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Activation and Induction of NUR77/NURR1 in Corticotrophs by CRH/cAMP: Involvement of Calcium, Protein Kinase A, and MAPK Pathways

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Nur factors are critical for proopiomelanocortin (POMC) induction by CRH in corticotrophs, but the pathways linking CRH to Nur are unknown. In this study we show that in AtT-20 corticotrophs CRH and cAMP induce Nur77 and Nurr1 expression and transcription at the NurRE site by protein kinase A (PKA) and calcium-dependent and -independent mechanisms. Calcium pathways depend on calmodulin kinase II (CAMKII) activity, and calcium-independent pathways are accounted for in part by MAPK activation (Rap1/B-Raf/MAPK-ERK kinase/ERK1/2), demonstrated by the use of molecular and pharmacological tools. AtT-20 corticotrophs express B-Raf, as do other cells in which cAMP stimulates MAPK. CRH/cAMP stimulated ERK2 activity and increased transcriptional activity of a Gal4-Elk1 protein, which was blocked by overexpression of dominant negative mutants and kinase inhibitors and stimulated by expression of B-Raf. The MAPK kinase inhibitors did not affect Nur77 and Nurr1 mRNA induction but blocked CRH or cAMP-stimulated Nur transcriptional activity. Moreover, MAPK stimulated phosphorylation and transactivation of Nur77. The functional impact of these pathways was confirmed at the POMC promoter. In conclusion, in AtT-20 corticotrophs the CRH/cAMP signaling that leads to Nur77/Nurr1 mRNA induction and transcriptional activation, and thus POMC expression, is dependent on protein kinase A and involves calcium/calmodulin kinase II (Nur induction/activation) and MAPK calcium-dependent and -independent (Nur phosphorylation-activation) pathways. (*Molecular Endocrinology* 16: 1638–1651, 2002)

FOLLOWING A STRESSFUL stimulus, CRH is synthesized in the hypothalamus and released into the portal system. Interaction of CRH with its receptor in corticotrophic cells leads to increased transcription of proopiomelanocortin (POMC) mRNA and secretion of ACTH into the circulatory system. POMC mRNA regulation has been described in detail both *in vivo* and in *vitro* (1). CRH induces POMC expression in pituitary primary cultures (1, 2) and in AtT-20 cells (3–5). In corticotroph cells, CRH leads to an increase in cAMP levels and activation of protein kinase A (PKA) (6–10). CRH elicits calcium entry through L-type voltage-dependent calcium channels, which in turn triggers the release of secretion vesicles anchored near the plasmatic membrane (8, 11). Calcium entry depends on PKA action and can be mimicked by exogenous cAMP treatment (8, 11). This is a rapid effect, and calcium spikes are produced 5 min after stimulation (12). In addition to its effect on the release of ACTH from vesicles, calcium is involved in the regulation of POMC mRNA (13) and is associated with the stimulation of c-*fos* expression by CRH (3).

Nur factors are the main mediators of CRH and cAMP stimulation of POMC in corticotrophs (14–16). Nur77, Nurr1, and NOR-1 are orphan nuclear receptors that belong to the Nur subfamily of transcription factors (17). They possess a highly conserved DNA binding domain that allows them to interact with the same DNA sequences in promoters, although specific and independent actions have also been described for these factors. Nur factors are particularly important in the regulation of the hypothalamic-pituitary-adrenal axis, acting on the hypothalamus (16), pituitary (14), and adrenal glands (18). In the pituitary, Nur77 and Nurr1 are involved in CRH-dependent induction of POMC mRNA, acting at two sites in the promoter: the NGFI-B response element (NBRE) site, which can bind one of these proteins as a monomer, and the Nur response element (NurRE) site, which can bind homodimeric Nur77/Nur77 or heterodimeric Nur77/Nurr1 proteins (14, 15, 19). The POMC NurRE (gTGATATT-

Abbreviations: CAMKII, Calmodulin kinase II; CAT, chloramphenicol acetyl transferase; CREB, cAMP response element binding protein; FCS, fetal calf serum; β-gal, β-galactosidase; LUC, luciferase; MBP, myelin basic protein; MEK, MAPK kinase; MEK DN, dominant negative mutant of MEK; MEK-EE, MEK constitutively active form expression vector; NA, noradrenaline; NBRE, NGFI-B response element; NIF, nifedipine; NurRE, Nur response element; POMC, proopiomelanocortin; PKA, protein kinase A; PKA DN, dominant negative mutant of PKA; RSV, rous sarcoma virus.

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TacctccAAATGCCA) is an inverted copy of two NBRE (AAAGGTCA) sites with two mutations each, separated by 6 bp. The POMC NurRE is more sensitive to CRH stimulation than the NBRE site and may be responsible for stimulation of the POMC promoter under physiological conditions (14, 15, 19). NOR-1, the third member of the Nur subfamily, although capable of interacting with the consensus NurRE and NBRE sites, can only bind the POMC NurRE site and activate transcription as heterodimers together with Nur77 (15). The mechanisms and pathways that lead to Nur77/Nurr1 mRNA induction and transcriptional activation by CRH remain unknown. As CRH exerts its effect in corticotrophs through the type-1 CRH receptor (7), which raises intracellular cAMP levels, and Nur77/Nurr1 factors are involved in POMC induction by CRH, acting mainly at the NurRE site, in this paper we addressed the issue of calcium involvement in CRH and cAMP stimulation of NurRE-directed transcription.

The MAPK pathway is regulated by cAMP in several cell types. In view of this, we hypothesized they might play a role in CRH-Nur-POMC pathways. Rap1 is a small G protein that is activated by cAMP and PKA (20, 21). Depending on the cell type, Rap1 either inhibits or activates the MAPK pathway. Rap1 was shown to block MAPK activation through Ras (22–24), and the inhibition of MAPK activity exerted by Rap1 is thought to occur by interaction with Ras downstream effectors forming an inactive complex (25). In NIH3T3 and Rat-1 cells, the Raf-1 MAPK-kinase kinase activation by Ras is blocked by cAMP and PKA-dependent phosphorylation of Raf (26, 27). In addition, Rap1 activation by cAMP or a constitutively active form produced by point mutation of Rap1 inhibits Raf-1 activity (22). However, in several other cell types, activation of Rap1 by cAMP leads to activation of MAPK signaling (25, 28). The protein responsible for Rap1-dependent MAPK activation has been characterized in PC12 cells, in which cAMP stimulates MAPK signaling. In these cells, B-Raf is directly activated by Rap1 and activates the MAPK kinase (MEK), bypassing the negative effect of Rap1 on Raf-1 activation (28). There is a strong correlation between B-Raf expression and MAPK activation or inhibition in several cell types. Thus, for example, neurons express B-Raf, and in these cells cAMP stimulates MAPK activity; however, in astrocytes, in which B-Raf is not expressed, cAMP inhibits MAPK activity (29). Furthermore, constitutive overexpression of B-Raf in an astrocytoma cell line, in which cAMP inhibits MAPK, renders these cells sensitive to MAPK activation by cAMP (29). Thus, B-Raf expression is necessary for and confers cAMP-dependent stimulation of MAPK in several cell types, but has not been examined in AtT-20 corticotrophs.

In this work we analyze the transduction pathways involved in NurRE-dependent transcriptional activation by CRH and cAMP in AtT-20 corticotrophs. We demonstrate that the CRH/cAMP signaling that leads to Nur77/Nurr1 mRNA induction and transcriptional activation, and thus POMC expression, is dependent on PKA and involves calcium/calmodulin kinase II (CAMKII) (Nur induction/activation) and MAPK calcium-dependent and -independent (Nur phosphorylation-activation) pathways.

RESULTS

Calcium Involvement in CRH and cAMP Regulation of Nur77 and Nurr1 mRNA Expression and Transcriptional Activity

Treatment with nifedipine (NIF), a specific L-type calcium channel inhibitor, blunted CRH-dependent stimulation of the POMC promoter (Fig. 1A), in agreement with the known fact that calcium is involved in CRH-induced POMC expression (13). NIF treatment also blocked the NurRE-dependent transcriptional increase induced by CRH but only partially inhibited cAMP stimulation (Fig. 1B). Another physiological stimulus of the cAMP/PKA pathway in the pituitary, noradrenaline (NA), was also tested. NA stimulation of NurRE activity was blocked by NIF, and simultaneous CRH+NA action was, as was the case for cAMP, only partially reversed by NIF (Fig. 1B). In all treatments, a 10-fold higher concentration of NIF produced the same partial inhibition (data not shown). Forced calcium entry (by depolarization with KCl), also led to an increase in NurRE activation, and this effect was potentiated by a calcium channel agonist, FPL64176 (Fig. 1B).

These results indicate that calcium is necessary for NurRE-dependent transcription by CRH. They also reveal a calcium-independent stimulation at the NurRE site, as evidenced by CRH+NA or cAMP treatment. In addition, they show that calcium entry through L-type channels is sufficient to activate NurRE-directed transcription.

We next examined whether this stimulation of NurRE-dependent transcriptional activity and calcium dependency is exerted at the level of Nur77 and Nurr1 mRNA expression. As shown in Fig. 2A, Nur77 and Nurr1 mRNA regulation correlate with NurRE-dependent transcriptional activity. CRH stimulation of Nur77 and Nurr1 mRNA was inhibited by NIF, and cAMP stimulation of Nur77 and Nurr1 mRNA was only partially inhibited (Fig. 2A). Calcium entry by depolarization with KCl was sufficient to induce Nur77 mRNA but not Nurr1 mRNA (Fig. 2A). The L-type calcium channel agonist FPL64176 increased the stimulation of Nur77 but did not modify Nurr1 expression (Fig. 2A).

These data show that calcium participates in Nur77/Nurr1 mRNA induction by CRH and is sufficient for Nur77 induction.

