Molecular and Cellular Endocrinology 336 (2011) 31–40

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Adrenocorticotropin hormone (ACTH) effects on MAPK phosphorylation in human fasciculata cells and in embryonic kidney 293 cells expressing human melanocortin 2 receptor (MC2R) and MC2R accessory protein (MRAP)β

Simon Roy[1], Sandra Pinard[1], Lucie Chouinard, Nicole Gallo-Payet*

Service d'Endocrinologie, Département de Médecine, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, Sherbrooke, Québec, Canada J1H 5N4

ARTICLE INFO

Article history:
Received 16 September 2010
Received in revised form
20 December 2010
Accepted 21 December 2010

Keywords:
Adrenocorticotropin hormone (ACTH)
Melanocortin 2 receptor (MC2R)
MC2R accessory protein (MRAP)
Mitogen-activated protein kinases (MAPKs)
cAMP
Arrestin

ABSTRACT

Adrenocorticotropin hormone (ACTH) exerts trophic effects on adrenocortical cells. We studied the phosphorylation of mitogen-activated proteins kinases (MAPKs) in human embryonic kidney cells stably expressing the ACTH receptor, MC2R, and its accessory protein MRAPβ and in primary cultures of human adrenal fasciculata cells. ACTH induced a maximal increase in p44/p42^{mapk} and of p38 MAPK phosphorylation after 5 min. Neither the overexpression of wild-type arrestin2, arrestin3 or their respective dominant negative forms affected p44/p42^{mapk} phosphorylation. However, preincubation with the recycling inhibitors brefeldin A and monensin attenuated both cAMP accumulation and p44/p42^{mapk} phosphorylation proportionally. Cyclic AMP-related PKA inhibitors (H89, KI(6–22)) and Rp-cAMPS decreased p44/p42^{mapk} phosphorylation but not ACTH-mediated cAMP production. The selective Epac1/2 activator, 8-pCPT-2′-O-MecAMP, did not modify the effect of ACTH. Thus, cAMP/PKA, but not cAMP/Epac1/2 pathways, or arrestin-coupled internalization of MC2R is involved in ACTH-induced p44/p42^{mapk} phosphorylation by human MC2R. Together, ACTH binding to MC2R stimulates PKA-dependent p44/p42^{mapk} phosphorylation.

1. Introduction

Adrenocorticotropin hormone (ACTH) is the major regulator of the adrenal cortex, having acute and chronic effects on steroid synthesis and secretion (Xing et al., 2010; Gallo-Payet and Payet, 2003; Sewer and Waterman, 2003; Forti et al., 2006). Although calcium and cytoskeleton-associated proteins participate in the effect of ACTH, the initial and most significant actions are mediated through cAMP and subsequent activation of protein kinase A (PKA) (Penhoat et al., 2001; Gallo-Payet and Payet, 2003).

Over the past several years, a role for mitogen-activated protein kinases (MAPKs) has also been suggested. This family of proteins includes the extracellular-signal-regulated kinases, ERK1/2 (p44/p42^{mapk}), the p38 MAPKs and the p54 c-Jun NH_2-terminal kinases (JNKs/stress-activated protein kinases) (Houslay and Kolch, 2000). However, published results in adrenocortical cells have been somewhat conflicting. Indeed, in the Y1 adrenocortical cell line, which exhibits properties resembling that of zona fasciculata cells (Lotfi et al., 1997; Le and Schimmer, 2001) and in H295R cells, a cell line corresponding more to zona glomerulosa cells (Janes et al., 2008), ACTH has been shown to stimulate p44/p42^{mapk} phosphorylation. In another study, Watanabe et al. (1997) rather observed an increase in JNK activity, whereas p44/p42^{mapk} phosphorylation was inhibited in response to ACTH, both in the adrenal cortex *in vivo* and in the Y1 adrenocortical cell line. Finally, in bovine and rat adrenocortical cells, Chabre et al. (1995) as well as Gallo-Payet et al. (1999) have shown that ACTH does not stimulate p44/p42^{mapk} activity under conditions where Ang II is effective. These latter findings are in agreement with prior observations in which p44/p42^{mapk} immunoreactivity was distributed throughout the zona glomerulosa (and in the medulla), but was not detectable (at least in basal

Abbreviations: DAPI, 4′,6′-diamino-2-phenylindole dihydrochloride; ACTH, adrenocorticotropin hormone; Rp-cAMPS, adenosine-3-5-cyclic monophosphorothioate, Rp isomer; BFA, brefeldin A; Epac, exchange protein directly activated by cAMP; ERK, extracellular regulated receptor kinase; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; FRT, *Flp* recombinase-mediated target site; FSK, forskolin; GPCR, G protein-coupled receptors; HEK, human embryonic kidney; IBMX, isobutyl 3-methylxanthine; MRAP, MC2R accessory protein; MC2R, melanocortin 2 receptor; MEM Eagle's medium, Minimum Essential Medium; MAPKs, mitogen-activated kinases; JNKs/stress-activated protein kinases, p54 c-Jun NH_2-terminal kinases; PMA, phorbol 12-myristate 13-acetate; PKA, protein kinase A.

* Corresponding author at: Service d'Endocrinologie, Département de Médecine, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, 3001, 12e Ave Nord, Sherbrooke, Québec, Canada J1H 5N4. Tel.: +1 819 564 5243; fax: +1 819 564 5292.
E-mail address: Nicole.Gallo-Payet@USherbrooke.ca (N. Gallo-Payet).
[1] Authors contributed equally to this work.

0303-7207/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2010.12.030

conditions) in the zona fasciculata and reticularis. Under ACTH stimulation, ERK1 (p44^{mapk}), but not ERK2 (p42^{mapk}) was increased in zona glomerulosa, but not in the inner zones (McNeill et al., 2005).

Furthermore, while MAPK activation by ACTH has been found in adrenocortical cells, the mechanisms involved in this activation are not yet clearly established. Indeed, in the Y1 cell line, cAMP and PKA are not responsible for the activation of p44/p42^{mapk} as the effect was not mimicked by forskolin and not modified in PKA-deficient cells (Lotfi et al., 1997). In H295R cells, p44/p42^{mapk} stimulation by ACTH appears to depend on receptor internalization, although identification of the signaling pathways involved is far from being established (Janes et al., 2008). As for many G protein-coupled receptors (GPCR), previous studies have shown that after ACTH binding, MC2R is desensitized and internalized, both in the Y1 cell line (Baig et al., 2001, 2002) and in M3 cells expressing

