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Adrenocorticotropin Induces Mitogen-Activated Protein Kinase Phosphatase 1 in Y1 Mouse Adrenocortical Tumor Cells

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ACTH signaling pathway includes the action of both protein kinases, mainly cAMP-dependent protein kinase (protein kinase A, PKA), and serine/threonine and tyrosine phosphatases. MAPK phosphatase-1 (MKP-1) is a dual activity protein phosphatase involved in the dephosphorylation of MAPK. To determine whether MKP-1 is a component of ACTH cascade, here we investigate the expression levels of MKP-1 gene in Y1 mouse adrenocortical tumor cells under ACTH stimulation. ACTH transiently increased MKP-1 mRNA and protein levels. MKP-1 mRNA increase occurred at 30 min, peaked at 1 h (6-fold), and returned to basal levels thereafter. The ACTH-mediated mRNA increase was blunted by actinomycin D and enhanced by cycloheximide. A cell permeable cAMP analog, 8-bromo-cAMP, also transiently induced MKP-1 mRNA (4-fold) and the PKA inhibitor *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamid abolished this effect. In contrast, *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamid only partially reduced the effect of ACTH, suggesting the participation of PKA-independent mechanisms in the hormone-induced MKP-1 expression. In addition, we show that the rise in intracellular Ca^{2+} and protein kinase C activation had a potent synergic effect on ACTH- and 8-bromo-cAMP-mediated MKP-1 induction. In summary, our findings demonstrate that MKP-1 is another component of ACTH signaling cascade and indicate that this hormone may potentially down-regulate MAPKs. (*Endocrinology* 144: 1399–1406, 2003)

MAPKs ARE A FAMILY of cytoplasmic serine/threonine kinases ubiquitously expressed that play a crucial role in transmitting transmembrane signals required for cell growth, differentiation, and apoptosis (1, 2). They can be classified into at least three subfamilies: ERKs, c-Jun NH_2-terminal protein kinases (JNKs) or stress-activated protein kinases (SAPKs), and p38 MAPKs. A common feature of MAPKs is the requirement of dual threonine and tyrosine phosphorylation to display maximal activity (2).

Because MAPK activation depends on phosphorylation processes, the magnitude and duration of their activity are linked to the activity of phosphatases capable to prompt MAPK dephosphorylation. MAPK phosphatases (MKPs) are a family of dual specificity (threonine/tyrosine) protein phosphatases specifically involved in the MAPK regulation (3, 4). Several distinct mammalian MKP family members have been identified and characterized and they can be divided in two broad classes. One group, typified by MKP-1, comprises nuclear enzymes rapidly induced by growth factors or stress signals (5, 6). The second group, typified by MKP-3, includes predominantly cytosolic enzymes, and their transcripts are induced with delayed kinetics by specific stimuli but not by environmental stress (7, 8).

ACTH is the main hormone involved in the regulation of both growth and endocrine activity of the adrenal gland. This hormone binds to specific G protein-coupled receptors on the cell surface and activates adenylyl cyclase, which produces intracellular cAMP levels increase and consequently, cAMP-dependent protein kinase (protein kinase A, PKA) activation (9–11). Although the acute and chronic regulation of adrenal function by ACTH (11–13) is primarily mediated by PKA-phosphorylated proteins, ACTH also prompts the activation of both SAPKs and ERK1/2 (14, 15), in addition to other kinases such as protein kinase C (PKC) (16) and Ca^{2+}-calmodulin-dependent protein kinase (17). Moreover, ACTH signaling pathway also includes protein dephosphorylation processes mediated by serine/threonine and tyrosine phosphatases (18, 19).

Because it is known that ACTH can regulate the activity of MAPKs and also of protein phosphatases (19, 20), in the present study we have examined the effect of this hormone on MKP-1 induction in serum-starved Y1 cells and demonstrated that ACTH stimulation results in a transient increase of MKP-1 mRNA followed by an increase in the protein levels.

Materials and Methods

Reagents

ACTH was provided by ELEA Laboratories (Buenos Aires, Argentina). Cell culture supplies were obtained from Life Technologies, Inc. BRL Plasticware was from Corning-Costar (Corning, NY). BSA, acrylamide, bis-acrylamide, actinomycin D (Act D), and cycloheximide were obtained from Sigma (St. Louis, MO). *N*-[2-(p-bomocinnamylamino)-ethyl]-5-isoquinolinesulfonamid (H-89), Ro-31–8220, phorbol-12,13-dibutyrate (PDBu), and A23187 were purchased from Calbiochem-Novabiochem (San Diego, CA). Specific polyclonal antibody against MKP-1 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against phospho-ERK1/2 (PP-ERK1/2) and ERK1/2 were from New England Biolabs, Inc. (Beverly, MA). Electrophoresis

Abbreviations: Act D, Actinomycin D; 8Br-cAMP: 8-bromo-3′, 5′-cAMP; CHX, cycloheximide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H-89, *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; JNKs, c-Jun NH_2-terminal protein kinases; MKPs: MAPK phosphatases; PDBu, phorbol-12,13-dibutyrate; PKA, protein kinase A, PKC, protein kinase C; PP-ERK1/2, phospho-ERK1/2; SAPKs, stress-activated protein kinases.

supplies, polyvinylidene difluoride membrane, and secondary antibody (horseradish peroxidase-conjugated goat antibody) were from Bio-Rad Laboratories, Inc. (Hercules, CA). Hybond-N^+ nylon membranes and ECL kit were provided by Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK) and [α-^{32}P]deoxy-CTP was from NEN Life Science Products (Boston, MA). All other chemicals were commercial products of the highest grade available.