To establish whether CRH, cAMP, and calcium regulation act at the Nur77 promoter, we transfected cells with a promoter Nur77-chloramphenicol acetyltransferase (CAT) construction. As expected from the above results, NIF treatment blocked CRH stimulation but only partially inhibited that of cAMP (Fig. 2B),

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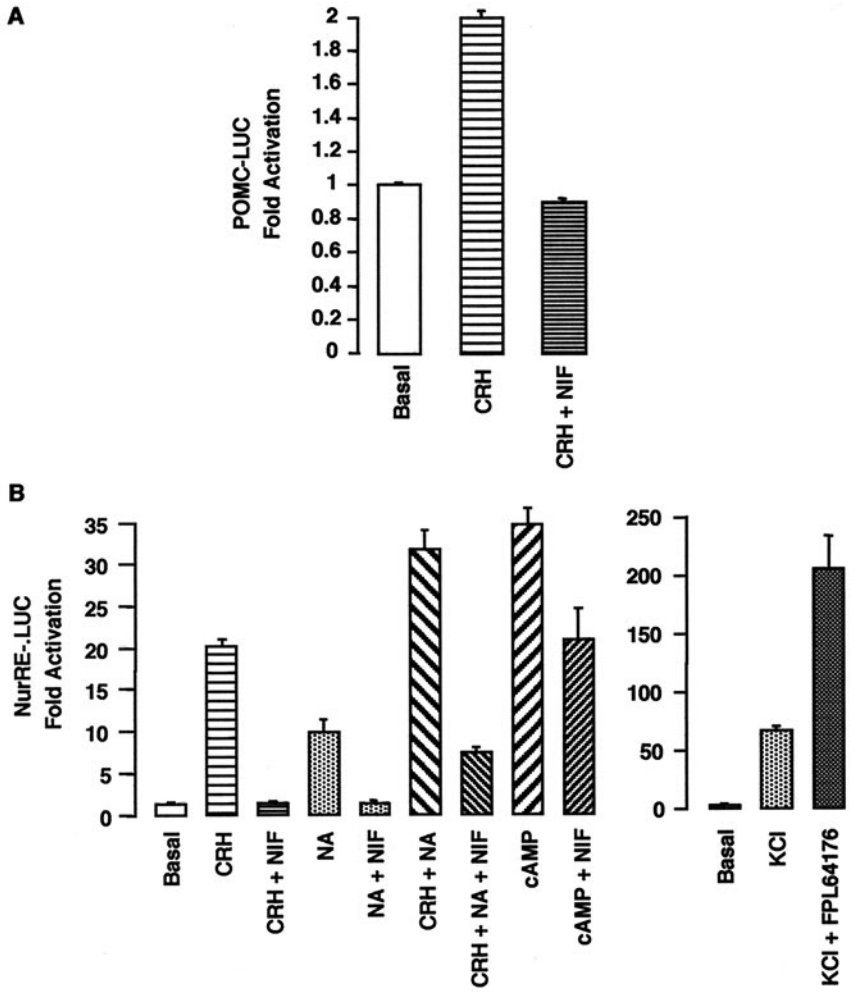

Fig. 1. Calcium-Dependent and -Independent Stimulation of NurRE by CRH and cAMP

A, AtT-20 cells were transfected with 500 ng of POMC-LUC plus 200 ng of RSV-β-gal. After 18 h in serum-free DMEM, cells were stimulated with 100 nM CRH or CRH plus 1 μM NIF for 6 h. Each value was normalized to β-gal activity, and results are expressed as mean $\pm$ SE (n = 4) of one representative of three independent experiments with similar results. B, AtT-20 cells were transfected with 500 ng of NurRE-LUC plus 200 ng of RSV-β-gal. After 18 h in serum-free DMEM, cells were stimulated as indicated with 100 nM CRH; 500 μM cAMP; 1 μM NA; 1 μM NIF; 36 mM KCl or KCl plus 2 μM of the L-type calcium channel agonist FPL64176 for 6 h. Each value was normalized to β-gal activity, and results are expressed as mean $\pm$ SE (n = 4) of one representative of four independent experiments with similar results. NIF was added 15 min before CRH, cAMP, or NA.

indicating that calcium regulation is exerted at the transcriptional level.

Protein Kinase Dependence of CRH and cAMP Regulation of NurRE-Dependent Transcriptional Activity and Nur77/Nurr1 mRNA Expression

We next analyzed protein kinase involvement in CRH and cAMP actions by using pharmacological inhibitors of CAMKII: KN62; PKA: H89; and adenylate cyclase: MDL12330A. CRH stimulation of NurRE-dependent transcription was inhibited by all three compounds (Fig. 3A). cAMP stimulation of NurRE-dependent transcription was completely blocked by the PKA inhibitor but only partially inhibited by the CAMKII inhibitor (Fig. 3B). The dependence on PKA was further confirmed by the inhibition of CRH or cAMP stimulation of Nur-RE by the expression of a dominant negative mutant of PKA (Fig. 3, A and B). Simultaneous treatment with the CAMKII inhibitor and NIF produced the same partial degree of inhibition as either treatment on its own (Fig. 3B). A 10-fold higher concentration of the CAMKII inhibitor produced the same partial inhibition (data not shown). The absence of synergistic or additive effects between these inhibitors suggests that they are blocking the same pathway, and that calcium exerts its effects through CAMKII. The existence of a calcium-independent pathway under cAMP stimulation is also confirmed. Concordant with the above mentioned effects on transcriptional activity, CRH induction of Nur77 is inhibited by the PKA and CAMKII inhibitors, and Nurr1 mRNA induction is inhibited by the

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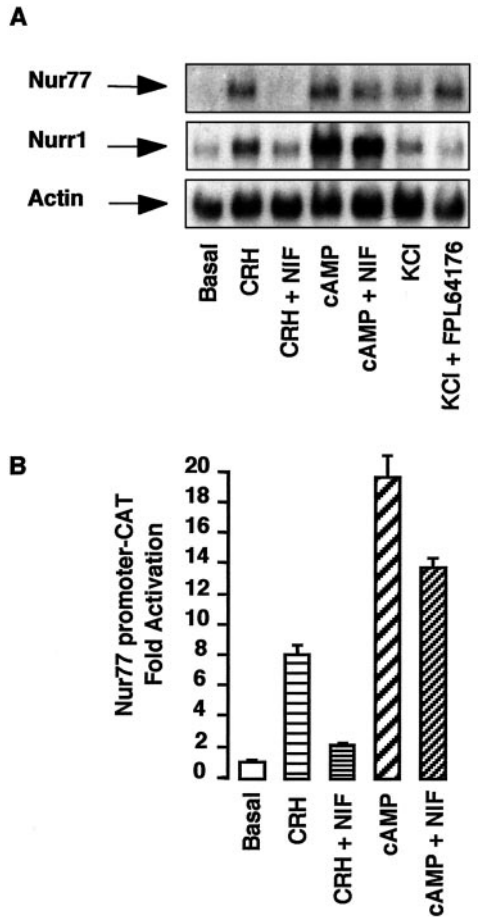

Fig. 2. Calcium-Dependent and -Independent Stimulation of Nur77 and Nurr1 mRNA Expression and Nur77 Promoter Regulation by CRH and cAMP

A, Northern blot analysis of Nur77 and Nurr1 mRNA in AtT-20 cells under different conditions: basal; 100 nM CRH; CRH plus 1 μM NIF; 500 μM cAMP; cAMP plus 1 μM NIF (added 15 min before CRH or cAMP); 36 mM KCl; and KCl plus 2 μM of the L-type calcium channel agonist FPL64176. After a 1-h stimulation, total mRNA was extracted and Northern blot analysis was performed. Nur77, Nurr1, and β-actin single bands were obtained; one of four independent experiments with similar results is shown. B, AtT-20 cells were transfected with 500 ng of Nur77promoter-CAT plus 200 ng of RSV-β-gal. After 18 h in serum-free DMEM, cells were stimulated with CRH (100 nM); CRH plus 1 μM NIF; 500 μM cAMP; or cAMP plus 1 μM NIF for 6 h. The mean $\pm$ SE CAT activities relative to β-gal levels are shown (n = 4 of one representative of two independent experiments with similar results).

CAMKII inhibitor (Fig. 3C). cAMP induction of Nur77 and Nurr1 mRNA is inhibited by the PKA inhibitor and only partially blocked by the CAMKII inhibitor (Fig. 3C).

MAPK Involvement in NurRE-Dependent Transcriptional Activation by CRH and cAMP

In addition to directly stimulating PKA and downstream targets, cAMP also regulates the MAPK pathway: depending on the cell type, cAMP may activate or inhibit MAPK (25–30). Thus, we analyzed MAPK involvement in CRH and cAMP-stimulated NurRE-dependent transcription. Treatment with the MEK inhibitors, PD98059 or UO126, inhibited CRH or cAMP stimulation of NurRE (Fig. 4), and the combined action of PD98059 and NIF produced a stronger inhibition of cAMP stimulation (Fig. 4). A 5-fold higher dose of PD98059 also resulted in a partial inhibition (data not shown). CRH or cAMP stimulation of NurRE-dependent transcription was also inhibited by a dominant negative mutant of MEK (Fig. 4). Furthermore, the transcriptional activity exerted by the overexpression of Nur77 was further stimulated by coexpression of a constitutively active form of MEK (Fig. 4). Different doses of a dominant negative form of Ras did not affect CRH or cAMP stimulatory action (not shown).

These results demonstrate that MAPK activity in corticotrophs mediates in part the NurRE-dependent transcriptional increases caused by CRH and cAMP. According to these results, MAPK activation may therefore account for the calcium-independent stimulation of NurRE by cAMP, as analyzed in further detail in the next section.

Characterization of the MAPK Cascade in AtT-20 Corticotrophs

To examine the activation of the MAPK transduction pathway by CRH and cAMP in corticotrophs, we studied their ability to activate Elk-1, a transcription factor activated by the ERK pathway. Cells were cotransfected with a Gal4 reporter plasmid and an expression vector for the chimeric Gal4 DNA binding domain fused to the transactivation Elk1 domain (Gal4-Elk1). CRH and cAMP increased Gal4-Elk1 activity (Fig. 5A); this increase was blocked by treatment with the MEK inhibitors PD98059 or UO126 or by expressing a dominant negative protein for MEK or Rap1b (RapN17) (Fig. 5A), which has been shown to block Rap1 effects (28, 31). The constitutive active form of Rap1b (RapG12V) stimulated Elk1 transcription (Fig. 5A). The presence of B-Raf has been shown to be necessary for MAPK activation by Rap1 in PC12 and other cell lines (28, 29, 32). We thus examined the expression of B-Raf mRNA in AtT-20 corticotrophs. We detected a single specific band by Northern blot, demonstrating constitutive expression of B-Raf (Fig. 5A, *inset*). Overexpression of B-Raf protein enhanced the CRH stimulatory action on Gal4-Elk1 (Fig. 5A).