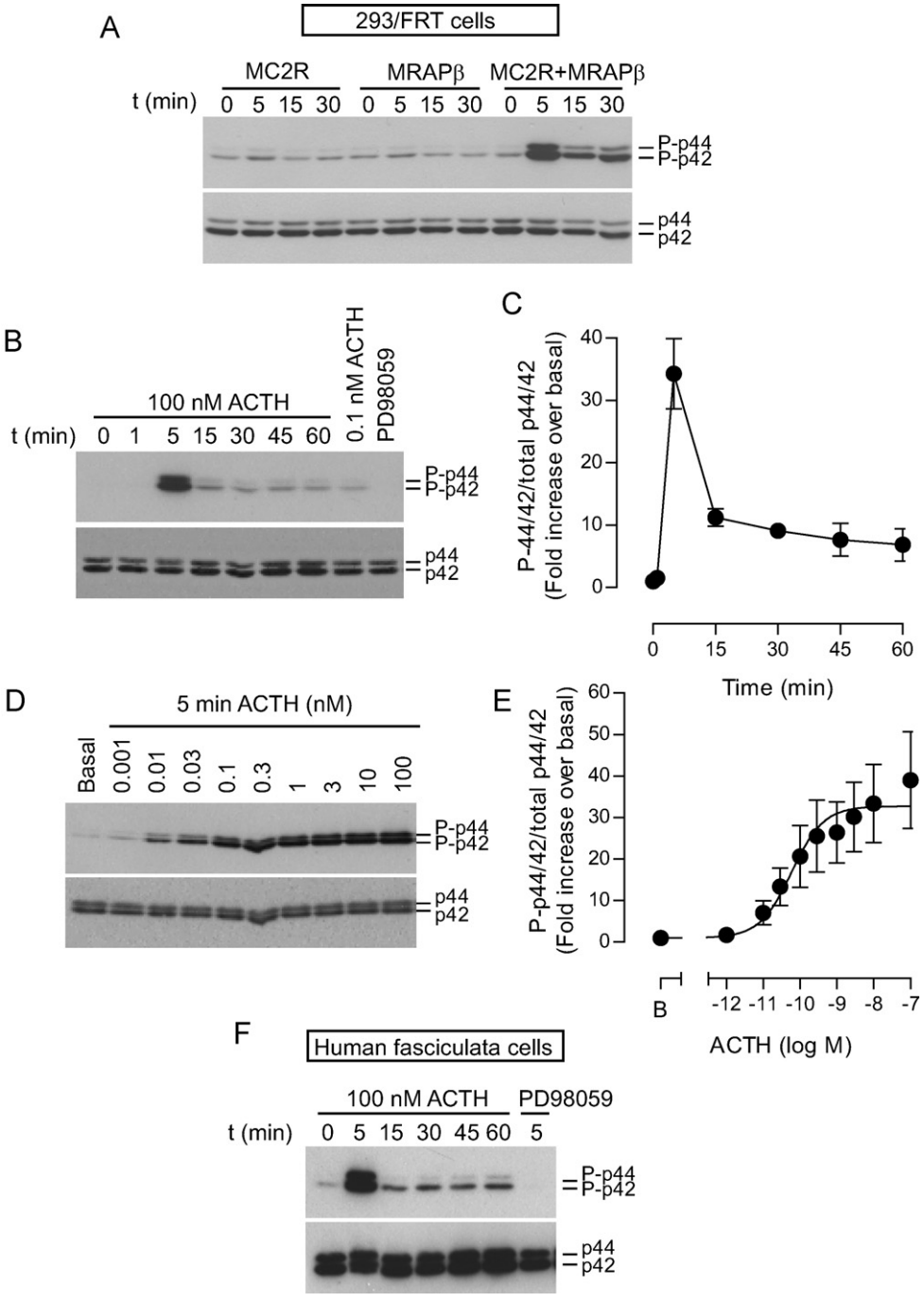

Fig. 1. Time-course and dose-dependent effect of ACTH on the phosphorylation of p44/p42^{mapk} in stable HEK cells (A–E) and in human fasciculata cells (F). (A) Native 293/FRT cells were transiently transfected with MC2R or MRAPβ alone or with both MC2R and MRAPβ. Cells were stimulated with 100 nM ACTH for 0, 5, 15 or 30 min. Cell lysates containing equal amounts of protein (30 μg) were subjected to Western blot analyses with antibodies against phosphorylated p44/p42^{mapk} (P-p44/p42) (upper panels of blots). Lower panels represent the same blots reprobed for total p44/p42 MAPK ($n = 2$). (B) Two-day cultured cells were stimulated in the absence or in the presence of 100 nM ACTH for intervals ranging from 1 to 60 min, or for 5 min in the presence of 20 μM PD98059 (a MEK inhibitor). (C) Time-course of p44/p42^{mapk} phosphorylation as analyzed by densitometry. (D) Cells were stimulated with various concentrations of ACTH ranging from 0.001 to 100 nM for 5 min. (E) Densitometric analysis of the dose–response effects on p44/p42^{mapk} phosphorylation. All data represent the mean ± S.E.M. of 3 different experiments. (F) Three-day cultured human fasciculata cells were stimulated in the absence or presence of 100 nM ACTH for intervals ranging from 1 to 60 min, or for 5 min in the presence of 20 μM PD98059 ($n = 2$).

MC2R (Kilianova et al., 2006) as well as in human embryonic kidney (HEK) cells stably transfected with the ACTH receptor, MC2R, and with its MC2R accessory proteins MRAPs (unpublished data). These studies have established that MC2R and arrestins are colocalized during ACTH-induced internalization (Kilianova et al., 2006). Desensitization and internalization are indeed known to be coupled to activation of the MAPK pathways (Ferguson, 2001; Shenoy and Lefkowitz, 2003). On the other hand, evidence also suggests that cAMP itself may serve as a stimulus (for the Rap1/B-raf cassette of signaling) or inhibitor (for the Ras/Raf-1 cassette of signaling) of p44/p42^{mapk} phosphorylation and activation (Houslay and Kolch, 2000).

Since the discovery of MC2R accessory proteins, MRAPs (Metherell et al., 2005), much progress has been achieved in the area of regulation of MC2R expression and function (Hinkle and Sebag, 2009; Webb and Clark, 2010). In recent years, our laboratory has developed and characterized a cell line system which fully reproduces dose–response curves of ACTH-induced cAMP production similar to that described in primary cultures of human and rat adrenocortical cells (Gallo-Payet and Escher, 1985; Gallo-Payet et al., 1996; Roy et al., 2007). This model relies on the stable coexpression of a single cytomegalovirus promoter-driven copy of Myc-tagged MC2R and Flag-tagged MRAP isoforms MRAPα or MRAPβ in human embryonic kidney (HEK) cells using *Flp* recombinase-mediated genome integration in the native 293/FRT host cells (Roy et al., 2007, 2010).

Thus, the aims of the present study were to compare time- and concentration-dependent effects of ACTH on MAPK phosphorylation in 293/FRT/Myc-MC2R/MRAPβ-Flag cells and in primary cultures of human fasciculata cells and to investigate whether p44/p42^{mapk} phosphorylation is dependent on cAMP production and/or on desensitization and internalization of the MC2R.

2. Materials and methods

2.1. Materials

The chemicals used in the present study were obtained from the following sources: Minimum Essential Medium (MEM Eagle's medium), OPTI-MEM and fetal bovine serum (FBS) and the *Flp* recombinase-mediated homologous recombination system (Flp-In™) were from Invitrogen (Burlington, ON, Canada); high glucose DMEM was from Wisent (St-Jean-Baptiste, QC, Canada). ACTH-(1–24) peptide (Cortrosyn) was purchased from Organon (Toronto, Canada). Ang II was from Bachem (Marina Delphen, CA); phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) 13.14.4E XP™ rabbit mAb, total p44/42 MAPK (Erk1/2) (137F5) rabbit mAb, phospho-p38 MAPK (Thr180/Tyr182) antibody, p38 MAPK antibody, phospho-SAPK/JNK (Thr183/Tyr185) (81E11) rabbit mAb, phospho-SAPK/JNK (Thr183/Tyr185) antibody, total SAPK/JNK antibody, horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were from New England Biolabs, Inc. (Mississauga, Ont, Canada). Alexa-Fluor coupled secondary antibodies were from Invitrogen. The enhanced chemiluminescence (ECL) detection system was from GE Healthcare (Baie d'Urfe, Quebec, Canada); PhoSTOP phosphatase inhibitor cocktail was from Roche (Laval, QC, Canada); 8-pCPT-2′-O-Me-cAMP, Rp-cAMPS and Sp-cAMPS were from Biolog Life Science Institute (Bremen, Germany); PKI(6–22) was purchased from EMD Chemicals (Gibbstown, NJ). Forskolin, ACTH (amino acids 1–39), isobutyl 3-methylxanthine (IBMX), phorbol 12-myristate 13-acetate

