity [30]. However, more recent reports indicate that H2R can also

Abbreviations: HR, histamine receptor; GPCR, G-protein coupled-receptor; ERK1/2, extracellular signal-regulated kinases; PI3K, phosphoinositide 3-kinase; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; DMEM/F12, Dulbecco's modified Eagle's medium/Ham F12; ECL, enhanced chemiluminescence; IBMX, 1-methyl-3-isobutylxanthine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DNDyn, dynamin dominant negative mutant; Akt-myr, constitutive active mutant of Akt1; PKI, PKA peptide inhibitor; PKImut, inactive mutant of PKI; Gαt, Gα-transducin; SRE, serum response element gene promoter.

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modulate other signaling pathways classically associated to tyrosine kinase receptors. In this context, H2R mediated histamine modulation of cell proliferation, migration, cellular permeability, gene expression, and memory have been proved to involve the extracellular signal-regulated kinases (ERK1/2) activity [9,25,34]. There is also evidence that some classical histamine actions mediated by H2R, such as acid gastric secretion, involves the modulation of PI3K (phosphoinositide 3-kinase) [35], which is a traditional mediator of growth factors and cytokines responses.

It is well known that there are multiple contact nodes shared by these two signaling pathways, whose coordinated action determines cell fate [2]. Hence, it is not surprising that the PI3K/Akt and Ras/MAPK pathways influence each other at different stages of signal propagation, both negatively and positively, resulting in a dynamic and complex crosstalk. The occurrence of these multiple regulatory feedbacks depends on cell type, the stage of cell differentiation, ligand type and dose [1]. Considering the importance of these two pathways on cell proliferation and cancer, and taking into account the relevant role of the H2R in regulating these processes [16,37,38,45], the aim of the present work is to elucidate the regulatory mechanism of PI3K and ERK pathways by the H2R agonist amthamine, their crosstalk, and their impact on cell proliferation.

Present findings show that in HEK293T cells transiently expressing the H2R, amthamine inhibits PI3K/Akt/mTOR and stimulates Ras/MEK/ERK pathways. Moreover, PI3K/Akt/mTOR signaling inhibition is necessary to achieve H2R mediated ERK activation. This balance affects, in consequence, Elk1 phosphorylation and the transcriptional activity of the serum response element, classically associated to proliferation. Consistently, amthamine-induced proliferation in Leydig MA-10 tumor cells, that endogenously expresses H2R, was enhanced by the inhibition of PI3K.

2. Materials and methods

2.1. Materials

Cell culture medium, antibiotic, bovine serum albumin (BSA), amthamine, ranitidine, tiotidine, and AG1478 were obtained from Sigma Chemical Company (St. Louis, MO). LY294002 (2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one), wortmannin (1*S*, 6b*R*,9a*S*,11*R*,11b*R*)11-(acetyloxy)-1,6b,7,8,9a,10,11,11b-octahydro-1-(methoxymethyl)-9a,11b-dimethyl-3*H*-furo[4,3,2-*de*]indeno[4,5,-*h*]-2-*h*]-2-benzopyran-3,6,9-trione), PD98059 (2-(2-Amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one) were from Tocris Cookson Inc. (Ballwin, MO). Fetal calf serum was from Natocor (Argentina) and horse serum from GIBCO. Rapamycin was obtained from LC Laboratories (Woburn, MA). Other chemicals used were of analytical grade and obtained from standard sources.

2.2. Plasmid constructions

pCEFL-HA-H2R was previously generated in our laboratory [52], pEGFP-PKI, pEGFP-PKI mut, pcDNA3-DNRAP1, pcDNA3-H-Ras N17, pcDNA3-K-Ras N17 and pCEFL-Gα transducin plasmids were generated in Dr. J. Silvio Gutkind laboratory. pACL4-AKT1-Δ4-129 was a gift from Dr. Virgina Novaro (Laboratorio de Carcinogénesis Hormonal, IBYME-CONICET, Buenos Aires, Argentina), pcDNA3-HA-dynaminK44A was a generous gifts from Dr. J. Benovic (Thomas Jefferson University, Microbiology and Immunology. Department, Kimmel Cancer Center, Philadelphia, PA).

2.3. Cell culture and transfections

HEK293T (Human embryonic kidney) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 5 μg/ml gentamicin. MA-10 Leydig cells [7] generously given by Dr. Omar P. Pignataro [19] were cultured in Dulbecco's modified Eagle's medium/Ham F12 (DMEM/F12) supplemented with 15% horse serum, 4.76 g/l of Hepes and 5 μg/ml gentamicin. All cultures were maintained at 37 °C in humidified atmosphere containing 5% CO_2.

For transient transfection, cells were grown to 80–90% confluency and the cDNA constructs were transfected using Lipofectamine 2000 (Life Technologies). The transfection protocol was optimized as recommended by the suppliers. Usually, assays were performed 48 h after transfection.

2.4. Western blot assay

For Western blot assays, cells were serum starved for 4 h, stimulated at the indicated period of time, washed and lysed in 50 mM Tris–HCl pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol, 10% glycerol and 0.05% bromophenol blue and sonicated to shear DNA. Total cell lysates were resolved by SDS-PAGE, blotted and incubated with the primary antibodies anti-ERK1/2, -pERK1/2, -Akt1/2/3, -pAkt1/2/3, -GAPDH, -pCREB, -tubulin, (Santa Cruz Biotechnology, CA), -actin, -pELK1, -pmTOR (Ser2448) or mTOR (Cell Signalling Technology, CST) followed by horseradish peroxidase conjugated anti-rabbit or anti-mouse (Vector Lab.) and developed by enhanced chemiluminescence (ECL) following the manufacturer's instructions (Amersham Life Science, England). Films were scanned and quantified using Scion Image® software from National Institutes of Health (NIH). Representative full-length blots are shown as Supplementary Fig. 1.

2.5. cAMP assay

For cAMP determination assays, cells were incubated 3 min in basal culture medium supplemented with 1 mM IBMX at 37 °C, followed by 9 min exposure to different concentration of amthamine, in the presence or not of H2R antagonist. The reaction was stopped by ethanol addition followed by centrifugation at 2000 g for 5 min. The ethanol phase was then dried and the residue resuspended in 50 mM Tris–HCl pH 7.4, 0.1% BSA. cAMP content was determined by competition of [^{3}H]cAMP for PKA, as previously described [18].

