

# A Calcium-Initiated Signaling Pathway Propagated through Calcineurin and cAMP Response Element-Binding Protein Activates Proenkephalin Gene Transcription after Depolarization

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## ABSTRACT

Essential components of a signal-transduction pathway regulating activity-dependent neuropeptide gene transcription have been identified. Proenkephalin (PENk) gene activation after depolarization of chromaffin cells with 40 mM KCl was blocked by the voltage-sensitive calcium-channel blocker methoxyverapamil (D600) (30  $\mu$ M) and by calcineurin inhibition with 100 nM cyclosporin A or ascomycin but not by inhibiting new protein synthesis with 0.5  $\mu$ g/ml cycloheximide. KCl-induced elevation of PENk mRNA was distinct from activation of the PENk gene by either cAMP or protein kinase C. Twenty-five micromolar forskolin- and 100 nM phorbol 12-myristate 13-acetate-induced elevations of PENk mRNA were cycloheximide-sensitive and were not blocked by cyclosporin A or ascomycin. KCl stimulated Ser-133 phosphorylation of cAMP response element-binding protein (CREB) in chromaffin cells, and CREB phosphorylation was blocked by both ascomycin and D600. A

reporter gene containing 193 bases of the PENk gene 5' flank driving luciferase gene expression (pENK12-Luc) transfected into chromaffin cells was transcriptionally activated by KCl depolarization. Activation was blocked by both ascomycin and D600 and required an intact CREB binding site (ENKCRE2). An oligonucleotide containing the PENk cAMP response element-2 was gel-shifted by both unstimulated and potassium-stimulated chromaffin cell nuclear extracts into a prominent complex supershifted by CREB antibodies. Finally, stimulation of transcription of the pENK12-Luc reporter by KCl in chromaffin cells was blocked by coexpression of the CREB antagonist A-CREB but not by the AP-1 antagonist A-Fos. Stimulus-transcription coupling after depolarization in chromaffin cells occurs via calcineurin-dependent activation of CREB, a pathway distinct from cAMP- or protein kinase C-initiated signaling and independent of immediate early gene regulation.

Stimulation of the splanchnic innervation of the adrenal medulla causes massive secretion of catecholamines and neuropeptides from dense-core vesicles of chromaffin cells (Fischer-Colbrie et al., 1988). Synaptic regulation of vesicular hormone secretion and biosynthesis has been studied using cultured chromaffin and PC12 cells as well as stress-induced activation of the adrenomedullary synapse in vivo as model systems (Ross et al., 1990; Wilson, 1991; Sabban et al., 1995; Wong et al., 2002). Stimulus-secretion synthesis coupling is the dual activation of exocytotic secretion and the transcription of genes encoding secreted polypeptides after activity-dependent postsynaptic calcium influx (Eiden et al., 1984). It permits maintenance of the neuropeptide phenotype in the face of secretory demand and is closely related to the plastic-

ity of neuropeptide phenotypes under physiological conditions of drastically altered synaptic signaling (MacArthur and Eiden, 1996; Zigmond and Sun, 1997).

Both elevated extracellular potassium and the cholinergic secretagogue nicotine stimulate calcium influx-dependent enkephalin secretion and gene transcription in chromaffin cells (Eiden et al., 1984; Siegel et al., 1985; Kley et al., 1986). Activity-dependent neuropeptide gene regulation throughout the neuroendocrine axis is mediated through signaling to gene-specific *cis*-regulatory elements (MacArthur and Eiden, 1996). One way this might occur is via protein kinase A-dependent activation (phosphorylation) of CREB. Neuropeptide genes examined so far that are transcriptionally responsive to cell depolarization [e.g., enkephalin, vasoactive intestinal

**ABBREVIATIONS:** CREB, cAMP response element-binding protein; PENk, proenkephalin; PMA, phorbol 12-myristate 13-acetate; D600, methoxyverapamil; VIP, vasoactive intestinal polypeptide; CRE, cAMP response element; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; bp, base pair(s); CHX, cyclohexamide; MSK1, mitogen- and stress-activated protein kinase isoform 1; IEG, immediate early gene; ERK, extracellular signal-regulated kinase; H89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; FK-506, the immunosuppressive macrolactam lactone Tacrolimus; FKBP12, FK-506 binding protein of 12 kDa; L-683590, C21-ethyl-FK-506; L-685818, C18-hydroxyl L-683590.

polypeptide (VIP), and substance P] contain a proximal cAMP response element (CRE) that can bind CREB and therefore mediate classic cAMP responsiveness via protein kinase A-dependent CREB phosphorylation (Montminy and Bilezikjian, 1987). CREB can also be activated by calcium via the stimulation of calmodulin kinase IV, which can directly phosphorylate and activate CREB at Ser-133 (Shaywitz and Greenberg, 1999). A second pathway for activity-dependent neuropeptide gene regulation involves the activation of immediate early genes (IEGs) such as Fos and Jun in response to calcium influx (Morgan and Curran, 1991). Fos and Jun, as members of AP-1 complexes, can transactivate neuropeptide genes at the same elements that bind CREB (Kobierski et al., 1991; Anouar et al., 1999). The proximal CRE of the proenkephalin A gene, called the ENKCRE-2, has been shown to mediate transcriptional responsiveness to both cAMP and increased intracellular calcium, which can be mimicked in F9 cells by cotransfection with junD, indicating that the ENKCRE-2 is capable of binding and transactivation through both CREB and AP-1 (Van Nguyen et al., 1990; Kobierski et al., 1991).