Cell cultures

Murine Y1 adrenocortical tumor cells, generously provided by Dr. Bernard Schimmer (University of Toronto, Toronto, Canada), were maintained in Ham-F10 medium, supplemented with 12.5% heat-inactivated horse serum and 2.5% heat-inactivated fetal bovine serum, 1.2 g/liter $NaHCO_3$, 200 IU/ml penicillin, and 200 mg/ml streptomycin sulfate and maintained in a 5% CO_2 humidified atmosphere (21). Cells were arrested early in the G1 phase of the cell cycle by transferring cells in the logarithmic phase of growth to serum-free medium for 72 h. After replacing the medium by fresh serum-free medium, the cultures were incubated with or without ACTH or 8-bromo-cAMP (8Br-cAMP), as stated in the legend of the corresponding figures. When indicated, the different reagents were added to the incubation medium 30 min (5 μg/ml Act D or 2 μg/ml cycloheximide) or 15 min (20 μM H-89, 10 μM A23187, 100 nM PDBu, or 5 μM Ro-31–8220) before ACTH or 8Br-cAMP stimulation. Following treatments, total RNA or cell lysates were obtained.

RNA isolation and Northern blot

Total RNA was isolated from Y1 cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA samples (20 μg/lane) were electrophoresed in a 1.2% agarose gel/2.2 M formaldehyde, and blotted onto Hybond-N^+, nylon membranes by capillarity. After prehybridization, blots were hybridized overnight at 42 C using [α-^{32}P]deoxy-CTP-radiolabeled cDNA probes. MKP-1 and 28S probes were labeled by random priming, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was labeled by PCR.

MKP-1 probe was obtained by RT-PCR from Y1 cells, using random primers for the RT and specific primers for the PCR. The forward (5′-GAGCTGTGCAGCAAACAGTCC-3′) and reverse (5′-CCGGTGGCAAGTGAAACTCC-3′) primers, designed according to the published sequence of mouse MKP-1 cDNA (GenBank accession no. NM_013642) were used to amplify a 741-bp fragment. The 741-bp amplified fragment corresponds to the region between bases 539 and 1280 of the mouse MKP-1 sequence. The RT-PCR product exhibited a molecular size that was in accordance with the primers used and the published sequence. The identity of the probe was confirmed by restriction analysis using *Apa*I, *Nco*I, and *Pst*I restriction enzymes, which cut at positions 659, 1113, 1059, respectively. Moreover, a sequence alignment analysis of the obtained probe showed no significant matches with other sequences.

The hybridization was performed in a solution containing 50% formamide, 5× SSPE (0.75 M NaCl; 0.05 M sodium phosphate; and 5 mM EDTA, pH 7.4), 5× Denhardt's, 0.5% sodium dodecyl sulfate, and 1 mg/ml denatured salmon sperm DNA. The filters were washed under high stringency conditions and analyzed with Storm PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) and scanner (Amersham Pharmacia Biotech). After signal quantification, the membranes were stripped and rehybridized with GAPDH or 28S rRNA radiolabeled probes. Relative mRNA levels were calculated after correcting the RNA loading by normalizing the primary hybridization signal with the GAPDH or 28S signal.

Western blot analysis

After the appropriate treatments, Y1 cells were washed with PBS and scrapped into a buffer containing 35 mM Tris (pH 7.4), 0.5% Triton X-100, 5 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol, 0.2 M sucrose, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 2 mM sodium orthovanadate. The suspension was incubated on ice for 10 min, vortexed for 1 min, and then centrifuged at 11,000 × *g* at 4 C for 10 min. Pellet was discarded and supernatant (total lysate) was subjected to Western blot analysis. Equal amounts of protein from total lysates (35 μg) were separated by 12% SDS-PAGE, as described by Laemmli (22) and transferred onto polyvinylidene difluoride membranes according to the procedure described by Towbin *et al.* (23). Western blot analysis was performed using polyclonal MKP-1 antibody and horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were detected using an enhanced chemiluminescence kit (ECL). The blots were subsequently stripped and reprobed using an antibody against the catalytic subunit of the PP2A protein phosphatase. PP2A catalytic subunit expression is tightly autocontrolled to ensure constant protein levels (24). Thus, we chose this procedure as loading control.

Western blot analysis of PP-ERK1/2 was performed using a polyclonal specific antibody and following the protocol described above. In this experiment, after the stripping, the membranes were reprobed with a specific polyclonal antibody that recognizes both the phosphorylated and nonphosphorylated forms of ERK1/2.

Determination of steroid production

Steroid production was determined as previously described (25, 26).

Protein determination

Protein concentration was determined by Bradford assay (27), using BSA as standard.

Statistics

Results are shown as the mean ± SD. Unless otherwise stated, statistical significance was evaluated using ANOVA followed by Tukey test. $P < 0.05$ was considered significant.

Results

Increase of MKP-1 mRNA levels by ACTH and cAMP in Y1 cells: time course and dose dependence

The Y1 mouse adrenocortical tumor cell line is widely used as model to study the regulation of adrenocortical function because these cells behave in many aspects like fasciculata cells from the normal adrenal cortex (13, 28). To analyze the potential effect of ACTH on MKP-1 expression, we chose as an experimental model this cell line cultured in similar conditions to those in which the effect of ACTH on both ERKs activity and cell growth was clearly established (15, 20). Thus, Y1 cells used in this work were arrested in G1 phase of the cell cycle by serum depletion for 72 h.

The effect of ACTH on MKP-1 mRNA levels in serum-starved Y1 cells was evaluated by Northern blot analysis. As shown in Fig. 1, ACTH treatment produced a transient increase in MKP-1 mRNA (Fig. 1). Kinetic analysis of the response revealed that ACTH-induced MKP-1 mRNA accumulation occurred at 30 min, peaked at 1 h, and returned to basal level thereafter (Fig. 1A). Quantitative analysis showed MKP-1 mRNA levels approximately 6-fold greater in ACTH-stimulated cells than in nonstimulated cells at 1 h of stimulation (Fig. 1B).