2.6. Reporter gene assay

HEK293T cells seeded on 12-well plates were cotransfected with the pSRE.L-luciferase reporter plasmid and pCEFLHA-H2R. In some experiments, cells were also co-transfected with the plasmid constructs previously detailed or an empty vector to maintain the total amount of DNA. After 6 h, cells were seeded in 96-well plates, and after 24 h deprived from serum for 16 h. Cells were then stimulated with diverse agents and luciferase activity was measured 6 h later with the Steady-Glo Luciferase Assay System according to the manufacturer's instructions (Promega Biosciences Inc. San Luis Obispo, CA, USA) using the FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, LLC). Experimental reporter activity was normalized to control activity.

2.7. Proliferation assay

Cell proliferation was assessed as a function of [^{3}H]thymidine incorporation. MA-10 cells (5×10^3/well) were seeded and cultured in 96-well plates for 24 h, and then growth medium was replaced with starvation medium (serum-free medium). Cells were incubated in these conditions for 24 h and then exposed for 48 h to different stimulus as indicated in the figures. Twelve hours before the end of the experiment [^{3}H]thymidine (0.25 μCi/ml) was added. The cells were then harvested using an automatic cell harvester (Nunc, Maryland, USA). The incorporation of the radioactive nucleotide was measured in a Pharmacia Wallac 1410 liquid scintillation counter and expressed as incorporation percentage with respect to a control group (cells grown in

culture medium). Assays were performed in triplicate and at least three independent experiments were conducted.

2.8. Statistical analysis

Statistical analysis was performed from at least three independent experiments.

Statistical of densitometric western blot analysis were carried out by one-way ANOVA or *t*-test followed by the Dunnett's or Tukey's Multiple Comparison post-test performed with GraphPad Prism 5.00 for Windows, GraphPad Software. Statistical analysis of reporter gene assay were performed by one-way ANOVA or two-way ANOVA followed by Dunnett's Multiple Comparison post-test.

3. Results and discussion

3.1. H2R activation leads to pAkt and pERK modulation

Histaminergic ligands trigger multiple intracellular mechanisms that go beyond the modulation of second messengers which ultimately determine cellular behavior and fate. Knowing the importance of H2R mediated biological responses in normal and abnormal cells, we aimed to characterize its involvement in the control of ERK and PI3K signaling pathways that govern cellular proliferation and survival.

In HEK293T cells transiently transfected with the H2R, kinetic experiments showed that histamine has opposite effects on these two signaling pathways. While causing a rapid decrease in pAkt levels, an increase in ERK1/2 phosphorylation was observed. After 5 min of stimulation, ERK1/2 reached the maximum phosphorylation level when pAkt levels were significantly decreased (Fig. 1A). Similar modulation profiles were obtained with the selective H2R agonist, amthamine (Fig. 1B). It is noteworthy that cells without treatment displayed high levels of pAkt indicating a high basal activity of Akt in this system, even when cells were starved for 4 h. High basal levels of pAkt were also observed in mock transfected HEK293T cells (Supplementary Fig. 2). These results indicate that specific H2R activation by histamine or amthamine modulates the activity of proteins involved in cell proliferation and survival, such as ERK1/2 and Akt, in an opposite manner. In this context, various reports described H2R-mediated ERK activation in several systems such as HUVEC cells, melanocytes and HEK293 cells which proved to be involved in cell permeability, migration and proliferation [25,34,50]. On the other hand, inhibition of PI3K pathway by LY294002 or wortmannin has been described to increase histamine-induced acid secretion [35]. Moreover, recently, time-course studies with histamine and amthamine revealed a rapid decrease in Akt2 phosphorylation levels in U937 leukemic cells [48].

3.2. pERK and pAkt modulation by H2R is independent of cAMP effectors

H2R activation leads to cAMP accumulation via the coupling to Gs proteins in various native cells (for review, see [21,39]) and cells expressing recombinant H2 receptors [3,4,20,36]. Consistently, HEK293T transfected with the H2R increased cAMP levels in an amthamine concentration-dependent manner. Amthamine-induced cAMP accumulation could be inhibited by H2R antagonists ranitidine and tiotidine indicating specific H2R activation by amthamine (Fig. 2A left). We next evaluated whether cAMP effectors were responsible for pAkt and pERK1/2 modulation. To address this, we evaluated the effect of co-transfecting HEK293T cells with PKI, a competitive PKA peptide inhibitor that binds the catalytic site of the kinase [14] and dampens phosphorylation by PKA. As expected, PKI blocked amthamine induced CREB phosphorylation. Moreover, in cells cotransfected with PKImut (peptide unable to compete with PKA) amthamine-induced CREB phosphorylation was not affected (Fig. 2A right) indicating that PKI is able to block PKA activity in our system. Although PKA inhibition significantly decreased basal Akt phosphorylation ($p < 0.01$), it did not modify the extent of the effect of amthamine treatment on pAkt levels (Fig. 2B). Comparable results were obtained when pERK were measured. Amthamine induced ERK activation was not diminished by co-transfection with PKI (Fig. 2B).

Besides PKA, cAMP was also described to activate EPAC, a guanine-nucleotide exchange factor (GEF) towards Rap1 in several systems. It was previously reported that depending on the cell-context, Rap1 could either block Ras-mediated ERK1/2 activation or activate ERK1/2 in a Ras independent manner [47]. There is also evidence of Rap1 mediated modulation of Akt in several systems [15,22,31]. With the aim to