Various, and probably cell-type-specific, mechanisms of calcium signaling may therefore drive neuropeptide gene activation during stimulus-secretion synthesis coupling. Electrophoretic mobility shift assays (EMSAs) have been used to identify binding to the ENKCRE-2 of CREB but not AP-1 in rat striatum (Konradi et al., 1993) and of AP-1 but not CREB in rat hippocampus (Sonnenberg et al., 1989). However, it was not possible to establish in these studies that the dominant gel-shift complex that was formed in highly heterogeneous brain nuclear extracts was composed of proteins contributed specifically by enkephalin-expressing cells. Nuclear protein extracts taken from chromaffin cells have been reported to form EMSA complexes with ENKCRE-2-containing oligonucleotides, which mainly contain AP-1 with smaller amounts of CREB-immunoreactive protein (Bacher et al., 1996; MacArthur, 1996). Because these cultures contain predominantly enkephalin-expressing cells, the probability that complex-forming proteins are present in the same cell population expressing the endogenous proenkephalin gene is higher than it is in brain tissue nuclear extracts. Nevertheless, these experiments, carried out in molar excess of the DNA target, do not provide information on the role of relative binding affinity in recruitment of a given transacting factor that is present in vast molar excess to the endogenous single-copy gene of interest. Furthermore, a direct link between endogenous enkephalin gene transcription and reporter gene transcriptional behavior has not been made in these cells, such that the results of EMSA with the ENKCRE-2 can be directly applied to the presumptive regulatory behavior of the endogenous neuropeptide gene. Here, we demonstrate parallel regulation of endogenous PEnk mRNA production and PEnk reporter gene transcription in response to depolarization of bovine chromaffin cells, allowing the direct application of reporter-gene behavior to hypotheses about depolarization-induced regulation of the cognate endogenous neuropeptide gene. Our results suggest that calcium-dependent transcriptional activation is mediated mainly through calcineurin-dependent phosphorylation of CREB, acting at the ENKCRE-2 of the proenkephalin gene.

## Materials and Methods

**Materials.** Forskolin was purchased from Calbiochem-Novabiochem (San Diego, CA). Collagenase was obtained from Worthington Biochemicals (Freehold, NJ); nystatin was from Invitrogen (Carlsbad, CA); ascomycin, rapamycin, cyclosporin A, methoxyverapamil (D600), DNase, cytosine arabinofuranoside, and cycloheximide were from Sigma Chemical (St. Louis, MO); L-683590 and L-685818 were from Merck (Whitehouse Station, NJ); and phorbol 12-myristate 13-acetate (PMA) was from LC Laboratories (Woburn, MA). Fos, Jun, and CREB antibodies were obtained from Santa Cruz Biochemicals (Santa Cruz, CA).

**Cell Culture and Drug Treatments.** Primary cultures of bovine chromaffin cells were prepared by perfusion of freshly obtained bovine (steer) adrenal glands with 0.1% Worthington collagenase and 30 units/ml DNase in standard release medium buffer with modifications that were described recently (Hahm et al., 1998). Cells were differentially plated in T150 flasks (approximately 100 million cells/flask) overnight, and nonadherent (chromaffin) cells were replated the next day at  $0.5 \times 10^6$  cells/well in 24-well plates (Costar, Cambridge, MA). Cells were cultured in Dulbecco's modified Eagle's medium with high glucose (Invitrogen) containing 5% heat-inactivated fetal bovine serum (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) and supplemented with 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin, 50  $\mu$ g/ml cytosine arabinofuranoside (Sigma), and 100 units/ml nystatin. In all experiments except as indicated, drug treatments were initiated 24 h after replating into 24-well dishes by removal of medium and addition of fresh medium containing inhibitors or vehicle. After a 30-min preincubation with inhibitors, medium was removed and replaced with inhibitors or vehicle containing 40 mM KCl (isotonic replacement of NaCl), 25  $\mu$ M forskolin, 0.1  $\mu$ M PMA, or medium alone. Medium and cells were harvested for peptide measurements 72 h later. Cells were harvested for RNA measurements 18 to 24 h later.

**Radioimmunoassay for Met-Enkephalin and VIP.** Met-enkephalin and VIP were assayed directly in aliquots of culture medium and in lyophilized 0.1 N HCl extracts of chromaffin cells as described previously (Hahm et al., 1998; Lee et al., 1999).