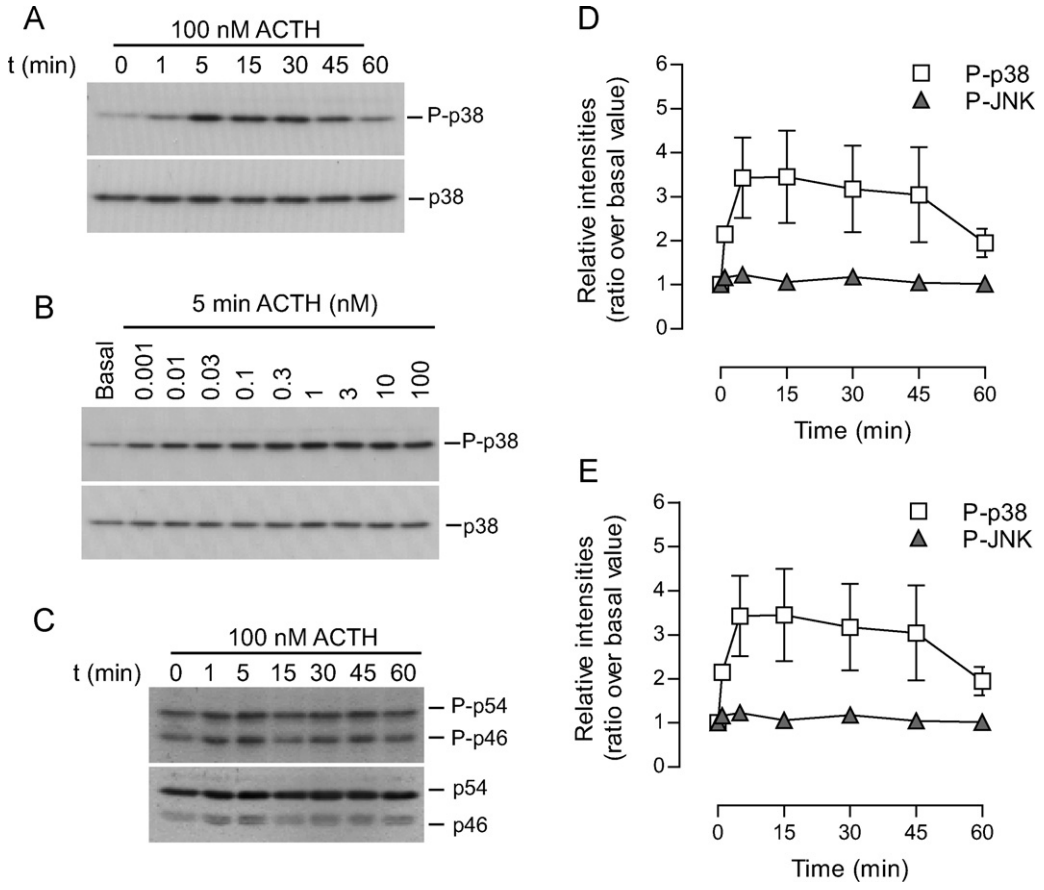

Fig. 2. Time-course and dose-dependent effect of ACTH on the phosphorylation of p38 MAPK in stable 293/FRT/Myc-MC2R/MRAPβ-Flag cells. (A) Three-day cultured cells were stimulated in the absence or in the presence of 100 nM ACTH for intervals ranging from 1 to 60 min. Cell lysates containing equal amounts of protein (30 μg) were subjected to Western blot analyses with antibodies against phosphorylated p38 MAPK (upper panels of blots). Lower panels represent the same blots reprobed for total p38 MAPK. (B) Cells were stimulated with various concentrations of ACTH ranging from 0.001 to 100 nM for 5 min. (C) Cells were stimulated in the absence or in the presence of 100 nM ACTH for intervals ranging from 1 to 60 min. Cell lysates containing equal amounts of protein (30 μg) were subjected to Western blot analyses with antibodies against phosphorylated p54/p46 JNK (upper panels of blots). Lower panels represent the same blots reprobed for total p54/p46 JNK. (D) Time-course of p38 MAPK and JNK phosphorylation as analyzed by densitometry. (E) Densitometric analysis of the dose–response effects on p38 MAPK and JNK phosphorylation. All data represent the mean ± S.E.M. of 3 different experiments.

(PMA), PD98059, SB203580, SP600125, monensin and brefeldin A (BFA) were from Sigma–Aldrich (Oakville, ON, Canada). All other chemicals were of A-grade purity.

2.2. Cell cultures

The *Flp* recombinase-mediated homologous recombination system (Flp-In™) was used to generate human embryonic kidney 293/FRT (*Flp* recombinase target site) cell lines stably expressing both Myc-MC2R and MRAPβ-Flag. Cell culture and characterization of these cell lines have been described previously (Roy et al., 2007). Only when required, cells were transfected with 0.5 μg/35 mm dish at 50% confluency with plasmid DNA coding for arrestins with Lipofectamine and PLUS reagent. To maintain similar transcriptional and translational activity when required, transfection of pEGFP was used as a control instead of an empty vector.

Human adult adrenal glands were obtained from renal transplant donors, through collaboration with the Quebec-Transplant Association. This project was approved by our institution's Human Subject Review Committee. Isolation and dissociation of cells from zona fasciculata were performed in MEM, as described previously (Côté et al., 2001). After a 20 min incubation at 37 °C with collagenase (2 mg/ml) and deoxyribonuclease (25 μg/ml), cells were disrupted by gentle aspiration, centrifuged (10 min at 100 × *g*) and cell pellets were resuspended in OPTI-MEM supplemented with 2% fetal bovine serum, 100 U/ml penicillin and 100 g/ml streptomycin. Cells were cultured in 35 mm Petri dishes at a concentration of 3–5 × 10^5 cells/dish and cultured at 37 °C in a humidified atmosphere (95% air with 5% CO_2). The culture medium was changed 24 h after initial addition of the medium, and cells were used after 2 days of culture for short term stimulation without or with ACTH, in the absence or presence of various compounds introduced 30 min prior to ACTH stimulation.

2.3. Measurement of p44/p42^{mapk} activity

Cells were starved in DMEM without FBS 30 min before ACTH stimulation. At the end of incubation, cells were washed once with ice-cold PBS and lysed in 1× lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Na pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1.5× PhoSTOP phosphatase inhibitor). The lysates were centrifuged 10 min at 12,000 × *g* at 4 °C, after which supernatants were aliquoted and stored at −80 °C. Five to 20 μg of samples were loaded into 10% bis-acrylamide gels in reducing SDS-PAGE conditions. Proteins were transferred onto nitrocellulose membranes, and probed with antibodies against the phosphorylated forms of p44/p42-, p38- or JNK-MAPK in 5% BSA TBS–Tween 0.05% and detected with HRP-linked secondary antibodies and ECL. Membranes were then stripped in 0.4 N NaOH in TBS–Tween and reblotted against the un-phosphorylated forms of the proteins of interest.

2.4. Fluorescent detection of p44/p42^{mapk}

Cells were cultured on poly-L-lysine glass coverslips placed in 35 mm dishes and were starved for 30 min with fresh DMEM one day later. The cells were then stimulated without or with 100 nM ACTH for 5 min or with 1 μM PMA for 15 min. After washing with PBS and fixation in MeOH, labeling for p44/p42^{mapk} phosphorylation was performed overnight using phospho-p44/p42^{mapk} (Thr202/Tyr204) (D13.14.4E) XP™ rabbit mAb (1:200) diluted in PBS containing 5% BSA. Indirect detection was performed using an anti-rabbit antibody coupled to Alexa-Fluo488. Subsequently DAPI was used to stain the nuclei. Images were recorded on an Olympus Fluoview 1000 (FV1000) laser-scanning confocal microscope (Olympus, Japan) built around an IX81-ZDC inverted microscope fitted with a U Plan S-Apo 60× (1.35 NA) oil immersion objective (Olympus URFL-T, MAG Biosystems). Emissions from each fluorophore were acquired sequentially to avoid fluorophore bleeding. Images were acquired with the exact same laser power and HV settings and fluorescence from PMA-treated cells which was considered as the maximal signal without saturation. All images were magnified 2–3 times in Photoshop CS3 (Adobe Systems Inc.). Of note, the very bright spots observed in human fasciculata cells are non-specific and represent autofluorescence.

2.5. Measurement of cAMP accumulation

For cAMP measurements, cells were loaded with tritiated adenine. Briefly, cells were washed once in Hank's buffered saline (130 mM NaCl, 3.5 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.5 mM $NaHCO_2$, 5 mM HEPES, pH 7.4, containing 1 g/l dextrose and 0.1% BSA) and subsequently stimulated with ACTH, in the presence of the phosphodiesterase inhibitor IBMX (iso-butyl-3-methyl-xanthine), at 37 °C. At the end of the stimulations, cells were lysed in 5% trichloroacetic acid (TCA), and intracellular cAMP accumulation was determined by measuring the conversion of [^{3}H]-ATP into [^{3}H]-cAMP eluted from Dowex and neutral alumina chromatography columns as described previously (Roy et al., 2007).

2.6. Data analysis

Results are presented as mean ± S.E.M. of the number of experiments indicated in parentheses, with each sample conducted in duplicate or triplicate. GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) was used for establishing EC_{50}, 95% confidence interval, maximal effects and other related parameters. *p*-Values on data