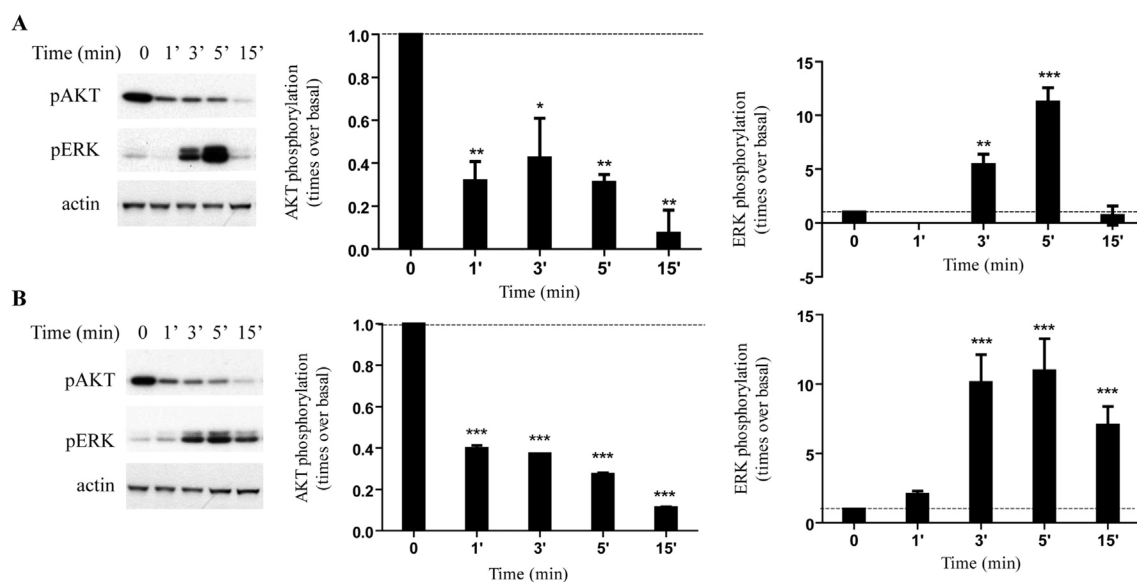

Fig. 1. Effect of H2R ligands on pERK and pAkt levels. HEK293T cells transfected with the H2R codifying plasmid were subjected to A) 100 μM histamine or B) 10 μM amthamine treatment for the indicated times. Concentration-response experiments were performed for p-ERK levels and shown as Supplementary Fig. 2. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with anti p-ERK, p-Akt and actin antibodies. Densitometric analysis was performed using ImageJ software and phosphorylation levels were normalized to actin. Data are presented as the means ± SD ($n = 3$). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ with respect to untreated cells.