The ACTH-triggered cAMP increase is a signal well recognized as key step in the hormonal control of adrenal function. Thus, next we tested the effect of this cyclic nucleotide on MKP-1 mRNA levels. As shown in Fig. 2, exposure of Y1 cells to 8Br-cAMP, a membrane-permeable cAMP derivative, also augmented the messenger levels, suggesting a role of cAMP as second messenger also in the ACTH-induced signaling toward MKP-1 induction. Kinetic profile of mRNA accumulation caused by 8Br-cAMP resembled the profile obtained under ACTH stimulation. Again, a significant in-

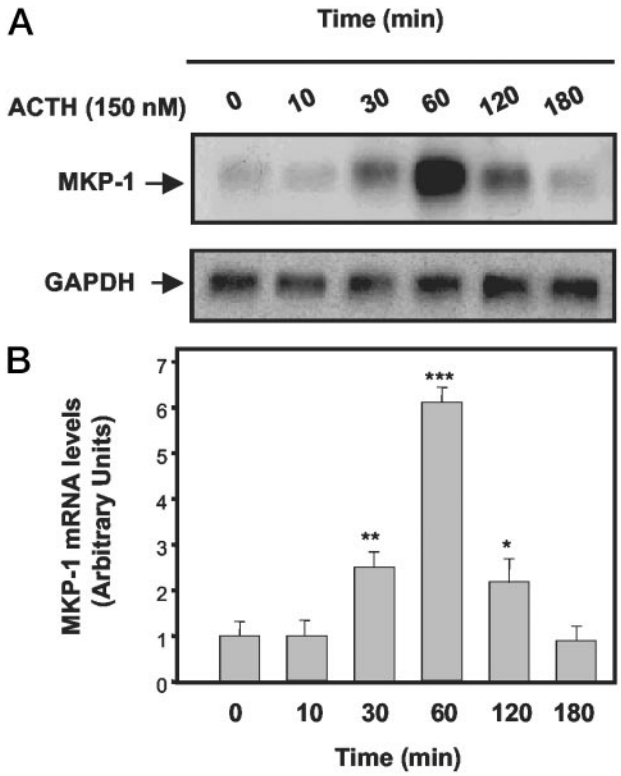

FIG. 1. Time course of ACTH-induced MKP-1 mRNA levels. The culture medium of serum-starved Y1 cells was replaced by fresh serum-free medium and stimulated with ACTH (150 nM) for the indicated times. After treatment, total RNA was isolated and samples containing equal quantities of RNA (20 μg) were subjected to Northern blot analysis using [α^{32}P]CTP-labeled cDNA probes for MKP-1 and GAPDH as described in *Materials and Methods*. A, Autoradiograms of a representative experiment, independently performed three times. B, The autoradiograms were quantitated by scanning densitometry, and the data were normalized against GAPDH mRNA. Presented values are expressed in arbitrary units and represent the mean ± SD of three independent experiments. *Asterisks* indicate significant differences from control (0 min). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

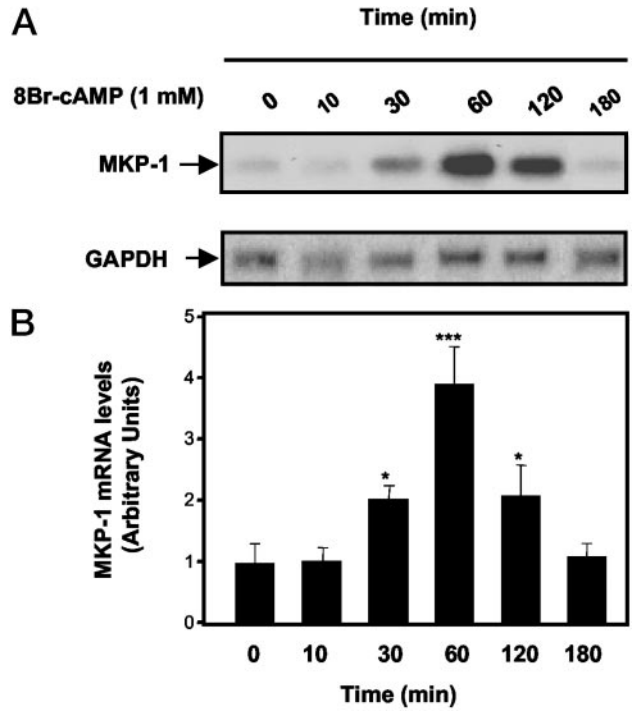

FIG. 2. Time-dependent effect of 8Br-cAMP on MKP-1 mRNA levels. Y1 cells were stimulated with 8Br-cAMP (1 mM) for the indicated times. After the treatment, total RNA was isolated and analyzed by Northern blot analysis using [α^{32}P]CTP-labeled cDNA probes for MKP-1 and GAPDH. The autoradiograms were quantitated by scanning densitometry, and the data were normalized against GAPDH mRNA and expressed in arbitrary units. A, Autoradiograms of a representative experiment, independently performed three times. B, Quantitative representation of the data obtained from three independent experiments (mean ± SD). *Asterisks* indicate significant differences from control (0 min). *, $P < 0.05$; ***, $P < 0.001$.

crease in MKP-1 mRNA levels was detected at 30 min, became maximal after 1 h (4-fold) and declined thereafter (Fig. 2). However, following 60 min of incubation, 8Br-cAMP produced a significantly lower ($P < 0.001$, two-way ANOVA followed by Bonferroni test) stimulation of MKP-1 mRNA abundance than ACTH (4-fold *vs.* 6-fold for 8Br-cAMP and ACTH, respectively).

To further establish the relationship between ACTH or cAMP and MKP-1 mRNA levels, we performed a dose-dependence analysis. In this experiment, as well as all the subsequent, MKP-1 mRNA-induced accumulation was assessed after 1 h of ACTH or 8Br-cAMP stimulation. Both ACTH and cAMP treatment increased MKP-1 mRNA levels in a concentration-dependent manner, with a detectable response at 1.5 nM ACTH or 0.1 mM 8Br-cAMP (Fig. 3).