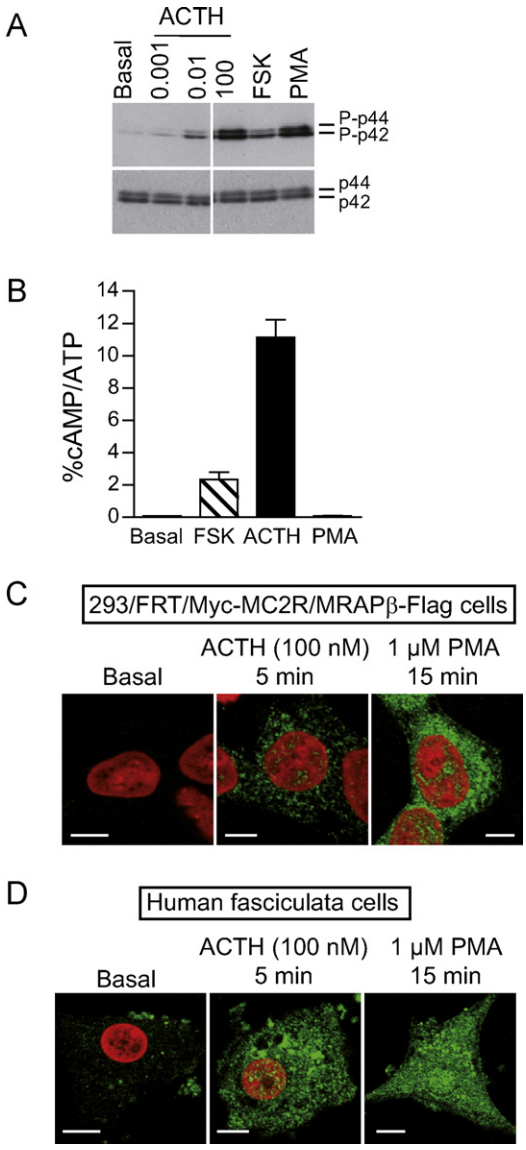

Fig. 3. Comparison of ACTH- and phorbol 12-myristate 13-acetate (PMA)-mediated effects on p44/p42^{mapk} phosphorylation. (A) Two-day cultured cells were stimulated in the absence or in the presence of ACTH (0.001, 0.01 and 100 nM), 10 μM forskolin (FSK) or 1 μM phorbol 12-myristate 13-acetate (PMA). Cell lysates containing equal amounts of protein (30 μg) were subjected to Western blot analyses with antibodies against phosphorylated p44/p42^{mapk} (P-p44/p42) (upper panels of blots). Lower panels represent the same blots reprobed for total p44/p42^{mapk}. (B) Two-day cultured cells were stimulated in the absence or in the presence of 100 nM ACTH, 10 μM forskolin (FSK) or 1 μM phorbol 12-myristate 13-acetate (PMA). Cyclic AMP accumulation was measured as described in Section 2 (B). (C and D) P44/p42^{mapk} phosphorylation evidenced by immunofluorescence microscopy in stable 293/FRT/Myc-MC2R/MRAPβ-Flag cells (C) and in human fasciculata cells (D). Cells were cultured on poly-L-lysine glass coverslips for 24 h, then incubated in the absence or in the presence of 100 nM ACTH for 5 or 15 min with 1 μM phorbol 12-myristate 13-acetate (PMA). After washing and fixation in MeOH, labeling of phosphorylated (P-p44/p42) was performed overnight using phospho-p44/p42^{mapk}, followed by DAPI staining to label the nuclei as detailed in Section 2. The indirect detection of phosphorylated p44/p42^{mapk} was performed using an anti-rabbit antibody coupled to Alexa-Fluo488. Images were recorded on an Olympus Fluoview 1000 (FV1000) laser-scanning confocal microscope. Data represent two experiments, with at least 6 cells examined in each condition. Scale bar, 10 μM.

fits were obtained with the extra sum-of-squares *F* test performed on curve fits. Statistical analysis was performed by ANOVA followed by Bonferonni's post hoc test.

3. Results

3.1. Effect of ACTH on p44/p42^{mapk} and on p38 MAPK phosphorylation in HEK cells and in human fasciculata cells

To assess whether ACTH-induced p42/p44^{mapk} phosphorylation was specific to the presence of a functional ACTH receptor in 293/FRT cells, native 293/FRT cells were transiently transfected with MC2R or MRAPβ alone or with both MC2R and MRAPβ. As shown in Fig. 1A, 100 nM ACTH induced a time-dependent increase in p44/p42^{mapk} phosphorylation only when both MC2R and MRAPβ were cotransfected in the same cells. Similarly, in cells stably expressing MC2R and MRAPβ the maximal effect was observed after 5 min, followed by a rapid decrease and a sustained lower plateau (Fig. 1B and C). The effect of ACTH was abolished by the MEK inhibitor PD98059 (20 μM) (Fig. 1B, right). The effect was concentration-dependent, with an EC_{50} value observed at 5.9 [1.6, 211] pM (Fig. 1D and E). Similar results were obtained in human fasciculata cells, where ACTH also induced a huge increase in p44/p42^{mapk} phosphorylation after a 5-min incubation (Fig. 1F).

Using identical experimental conditions, ACTH also induced a time-dependent and sustained increase in p38 MAPK. The maximal effect was observed after 5 min and was sustained for 45 min (Fig. 2A and D). A plateau reached at a concentration of 0.1 nM, with a smaller increase compared to p44/p42^{mapk} phosphorylation (Fig. 2B and E). In these cells, ACTH did not stimulate JNK (Fig. 2C–E).

3.2. Mechanisms involved in ACTH-induced p44/p42^{mapk} phosphorylation

Since the most significant effect of ACTH on MAPK activity was observed on p44/p42^{mapk}, the following experiments were aimed at understanding the underlying mechanisms involved in

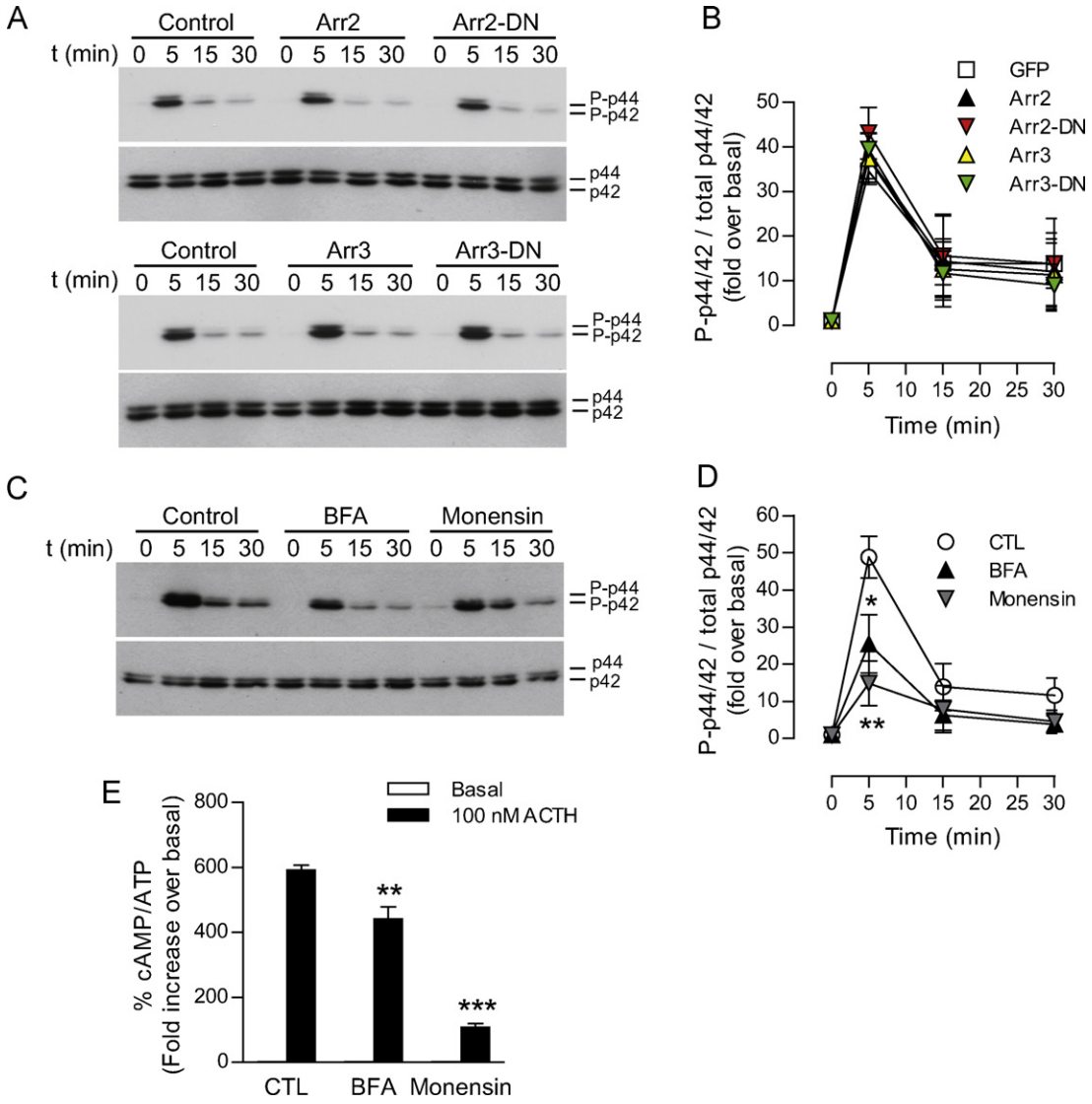

Fig. 4. Influence of internalization and recycling inhibitors on p44/p42^{mapk} phosphorylation. (A) In 293/FRT/Myc-MC2R/MRAPβ-Flag cells overexpressing either wild-type (Arr2, Arr3) or dominant-negative arrestin2 or arrestin3 (Arr2-DN, Arr3-DN), p44/p42^{mapk} phosphorylation and total p44/p42^{mapk} were measured by Western blotting following 100 nM ACTH stimulation for the indicated time periods. (B) Densitometric analysis of the results illustrated in (A). Data represent the mean ± S.E.M. of 3 different experiments. (C) Cells were stimulated with 100 nM ACTH in the absence or in the presence of 10 μg/ml brefeldin A (BFA) (a Golgi-disrupting agent) or 25 μM monensin (an endosome recycling inhibitor); p44/p42^{mapk} phosphorylation and total p44/p42^{mapk} were measured as aforementioned. (D) Densitometric analysis of the results illustrated in (C). Data represent the mean ± S.E.M. of 3 different experiments. (E) Cyclic AMP accumulation induced by 100 nM ACTH alone or in the presence of BFA or monensin. Results represent the mean ± S.E.M. of 3 different experiments. $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$, statistical significance compared with control cells.

this action. Experiments were conducted at the peak of activation (5 min) and at the plateau (15 min) phase, using various tools. A transient p44/p42^{mapk} phosphorylation is generally linked to phosphoinositide breakdown and subsequent diacylglycerol/protein kinase C signaling, which in turn activates the Ras/Raf-1 cascade, whereas a sustained activation is rather linked to cAMP/PKA or Epac/PKA and to the Rap1/B-Raf signaling cassette (Stork and Schmitt, 2002). As shown in Fig. 3A, incubation with 1 μM PMA (phorbol 12-myristate 13-acetate), a general activator of protein kinase C mimicking diacylglycerol and used as control for measuring the ability of the cells to stimulate MEK, increased p44/p42^{mapk} phosphorylation. In addition, a 5-min incubation with 10 μM forskolin, a direct activator of adenylyl cyclases, induced a smaller increase in p44/p42^{mapk} phosphorylation, as well as a smaller increase in cAMP accumulation, as compared to 100 nM ACTH; PMA on the other hand had no effect on cAMP accumulation (Fig. 3B).