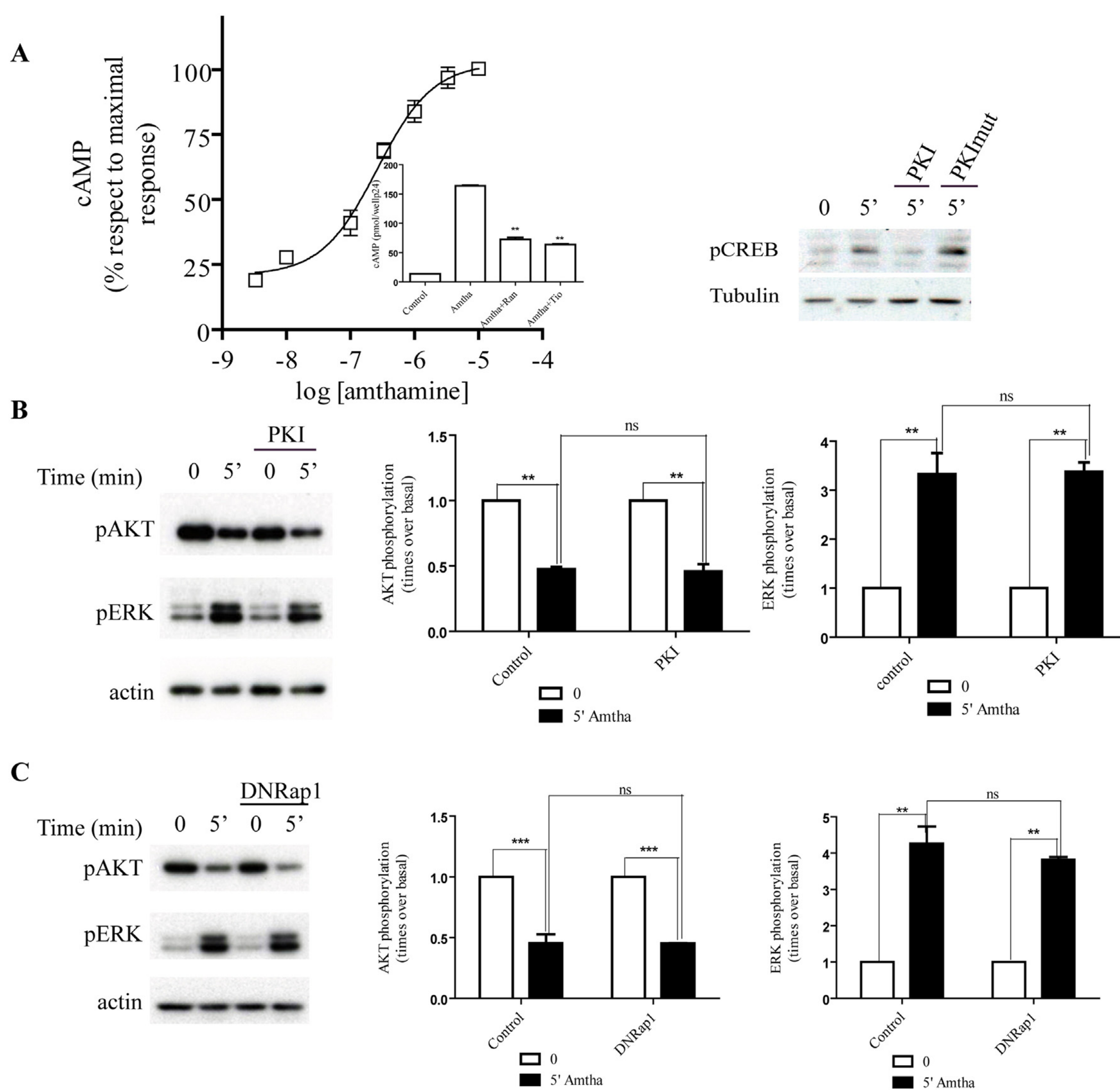

Fig. 2. Effect of PKA and Rap1 on amthamine modulation of pERK and pAkt levels. A-Left) HEK293T cells transfected with the H2R codifying plasmid were stimulated with different concentration of amthamine for 9 min. in the presence of IBMX. *Inset*: HEK293T cells transfected with the H2R codifying plasmid were stimulated or not (Basal) with 10 μM amthamine (Amtha), 10 μM ranitidine (Ran), 10 μM tiotidine (Tio) as indicated for 9 min. in the presence of IBMX. cAMP was determined as is detailed in Methods. A-Right) HEK293T cells transfected with the H2R codifying plasmid (lanes 1 and 2) or cotransfected with PKI (lane 3) or PKI mut (lane 4) were treated with 10 μM amthamine for 5 min. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with anti pCREB and tubulin antibodies. B and C) HEK293T cells transfected with the H2R codifying plasmid (Control) or cotransfected with B) PKI or C) DNRap1 were subjected to 10 μM amthamine treatment for 5 min. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with anti p-ERK, p-Akt and actin antibodies. A-C) Densitometric analysis was performed using ImageJ and phosphorylation levels were normalized to tubulin or actin. Basal correspond to amthamine untreated cells for each transfection. Data is presented as the means ± SD ($n = 3$). ***$p < 0.001$, **$p < 0.01$, ns: not significant.

determine whether Rap1 pathway is involved in amthamine induced pERK1/2 and pAkt modulation, we used a dominant negative mutant of Rap1 that blocks Rap1 action [12]. Fig. 2C shows that ERK1/2 and Akt activity was not modified in the presence of the construct, ruling out the participation of Rap1 in these pathways. Overall these results point to other signaling components over Gs/AMPc/PKA/Epac as responsible for ERK and Akt activity modulation.

3.3. Amthamine induced pERK modulation is mediated by dynamin and Ras proteins

Receptor internalization has been described as a trigger for the activation of Ras/MEK/ERK pathway for several GPCRs such as the dopamine D2S, chorticotrophine releasing hormone and α1 adrenergic receptors, among others [8,26,40]. However, we have previously determined that Ras/MEK/ERK activation by amthamine is independent of H2R internalization, though the latter is blocked by the dynamin dominant negative mutant (DNDyn) [5]. Present results show that although DNDyn completely dampened amthamine induced ERK1/2 phosphorylation it has no effect on the decrease in pAkt levels induced by amthamine. However, as already observed for PKI, this construct led to a significant decrease in basal pAkt levels ($p < 0.01$) suggesting that dynamin activity is, at least partially, responsible for basal Akt phosphorylation (Fig. 3A). Similar results have been obtained by Jimenez-Sainz and coworkers who showed that ERK activation induced by MCP-1 agonist is independent of CCR2B receptor internalization but it was blocked in the presence of DNDyn [23]. Moreover, Xu and colleagues showed in HEK293 cells transfected with H2R expression vector that ERK activation promoted by another H2R agonist, dimaprit, is also mediated by dynamin [50].

Classically, dynamin regulation of GPCRs was associated to receptor internalization mediated by its GTPase activity. However, other functions of dynamin acting as a scaffold molecule or as a mediator of general endocytic complex may also regulate GPCRs signaling. In this context, it has been described that dynamin is required for MAPK modulation by promoting the direct contact between activated MEK