Induction of MKP-1 mRNA by ACTH and cAMP depends on transcriptional activation

To show that ACTH- or 8Br-cAMP-induced MKP-1 mRNA increase is linked to transcriptional activation of the gene, we analyzed the effect of a transcription inhibitor, Act D, on MKP-1 mRNA levels in ACTH- and 8Br-cAMP-stimulated Y1 cells. Given that MKP-1 is an immediate early gene product (29) and that the protein synthesis inhibitors are able to prolong the half-life of mRNA encoded by these genes (30), we also analyzed the effect of a protein synthesis inhibitor, cycloheximide (CHX). As shown in Fig. 4, Act D (5 μg/ml), added 30 min before 60-min exposure to ACTH or 8Br-cAMP, completely abolished MKP-1 induction triggered by the hormone or the second messenger (Fig. 4A). In contrast, preincubation for 30 min with CHX (2 μg/ml) did not avoid MKP-1 induction by ACTH or 8Br-cAMP. Moreover, CHX enhanced the response to both ACTH and 8Br-cAMP. In addition, CHX *per se* increased MKP-1 mRNA, suggesting that protein synthesis is involved in the rapid decay of MKP-1 mRNA. This is in agreement with previous reports demonstrating the existence of destabilizing motifs in the 3′ untranslated region of MKP-1 mRNA (31, 32). Together, these results indicate that the effect of ACTH or 8Br-cAMP is due to their action on gene transcription. However, MKP-1 mRNA stabilization by ACTH through a cAMP-dependent mechanism cannot be ruled out.

ACTH induces MKP-1 protein

To test whether the effect of ACTH on MKP-1 mRNA amount is reproduced also on the protein, MKP-1 protein

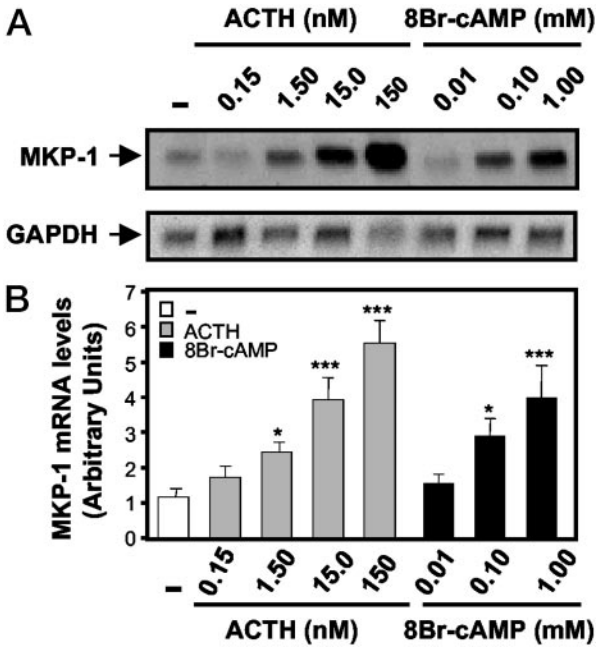

FIG. 3. Concentration-dependent effect of ACTH and 8Br-cAMP on MKP-1 mRNA levels. Y1 cells were incubated for 1 h in serum-free medium in the absence (control) or presence of increasing concentrations of ACTH (0.15–150 nM) or 8Br-cAMP (0.01–1 mM). Total RNA was isolated and MKP-1 and GAPDH mRNA were analyzed by Northern blot using ^{32}P-labeled specific probes. A, Autoradiograms of a representative experiment, independently performed three times. B, Quantitative representation of the normalized MKP-1 mRNA data. Presented values are expressed in arbitrary units and represent the mean ± SD of three independent experiment. *Asterisks* indicate significant differences from nonstimulated cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

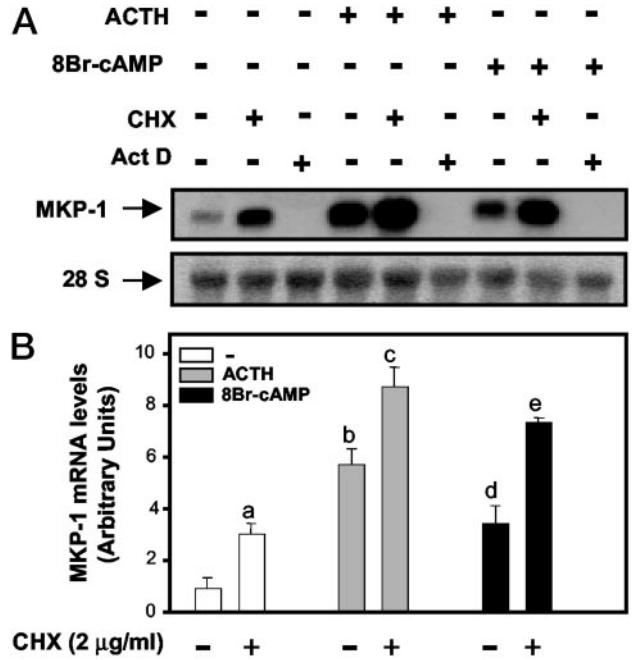

FIG. 4. Effect of Act D and CHX on the ACTH and 8Br-cAMP-mediated MKP-1 mRNA. The cells were incubated in the absence or presence of Act D (5 μg/ml) or CHX (2 μg/ml) for 30 min before the addition of ACTH (150 nM) or 8Br-cAMP (1 mM). After 1 h of stimulation, total RNA was isolated and analyzed by Northern blot using ^{32}P-labeled cDNA probes for MKP-1 and 28S mRNA as described in *Materials and Methods*. The autoradiograms were quantitated by scanning densitometry, and the data were normalized against 28S mRNA and expressed in arbitrary units. A, Autoradiograms of a representative experiment, independently performed three times. B, Quantitative representation of the data obtained from three independent experiments. (mean ± SD). Significant differences are reported in figure as: a, $P < 0.05$; b, $P < 0.001$; and d, $P < 0.05$ *vs.* nonstimulated cells incubated without CHX; c, $P < 0.001$ *vs.* ACTH; e, $P < 0.001$ *vs.* 8Br-cAMP.

levels in cell lysates obtained from Y1 cells exposed to ACTH for various times were monitored by Western blot analysis. MKP-1 antibody recognized a band migrating with a relative molecular mass of 39,000 as was reported (33). As shown in Fig. 5, ACTH treatment caused a transient increase of MKP-1 protein that was evident after 1 h of stimulation, peaked at 2 h, and declined thereafter. In contrast, MKP-1 protein was barely detected in control cells (Fig. 5). Translation of MKP-1 mRNA correlates with transcription of the MKP-1 gene.