In correlation with the increased Elk1 transactivation, CRH and cAMP stimulated ERK2 kinase activity and phosphorylation of ERK1 and ERK2 (Fig. 5B). Similar amounts of ERK2 protein were detected for each condition (Fig. 5B). cAMP stimulated ERK2 activity at 2 min but not at 5 or 10 min, a kinetic similar to that recently reported for cAMP treatment of GH3 cells (33). These results show that CRH and cAMP stimulation of the MAPK pathway in corticotrophs involves Rap1, MEK, and ERK2 activities.

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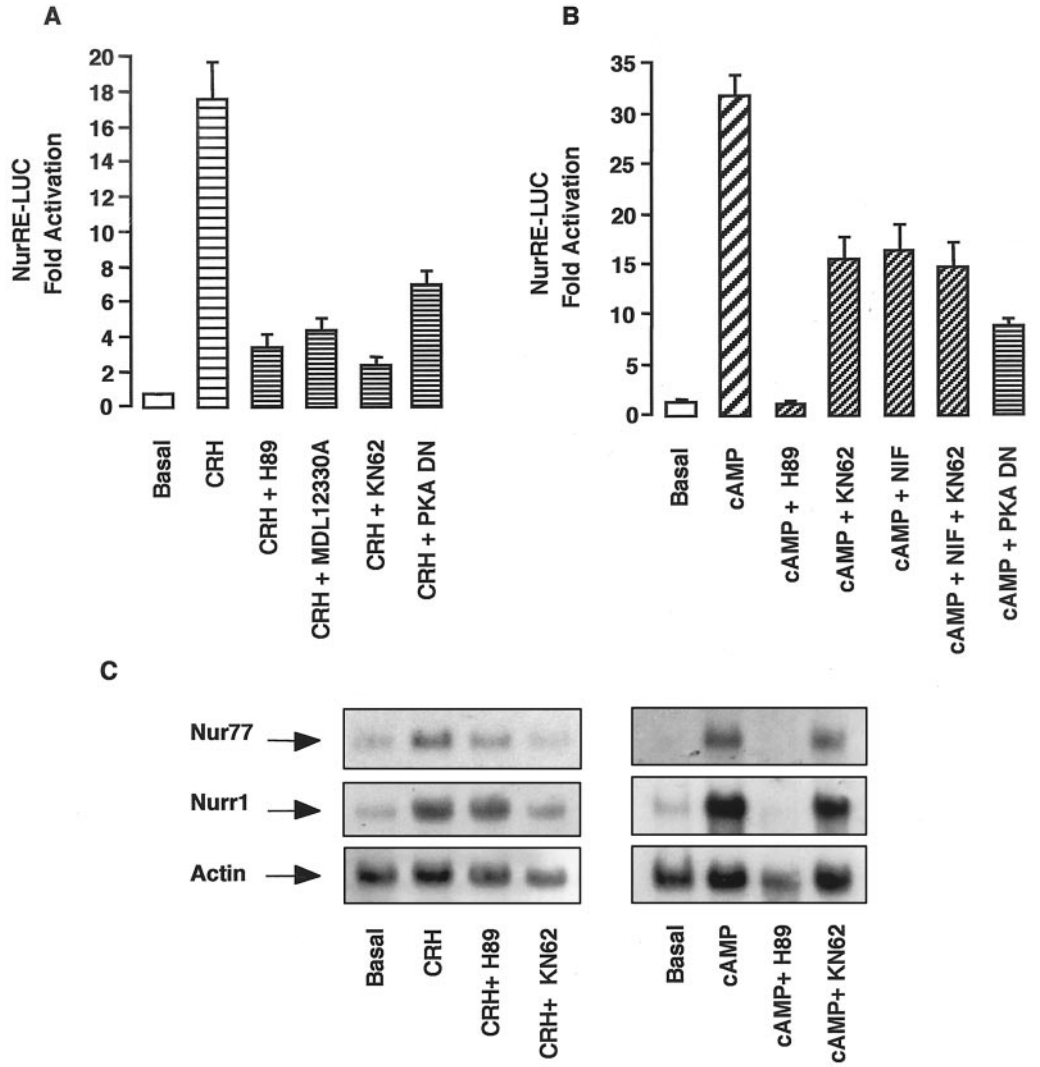

Fig. 3. PKA, CAMKII, and Calcium Dependence of CRH and cAMP Stimulation of NurRE-Directed Transcription and Nur77/Nurr1 mRNA Expression

A and B, AtT-20 cells were transfected with 500 ng of NurRE-LUC plus 200 ng of RSV-β-gal. After 18 h in serum-free DMEM, cells were treated with 10 μM PKA inhibitor, H89; 10 μM adenylate cyclase inhibitor, MDL; 10 μM CAMKII inhibitor, KN62, 1 μM NIF; or combinations where indicated. After 15 min, cells were stimulated with 100 nM CRH (panel A) or 500 μM cAMP (panel B) for 6 h. Where indicated, cells were transfected with 300 ng of PKA DN (dominant negative mutant of PKA). Each value was normalized to β-gal activity, and results are expressed as mean $\pm$ SE (n = 4) of one representative of four independent experiments with similar results. C, Northern blot analysis of Nur77 and Nurr1 mRNA in AtT-20 cells after 1-h stimulation with: 100 nM CRH; CRH plus 10 μM PKA inhibitor, H89; CRH plus 10 μM CAMKII inhibitor, KN62; 500 μM cAMP; cAMP plus 10 μM PKA inhibitor, H89; cAMP plus 10 μM CAMKII inhibitor, KN62. After 1 h stimulation, total mRNA was extracted and Northern blot analysis performed. Nur77, Nurr1, and β-actin single bands were obtained; one of four independent experiments with similar results is shown.

To establish whether activation of Rap1 by CRH and cAMP is involved in the activation cascade that stimulates NurRE, we analyzed CRH and cAMP stimulation of NurRE-dependent transcription in the presence of RapN17 and RapG12V. RapN17 expression inhibited NurRE activation by CRH and cAMP, whereas RapG12V potentiated CRH but not cAMP stimulation (Fig. 5C). A higher RapN17 concentration produced no greater inhibition of cAMP stimulation (data not shown). This result is in concordance with the lower activation of MAPK exerted by CRH with respect to cAMP (Fig. 5, A and B); under a lower stimulation level of MAPK, RapG12V may increase the stimuli because the transduction pathway is not fully activated. RapN17 and RapG12V effects were highly specific insofar as: 1) these plasmids did not affect basal or IL-1-induced expression of kB-luciferase (LUC) reporter, a nuclear factorκB responding element (data not shown), ruling out nonspecific or toxic effects, and 2) overexpression of a dominant negative mutant of cAMP response element binding protein (CREB S133A) did not affect NurRE stimulation by CRH or

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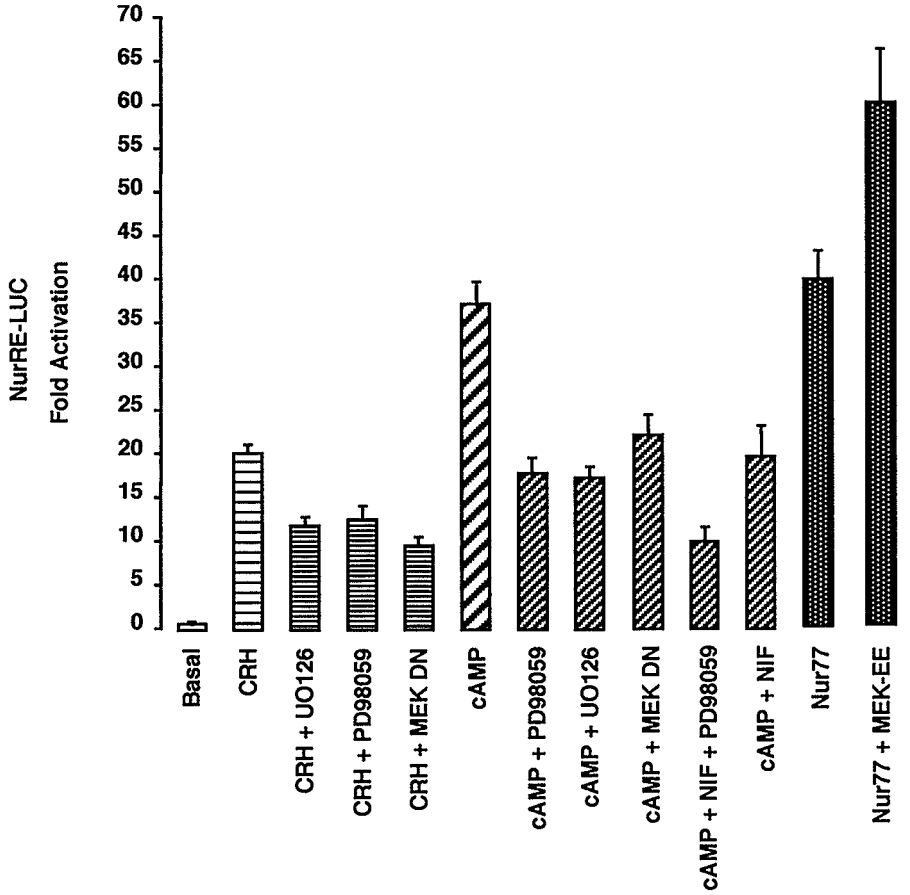

Fig. 4. MAPK Dependence of CRH and cAMP Stimulation of NurRE-Directed Transcription and Nur77

AtT-20 cells were transfected with 500 ng of NurRE-LUC plus 200 ng of RSV-β-gal. After 18 h in serum-free DMEM, cells were treated with the inhibitors of MEK, 50 μM PD98059, or 10 μM UO126; 1 μM NIF; or combinations when indicated. After 15 min, cells were stimulated with 100 nM CRH or 500 μM cAMP for 6 h. Where indicated, cells were transfected with 300 ng of MEK DN or the expression vector of Nur77 (Nur77) or 30 ng of MEK-EE. MEK-EE treatment alone did not increase NurRE activation (data not shown). Each value was normalized to β-gal activity, and results are expressed as mean $\pm$ SE (n = 4) of one representative of four independent experiments with similar results.

cAMP (data not shown), confirming the specificity of the RapN17 action. These data indicate that Rap1 is involved in the MAPK pathway leading to NurRE activation in corticotrophs.