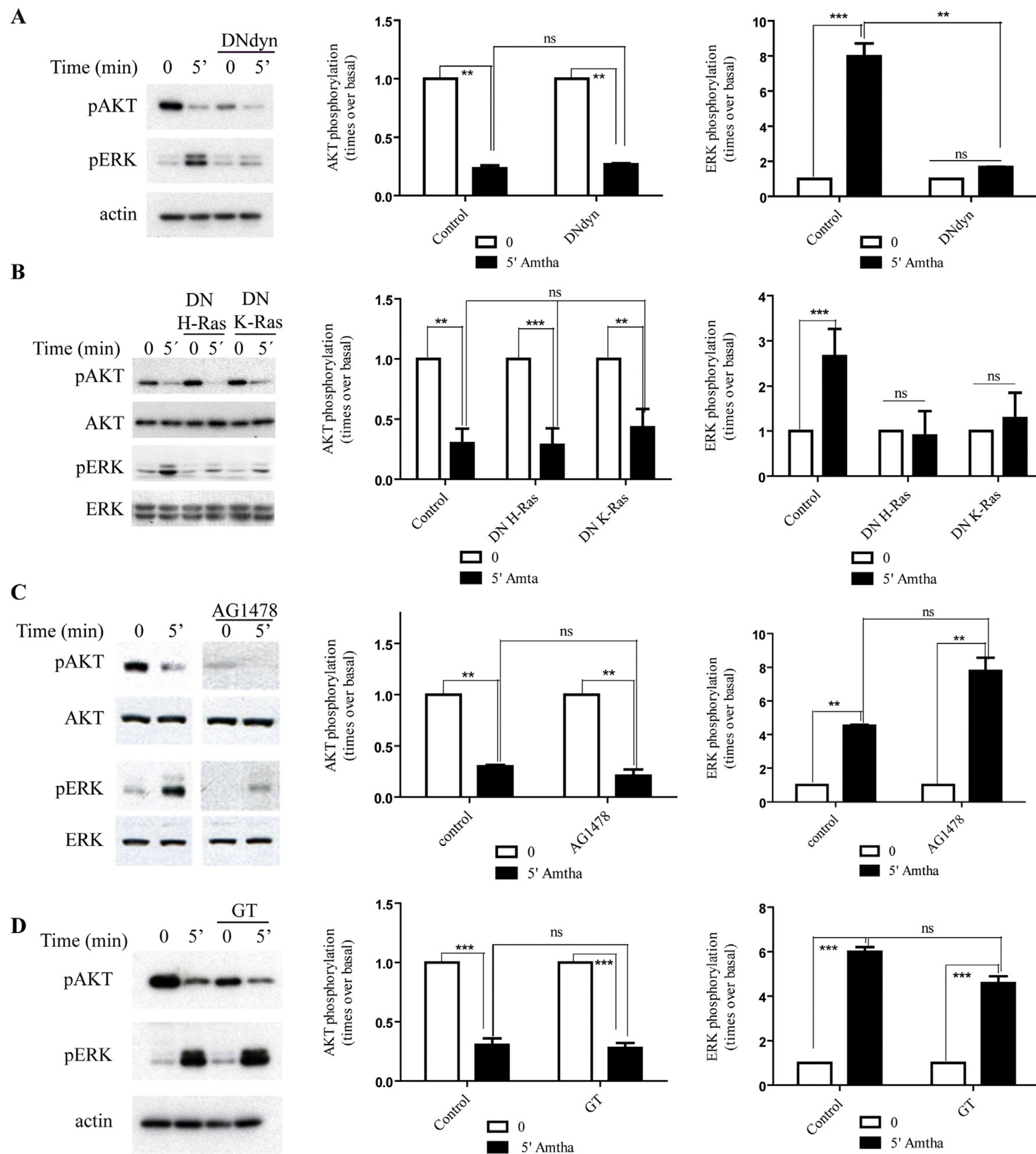

Fig. 3. Effect of dynamin, Ras, EGF transactivation and Gβγ on amthamine modulation of pERK and pAkt levels. HEK293T cells transfected with the H2R codifying plasmid (Control) or cotransfected with A) Dndyn, B) DNH-Ras or DNK-Ras, D) G transducin (GT) were subjected to 10 μM amthamine treatment for 5 min. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with anti p-ERK, p-Akt, ERK, Akt and actin antibodies. C) HEK293T cells transfected with the H2R codifying plasmid (control) were subjected to 1 μM AG1478 pretreatment for 50 min and stimulated with 10 μM amthamine for 5 min. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with anti p-ERK, p-Akt, ERK and Akt antibodies. A-D) Densitometric analysis were performed with ImageJ and normalized to actin, ERK or Akt levels as is indicated in each case. Basal correspond to amthamine untreated cells for each transfection or pretreatment. Data is presented as the means ± SD ($n = 3$). ***$p < 0.001$, **$p < 0.01$, ns: not significant.

and its target ERK in the cytosol [27] and that DNDyn may inhibit the endocytosis of the H-Ras/Raf complex preventing activation of MAPK and other Ras-dependent functions [42].

Interestingly, the dominant negative mutants of K-Ras and H-Ras completely abolished ERK1/2 activation by amthamine (Fig. 3B). On the other hand, the decrease in pAkt levels induced by amthamine was not affected by these mutants. These results clearly demonstrate that Ras proteins are involved in pERK1/2 modulation but not in pAkt regulation induced by amthamine. In this context, the dependence of H2R mediated ERK activation on both dynamin and Ras activity could be explained in terms of dynamin action as scaffold or general endocytosis mediator.

On the other hand, EGF receptor transactivation pathway has been implicated in linking a variety of G-protein coupled receptors to MAPK cascades [49,51]. Here, we further investigate whether amthamine induced Ras/ERK and AKT modulation pathways would be mediated through EGFR transactivation. In H2R transfected cells, the pretreatment with the EGFR inhibitor, AG1478, which blocked ERK1/2 activation by EGF (data not shown) did not modified amthamine induced pERK1/2 and pAkt modulation (Fig. 3C). The basal phosphorylation levels of both, ERK1/2 and Akt, were drastically reduced in presence of AG1478, likely due to the blockage of remaining growth factors present in the cell culture that may account for basal activity maintenance of these pathways.

Gβγ signaling was classically associated to PI3K/Akt modulation by GPCRs [11,24]. Besides, this dimer proved to be involved in the activation of ERK mediated by H2R inverse agonists [5]. In order to evaluate the potential implication of Gβγ in the effects of amthamine

on these signaling pathways, pERK1/2 and pAkt modulation was evaluated in HEK293T cells cotransfected with H2R and Gα-transducin (Gαt), a widely used scavenger of Gβγ [17,32]. In the presence of Gαt, basal pAkt levels were significantly diminished ($p < 0.001$), suggesting a role of Gβγ dimer in the maintenance of basal activity of PI3K. However, amthamine induced pERK1/2 and pAkt modulation were not modified (Fig. 3D).

Concerning this, it has been previously described that GPCRs may inhibit Akt pathway by activating the negative regulator of the PI3K signaling, PTEN (tumor suppressor with sequence homology to protein tyrosine phosphatases) [53]. Furthermore, Bousquet and coworkers showed evidence of a physical interaction between a GPCR, somatostatin receptor 2 and the PI3K regulatory subunit p85 [10].

3.4. PI3K/AKT/mTOR pathway inhibition is necessary to ERK1/2 activation

Ras/ERK and PI3K/mTOR were originally thought as linear signaling pathways activated by different stimuli. Today, it is well known that both paths interact to regulate each other and co-regulate downstream functions. There is a great interest in the study of this crosstalk considering the relevance of the cellular processes involved, its extent and significance in cancer therapeutics [13]. Dysregulation of both signal transduction pathways leads to uncontrolled cellular proliferation, survival, invasion, and metastases frequently observed in patient with some type of cancers and in general, associated with a poorer prognosis [33,43,44]. Taking into account that amthamine modulates pERK1/2 and pAkt levels in an opposite manner and that pAkt is modulated faster