Inhibition of PKA activity partially reduces MKP-1 mRNA induction by ACTH

The results obtained with 8Br-cAMP suggested that PKA activation mediates the induction of MKP-1 gene transcription by ACTH. However, the fact that the effect of ACTH is stronger than that produced by 8Br-cAMP suggests that PKA-independent signaling events could be also acting on the hormonal regulation of MPK-1 expression. Thus, in the next experiments we tested the contribution of PKA activity on MKP-1 mRNA induction by ACTH and 8Br-cAMP in Y1 cells exposed to the isoquinolinesulfonamide H-89 (20 μM), a potent PKA inhibitor (inhibition constant = 0.05 μM; Ref. 34). At that concentration, H-89 does not affect PKC, myosin light chain kinase, Ca^{2+}-calmodulin-dependent protein kinase, and casein kinase I and II, but it may exert an inhibitory effect on cyclic GMP-dependent protein kinase (inhibition constant = 0.5 μM; Ref. 35). However, given that the ACTH signaling pathway does not involve cyclic GMP-dependent protein kinase action, in our system H-89 (20 μM) is acting as a specific PKA inhibitor. This inhibitor reduced MKP-1 mRNA levels induced by 8Br-cAMP to the baseline but only partially reduced gene expression triggered by ACTH (Fig. 6), suggesting that in addition to PKA, other components of ACTH signaling pathway could be responsible for the action of this hormone on MKP-1 mRNA induction. The difference between the inhibition produced by H-89 treatment of ACTH- or 8Br-cAMP-stimulated cells is not due to a lack of inhibition of PKA activity in ACTH-treated cells. At the concentration used, H-89 completely blocked PKA activity in ACTH- or 8Br-cAMP-stimulated cells (data not shown).

Calcium and PKC signals enhance the effect of ACTH and 8Br-cAMP on MPK-1 mRNA levels in Y1 cells

Previous reports underline the essential role played by Ca^{2+} in the regulation of MKP-1 expression (36, 37). In addition, PKC activation has also been reported as mediator of

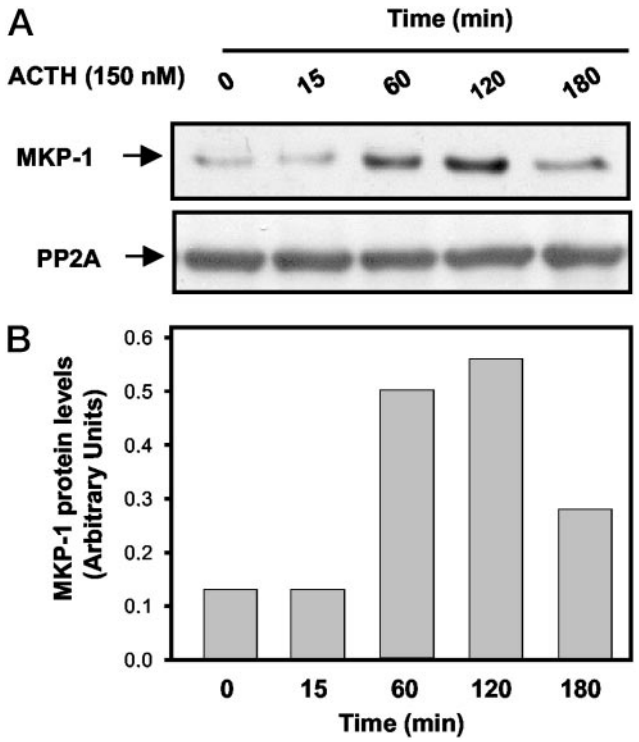

FIG. 5. Temporal profile of MPK-1 protein induction by ACTH. The culture medium of serum-starved Y1 cells was replaced by fresh serum-free medium and the cells were immediately processed or incubated for the times indicated in the absence or presence of ACTH (150 nM). After treatment, the cells were lysated and total cellular proteins were electrophoresed on 12% SDS-PAGE, transferred to membranes, and probed using an antibody against MKP-1 protein. After stripping, the membranes were reprobed using an antibody against PP2A catalytic subunit. Specific bands were detected by enhanced chemiluminescence. A, Western blot representative of two independent experiments. B, Quantitative representation of the data from A, normalized with PP2A levels.