The PKA inhibitor completely blocked cAMP effects on Gal4-Elk1 transcriptional activation, but both NIF and the CAMKII inhibitor, as was the case for NurRE-LUC activity, only partially inhibited the stimulation by cAMP (Fig. 5D). NIF treatment or CAMKII or PKA inhibition almost completely blocked CRH stimulation (Fig. 5D). These results show that activation of MAPK in AtT-20 cells is fully dependent on PKA activity, that calcium contributes to ERK activity and that cAMP activates the pathway also independently of calcium.

Direct MAPK Regulation of Nur77 Transactivation and Phosphorylation

Inhibition of MEK only slightly inhibited Nur77 and Nurr1 mRNA induction by cAMP and not by CRH (Fig. 6A). Thus, MAPK-dependent stimulation of NurRE by CRH is most probably exerted at a level other than that of Nur77 or Nurr1 expression. To determine whether stimulation of NurRE-dependent transcription by CRH, cAMP, and MAPK is exerted by direct regulation of Nur77 transactivation, we developed a chimeric Gal4-Nur77 construct and cotransfected cells with the Gal4 reporter plasmid. Both CRH and cAMP slightly but consistently increased Gal4-Nur77 basal transcriptional activity in AtT-20 cells (Fig. 6B). Inhibition of MEK dramatically reduced basal transactivation activity of the Gal4-Nur77 protein and blocked CRH and cAMP stimulation (Fig. 6B). The absence of action of the MEK inhibitor PD98059 on constitutive β-galactosidase (β-gal) expression (data not shown) and on CRH-induced Nur77/Nurr1 or Actin mRNA (Fig. 6A) assures the specificity and absence of toxicity of the effect with this inhibitor. These results demonstrate that the regulation on NurRE activity by CRH and cAMP is controlled not only by induction of Nur77 and Nurr1 mRNAs but also by regulation of Nur77 trans-

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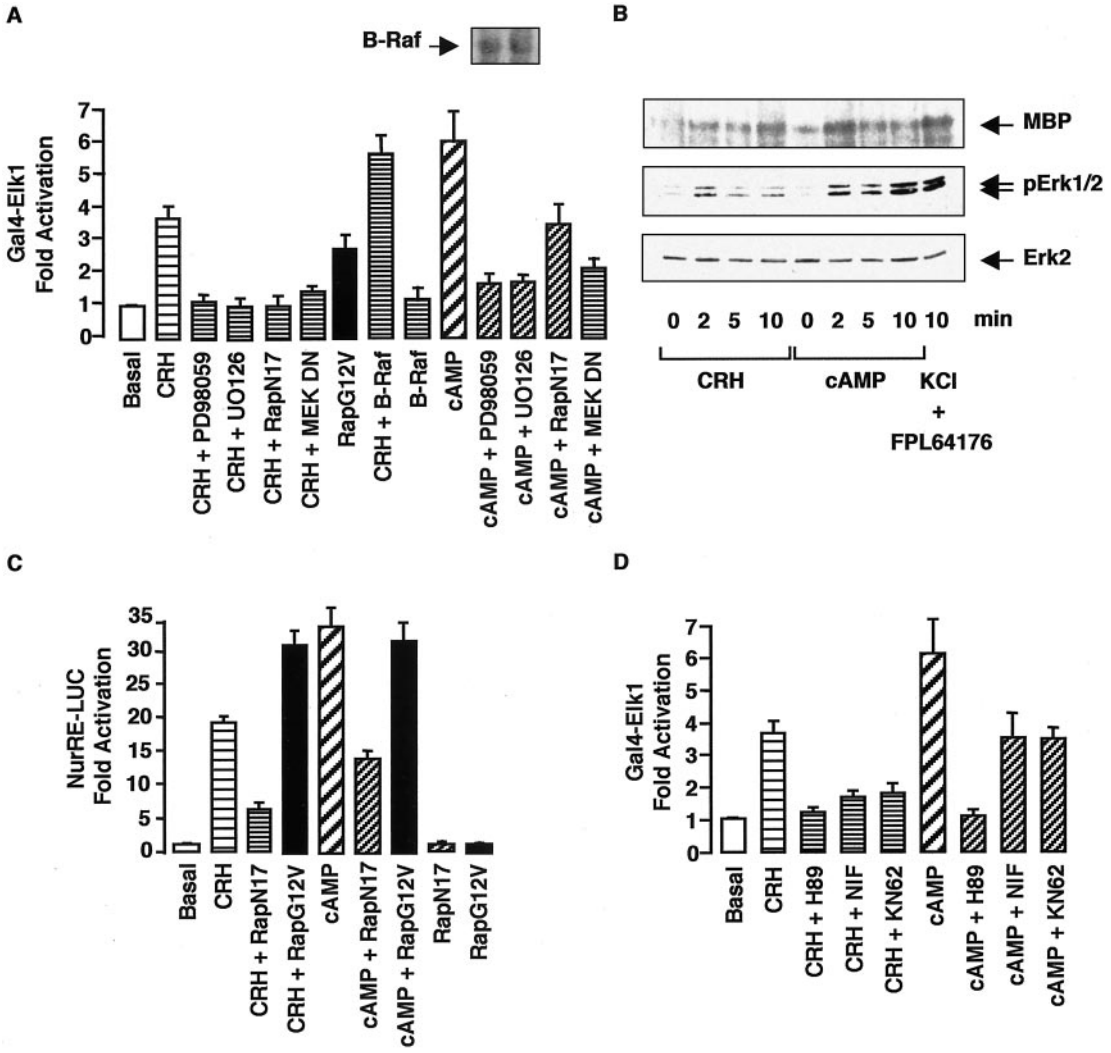

Fig. 5. CRH and cAMP Stimulate the MAPK Pathway in AtT-20 Cells

A, AtT-20 cells were transfected with 500 ng of pG5-LUC, 300 ng of Gal4-Elk1, plus 200 ng of RSV-β-gal. Where indicated, cells were transfected with 300 ng of RapN17, RapG12V, MEK DN, or B-Raf expression vector. After 18 h in serum-free DMEM, cells were left untreated or treated with 100 nM CRH or 500 μM cAMP in the absence or presence (15 min before) of the inhibitors of MEK, 50 μM PD98059, or 10 μM UO126 for 6 h. Each value was normalized to β-gal activity, and results are expressed as mean $\pm$ SE (n = 4) of one representative of five independent experiments with similar results. *Inset*, Northern blot analysis of B-Raf mRNA in unstimulated AtT-20 cells. AtT-20 cells were allowed to stand 18 h in serum-free medium, after which the mRNA was extracted and Northern blot analysis was performed in duplicate to detect basal expression of B-Raf; a single specific band was obtained. B, AtT-20 cells were stimulated with 100 nM CRH, 500 μM cAMP, or KCl plus the 2 μM L-type calcium channel agonist FPL64176 for different stimulation times as indicated. Cellular extracts were immunoprecipitated with the anti-ERK2 antibody, and the kinase assay was performed as detailed in *Materials and Methods*. *In vitro* phosphorylation of the MBP (substrate) is shown for each condition. Western blot analysis of phosphorylated ERK1 and ERK2 (pErk1/2) and total ERK2 protein levels are shown for each condition. One of three independent experiments with similar results is shown. C, AtT-20 cells were transfected with 500 ng of NurRE-LUC plus 200 ng of RSV-β-gal. Where indicated, cells were transfected with 300 ng of RapN17 or RapG12V. After 18 h in serum-free DMEM, cells were left untreated or treated with 100 nM CRH or 500 μM cAMP for 6 h. Each value was normalized to β-gal activity, and results are expressed as mean $\pm$ SE (n = 4) of one representative of four independent experiments with similar results. D, AtT-20 cells were transfected with 500 ng of pG5-LUC, 300 ng of Gal4-Elk1, plus 200 ng of RSV-β-gal. After 18 h in serum-free DMEM, cells were treated, as indicated, with 1 μM NIF; 10 μM PKA inhibitor, H89; 10 μM CAMKII inhibitor, KN62. After 15 min, cells were stimulated with 100 nM CRH or 500 μM cAMP for 6 h. Each value was normalized to β-gal activity, and results are expressed as mean $\pm$ SE (n = 4) of one representative of three independent experiments with similar results.

activation and that the MAPK pathway is involved in both constitutive and CRH- or cAMP-stimulated transactivation activity of Nur77.

MAPK has been shown to regulate transcriptional activity by phosphorylation of the transactivation domain of different transcription factors (*e.g*. JNK/c-Jun,

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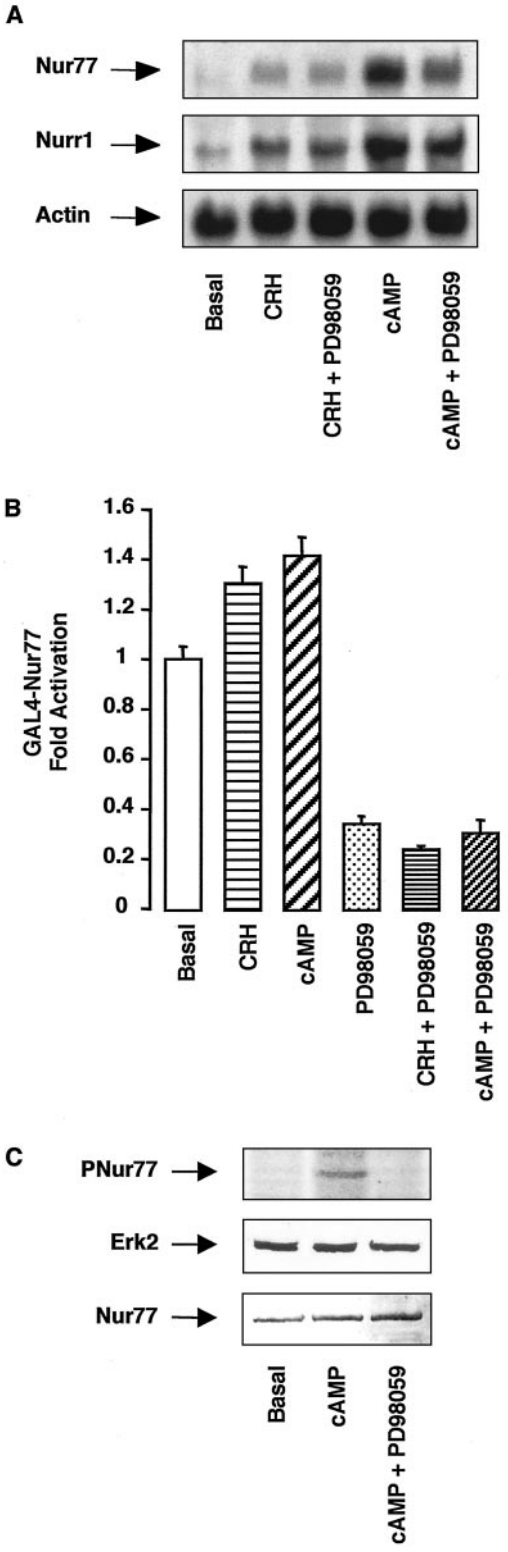