The subcellular distribution of activated p44/p42^{mapk} is important in defining its function, whereby proliferation is generally associated with localization in the nucleus and tissue functionality with localization in the cytoplasm (Schaeffer and Weber, 1999; Pouyssegur et al., 2002; Stork and Schmitt, 2002; May and Hill, 2008). The subcellular distribution of phosphorylated p44/p42^{mapk} was therefore monitored by confocal microscopy. As shown in Fig. 3C and D, after a 5 min incubation, ACTH induced an increase in phosphorylated p44/p42^{mapk} fluorescence in the cytoplasm in both 293/FRT/Myc-MC2R/MRAPβ-Flag cells and human fasciculata cells, which was also accompanied by a lower increase in the nucleus. This increase was transient and no longer observed at 15 min of ACTH stimulation. PMA, in similar experimental conditions, induced a massive increase both in the cytoplasm and in the nucleus (Fig. 3C and D).

In a previous study, we had shown that MC2R and arrestins colocalized and interacted during ACTH-induced internalization (Kilianova et al., 2006, unpublished data). Since arrestins (arrestin2, also called β-arrestin1 and arrestin3, also called β-arrestin2) are often found to be mediators of GPCR signaling to the MAPK pathways (Lefkowitz and Shenoy, 2005), we next examined whether arrestin overexpression could modulate ACTH-induced p44/p42^{mapk} phosphorylation. Neither the overexpression of wild-type β-arrestins, arrestin2, arrestin3 or their respective dominant negative forms, arrestin2-DN and arrestin3-DN, affected the profile of p44/p42^{mapk} phosphorylation (Fig. 4A and B). On the other hand, preincubation with the recycling inhibitors brefeldin A (BFA) and monensin reduced ACTH-induced p44/p42^{mapk} phosphorylation (Fig. 4C and D). These results indicate that arrestin-coupled internalization is not involved in p44/p42^{mapk} phosphorylation by ACTH. To verify whether there was a correlation between cAMP accumulation and p44/p42^{mapk} phosphorylation, cAMP production was measured in cells preincubated with brefeldin A (BFA) and monensin. As shown in Fig. 4E, both inhibitors significantly reduced cAMP levels, as observed with p44/p42^{mapk} phosphorylation, thus corroborating the correlation between these two events (cAMP accumulation and p44/p42^{mapk} activation).

To verify whether p44/p42^{mapk} phosphorylation may be dependent on the cAMP pathway, both 293/FRT/MC2R/MRAPβ and human fasciculata cells were stimulated with 100 nM ACTH alone or in the presence of 10 μM H89, added 30 min prior to ACTH stimulation. At this concentration, H89 is generally considered as a specific inhibitor of PKA (Tremblay et al., 1991; Baig et al., 2001; Kilianova et al., 2006; Liu et al., 2008; Aumo et al., 2010). In the presence of H89, p44/p42^{mapk} phosphorylation was significantly reduced in both cell types, while, under the same conditions, GF109203X, an inhibitor of PKC, had no effect on ACTH response

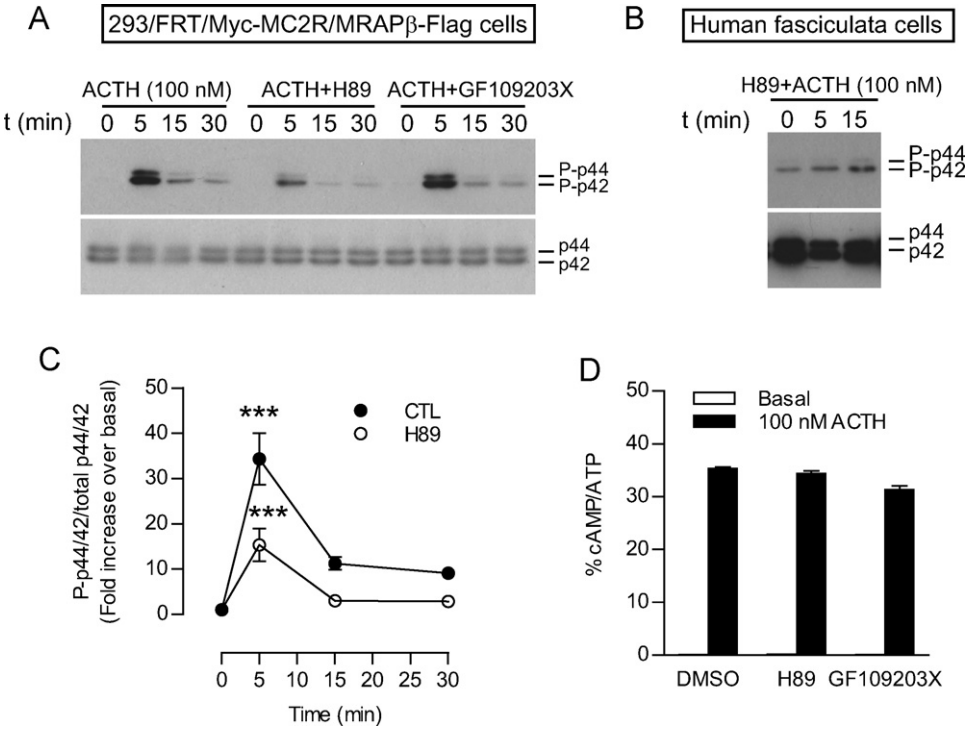

Fig. 5. Influence of H89 on p44/p42^{mapk} phosphorylation and on cAMP accumulation. (A) Two-day cultured 293/FRT/Myc-MC2R/MRAPβ-Flag cells (A) were stimulated for the indicated time periods in the absence or in the presence of 100 nM ACTH and in the absence or in the presence of 10 μM H89 (inhibitor of PKA) or 10 μM GF109203X (a general inhibitor of protein kinase C). (B) Human adrenal fasciculata cells in primary culture were stimulated for the indicated time periods in the presence of 100 nM ACTH and 10 μM H89 (inhibitor of PKA); cells stimulated with ACTH alone are shown in Fig. 1F; p44/p42^{mapk} phosphorylation and total p44/p42^{mapk} were measured as aforementioned. (C) Densitometry analysis of H89 effects illustrated in (A). Data represent the mean ± S.E.M. of 3 different experiments. ***$p < 0.001$, statistical significance compared with control cells. (D) Cyclic AMP accumulation induced by 100 nM ACTH alone or in the presence of 10 μM H89 or 10 μM GF109203X. Results are representative of 3 independent experiments.