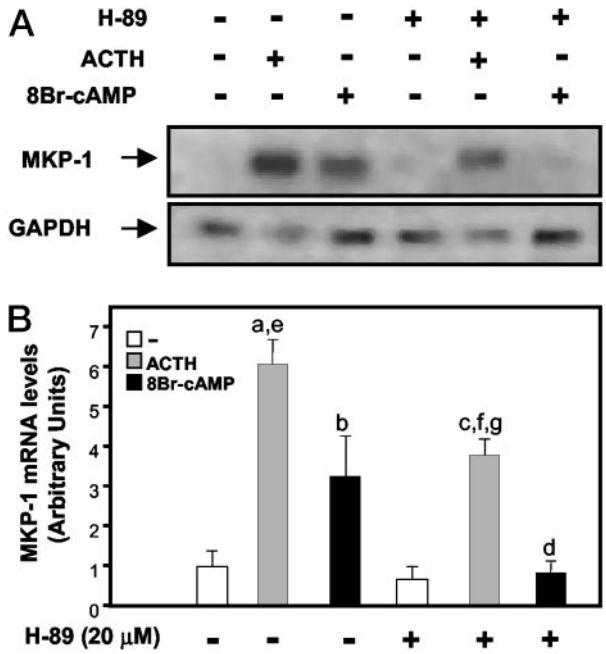

FIG. 6. Effect of H-89 on MKP-1 gene expression induced by ACTH or 8Br-cAMP. MKP-1 mRNA from Y1 cells exposed to the PKA inhibitor H-89 was analyzed by Northern blot. The inhibitor was added to the culture medium 15 min before the addition of ACTH (150 nM) or 8Br-cAMP (1 mM). After 1 h of stimulation cell monolayers were processed and total RNA was isolated. MKP-1 and GAPDH mRNA were detected using ^{32}P-labeled specific probes. A, Autoradiograms of a representative experiment, independently performed three times. B, Quantitative representation of the normalized MKP-1 mRNA data. Presented values are expressed in arbitrary units and represent the mean ± SD of three independent experiments. Significant differences are reported in figure as: a, $P < 0.001$; b, $P < 0.01$; and g, $P < 0.001$ *vs.* nonstimulated cells incubated without H-89; c, $P < 0.01$ *vs.* ACTH; d, $P < 0.01$ *vs.* 8Br-cAMP; e, $P < 0.001$ *vs.* 8Br-cAMP; f, $P < 0.001$ *vs.* 8Br-cAMP plus H-89.

agonist induced-MKP-1 mRNA increase in several cell types (38). As shown in Fig. 7, in Y1 cells both the increase of intracellular Ca^{2+} prompted by the ionophore A23187 (10 μM; panels A and D) as well as PKC activation produced by PDBu (100 nM; panels B and E) were able to enhance MKP-1 mRNA induction provoked by ACTH and 8Br-cAMP. However, in Y1 cells incubated in the absence of ACTH or 8Br-cAMP, the ionophore A23187 was able to produce a slight increase on MKP-1 mRNA levels (panels A and D), whereas PDBu was ineffective (panels B and E). In spite of these differences, our observations attribute a potential role to intracellular Ca^{2+} and PKC activity as mediators of MKP-1 gene transcription regulation in Y1 cells. In addition, we have evaluated the rate of MKP-1 mRNA in ACTH- or 8Br-cAMP-stimulated Y1 cells in the presence of Ro-31–8220, a widely used PKC inhibitor. The incubation of the cells with 5 μM Ro-31–8220 significantly reduced the levels of ACTH-induced MKP-1 mRNA (Fig. 7, C and F). At the concentrations used, Ro-31–8220 reduced also the effect of 8Br-cAMP. Nevertheless, this PKC inhibitor had a more pronounced effect on ACTH- than on 8Br-cAMP-induced MKP-1 mRNA levels supporting the notion that PKC could contribute to ACTH-induced MKP-1 expression.

ACTH promotes ERK1/2 phosphorylation and cycloheximide delays ERK1/2 dephosphorylation

The ERK1/2 cascade is an important regulatory pathway in cell cycle progression, and it is widely used as a biochemical marker to evaluate the mitogenic potential of hormones and growth factors. It has been demonstrated that a brief pulse of ACTH (up to 2 h) has a growth-promoting effect in arrested Y1 cells and that this event is preceded by ERK1/2 phosphorylation (39). The fact that the highest mitogenic effect of ACTH is detected at the same time that MKP-1 protein expression is maximal, lead us to speculate that the decrease of ACTH mitogenic effect may be related to MKP-1-dependent ERK1/2 dephosphorylation. To test this hypothesis, we measured the time course of ACTH-induced ERK1/2 phosphorylation in the presence or the absence of 2 μg/ml CHX to abolish MKP-1 induction (40). As shown in Fig. 8, ACTH promoted the phosphorylation of ERK1/2 in a transient fashion. The effect peaked at 15 min and decayed progressively after 1 h, coinciding with the appearance of MKP-1 (see Fig. 5). Impairment of MKP-1 synthesis by CHX caused a slower rate of ERK1/2 dephosphorylation (Fig. 8). Taken together, our results suggest that a putative physiological role for ACTH-mediated MKP-1 induction could be to counteract the effect of the hormone on cell growth.