Fig. 6. MAPK Phosphorylates and Stimulates Nur77 Transactivation

A, Northern blot analysis of Nur77 and Nurr1 mRNA in AtT-20 cells: basal; 100 nM CRH; CRH plus 50 μM of the MEK inhibitor, PD98059; 500 μM cAMP; cAMP plus 50 μM PD98059. After 1-h stimulation, total mRNA was extracted and Northern blot analysis was performed. Nur77, Nurr1, and β-actin single bands were obtained; one of three independent experiments with similar results is shown. Similar results were obtained with UO126 (10 or 20 μM). B, AtT-20 cells were transfected with 500 ng of pG5-LUC, 300 ng of Gal4-Nur77, plus 200 ng of RSV-β-gal. After 18 h in serum-free DMEM, cells were left untreated or treated for 6 h with 100 nM CRH or 500 μM cAMP in the absence or presence (15 min before) of 50 μM of the MEK inhibitor (inhMEK) PD98059. Each value was normalized to β-gal activity, and results are expressed as mean $\pm$ SE (n = 4) of one representative of three independent experiments with similar results. C, AtT-20 cells were unstimulated or stimulated with 500 μM cAMP or cAMP plus 50 μM PD98059 for 10 min, cellular extracts were immunoprecipitated with the anti-ERK2 antibody, and *in vitro* phosphorylation of Nur77 (PNur77) assay was performed with substrate immunoprecipitated from AtT-20 cells transfected with Gal4-Nur77, as detailed in *Materials and Methods*. Western blot analysis of Gal4-Nur77 (Nur77) and total ERK2 (Erk2) are shown for each condition as control for protein load. No PNur77 band was observed in the absence of Gal4-Nur77 immunoprecipitates or when a Gal4 empty plasmid (without Nur77) was used instead of the Gal4-Nur77 construct. One of three independent experiments with similar results is shown.

p38/ATF-2). Immunoprecipitated Nur77 was phosphorylated by immunoprecipitated ERK2, showing the direct phosphorylation of Nur77 by ERK2 (Fig. 6C).

Calcium, PKA, and MAPK Regulate the POMC Promoter

To establish whether NurRE-dependent transcriptional regulation by different protein kinases, including MAPK, also occurs in the context of the POMC promoter, we transfected AtT-20 cells with the POMC-LUC plasmid and tested the pharmacological inhibitors and dominant negative mutants on CRH and cAMP stimulation. Similar to their effect on NurRE-dependent transcription, the PKA and CAMKII inhibitors blocked POMC promoter activation by CRH, whereas the MEK inhibitors had only partial inhibitory effects (Fig. 7A). cAMP stimulation of the promoter was completely blunted by the PKA inhibitor and partially inhibited by the MEK and CAMKII inhibitors, although the latter had a much stronger effect than the former (Fig. 7B). The expression of the dominant negative mutant for MEK or RapN17 inhibited both CRH and cAMP stimulation of the POMC promoter (Fig. 7, A and B). Moreover, the constitutively active Rap1 (RapG12V) or MEK (MEK-EE) further increased the stimulation of POMC by Nur77, supporting the functional relevance of this pathway (Fig. 7B). These results confirm that POMC regulation by PKA, CAMKII, and MAPK parallels that of Nur77/Nurr1 expression and transcriptional activation at the NurRE site.

DISCUSSION

Our results show that in AtT-20 corticotrophs two main signal transduction pathways are involved down-

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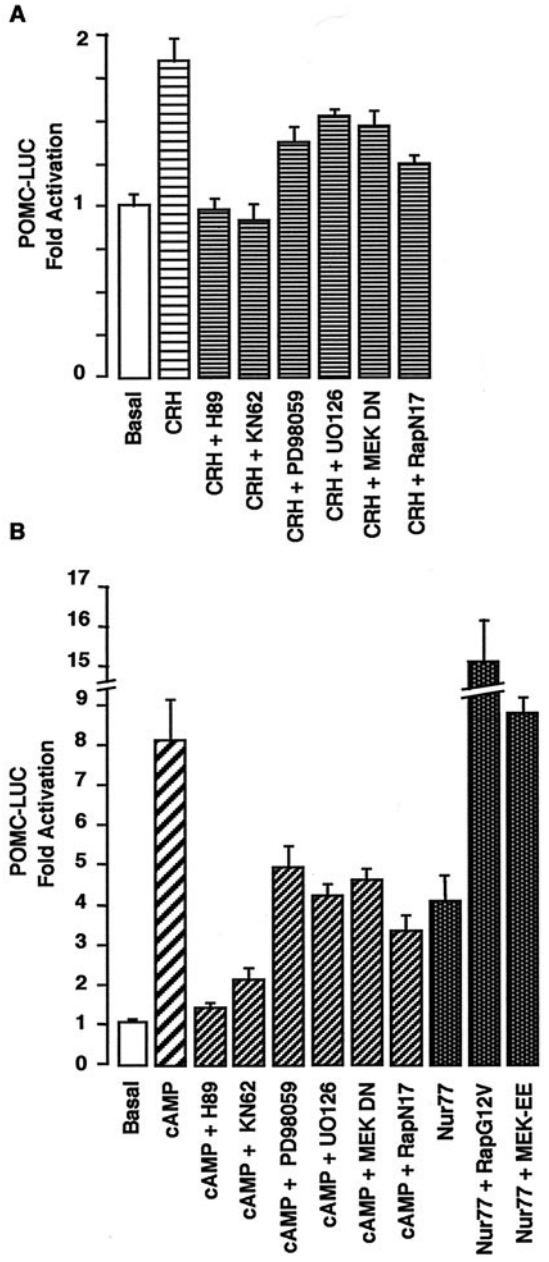

Fig. 7. PKA, CAMKII, and MEK Dependence of CRH and cAMP Stimulation of the POMC Promoter

A and B, AtT-20 cells were transfected with 500 ng of POMC-LUC plus 200 ng of RSV-β-gal. Where indicated, cells were transfected with 300 ng of a dominant negative mutant of MEK (MEK DN), RapN17, RapG12V, or the expression vector of Nur77 (Nur77) or 30 ng of MEK-EE. After 18 h in serum-free DMEM, cells were treated with 10 μM of the CAMKII inhibitor, KN62; 10 μM PKA inhibitor, H89; or the inhibitors of MEK, 50 μM PD98059 or 10 μM UO126, as indicated. After 15 min, cells were stimulated with 100 nM CRH (panel A) or 500 μM cAMP (panel B) for 6 h. Each value was normalized to β-gal activity, and results are expressed as mean $\pm$ SE (n = 4) of one representative of five independent experiments with similar results.

stream of PKA activation by cAMP and CRH (Fig. 8). One of these pathways is dependent on calcium entry through calcium-dependent voltage channels at the plasma membrane, involves CAMKII, and regulates Nur77 and Nurr1 induction and activity. The other pathway is independent of CAMKII and is induced by CRH/NA or cAMP stimulation. Both involve a MAPK pathway that is activated by CRH and cAMP, is dependent on PKA action, and involves activation of the small G protein Rap1 and B-Raf. Activation of MAPK stimulates the POMC promoter by regulating Nur77 transactivation and not Nur77 and Nurr1 mRNA induction.

In endocrine cells cAMP actions may be dependent or independent of PKA (34). We show that In AtT-20 cells cAMP and CRH actions on Nur77/POMC, including the activation of MAPK, are dependent on PKA.

We demonstrate that Nur77 induction by CRH is dependent on calcium entry, and this effect is exerted at the level of the Nur77 promoter. The Nur77 promoter possesses no consensus cAMP response element sites that could be responsive to cAMP-PKA and calcium-CAMKII stimuli. The regulation of the Nur77 promoter in T cells by calcium involves two MEF2 sites located at positions -295 and -255 bp (35–37) and NFAT sites (37). Calcium-independent regulation also exists and involves different positions on the promoter. In contrast, the Nurr1 promoter contains consensus cAMP response element sites (38) that may confer both cAMP and calcium sensitivity.

The calcium-independent mechanism for Nur77/Nurr1 activation in AtT-20 cells can be shown with either CRH/NA or cAMP treatment. The fact that NA is necessary may be related to cAMP levels or PKA activation. cAMP levels are increased only 2-fold by CRH (39), and this level may not be sufficient for stimulation of POMC expression and Nur77/Nurr1 activation in the absence of calcium. Both calcium-dependent and -independent cAMP actions in AtT-20 cells have been demonstrated using specific protein phosphatases blockers (39). Furthermore, AtT-20 cells possess two isoforms of the PKA-regulatory subunits, type I and type II, the latter being the most abundant. Although cAMP treatment stimulates both isoforms, CRH only activates the type I isoform (9, 40). Thus, different PKA activation levels obtained by CRH, CRH/NA, or cAMP in AtT-20 cells may explain why the calcium-independent pathway is evidenced by cAMP or CRH/NA.

CRH- or cAMP-induced NurRE activation involves the MAPK signaling pathway, as demonstrated using dominant negative mutants of Rap1 or MEK, overexpression of MEK, or the MEK inhibitors PD98059 and UO126. Similarly, by using a Gal4-Elk1 transcription factor and ERK kinase assays, we show that MAPK activity is increased by CRH and cAMP treatments and depends on Rap1 activation. In addition, MAPK regulation of NurRE-directed transcription does not depend on induction of Nurr1 and Nur77 mRNA but on an increased transactivational activity of Nur77.