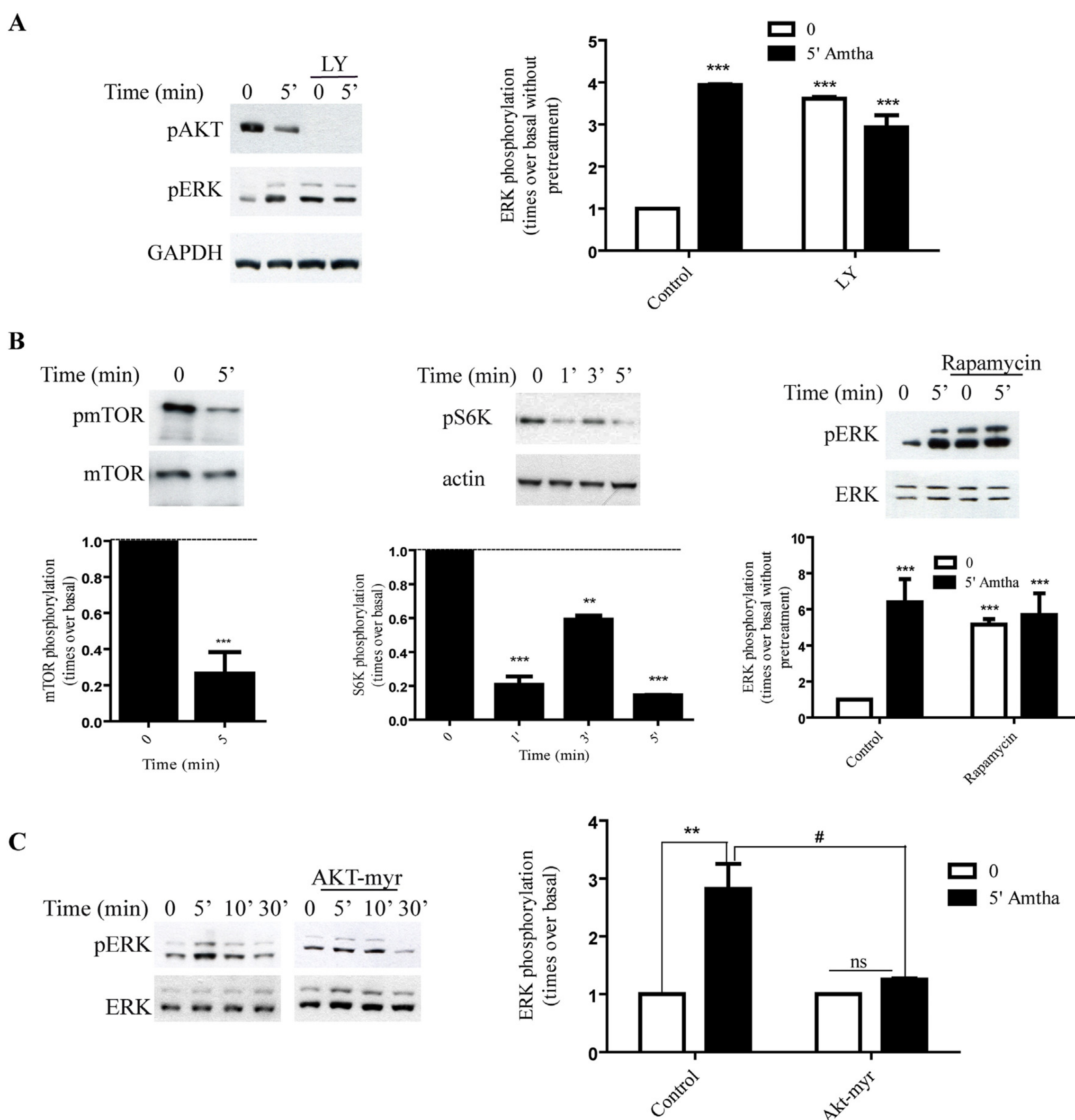

Fig. 4. Effect of PI3K/Akt/mTOR signaling pathway on amthamine modulation of pERK levels. A) HEK293T cells transfected with the H2R codifying plasmid (Control) were subjected to 50 μM LY294002 pretreatment for 50 min and stimulated with 10 μM amthamine for 5 min. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with anti p-ERK, p-Akt and GAPDH antibodies. B) *Left*: HEK293T cells transfected with the H2R codifying plasmid were subjected to 10 μM amthamine treatment for the indicated times. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with anti p-mTOR and mTOR antibodies. *Middle*: HEK293T cells transfected with the H2R codifying plasmid were subjected to 10 μM amthamine treatment for the indicated times. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with anti p-S6 K and actin antibodies. *Right*: HEK293T cells transfected with the H2R codifying plasmid were subjected to 1 μM rapamycin pretreatment for 50 min and stimulated with 10 μM amthamine for 5 min. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with anti p-ERK, and ERK antibodies. C) HEK293T cells transfected with the H2R codifying plasmid (Control) or cotransfected with Akt-myr were subjected to 10 μM amthamine treatment for different times of period. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with anti p-ERK and ERK antibodies. A-C) Densitometric analysis were performed with ImageJ and normalized to GAPDH, actin or ERK levels. Data are presented as the means ± SD ($n = 3$). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ with respect to amthamine untreated control cells.