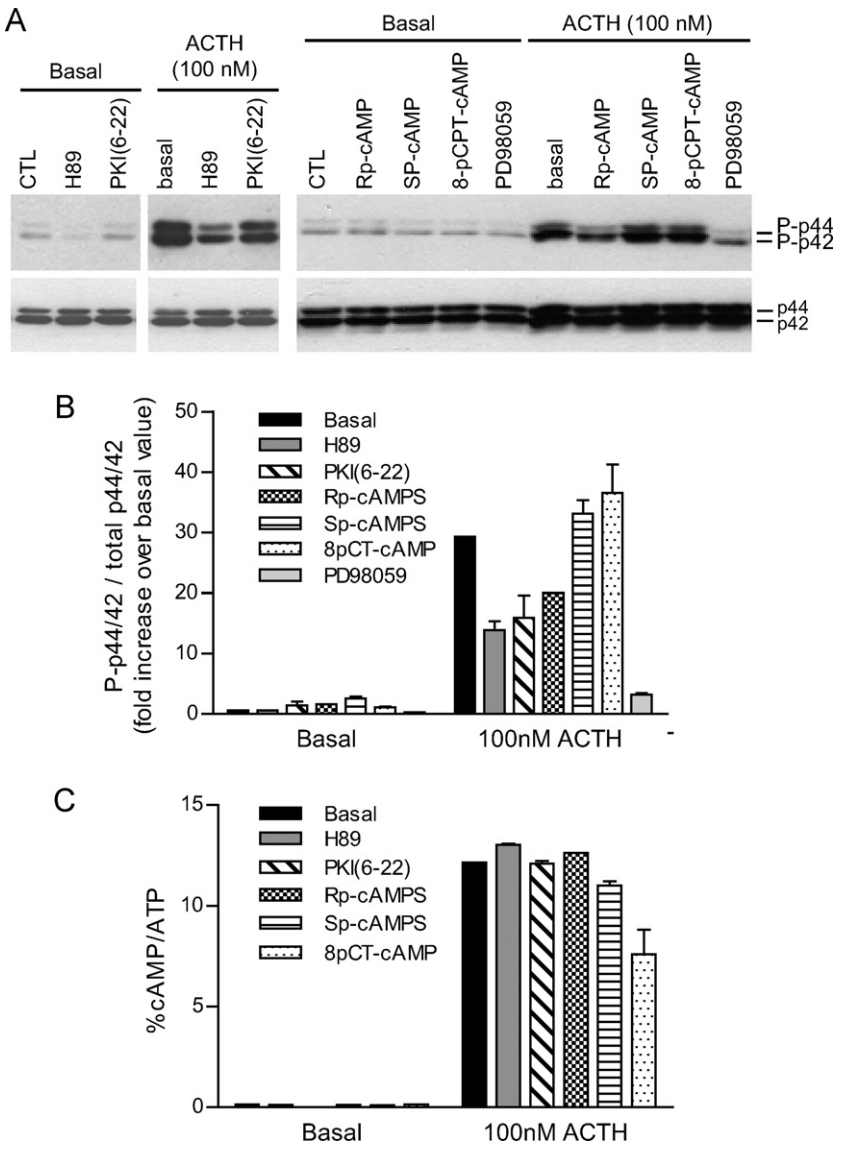

Fig. 6. Influence of various cAMP-dependent inhibitors and activators on p44/p42^{mapk} phosphorylation. (A) In two-day cultured 293/FRT/Myc-MC2R/MRAPβ-Flag cells, p44/p42^{mapk} phosphorylation and total p44/p42 were measured by Western blotting following 100 nM ACTH stimulation conducted for 5 min in the absence or in the presence of H89 (10 μM) (inhibitor of PKA), PKI(6–22) (a cAMP-dependent PKA inhibitor) (4 μM), Rp-cAMPS (adenosine-3-5-cyclic monophosphorothioate, Rp isomer) (50 μM) (which competitively inhibits cAMP activation of PKA), Sp-cAMPS (50 μM) (the inactive form of Rp-cAMPS), 8-pCPT-2′-O-Me-cAMP (100 μM) (8pCT-cAMP) (a selective Epac1/2 activator) and PD98059 (20 μM) (a MEK inhibitor). (B) Densitometry analysis of independent experiments, one being illustrated in panel (A). (C) Cyclic AMP accumulation induced by 100 nM ACTH alone or in the presence of the drugs indicated above. Results are representative of 2 or 3 independent experiments, each conducted in triplicate.

(Fig. 5A–C). These results thereby indicate the participation of cAMP-dependent PKA in ACTH response. In similar experimental conditions, these drugs did not modify cAMP accumulation by ACTH (Fig. 5D).

Other more specific inhibitors of cAMP-dependent protein kinase A were also used to gain a better understanding of the involvement of this particular pathway, namely PKI(6–22) (4 μM), Rp-cAMPS (adenosine-3-5-cyclic monophosphorothioate, Rp isomer) (50 μM) (which competitively inhibits cAMP activation of PKA), as well as its inactive form, Sp-cAMPS (50 μM) and pCPT-2′-O-Me-cAMP (a selective Epac1/2 activator) (Liu et al., 2008). As shown in Fig. 6A, alone, the various compounds had either no effect or only a small effect on p44/p42^{mapk} phosphorylation. However, after a 30 min preincubation, before addition of ACTH, the cAMP-dependent PKA inhibitor PKI(6–22) and Rp-cAMPS decreased the ACTH response by 50%, while, in similar conditions, PD98059 abrogated the ACTH effect. Furthermore, Sp-cAMPS (50 μM) or CPT-2′-O-Me-cAMP, did not stimulate p44/p42^{mapk} phosphorylation and did not affect the ACTH response. On the other hand, none of these agents affected basal or ACTH-induced cAMP stimulation (Fig. 6E). Together, these results indicate ACTH binding to MC2R. MC2R stimulates PKA-dependent p44/p42^{mapk} phosphorylation, although cAMP itself had no effect.

4. Discussion

As previously described in Y1 adrenocortical cells (Lotfi et al., 1997; Le and Schimmer, 2001) in H295R cells (Janes et al., 2008) and more recently in MC2R-transfected Chinese hamster ovary cells (Sebag and Hinkle, 2010), our results show that ACTH induces a rapid increase in p44/p42^{mapk} phosphorylation while also promoting a lower, but sustained and concentration-dependent p38 MAPK phosphorylation. The JNK pathway, on the other hand,

was not stimulated under these same conditions. These findings indicate that p44/p42^{mapk} activation by MC2R is promoted by a mechanism that is independent from interaction with arrestins, but rather dependent, at least partly, on ACTH-induced cAMP production.

Because native 293/FRT cells transfected with either MC2R or MRAPβ alone did not show significant levels of p42/p44^{mapk} phosphorylation after ACTH stimulation, our results demonstrate that ACTH-induced p42/p44^{mapk} phosphorylation requires a functional ACTH receptor expressed at the plasma membrane (i.e. MC2R + MRAPβ) in order to bind ACTH with high affinity (Roy et al., 2007). A number of arguments suggest that activation of p44/p42^{mapk} by ACTH is partly mediated by cAMP-dependent processes, rather than from MC2R internalization. Firstly, neither the overexpression of the wild-type β-arrestin, arrestin2, arrestin3 or their respective dominant negative forms, arrestin2-DN and arrestin3-DN, affected the profile of p44/p42^{mapk} phosphorylation. This result strongly suggests that p44/p42^{mapk} phosphorylation is not arrestin-mediated. Secondly, drugs used to inhibit receptor recycling (brefeldin A and monensin) conversely reduced p44/p42^{mapk} phosphorylation, with a concomitant decrease in cAMP production. Similar results have also been reported for H295R cells (Janes et al., 2008). In addition, the present results show that forskolin induced only a small increase in p44/p42^{mapk} while the cAMP-dependent PKA inhibitors (H89, PKI(6–22)) failed to completely abolish p44/p42^{mapk} phosphorylation. These results suggest that cAMP participates, but does not reproduce p44/p42^{mapk} activation by ACTH. Furthermore, the selective Epac1/2 activator, 8-pCPT-2′-O-Me-cAMP, did not modify the effect of ACTH, thereby indicating that Epac1/2 did not contribute in the MEK1 signaling cassette. Altogether, stimulation of p44/p42^{mapk} phosphorylation by ACTH thus appears to be linked mainly to stimulation of the cAMP/PKA pathway.

The massive accumulation of phosphorylated p44/p42^{mapk} observed herein in the cytoplasm supports the view that localization of p44/p42^{mapk} in the cytoplasm may be associated with cellular differentiation as well as various cell functions, such as proliferation, migration, hypertrophy (Houslay and Kolch, 2000; Pouyssegur et al., 2002) or steroid biosynthesis (Poderoso et al., 2008). Indeed, we have previously shown that both p44/p42^{mapk} and p38 MAPK are required for increasing protein synthesis and cell hypertrophy (Otis et al., 2005). Cyclic AMP-mediated steroid biosynthesis in MA10 Leydig cells requires the phosphorylation of the steroidogenic acute regulatory protein (StAR) by p42/p44 and PKA (Poderoso et al., 2008). The smaller increase of phosphorylated p44/p42^{mapk} in the nucleus may be associated with increased expression of transcription factors necessary for supporting functionality, rather than to initiate proliferation (Marshall, 1995; Pouyssegur et al., 2002), a notion further supported by the observation that the transient increase in the nucleus is generally associated with activation, not inhibition, of proliferation (Marshall, 1995; Pouyssegur et al., 2002; May and Hill, 2008). Finally, our results demonstrate that signaling following ACTH binding to its receptor is more efficient in increasing p44/p42^{mapk} phosphorylation than forskolin or cAMP analogs. Such observations are not surprising, since it is well known that ACTH action may result from a variety of interactions, in particular with extracellular matrix, integrins and cytoskeleton (Côté et al., 1997; Otis et al., 2007; Sewer and Li, 2008), all of which can initiate various routes of p44/p42^{mapk} activation. Alternatively, ACTH-mediated cAMP production may occur in subplasmalemmal microdomains (Szaszak et al., 2008) where cAMP, PKA and p44/p42^{mapk} activities may be compartmentalized instead of rising globally by the broad-range action of forskolin and cAMP analogs. Thus, regardless of the mechanisms at play, and despite the fact that ACTH is well known for inhibiting adrenocortical cell proliferation and DNA