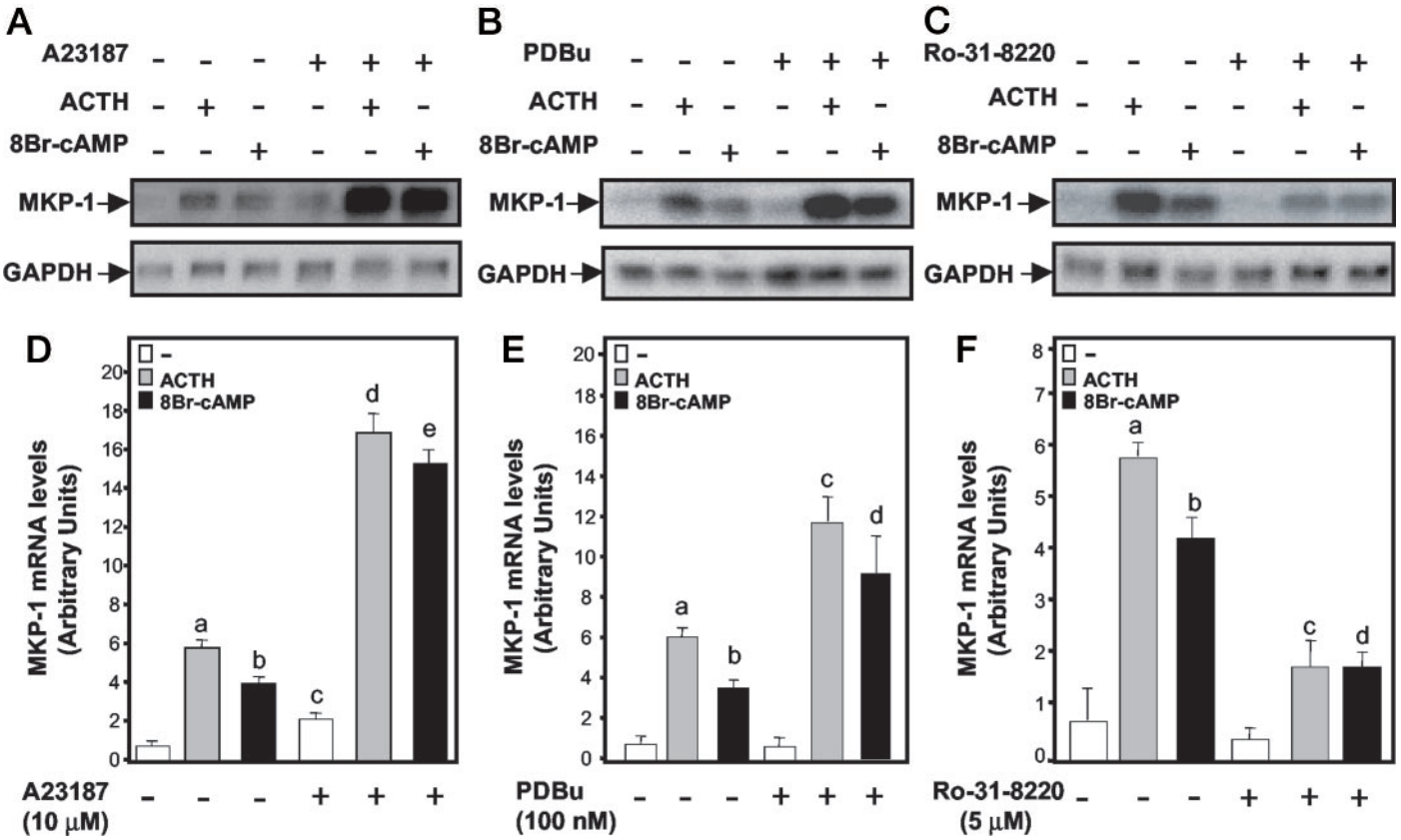

FIG. 7. Effects of A23187, PDBu, and Ro-31–8220 on MKP-1 gene expression induced by ACTH or 8Br-cAMP. MKP-1 mRNA from Y1 cells exposed to 10 μM A23187 (A and D), 100 nM PDBu (B and E), or 5 μM Ro-31–8220 (C and F) was analyzed by Northern blot. The reagents were added to the culture medium 15 min before the addition of ACTH (150 nM) or 8Br-cAMP (1 mM). After 1 h of stimulation, cell monolayers were processed and total RNA was isolated. MKP-1 and GAPDH mRNA were detected using ^{32}P-labeled specific probes. A–C, Autoradiograms of representative experiments, independently performed three times. D–F, Quantitative representation of the normalized MKP-1 mRNA data. Presented values are expressed in arbitrary units and represent the mean ± SD of three independent experiments. Significant differences are reported in panel D as: a, $P < 0.001$; b, $P < 0.01$; and c, $P < 0.05$ *vs.* nontreated cells; d, $P < 0.001$ *vs.* ACTH; e, $P < 0.001$ *vs.* 8Br-cAMP. E, a, $P < 0.001$; and b, $P < 0.01$ *vs.* nontreated cells; c, $P < 0.001$ *vs.* ACTH; d, $P < 0.001$ *vs.* 8Br-cAMP. F, a, $P < 0.001$; b, $P < 0.001$ *vs.* nontreated cells; c, $P < 0.001$ *vs.* ACTH; d, $P < 0.001$ *vs.* 8Br-cAMP.

In addition, we observed that, in serum-starved Y1 cells, the capacity of ACTH to promote cell growth during the first 2 h of stimulation contrasts with the action of the hormone on steroid synthesis. In fact, the quantification of steroid production demonstrated that nonstarved Y1 cells produce a full steroidogenic response to ACTH after 8 h of stimulation. On the contrary, ACTH-induced steroidogenesis is reduced 50% at 2 and 8 h of stimulation in starved Y1 cells compared with nonstarved Y1 cells. The steroidogenic capacity of serum-starved Y1 cells was recovered after 24 h of ACTH stimulation. The same results were obtained when the cells were stimulated with 8Br-cAMP.

In view of the fact that the cells recover steroid-producing ability following MKP-1 protein induction and once ACTH's mitogenic activity is halted, it is possible that MKP-1 protein could act as a switch to control whether the cell enters the mitogenic cycle or it commits to the production of steroid hormones.

Discussion

The present study demonstrates that ACTH induces MKP-1 in Y1 mouse adrenocortical tumor cells. The effect of ACTH on MKP-1 mRNA induction is rapid and transient, and it is partially mimicked by 8Br-cAMP. In addition, we show here that the effect of the hormone and the second messenger is reduced by exposure of the cells to H-89 in a concentration that preferentially inhibits PKA activity. Taken together, our results indicate that ACTH regulates MKP-1 gene expression, at least in part, through PKA activation. These results are in accordance with previous reports that show PKA-dependent MKP-1 induction triggered by glucagon and cAMP-elevating agents in rat hepatocytes (41) and PC12 cells (42), respectively. Moreover, analysis of the 5′-flanking region of both murine and human MKP-1 genes revealed two Ca^{2+}/cAMP-responsive elements (CREs) in its promoter (43), strongly supporting that ACTH regulates MKP-1 expression by a PKA-dependent mechanism.