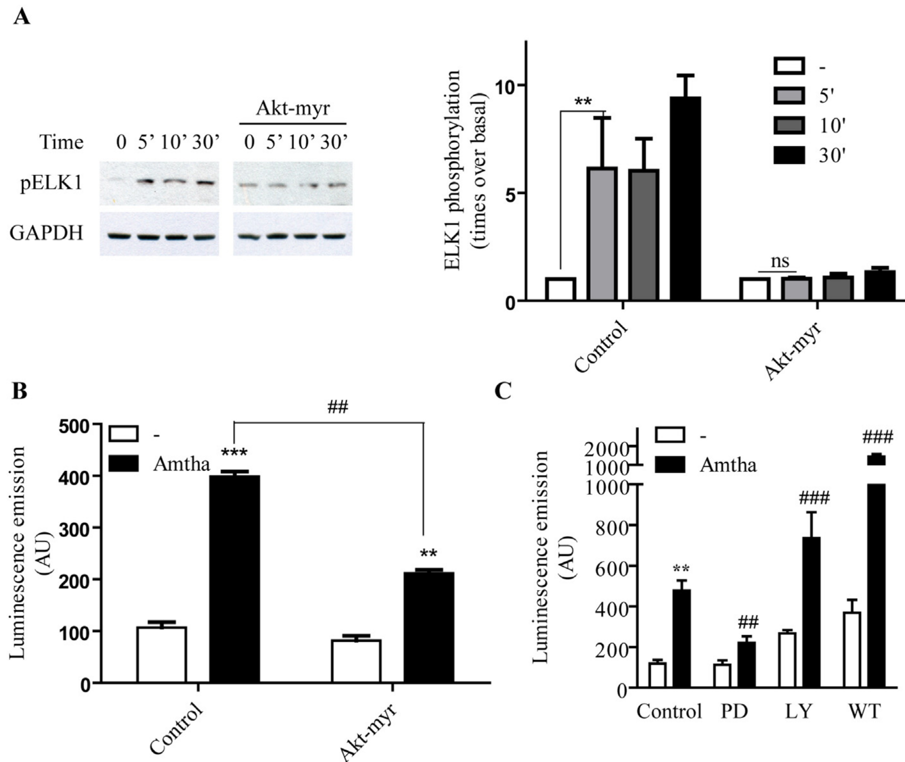

Fig. 5. Effect of PI3K/Akt/mTOR pathway on downstream effectors of ERK1/2. A) HEK293T cells transfected with the H2R codifying plasmid (Control) or cotransfected with Akt-myr were subjected to 10 μM amthamine treatment for the indicated times. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with anti p-Elk1 and GAPDH antibodies. Densitometric analysis were performed with ImageJ and normalized to GAPDH levels. Data are presented as the means ± SD ($n = 3$). ***$p < 0.001$, **$p < 0.01$ with respect to untreated cells. B) HEK293T cells transfected with the H2R codifying plasmid (Control) or cotransfected with Akt-myr were incubated with 10 μM amthamine for 16 h. Luciferase activity was determined as stated in methodology section. Data were calculated as the mean ± SD of assay triplicates. Similar results were obtained in at least three independent experiments. **$p < 0.01$. ***$p < 0.001$ with respect to unpretreated control cells, ##$p < 0.01$. C) HEK293T cells cotransfected with the reporter SRE—Luc and the H2R codifying plasmid were pretreated for 50 min with 50 μM PD98059 (PD), 50 μM LY294002 (LY) or 5 μM wortmannin (WT) and incubated with 10 μM amthamine for 16 h. Luciferase activity was determined as stated in methodology section. Data were calculated as the mean ± SD of assay triplicates. Similar results were obtained in at least three independent experiments. **$p < 0.01$ with respect to unpretreated control cells, ##$p < 0.01$, ###$p < 0.001$ with respect to unpretreated amthamine stimulated cells.

than pERK (Fig. 1B), we analyzed whether a crosstalk between these two pathways occurs. In order to do that, H2R-transfected HEK293T cells were pretreated with the PI3K inhibitor, LY294002, and stimulated with amthamine. As expected, the presence of LY294002 turned undetectable the levels of pAkt. Interestingly, LY294002 treatment significantly increased pERK basal levels which reached the same degree of phosphorylation as the observed after amthamine stimulation (Fig. 4A), indicating that the sole inhibition of the basal activity of PI3K/Akt pathway is able to induce ERK phosphorylation. Considering that mTOR is a kinase activated downstream Akt, that is implicated in the control of transcription initiation, organization of actin cytoskeleton and regulation of cell proliferation and death, we next analyzed the role of mTOR in amthamine induced signaling pathway. As a first approach, the modulation of mTOR activity by the H2R was evaluated by its phosphorylation and the phosphorylation of its target, S6K. Consistently with pAkt modulation, amthamine treatment also decreased pmTOR and pS6K levels (Fig. 4B left and middle). In order to determine whether mTOR participates in the induced ERK phosphorylation observed after

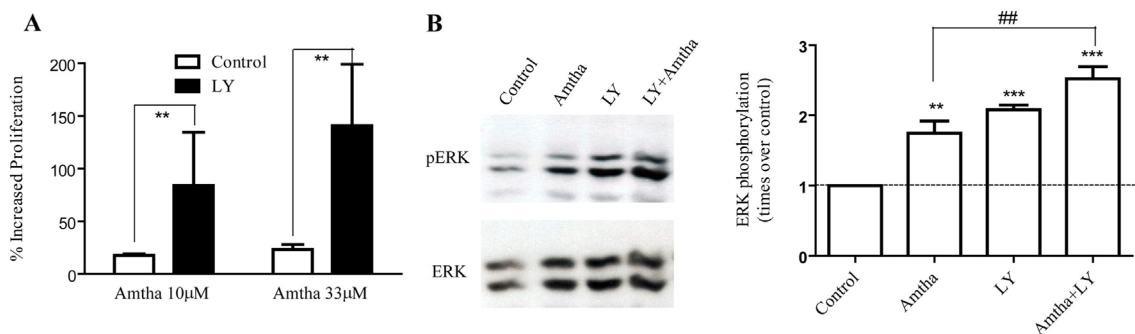

Fig. 6. Effect of PI3K/Akt pathway inhibition on amthamine stimulated MA-10 cells proliferation. A) MA-10 Leydig cells were seeded and cultured in 96-well plates for 24 h, starved and then pretreated for 50 min with 50 μM LY294002 (LY) or not (Control) and exposed for 48 h to amthamine as indicated in the figures. Twelve hours before the end of the experiment [^{3}H]thymidine was added. The incorporation of the radioactive nucleotide was expressed as percentage increased proliferation with respect to cells without amthamine treatment. Data were calculated as the mean ± SD of assay triplicates. Similar results were obtained in at least three independent experiments. **$p < 0.01$. B) MA-10 cells were subjected to 50 μM LY294002 pretreatment for 50 min and stimulated with 10 μM amthamine for 5 min. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with anti p-ERK and ERK antibodies. Densitometric analysis was performed using ImageJ software and phosphorylation levels were normalized to ERK levels. Basal correspond to control cells. Data is presented as the means ± SD ($n = 3$). ***$p < 0.001$, **$p < 0.01$, with respect to control cells. ##$p < 0.01$.

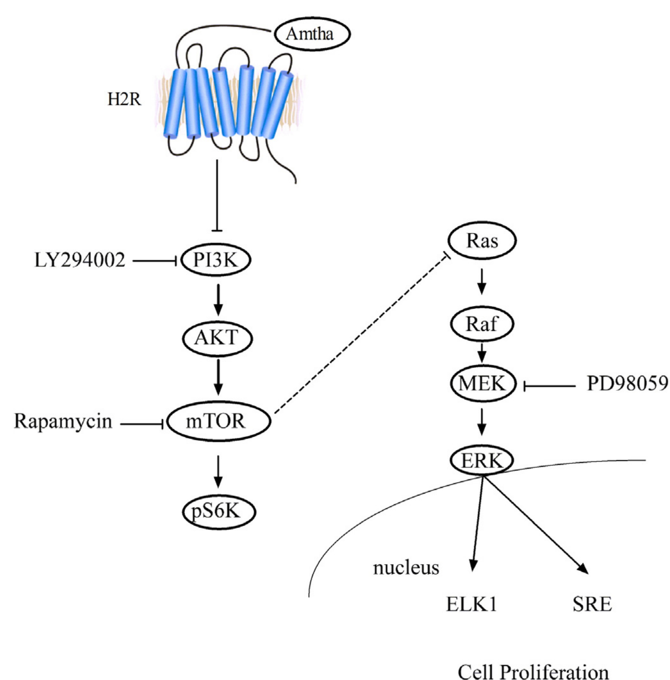

Fig. 7. Scheme of crossregulation of PI3K/Akt and ERK cascades by amthamine H2R activation. Arrows show activating and blunt-end lines inhibitory interactions. Dotted lines indicate suggested interaction.