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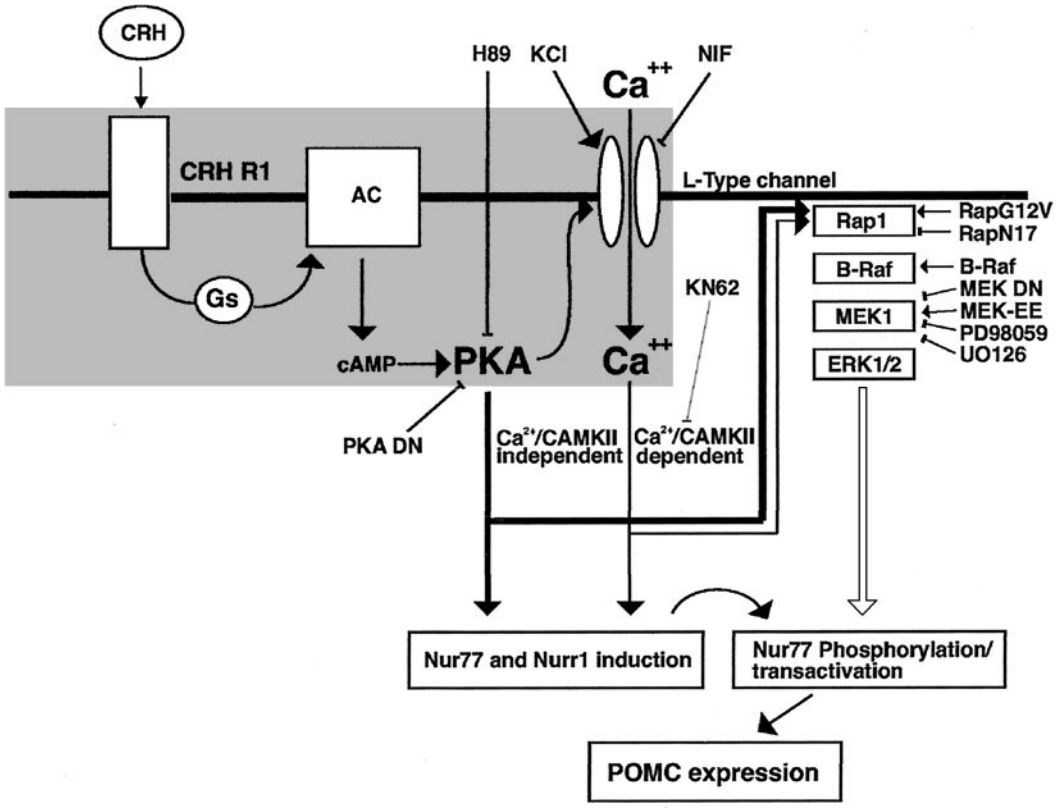

Fig. 8. Pathways Involved in CRH and cAMP Induction and Activation of Nur Factors and POMC Expression

PKA activation by CRH and cAMP activates two main transduction pathways: one of these depends on calcium entry at the plasmatic membrane and involves CAMKII activity; the other pathway is calcium independent. The MAPK pathway is also activated by CRH and cAMP by calcium-dependent and -independent mechanisms and involves Rap1, B-Raf, MEK, and ERK activities. These different transduction pathways regulate POMC expression by different mechanisms: 1) calcium-dependent and -independent induction of Nur77 and Nurr1, which does not involve MAPK activity; and 2) a MAPK stimulation of transcription and activity of Nur77. The different points at which inhibitors act are indicated. H89: PKA inhibitor; KN62: CAMKII inhibitor; NIF: L-type Ca^{2+} channel antagonist; PD98059 and UO126: MEK inhibitors; RapN17 (dominant negative form of Rap1), RAPG12V (Rap1 expression vector), B-Raf (B-Raf expression vector), MEK DN, MEK-EE, and PKA DN. The *shaded area* corresponds to the pathway described previously for POMC-ACTH, which acts also for Nur factors, as we have demonstrated. The *nonshaded area* corresponds to new pathways described in this study for Nur factors and POMC transcription.

PKA-dependent and -independent activation of Rap1 by cAMP has been described in several models (21, 41, 42); our results show that in AtT-20 cells such activation is PKA dependent. B-Raf expression in AtT-20 cells is instrumental for cAMP-MAPK activation because it is the link that allows activation of MAPK by Rap1 (28, 32, 42). We show that the expression of B-Raf increases the CRH stimulation of the MAPK pathway. In several types of cells cAMP induces phosphorylation of Rap by PKA and inhibition of Raf activation by Ras (26, 27). B-Raf presence and activity allow the cells to bypass the inhibitory effect of Rap1 on Raf-1 and, consequently, on MAPK activity. B-Raf expression is cell type specific, and a correlation between stimulation of MAPK by cAMP and B-Raf presence can be drawn (32). In neurons that express B-Raf, cAMP treatment stimulates MAPK activity, whereas in astrocytes that lack B-Raf expression, cAMP treatment inhibits MAPK activity (29). Furthermore, constitutive expression of B-Raf in astrocytoma cells renders these cells sensitive to cAMP/Rap1-dependent stimulation of MAPK (29). Using the constitutive active form of Rap1 (RapG12V), we demonstrate that in AtT-20 cells the pathway linking Rap1 activation to ERK and increased Elk1 transcriptional activity exists and may be attributed to B-Raf presence, as was previously seen in PC12 cells (28). Furthermore, we show that RapG12V can activate basal Elk1 transcriptional activity and ERK activity, but does not stimulate the NurRE-LUC construct under basal conditions. This result is consistent with the fact that MAPK activity, in contrast to calcium and cAMP, does not increase Nur77 or Nurr1 expression levels, and in the absence of Nur77 protein no regulation by MAPK can be seen.

We show that in AtT-20 corticotrophs B-Raf is expressed, and using pharmacological (PD98059, UO126), biochemical (kinase and Nur77 phosphorylation assays), and molecular (Gal4-Elk1, RapN17, MEK dominant negative, and MEK expression vec-

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tors) tools, we demonstrate MAPK (Rap1/B-Raf/MEK/ERK1/2) induction by CRH and cAMP in those cells.

The studies with NIF show that downstream of PKA stimulation by CRH and cAMP, MAPK activation in AtT-20 cells involves not only calcium-dependent but also calcium-independent pathways, which may be particularly important for the calcium-independent stimulation of NurRE.

In addition to MAPK calcium-independent actions, it has been shown that in PC12 cells, MAPK activation by nerve growth factor is inhibited by an inhibitor of calmodulin, and that this effect is due to blockage of Raf-1 and B-Raf kinase activity (43). Calcium-dependent and -independent phosphorylation of CREB has also been described in PC12 cells; in this case, too, calcium-dependent phosphorylation requires the activation of the Rap1-B-Raf-MEK-ERK pathway (44). Thus, common regulatory pathways for MAPK regulation by calcium-dependent and -independent transduction signals seem to exist in corticotrophs and neuronal cells.

MAPK regulates Nur77 transactivation but not its induction. Both the Gal4-Nur77 basal and stimulated activities were blunted when cells were treated with the MEK inhibitor, indicating that both basal and stimulated activities of Nur77 are controlled by MAPK. Moreover, there is a direct regulatory action exerted by phosphorylation of Nur77 by ERK-2. In fact, Nur77 possesses two ERK phosphorylation sites at positions 108 and 325.

Basal MAPK activity may depend on autocrine and paracrine factors present in the culture medium. Overexpression of Nur77 without further stimulation is capable of increasing transcription at the NurRE and NBRE sites (14), indicating that the overexpressed protein is, at least in part, transcriptionally active. Basal MAPK activity is probably necessary for transcriptional activity of NurRE, since treatment with the MEK inhibitor severely decreases the transactivation of Gal4-Nur77. In NIH 3T3 fibroblasts, activation of CREB is dependent on basal MAPK activity. In these cells, although the MAPK pathway (Raf-MEK-ERK) is not stimulated by forskolin treatment, inhibition of the basal MAPK pathway by a dominant negative Raf protein or the pharmacological inhibitor of MEK (PD98059) can inhibit transcription of CREB without affecting Ser133 phosphorylation (45).

In conclusion, we show that CRH and cAMP trigger an array of calcium-dependent and -independent transduction signals, including a MAPK kinase pathway (Fig. 8). These different signals combine to regulate Nur77 and Nurr1 expression and activity, resulting in a unified control of POMC expression. As very recently stated, cAMP acts by different pathways including one through which PKA impacts the Rap-Raf-MAPK pathway in certain cell types and stages of differentiation (34). Furthermore, it is possible that in some cells both PKA-mediated phosphorylation of CREB and phosphorylation by other protein kinases (3-phosphoinositide-dependent kinase 1, protein kinase B, or MAPK) may be necessary for full activation of transcription (34). In this paper we demonstrate that AtT-20 corticotrophs constitute one important endocrine cell in which this pathway is relevant for control of POMC expression by Nur factors.

MATERIALS AND METHODS

Materials

Materials and reagents, unless stated, were obtained from Seromed (Berlin, Germany), Life Technologies, Inc. (Karlsruhe, Germany), Flow (Meckenheim, Germany), Falcon (Heidelberg, Germany), Nunc (Wiesbaden, Germany), and Sigma (St. Louis, MO).

Cell Culture and Stimulation

AtT-20 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured as previously described (46) under standard culture conditions in DMEM (Life Technologies, Inc.), with 10% fetal calf serum (FCS) supplemented with glutamine and antibiotics. Cells were treated as indicated in each experiment with human/rat CRH (100 nM) (Bachem, Heidelberg, Germany), cAMP (500 μM) (chlorophenylthio-cAMP), or KCl (36 mM) plus the L-type calcium channel agonist FPL64176 (2 μM) (RBI, Natick, MA). When used, NIF (1 μM) and the inhibitors of protein kinases, MDL 12330 (10 μM) (RBI), KN-62 (10 μM) (RBI), H89 (10 μM) (Calbiochem, San Diego, CA), UO126 (10–20 μM) (Calbiochem), and PD98059 (50 μM) (Calbiochem) were added 15 min before other stimulatory treatment.