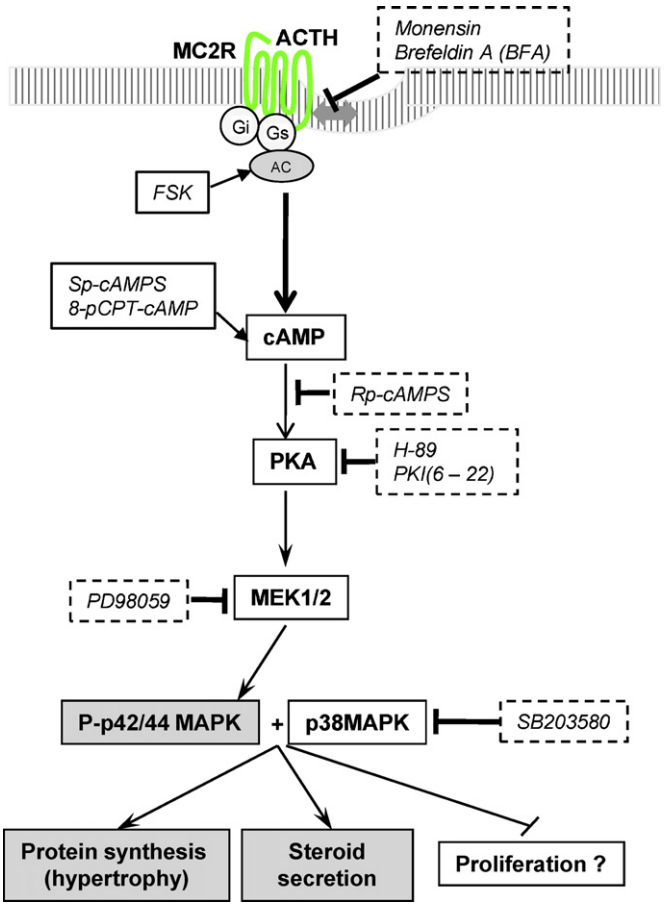

Fig. 7. Schematic representation to the potential pathways involved in p44/p42^{mapk} activation by the ACTH receptor, MC2R. Results obtained herein, using various tools, indicate that in 293/FRT/Myc-MC2R/MRAPβ-Flag cells, ACTH stimulated p44/p42^{mapk} and p38 MAPK, but not JNK phosphorylation. The mechanisms implicated in p44/p42^{mapk} activation involved downstream effectors of cAMP, PKA. Furthermore, drugs which inhibit MC2R recycling (endosome and Golgi transport) decreased both cAMP and ERK activation, suggesting correlation between the two events. On the other hand, β-arrestin-coupled internalization was not involved in p44/p42^{mapk} phosphorylation. Finally, p44/p42^{mapk} activation by ACTH was more intense than that obtained with various drugs stimulating cAMP directly, thus indicating that interaction of ACTH with its MC2 receptor mobilizes scaffold proteins which could participate in the activation of PKA and Epac. Tools used to investigate the various pathways were as follows: (1) for the cAMP-PKA-dependent pathways: forskolin, a direct activator of adenylyl cyclases; H89, an inhibitor of protein kinase A (PKA), which competes with ATP for its binding site on PKA; PKI(6–22), a highly specific inhibitor of cAMP-dependent PKA; Rp-cAMPS (adenosine-3-5-cyclic monophosphorothioate, Rp isomer) which competitively inhibits cAMP activation of PKA, but not Epac; Sp-cAMPS, a potent and specific activator of cAMP-dependent protein kinases. (2) For the endocytosis pathway: monensin and brefeldin A (BFA) are recycling inhibitors; (3) PD98059, an inhibitor of MEK1/2; SP600125, a JNK inhibitor; and SB203580, an inhibitor of the p38 pathway.

synthesis *in vitro* (Ramachandran and Suyama, 1975; Duperray and Chambaz, 1980; Hornsby, 1985), it is also well established that hypophysectomy decreases the volume of zona fasciculata (Cater and Stack-Dunne, 1953), whereas ACTH treatment increases the overall volume of the cortex (Mazzocchi et al., 1986). These animal studies indicating a trophic role of ACTH have been further corroborated in humans by the observation that patients with homozygous defects of the ACTH receptor (called melanocortin 2 receptor, MC2R) exhibit a very atrophic zona fasciculata and reticularis (for review see Clark and Weber, 1998).

In conclusion, as summarized in Fig. 7, results of the present study indicate that in 293/FRT/Myc-MC2R/MRAPβ-Flag cells as well as in human fasciculata cells, ACTH stimulates p44/p42^{mapk} phosphorylation and that PKA is mainly involved in this activa-

tion. Furthermore, drugs which inhibit the endosome and Golgi trafficking decrease both cAMP and ERK activation, suggesting a correlation between these two events. Such findings further support not only a key role for cAMP in mediating ACTH responses, but also other initial interacting proteins, which remain to be identified. Several proteins could be proposed to participate in the activation of PKA by MC2R, such as cytoskeleton- or integrins-associated scaffold proteins (Sewer and Waterman, 2003).

Finally, the cell model used herein, which fully reproduces human fasciculata cells in terms of cAMP production and ERK activation by ACTH, thus appears appropriate for gaining insight in our understanding of the precise inner workings of ACTH after binding to MC2R. Since proliferation, migration, hypertrophy and differentiation are all controlled by both cAMP and p44/p42^{mapk}, and are important for adrenal organization and function, understanding the mechanisms of ACTH action following binding to its MC2 receptor, as well as through its main second messenger cAMP, represent an important challenge which certainly warrants further clarification. Nevertheless, from all the studies conducted to date, it appears that the potency and duration of cAMP accumulation are the key factors in explaining the differences in p44/p42^{mapk} responses to ACTH. Thus, all of the mechanisms likely modulating cAMP levels also likely modulate p44/p42^{mapk} activation and function. This is perhaps why the absence of mutations in MC2R (review in Clark and Weber, 1998) or the absence of the key markers of zona glomerulosa (Lee et al., 2005) or zona fasciculata (Mullins et al., 2009) affect not only steroidogenesis, but also the structural organization of the gland.

Acknowledgments

We thank all the members of the laboratory for the various aspects related to the present work. We thank Drs. Robert Dumaine and Philippe Sarret for use of their confocal microscope and Jean Lainé for help with confocal studies. We thank Dr. Roger D. Cone (Vollum Institute, Oregon Health and Science University, Portland, Oregon) for providing us with the human MC2R cDNA. We are also grateful to Pierre Pothier for critical review of the manuscript. This work was supported by grants from the Canadian Institutes for Health Research to N.G.-P. (MOP-10998) and by the Canada Research Chairs Program. N.G.-P. is a recipient of a Canada Research Chair in Endocrinology of the Adrenal Gland and is a member of the FRSQ-funded Centre de Recherche Clinique Étienne-le Bel. S.R. of a studentship from the Fonds de la Recherche en Santé du Québec.

References

Aumo, L., Rusten, M., Mellgren, G., Bakke, M., Lewis, A.E., 2010. Functional roles of protein kinase A (PKA) and exchange protein directly activated by 3′,5′-cyclic adenosine 5′-monophosphate (cAMP) 2 (EPAC2) in cAMP-mediated actions in adrenocortical cells. Endocrinology 151, 2151–2161.

Baig, A.H., Swords, F.M., Noon, L.A., King, P.J., Hunyady, L., Clark, A.J., 2001. Desensitization of the Y1 cell adrenocorticotropin receptor: evidence for a restricted heterologous mechanism implying a role for receptor–effector complexes. J. Biol. Chem. 276, 44792–44797.

Baig, A.H., Swords, F.M., Szaszak, M., King, P.J., Hunyady, L., Clark, A.J., 2002. Agonist activated adrenocorticotropin receptor internalizes via a clathrin-mediated G protein receptor kinase dependent mechanism. Endocr. Res. 28, 281–289.

Cater, D.B., Stack-Dunne, M.P., 1953. The histological changes in the adrenal of the hypophysectomised rat after treatment with pituitary preparations. J. Pathol. Bacteriol. 66, 119–133.

Chabre, O., Cornillon, F., Bottari, S.P., Chambaz, E.M., Vilgrain, I., 1995. Hormonal regulation of mitogen-activated protein kinase activity in bovine adrenocortical cells: cross-talk between phosphoinositides, adenosine 3′,5′-monophosphate, and tyrosine kinase receptor pathways. Endocrinology 136, 956–964.

Clark, A.J., Weber, A., 1998. Adrenocorticotropin insensitivity syndromes. Endocr. Rev. 19, 828–843.

Côté, M., Guillon, G., Payet, M., Gallo-Payet, N., 2001. Expression and regulation of adenylyl cyclase isoforms in the human adrenal gland. J. Clin. Endocrinol. Metab. 86, 4495–4503.

Côté, M., Payet, M.-D., Gallo-Payet, N., 1997. Association of alpha S-subunit of the GS protein with microfilaments and microtubules: implication during adrenocorticotropin stimulation in rat adrenal glomerulosa cells. Endocrinology 138, 69–78.