pAkt reduction we pretreated the cells for 6 h with the mTOR inhibitor rapamycin and pERK levels were evaluated after amthamine treatment. As with LY294002, rapamycin per se also increased pERK levels in the same degree that amthamine and to an extent where amthamine was no longer effective to increase the phosphorylation levels of ERK1/2 (Fig. 4B right). Hence, the reduction in PI3K/Akt/mTOR signaling induced by the H2R agonist seems to be sufficient to achieve the increase in pERK levels in our system.

In order to confirm this last observation, the effect of amthamine was evaluated in the presence of a constitutive active mutant of Akt1 (Akt-myr) which contains a myristoylation signal instead of the regulatory sequence by PI3K (pH domain). In accordance with our results showed previously, amthamine failed to increase pERK in cells overexpressing Akt-myr (Fig. 4C). These results prove that the amthamine mediated inhibition of the PI3K/Akt/mTOR pathway is not only sufficient but necessary to achieve activation of the ERK1/2 pathway. In this context, recent work by Aksamitiene and colleagues showed that Akt and its downstream effector mTOR negatively modulate ERK signaling cascade, and this crosstalk occurs by inhibiting Ras activity [1,2]. Based on these results it can be hypothesized that mTOR inhibition induced by amthamine allows Ras proteins activation with subsequent phosphorylation of ERK. Moreover, Wang and colleagues demonstrated that rapamycin treatment increases basal ERK phosphorylation and suggested that this response is due to a compensatory mechanism of the Ras/MEK/ERK and PI3K/AKT/mTOR pathways [46].

Concernig ERK activation associated with the H2R, it is important to highlight that this and previous studies of our group [5,6], revealed functional selectivity of H2R. We have shown that both, agonist and inverse agonists with opposite efficacies for cAMP modulation, display positive efficacy regarding ERK1/2 phosphorylation but the underlying mechanisms are different. Although ERK1/2 phosphorylation by inverse agonists involves Gβγ and PI3K activation and is independent of dynamin, amthamine stimulated ERK1/2 phosphorylation is mediated by the inhibition of PI3K/Akt pathway, the activation of dynamin, and is independent of Gβγ subunits.

In an attempt to evaluate the effect of PI3K/Akt/mTOR pathway on downstream effectors of ERK1/2, we evaluated the phosphorylation of the transcription factor Elk1 and its downstream activity over the serum response element gene promoter (SRE). As expected, pretreatment with the MEK inhibitor PD98059 abolished SRE promoter activity indicating that amthamine effect is mediated by MEK/ERK signaling cascade (Fig. 5C). Consistently with our previous results, both Elk1 phosphorylation and SRE reporter activation by amthamine treatment were blocked in the presence of Akt-myr (Fig. 5A-B).

To determine the involvement of PI3K in SRE modulation, cells were pretreated with the PI3K inhibitors LY294002 and wortmannin. As shown in Fig. 5C, pretreatment with PI3K inhibitors increased basal luciferase activity, while amthamine treatment in presence of LY294002 or wortmannin led to higher increases in luciferase activity suggesting a potentiating effect of the PI3K inhibitors. Overall, these results indicate that ERK1/2 mediated modulation of SRE activity by amthamine involves the inhibition of the PI3K/Akt pathway confirming the existence of a crossregulation between these two pathways.

Finally, knowing that in native Leydig MA-10 cells, H2R mediated proliferation involves ERK activity [38], we evaluated whether the inhibition of PI3K/Akt pathway modifies amthamine stimulated MA-10 proliferation. In [^{3}H]thymidine incorporation assays, LY294002 significantly potentiate amthamine stimulated proliferation (Fig. 6A). In concordance, LY294002 significantly potentiated amthamine stimulated ERK phosphorylation (Fig. 6B). In a similar way, although amthamine alone did not induced proliferation in HEK293T cells transfected with the H2R, cotreatment with LY294002 induced cell proliferation in this system (Supplementary Fig. 4).

The activation of PKB/Akt is crucial for the regulation of numerous downstream targets involved in cell growth, proliferation and survival. However, our results, obtained in MA10 cells, show the need of the reduction of PI3K activity to fully activate ERK pathway and induce cell proliferation. These observations, although divergent are not mutually exclusive and the cell context could be decisive for the biological role of the kinase activity. Moreover, although we show that PI3K inhibition is necessary for H2R-mediated ERK activation, and consequent induction of cell proliferation, this results does not rule out that PI3K activation also result in an increment of cell division.

These results indicate that the balance between these two interconnected signaling pathways can determine cell behavior in response to H2R stimulation. The knowledge of the interrelation between these signaling pathways in the specific context of each cellular system may account for the differences in the proliferation response observed in the H2R response to histamine.

4. Conclusions

In the present work, we examined the hypothesis that a crosstalk between ERK and PI3K pathway occurs when H2R was activated by its specific agonist amthamine. Using HEK293T cells transiently transfected with human H2R as a model system, we provide here several lines of evidence summarized in Fig. 7, indicating that inhibition of PI3K/Akt/mTOR pathway is needed for MEK/ERK activation. Specifically: 1) -Treatment with amthamine induced a striking enhancement of ERK phosphorylation preceeded by a decrease in Akt and mTOR phosphorylation; 2) Enhancement of ERK phosphorylation was elicited in cells treated with LY294002 or rapamycin, PI3K and mTOR inhibitors respectively; 3) A sustained Akt activation elicited by Akt-myr transfection prevented amthamine induced ERK phosphorylation.; 4) A potentiating effect on amthamine ERK dependent SRE activity was elicited by the PI3K inhibitor, LY294002; 5) In MA-10 cells endogenously expressing H2R cells, LY294002 significantly potentiate amthamine stimulated proliferation mediated by ERK.

Disclosure statement

The authors have no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2016.06.016.

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