Plasmid Constructs

The plasmid constructs that we used were kindly provided and/or previously described: the NurRE-LUC, which contains three copies of NurRE sites coupled to the minimal POMC (−34/+63) promoter, and the Nur77 expression vector (pCMX-Nur77) were provided by Dr. Jacques Drouin (14, 19); the Nurr1 plasmid was obtained from Dr. Naganar Ohkura (47); the Nur77 promoter (−480) coupled to the CAT gene (Nur77promoter-CAT) came from Dr. Astar Winoto (35); the (770-bp fragment) POMC-LUC was obtained from Dr. Malcom Low (48); the Gal4-Elk1 expression vector and the B-Raf plasmid were provided by Dr. Phillip Stork (28); the expression vectors for RapN17 and RapG12V were supplied by Dr. Daniel Altschuler (49); the kB-LUC plasmid came from Dr. Michael Bell (50); the CREBS133A was provided by Dr. Beat Lutz (51); the MEK DN (dominant negative mutant of MEK) was supplied by Dr. Sivio Gutkind; the pBIND and pG5-LUC were obtained from Promega Corp. (Madison, WI); pMt-REV-PKA DN (dominant negative mutant of PKA) and pcDNA3 Ras N17, a dominant negative form of Ras and pcDNA3 MEK-EE, a constitutively active form of MEK, were described previously (52, 53). The Gal4-Nur77 plasmid was constructed as follows: the coding cDNA of pCMX-Nur77 plasmid was amplified with PCR using the CgCTCTAgAATgCCCTgTaTCCAAgCTC and ggggTACCTCAgAAAgACAAggTg primers. The fragment containing the coding Nur77 sequence was cloned using the *Nco*I (blunt) and *Kpn*I enzymes in-frame to the pBIND vector previously cut with *Eco*RV and *Kpn*I. Correct in-frame insertion was verified by sequencing.

Northern Blot

For Northern blot analysis, cells were plated on six-well plates, at a density of 400,000 cells per well in DMEM 10%

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FCS supplemented. After overnight attachment of cells, medium was changed for DMEM without serum and left overnight. After overnight washout, cells were treated with different stimuli. At the indicated times total RNA was isolated by the guanidine isothiocyanate-phenol-chloroform method. RNA extraction, denaturing gel electrophoresis, Northern blotting, preparation of radiolabeled probes, and hybridization procedures were performed as described previously (46). Briefly, 5- to 10-μg RNA samples, as measured at 260 nM, were denatured with glyoxal, electrophoresed on a 1.2% agarose gel, and transferred overnight to a Hybond-N nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The filters were baked for 2 h at 80 C, prehybridized for 4 h at 60 C, and then hybridized in the same solution (50% formamide, 5× sodium chloride/sodium phosphate/EDTA, 5× Denhardt's solution, 0.1% sodium dodecyl sulfate, 100 μg/ml denatured salmon sperm DNA) with the addition of the radiolabeled probe at the same temperature for 12 h. Each blot was washed under increasingly stringent conditions of salt and temperature with a final wash of 30 min at 60 C in 0.1× sodium chloride/sodium citrate containing 1% sodium dodecyl sulfate. Dried filters were exposed to Kodak XAR5 film (Eastman Kodak Co., Rochester, NY) at −70 C with intensifying screens for 6 h to 4 d.

The following probes were used: a fragment of 1.4 kb (*Pvu*II digest) from Nur77 cDNA (14, 19); the B-Raf cDNA (640 bp) (28), a fragment of 500 bp of Nurr1 cDNA (*Hin*dIII, *Pst*I digest) (47), and a 1-kb Pst-1 digest cDNA for actin (54). The cDNA probes were labeled with a random-primed kit with α-^{32}P-dCTP (Amersham Pharmacia Biotech). The autoradiograms were scanned with an ultroscan II laser densitometer (LKB, Rockville, MD). The blots were reprobed after eluting the first radiolabeled probe by washing according to the manufacturer's instructions in 5 mM Tris/HCl (pH 8.0), 2 mM EDTA, 0.1× Denhardt's solution, at 65 C for 2 h. After the previous signal was removed, confirmed by reexposure of the autoradiogram, the blots were prehybridized and hybridized following the methods described above.

Transient Transfection and Reporter Luciferase, CAT, and β-gal Assays

Cell transfection was performed with lipofectamine using standard procedures, as previously described (55). After plating the cells in six-well plates, the cells were transfected for 6 h in OPTIMEM (Life Technologies, Inc.) using 8 μl of lipofectamine (Life Technologies, Inc.) per well, with the following plasmids, as indicated: NurRE-LUC (500 ng), POMC-LUC (500 ng), Nur77promoter-CAT (500 ng), Gal4-Elk1 (300 ng) or Gal4-Nur77 (300 ng) plus the reporter for Gal4 pG5-LUC (500 ng), RapN17, RapG12V, PKA DN, MEK DN, RasN17, B-Raf expression vectors (300 ng), and MEK-EE (30 ng), in conjunction with a control rous sarcoma virus (RSV)-β-gal construction (200 ng). Cells were then washed and left overnight in DMEM without serum. The next day cells were washed and stimulated for 6 h in serum-free DMEM with the indicated stimuli. After treatment, LUC activity in cell lysates was measured as previously described (55) using the Luciferase measure kit (Promega Corp.) with a Junior luminometer (Berlthold, Bad Wildbad, Germany). CAT and β-gal activities were measured as previously described (55), the last as control for transfection efficiency. Results were standardized for β-gal activity. Empty vectors for POMC-LUC and NurRE-LUC constructs showed no stimulation by CRH or cAMP (data not shown).

MAPK Assay, Nur77 Phosphorylation, and Western Blot

AtT-20 cells were cultured in six-well plates in DMEM 10% FCS supplemented with glutamine and antibiotics; 24 h later cells were washed out and left for another 24 h in serum-free DMEM. Stimulation was performed for 2, 5, or 10 min with CRH, cAMP, or KCl plus the L-type calcium channel agonist FPL64176. Cellular extracts were collected using a kinase lysis buffer (HEPES, 20 mM, pH 7.5; EGTA, 10 mM; β-glycerolphosphate, 40 mM; NP-40, 1%; $MgCl_2$, 2.5 mM; orthovanadate, 2 mM; phenylmethylsulfonyl fluoride, 1 mM; dithiothreitol, 1 mM; leupeptin, 20 μg/ml). After centrifugation, supernatants were split to detect total (phosphorylated and unphosphorylated) ERK2 protein and phosphorylated ERK1/2 protein contents by Western blot as described previously (53). Briefly, after separation of proteins by SDS-PAGE (10% polyacrylamide) and blotting onto nitrocellulose membranes, immunoblot analysis was performed with antiphospho-ERK antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After stripping, the membranes were treated with the anti-ERK2 antibody (Santa Cruz Biotechnology, Inc.). Detection was performed by chemiluminescence (Amersham Pharmacia Biotech) for antiphospho-ERK antibody and with the phosphatase alkaline method (Life Technologies, Inc.) for the anti-ERK2 antibody. Extracts were used for immunoprecipitation and kinase assay as described previously (56). Briefly, the supernatants were immunoprecipitated with anti-ERK2 (Santa Cruz Biotechnology, Inc.) antibody. After incubation at 0 C for 90 min, protein A/G plus-Agarose beads (Santa Cruz Biotechnology, Inc.) were added and left in agitation at 4 C for 30 min. After precipitation the pellet was washed three times with 500 μl of wash buffer 1 (PBS; NP-40 1%; vanadate, 2 mM), one time with 500 ml of wash buffer 2 (Tris, 100 mM, pH 7.5; LiCl, 0.5 M) and one time with the kinase reaction buffer [3-(*N*-morpholino)propane sulfonic acid, 4.2 mM; β-glycerophosphate, 4.2 mM; $MgCl_2$, 2.5 mM; EGTA 0.2 mM; sodium fluoride, 0.2 mM; vanadate, 0.2 mM).

Each pellet was incubated with 30 μl of the reaction buffer plus γ^{32}ATP (10 μCi), myelin basic protein (MBP) (10 μg), dithiothreitol (3 mM), and rATP at 37 C for 30 min. The cracking buffer (10 μl) was added and the products boiled for 5 min to denaturate the MBP. After electrophoresis, the gel was dried for 2 h at 80 C and exposed to an autoradiographic Kodak XAR5 film at −70 C with intensifying screens for 6 h to 4 d. For phosphorylation of Nur77 protein the assay was performed *in vitro* as follows: AtT-20 cells were transiently transfected with a 500 ng of the Gal4-Nur77 construction and, after 44 h, cells were treated with 50 μM PD98059 for 4 h. Cellular extracts were obtained and the protein was immunoprecipitated using polyclonal goat IgG anti the C-terminal part of Nur77 antibody (Santa Cruz Biotechnology, Inc.), as described above. The immunoprecipitated protein was used as *in vitro* substrate for the kinase reaction (performed as described above) with mouse ERK2 immunoprecipitated from AtT-20 cells treated under different conditions. Western blot analysis for Gal4-Nur77 was performed using the polyclonal IgG anti-Nur77 antibody already described and a second antigoat coupled to phosphatase alkaline antibody (Santa Cruz Biotechnology, Inc.).