Duperray, A., Chambaz, E.M., 1980. Effect of prostaglandin E1 and ACTH on proliferation and steroidogenic activity of bovine adreno-cortical cells in primary culture. J. Steroid Biochem. 13, 1359–1364.

Ferguson, S.S., 2001. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. Pharmacol. Rev. 53, 1–24.

Forti, F.L., Dias, M.H., Armelin, H.A., 2006. ACTH receptor: ectopic expression, activity and signaling. Mol. Cell. Biochem. 293, 147–160.

Gallo-Payet, N., Côté, M., Chorvatova, A., Guillon, G., Payet, M., 1999. Cyclic AMP-independent effects of ACTH on glomerulosa cells of the rat adrenal cortex. J. Steroid. Biochem. Mol. Biochem. 69, 335–342.

Gallo-Payet, N., Escher, E., 1985. Adrenocorticotropin receptors in rat adrenal glomerulosa cells. Endocrinology 117, 38–46.

Gallo-Payet, N., Grazzini, E., Côté, M., Bilodeau, L., Chorvatova, A., Payet, M.D., Chouinard, L., Guillon, G., 1996. Role of calcium in the mechanism of action of ACTH in human adrenocortical cells. J. Clin. Invest. 98, 460–466.

Gallo-Payet, N., Payet, M.D., 2003. Mechanism of action of ACTH: beyond cAMP. Microsc. Res. Tech. 61, 275–287.

Hinkle, P.M., Sebag, J.A., 2009. Structure and function of the melanocortin2 receptor accessory protein (MRAP). Mol. Cell. Endocrinol. 300, 25–31.

Hornsby, P., 1985. Regulation of adrenocortical cell proliferation in culture. Endocr. Res. 10, 259–281.

Houslay, M.D., Kolch, W., 2000. Cell-type specific integration of cross-talk between extracellular signal-regulated kinase and cAMP signaling. Mol. Pharmacol. 58, 659–668.

Janes, M.E., Chu, K.M., Clark, A.J., King, P.J., 2008. Mechanisms of adrenocorticotropin-induced activation of extracellularly regulated kinase 1/2 mitogen-activated protein kinase in the human H295R adrenal cell line. Endocrinology 149, 1898–1905.

Kilianova, Z., Basora, N., Kilian, P., Payet, M.D., Gallo-Payet, N., 2006. Human MC2R expression and functionality. Effect of PKA and PKC on desensitization and internalization. Endocrinology 147, 2325–2337.

Le, T., Schimmer, B.P., 2001. The regulation of MAPKs in Y1 mouse adrenocortical tumor cells. Endocrinology 142, 4282–4287.

Lee, G., Makhanova, N., Caron, K., Lopez, M.L., Gomez, R.A., Smithies, O., Kim, H.S., 2005. Homeostatic responses in the adrenal cortex to the absence of aldosterone in mice. Endocrinology 146, 2650–2656.

Lefkowitz, R.J., Shenoy, S.K., 2005. Transduction of receptor signals by beta-arrestins. Science 308, 512–517.

Liu, H., Enyeart, J.A., Enyeart, J.J., 2008. ACTH inhibits bTREK-1 K^+ channels through multiple cAMP-dependent signaling pathways. J. Gen. Physiol. 132, 279–294.

Lotfi, C.F., Todorovic, Z., Armelin, H.A., Schimmer, B.P., 1997. Unmasking a growth-promoting effect of the adrenocorticotropic hormone in Y1 mouse adrenocortical tumor cells. J. Biol. Chem. 272, 29886–29891.

Marshall, C.J., 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80, 179–185.

May, L.T., Hill, S.J., 2008. ERK phosphorylation: spatial and temporal regulation by G protein-coupled receptors. Int. J. Biochem. Cell Biol. 40, 2013–2017.

Mazzocchi, G., Malendowicz, L.K., Rebuffat, P., Robba, C., Gottardo, G., Nussdorfer, G.G., 1986. Short- and long-term effects of ACTH on the adrenal zona glomerulosa of the rat. A coupled stereological and enzymological study. Cell Tissue Res. 243, 303–310.

McNeill, H., Whitworth, E., Vinson, G.P., Hinson, J.P., 2005. Distribution of extracellular signal-regulated protein kinases 1 and 2 in the rat adrenal and their activation by angiotensin II. J. Endocrinol. 187, 149–157.

Metherell, L.A., Chapple, J.P., Cooray, S., David, A., Becker, C., Ruschendorf, F., Naville, D., Begeot, M., Khoo, B., Nurnberg, P., Huebner, A., Cheetham, M.E., Clark, A.J., 2005. Mutations in MRAP, encoding a new interacting partner of the ACTH receptor, cause familial glucocorticoid deficiency type 2. Nat. Genet. 37, 166–170.

Mullins, L.J., Peter, A., Wrobel, N., McNeilly, J.R., McNeilly, A.S., Al-Dujaili, E.A., Brownstein, D.G., Mullins, J.J., Kenyon, C.J., 2009. Cyp11b1 null mouse, a model of congenital adrenal hyperplasia. J. Biol. Chem. 284, 3925–3934.

Otis, M., Campbell, S., Payet, M.D., Gallo-Payet, N., 2005. Angiotensin II stimulates protein synthesis and inhibits proliferation in primary cultures of rat adrenal glomerulosa cells. Endocrinology 146, 633–642.

Otis, M., Campbell, S., Payet, M.D., Gallo-Payet, N., 2007. Expression of extracellular matrix proteins and integrins in rat adrenal gland: importance for ACTH-associated functions. J. Endocrinol. 193, 331–347.

Penhoat, A., Naville, D., Begeot, M., 2001. The adrenocorticotrophic hormone receptor. Curr. Opin. Endocrinol. Diabetes 8, 112–117.

Poderoso, C., Converso, D.P., Maloberti, P., Duarte, A., Neuman, I., Galli, S., Maciel, F.C., Paz, C., Carreras, M.C., Poderoso, J.J., Podesta, E.J., 2008. A mitochondrial kinase complex is essential to mediate an ERK1/2-dependent phosphorylation of a key regulatory protein in steroid biosynthesis. PLoS ONE 3, e1443.

Pouyssegur, J., Volmat, V., Lenormand, P., 2002. Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling. Biochem. Pharmacol. 64, 755–763.

Ramachandran, J., Suyama, A., 1975. Inhibition of replication of normal adrenocortical cells in culture by adrenocorticotropin. Proc. Natl. Acad. Sci. U.S.A. 72, 113–117.

Roy, S., Perron, B., Gallo-Payet, N., 2010. Role of asparagine-linked glycosylation in cell surface expression and function of the human adrenocorticotropin receptor (melanocortin 2 receptor) in 293/FRT cells. Endocrinology 151, 660–670.

Roy, S., Rached, M., Gallo-Payet, N., 2007. Differential regulation of the human adrenocorticotropin receptor [melanocortin-2 receptor (MC2R)] by human MC2R accessory protein isoforms alpha and beta in isogenic human embryonic kidney 293 cells. Mol. Endocrinol. 21, 1656–1669.

Szaszak, M., Christian, F., Rosenthal, W., Klussmann, E., 2008. Compartmentalized cAMP signalling in regulated exocytic processes in non-neuronal cells. Cell. Signal. 20, 590–601.

Schaeffer, H.J., Weber, M.J., 1999. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol. Cell. Biol. 19, 2435–2444.

Sebag, J.A., Hinkle, P.M., 2010. Regulation of G protein-coupled receptor signaling: specific dominant-negative effects of melanocortin 2 receptor accessory protein 2. Sci. Signal. 3, ra28.

Sewer, M.B., Waterman, M.R., 2003. ACTH modulation of transcription factors responsible for steroid hydroxylase gene expression in the adrenal cortex. Microsc. Res. Tech. 61, 300–307.

Sewer, M.B., Li, D., 2008. Regulation of steroid biosynthesis by the cytoskeleton. Lipids 43, 1109–1115.

Shenoy, S.K., Lefkowitz, R.J., 2003. Multifaceted roles of beta-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling. Biochem. J. 375, 503–515.

Stork, P.J., Schmitt, J.M., 2002. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol. 12, 258–266.

Tremblay, E., Payet, M.D., Gallo-Payet, N., 1991. Effects of ACTH and angiotensin II on cytosolic calcium in cultured adrenal glomerulosa cells. Role of cAMP production in the ACTH effect. Cell Calcium 12, 655–673.

Watanabe, G., Pena, P., Albanese, C., Wilsbacher, L.D., Young, J.B., Pestell, R.G., 1997. Adrenocorticotropin induction of stress-activated protein kinase in the adrenal cortex in vivo. J. Biol. Chem. 272, 20063–20069.

Webb, T.R., Clark, A.J., 2010. Minireview: the melanocortin 2 receptor accessory proteins. Mol. Endocrinol. 24, 475–484.

Xing, Y., Parker, C.R., Edwards, M., Rainey, W.E., 2010. ACTH is a potent regulator of gene expression in human adrenal cells. J. Mol. Endocrinol. 45, 59–68.