Even when both ACTH and 8Br-cAMP induce MKP-1 gene expression with similar kinetics, the effect of the hormone is quantitatively stronger. We have used concentrations of ACTH and 8Br-cAMP high enough to produce full PKA activation (data not shown). Thus, the stronger induction of MKP-1 exerted by ACTH suggests that the effect of this hormone may be mediated by both PKA-dependent and

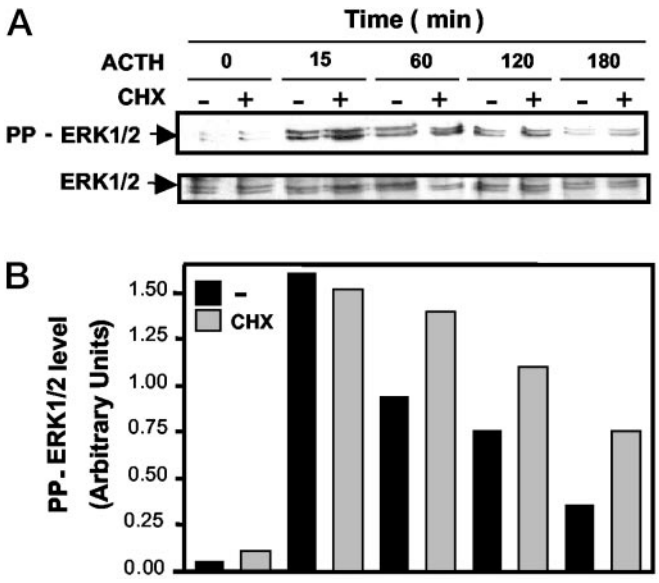

FIG. 8. Time course of ACTH-stimulated phosphorylation of ERK1/2: effect of cycloheximide. Starved-Y1 cells were pretreated with or without 2 μg/ml cycloheximide for 30 min before stimulation with ACTH (150 nM) for the indicated times. Following lysis, proteins were separated by 12% SDS-PAGE and Western blotted with anti-PP-ERK1/2. After stripping the membranes were reprobed using an antibody against ERK1/2. Specific bands were detected by enhanced chemiluminescence. A, Western blots representative of two independent experiments. B, Scanning densitometry quantification of autoradiograms shown in panel A, normalized against ERK1/2.

independent signaling events. This notion is supported by the fact that H-89 abolishes 8Br-cAMP induction but only partially reduces the effect of ACTH on MKP-1 mRNA.

MKP-1 is regulated at transcriptional levels and is induced by multiple stimuli (37, 41, 42, 44, 45). Taking into account the broad spectrum of MKP-1 inducers, the involvement of multiple signals in the regulation of MKP-1 gene transcription could be expected. The effect of ACTH was reduced by Ro-31–8220, an ATP-competitive protein kinase inhibitor that acts mainly on PKC (35), indicating a putative contribution of PKC on the action of ACTH on MKP-1 gene expression. Also, we observed an effect of the Ca^{2+} ionophore A23187, which suggests the participation of increases in intracellular Ca^{2+} concentrations in the regulation of MKP-1 mRNA induction. The finding that this compound also enhanced the effect of ACTH and 8Br-cAMP may suggests that the intracellular Ca^{2+} levels reached in ACTH-stimulated cells are not sufficient to produce maximal induction of MKP-1 gene expression.

We show here that PDBu *per se* was not effective as MKP-1 inducer in Y1 cells. This will suggest that PKC activation is, in itself, insufficient to induce MKP-1 in G1 phase arrested Y1 cells. Because PDBu-induced PKC activation enhanced the effect of both 8Br-cAMP and ACTH, a sequential activation of PKA-PKC that promotes MKP-1 induction seems likely.

A central question raised by our findings is the functional significance of MPK-1 induction by ACTH. MKP-1 protein is able to interact selectively with the MAPKs family members (3). Thus, its activity is linked exclusively to the regulation of MAPKs pathways. ACTH-mediated ERK1/2 and SAPKs activation is already demonstrated (14, 15). Thus, the increase in MKP-1 protein levels could regulate both the mitogenic activity and the stress response of adrenocortical cells to ACTH by balancing the activity of ERK1/2 and SAPKs.

A short pulse of ACTH (up to 2 h) produces a mitogenic effect on both Y1 and Kin-8 cells arrested in the G1 phase of the cell cycle, an action preceded by the rapid ERK1/2 phosphorylation in both cell types (39). By contrast, continuous ACTH treatment causes a growth-inhibitory effect on the cells via a cAMP-dependent pathway (39). Thus, this picture could indicate that the transient expression of MKP-1 induced by ACTH contributes to limit the temporal action of ERK1/2. Our results demonstrating a slower rate of ERK1/2 dephosphorylation when MKP-1 synthesis was impaired by CHX supports that notion.

Sewer and Waterman (46) have recently demonstrated that ACTH- and cAMP-mediated transcription of human CYP17, a gene encoding a hydroxylase that participates in corticosteroid synthesis, is dependent on both tyrosine and serine/threonine phosphatase activities. Based on this observation, the authors suggest that a dual function phosphatase could be involved in the induction of CYP17 by ACTH (46). MKP-1 could serve also in this regulatory mechanism, and the control of steroidogenesis constitutes another possible physiological role of the enzyme. The induction of enzymes and/or transcription factors by ACTH through an MKP-1-mediated mechanism in G1-arrested Y1 cells could explain our results showing a reduced steroidogenic response to ACTH and the recovery after 24 h of ACTH stimulation in serum-starved cells.

In summary, the present findings clearly demonstrate that ACTH transiently induces MKP-1 expression in Y1 cells mainly by a PKA-dependent mechanism. Even when increases in intracellular Ca^{2+} concentrations and other kinases can cooperate with PKA to produce the maximal effect of ACTH on MKP-1 mRNA levels, our findings underline the essential role of PKA in this process. Further studies should be carried out to characterize the PKA-independent events that contribute to MKP-1 induction by ACTH. Also, a study of the functional role of the hormonal regulation of MKP-1 on adrenocortical cell biology will be undertaken.

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