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REFERENCES

1. Lundblad JR, Roberts JL 1988 Regulation of proopiomelanocortin gene expression in pituitary. Endocr Rev 9:135–158
2. Levin N, Blum M, Roberts JL 1989 Modulation of basal and corticotropin-releasing factor-stimulated proopiomelanocortin gene expression by vasopressin in rat anterior pituitary. Endocrinology 125:2957–2966
3. Boutillier AL, Sassone-Corsi P, Loeffler JP 1991 The protooncogene c-*fos* is induced by corticotropin-releasing factor and stimulates proopiomelanocortin gene transcription in pituitary cells. Mol Endocrinol 5:1301–1310
4. Boutillier AL, Monnier D, Lorang D, Lundblad JR, Roberts JL, Loeffler JP 1995 Corticotropin-releasing hormone stimulates proopiomelanocortin transcription by cFos-dependent and -independent pathways: characterization of an AP1 site in exon 1. Mol Endocrinol 9:745–755
5. Usui T, Nakai Y, Tsukada T, Fukata J, Nakaishi S, Naitoh Y, Imura H 1989 Cyclic AMP-responsive region of the human proopiomelanocortin (POMC) gene. Mol Cell Endocrinol 62:141–146
6. Schecterson LC, Mcknight GS 1991 Role of cyclic adenosine 3′,5′-monophosphate-dependent protein kinase in hormone-stimulated β-endorphin secretion in AtT20 cells. Mol Endocrinol 5:170–178
7. Timpl P, Spanagel R, Sillaber I, Kresse A, Reul JM, Stalla GK, Blanquet V, Steckler T, Holsboer F, Wurst W 1998 Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor. Nat Genet 19:162–166
8. Luini A, Lewis D, Guild S, Corda D, Axelrod J 1985 Hormone secretagogues increase cytosolic calcium by increasing cAMP in corticotropin-secreting cells. Proc Natl Acad Sci USA 82:8034–8038
9. Litvin Y, PasMantier R, Fleischer N, Erlichman J 1984 Hormonal activation of the cAMP-dependent protein kinases in AtT20 cells. J Biol Chem 259:10296–10302
10. Stalla GK, Stalla J, Huber M, Loeffler JP, Hollt V, von Werder K, Muller OA 1988 Ketoconazole inhibits corticotropic cell function *in vitro*. Endocrinology 122: 618–623
11. Lee AK, Tse A 1997 Mechanism underlying corticotropin-releasing hormone (CRH) triggered cytosolic Ca rise in identified rat corticotrophs. J Physiol 504: 367–378
12. Clark TP, Kemppainen RJ 1994 Glucocorticoids do not affect intracellular calcium transients in corticotrophs: evidence supporting an effect distal to calcium influx. Neuroendocrinology 60:273–282
13. Von Dreden G, Loeffler JP, Grimm C, Hollt V 1988 Influence of calcium ions on proopiomelanocortin mRNA levels in anterior clonal pituitary cells. Neuroendocrinology 47:32–37
14. Philips A, Lesage S, Gingras R, Maira MH, Gauthier Y, Hugo P, Drouin J 1997 Novel dimeric Nur77 signaling mechanism in endocrine and lymphoid cells. Mol Cell Biol 17:5946–5951
15. Maira M, Martens C, Philips A, Drouin J 1999 Heterodimerization between members of the Nur subfamily of orphan nuclear receptors as a novel mechanism for gene activation. Mol Cell Biol 19:7549–7557
16. Murphy EP, Coneely OM 1997 Neuroendocrine regulation of the hypothalamic pituitary adrenal axis by the nurr1/nur77 subfamily of nuclear receptors. Mol Endocrinol 11:39–47
17. Emmark E, Gustafsson JA 1996 Orphan nuclear receptors-the first eight years. Mol Endocrinol 10:1293–1307
18. Wilson TE, Mouw AR, Weaver CA, Milbrandt J, Parker KL 1993 The orphan nuclear receptor NGFI-B regulates expression of the gene encoding steroid-21-hydroxylase. Mol Cell Biol 13:861–868
19. Philips A, Maira M, Mullick A, Chamberland M, Lesage S, Hugo P, Drouin J 1997 Antagonism between Nur77 and glucocorticoid receptor for control of transcription. Mol Cell Biol 17:5952–5959
20. Altschuler D, Lapetina EG 1993 Mutational analysis of the cAMP-dependent protein kinase-mediated phosphorylation site of Rap1b. J Biol Chem 268: 7527–7531
21. Rooij J, Zwartkruis FJT, Verheijen MHG, Cool RH, Nijman SMB, Wittinghofer A, Bos JL 1998 Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature 396: 474–477
22. Cook SJ, Rubinfeld B, Albert I, McCormick F 1993 RapV12 antagonizes Ras-dependent activation of ERK1 and ERK2 by LPA and EGF in rat-1 fibroblasts. EMBO J 12:3475–3485
23. Li W, Whaley CD, Mondino A, Müller DL 1996 Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4+ T cells. Science 271:1272–1276
24. Fields PE, Gajewski TF, Fitch FW 1996 Blocked Ras activation in anergic CD4+ T cells. Science 271: 1276–1278
25. Bos JL 1998 All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral. EMBO J 17:6776–6782
26. Cook SJ, McCormick F 1993 Inhibition by cAMP of Ras-dependent activation of Raf. Science 262:1069–1072
27. Wu J, Dent P, Jelinek T, Wolfman A, Weber MJ, Sturgill TW 1993 Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3′,5′-monophosphate. Science 262:1065–1069
28. Vossler MR, Yao H, York RD, Pan MG, Rim CS, Stork PJS 1997 cAMP activates MAP kinase and Elk-1 through a B-Raf-and Rap1-dependent pathway. Cell 89:73–82
29. Dugan LL, Kim JS, Zhang Y, Bart RD, Sun Y, Holtzman DM, Gutmann DH 1999 Differential effects of cAMP in neurons and astrocytes. Role of B-raf. J Biol Chem 274: 25842–25848
30. Young SW, Dickens M, Tavare JM 1994 Differentiation of PC12 cells in response to a cAMP analogue is accompanied by sustained activation of mitogen-activated protein kinase: comparison with the effects of insulin, growth factors and phorbol esters. FEBS Lett 338:212–216
31. York RD, Yao H, Dillon T, Ellig CL, Eckert SP, McCleskey EW, Stork PJS 1998 Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. Nature 392:622–626
32. Qiu W, Zhuang S, von Lintig FC, Boss GR, Pilz RB 2000 Cell type-specific regulation of B-raf kinase by cAMP and 14–3-3 proteins. J Biol Chem 275:31921–31929
33. Kievit P, Lauten JD, Maurer RA 2001 Analysis of the role of the mitogen-activated protein kinase in mediating cyclic-adenosine 3′,5′-monophosphate effects on prolactin promoter activity. Mol Endocrinol 15:614–624
34. Richards JS 2001 New signaling pathways for hormones and cyclic adenosine 3′,5′-monophosphate action in endocrine cells. Mol Endocrinol 15:209–218
35. Woronicz JD, Lina A, Calnan BJ, Szychowski S, Cheng L, Winoto A 1995 Regulation of the Nur77 orphan steroid receptor in activation-induced apoptosis. Mol Cell Biol 15:6364–6376
36. Youn HD, Sun L, Prywes R, Liu JO 1999 Apoptosis of T cells mediated by Ca-induced release of the transcription factor MEF2. Science 286:790–793

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37. Blaeser F, Ho N, Prywes R, Chatila TA 2000 Ca(2+)-dependent gene expression mediated by MEF2 transcription factors. J Biol Chem 275:197–209
38. Castillo SO, Xiao Q, Lyu MS and A KC 1997 Organization, sequence, chromosomal localization, and promoter identification of the mouse orphan nuclear receptor Nurr1 gene. Genomics 41:250–257
39. Antaraki A, Ang KL, Antoni FA 1997 Involvement of calyculin A inhibitable protein phosphatases in the cyclic AMP signal transduction pathway of mouse corticotroph tumor (AtT20) cells. Br J Pharmacol 121:991–999
40. Erlichman J, Litvin Y, Fleischer N 1984 Immunological and molecular characterization of cAMP-dependent protein kinases in AtT-20 cells. J Biol Chem 259: 10289–10295
41. Tsygankova OM, Saavedra A, Rebhun JF, Quilliam LA, Meinkoth JL 2001 Coordinated regulation of Rap1 and thyroid differentiation by cyclic AMP and protein kinase A. Mol Cell Biol 21:1921–1929
42. Grewal SS, Horgan AM, York RD, Withers GS, Bankers GA, Stork PJ 2000 Neuronal calcium activates Rap1 and B-Raf signaling pathway via the cyclic adenosine monophosphate-dependent protein kinase. J Biol Chem 275: 3722–3728
43. Egea J, Espinet C, Soler RM, Peiro S, Rocamora N, Comella J 2000 Nerve growth factor activation of the extracellular signal-regulated kinase pathway is modulated by Ca+ and calmodulin. Mol Cell Biol 20: 1931–1946
44. Grewal SS, Fass DM, Yao H, Ellig CL, Goodman RH, Stork PJS 2000 Calcium and cAMP signals differentially regulate cAMP-responsive element-binding protein function via a Rap1-extracellular signal-regulated kinase pathway. J Biol Chem 275:34433–34441
45. Seternes OM, Johansen B, Moens U 1999 A dominant role for the Raf-MEK pathway in forskolin, 12-O-tetradecanoyl-phorbol acetate, and platelet-derived growth factor-induced CREB (cAMP-responsive element-binding protein) activation, uncoupled from serine 133 phosphorylation in NIH 3T3 cells. Mol Endocrinol 13: 1071–1083
46. Arzt E, Stelzer G, Renner U, Lange M, Müller OA, Stalla GK 1992 Interleukin-2 and interleukin-2 receptor expression in human corticotrophic adenoma and murine pituitary cell cultures. J Clin Invest 90:1944–1951
47. Ohkura N, Hosono T, Maruyama K, Tsukada T, Yamaguchi K 1999 An isoform of Nurr1 as a negative inhibitor of the NGFI-B family signaling. Biochim Biophys Acta 1444: 69–79
48. Liu B, Mortrud M, Low MJ 1995 DNA elements with AT-rich core sequences direct pituitary cell-specific expression of the pro-opiomelanocortin gene in transgenic mice. Biochem J 312:827–832
49. Peterson SN, Trabalzini L, Brtva TR, Fischer T, Altschuler DL, Martelli P, Lapetina EG, Der CJ, White GC 1996 Identification of a novel RalGDS-related protein as a candidate effector for Ras and Rap1. J Biol Chem 271: 29903–29908
50. McKean DJ, Bell M, Huntoon C, Rastogi S, Norstrand MV, Podzorski R, Nilson A, Paya C 1995 IL-1 receptor and TCR signals synergize to activate NF-kB mediated gene transcription. Int Immunol 7:9–20
51. Rammes G, Steckler T, Lresse A, Schutz G, Zieglgansberger W, Lutz B 2000 Synaptic plasticity in the basolateral amygdala in transgenic mice expressing dominant-negative cAMP response element-binding protein (CREB) in forebrain. Eur J Neurosci 12:2534–2546
52. Clegg CH, Abrahamsern MS, Degen JL, Morris DR, McKnight GS 1992 Cyclic AMP-dependent protein kinase controls basal gene activity and steroidogenesis in Y1 adrenal tumor cells. Biochemistry 31:3720–3726
53. Coso OA, Chiariello M, Yu JC, Teramoto H, Crespo P, Xu N, Miki T, Gutkind JS 1995 The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell 81:1137–1146
54. Arzt E, Sauer J, Pollmacher T, Labeur M, Holboer F, Reul JMHM, Stalla GK 1994 Glucocorticoids suppress interleukin-1 receptor antagonist synthesis following induction by endotoxin. Endocrinology 134:672–677
55. Costas M, Trapp T, Paez Pereda M, Sauer J, Rupprecht R, Nahmod VE, Reul JMHM, Holsboer F, Arzt E 1996 Molecular and functional evidence for *in vitro* cytokine enhancement of human and murine target cell sensitivity to glucocorticoids. J Clin Invest 98:1409–1416
56. Crespo P, Xu N, Simonds WF, Gutkind JS 1994 Ras-dependent activation of MAPK pathway mediated by G-protein $\beta\gamma$ subunits. Nature 369:418–420

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