**Northern Blot Analysis.** Northern blot analysis of proenkephalin A mRNA was performed using total RNA isolated from cells maintained in 24-well plates by extracting with SDS-EDTA-Tris-proteinase K buffer containing 10 mM Tris, pH 7.4, 1% SDS, 5 mM EDTA, and 100  $\mu$ g/ml proteinase K, as described previously (Hahm et al., 1998). Total RNA samples isolated from  $0.5 \times 10^6$  cells/well were denatured and separated using 1% agarose-formaldehyde gel, electrophoretically transferred onto a nylon membrane, and hybridized with  $^{32}$ P-labeled bovine proenkephalin A cDNA probe. The membrane was washed and autoradiographed, and proenkephalin mRNA bands were quantified by densitometric scanning of autoradiograms as described previously (Hahm et al., 1998). Uniformity of total chromaffin cell RNA per lane was ensured by quantification of ethidium bromide-stained ribosomal (18S and 28S) RNA before electrophoretic transfer of RNA onto the nylon membrane.

**Quantitative Reverse Transcriptase-Polymerase Chain Reaction.** Quantitative reverse transcriptase-polymerase chain reaction was used to assess proenkephalin mRNA levels in some experiments. RNA isolation, reverse transcription, and quantification of proenkephalin A transcripts was carried out as described previously (Hamelink et al., 2002a) using primer/probe sets optimized for bovine PEnk designed with the Primer Express software package (PerkinElmer Life and Analytical Sciences, Boston, MA) as follows: forward primer, TCCCTTTCCCATCAGTGAC; reverse primer, CCAGCGCAGCAGTCTTTCA; and probe, CAGAAGCCTTCCTCT-GCCCC.

**Nuclear Extract Preparation.** Chromaffin cell nuclear extracts were prepared from cells treated with 40 mM KCl (or with vehicle) for 10 h and maintained in 6-well plates precoated with poly-D-lysine (1 ml of 100  $\mu$ g/ml for 7 min/well) at a density of  $4 \times 10^6$  cells/well,

as described earlier (MacArthur et al., 1993), with minor modifications. All the steps were taken at 4°C. Cells maintained in six-well plates were washed two times with Tris-buffered saline and scraped off the plate using 0.3 ml of freshly prepared buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 5 µg/ml leupeptin, 5 µg/ml aprotinin, and 2 µg/ml pepstatin), transferred to a microcentrifuge tube and allowed to swell on ice for 15 min. Cells were then lysed by adding 10 µl of 10% NP40 and vortexing four times for 1 s each. Samples were centrifuged for 30 s at 13,000 rpm, and nuclear pellets were resuspended in 35 µl of buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors as described above). Nuclear proteins were extracted by vigorously rocking the sample containing nuclei for 15 min. Samples were then centrifuged for 5 min at 13,000 rpm, and the supernatants containing nuclear proteins were stored at -80°C in small aliquots.

**Electrophoretic Mobility Shift Assays.** For gel-shift assays, binding reactions were performed in the presence of 3 µg of nuclear proteins and 100,000 to 140,000 cpm of double-stranded probe, which was produced by labeling annealed complementary oligonucleotides for the ENK-CRE2 sequence (ggcgctgcgtcaacagcag) with [<sup>32</sup>P]ATP using T4 polynucleotide kinase (Promega, Madison, WI) in a 10-µl reaction containing 10 mM Tris, pH 7.5, 25 mM NaCl, 1 mM DTT, 1 mM EDTA, and 5% glycerol at room temperature for 20 min. Unincorporated nucleotides were removed using the Sephadex G-50 column-purification method. In supershift assays, nuclear extract was preincubated with the probe for 10 min and incubated for an additional 20 min after mixing with 1 µl of Transcruz antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The specificity of antibodies used was shown previously (Anouar et al., 1999). Samples were resolved using 5% polyacrylamide gels at 4°C, and the gels were dried and autoradiographed.

**Transient Transfection Experiments.** Chromaffin cells were transfected using the Profection mammalian transfection system (Promega). Cells were plated in poly-D-lysine-coated 12-well plates at a density of  $1 \times 10^6$  cells/well and were allowed to attach for approximately 16 h. Cells were transfected for 15 h according to the manufacturer's instructions, with 1.5 µg/well of either pENK12-luc reporter construct or pENK12-mCRE2 construct, which contains a double base-pair mutation within the ENK-CRE2 sequence, together with 1.5 µg of pRC-RSV-β-galactosidase construct. In cotransfection experiments, an additional 2.0 µg of the dominant-negative expression vector for CREB or Fos (or an empty vector) was included. After transfection, cells were carefully washed two times with complete medium and allowed to recover for 5 to 8 h before treating with KCl. Cells were harvested 40 to 48 h after treatment using 200 µl of reporter lysis buffer, and 20 µl of the lysate was used (in duplicate) for the luciferase assay.

**Reporter Constructs and Expression Vectors.** pENK12-Luc was constructed by subcloning the EcoRI/HincII fragment of pENKAT-12 (Comb et al., 1986), obtained from Dr. Steven Hyman (Harvard University, Boston, MA) and containing 406 bp of the human proenkephalin gene, including 193 bp of the 5' flank, exon I (70 bp), intron A (87 bp), and exon II (53 bp), into the multiple cloning site of the pGL3-basic luciferase reporter (Promega). pENK-mCRE2 was constructed using the Transformer site-directed mutagenesis kit (BD Biosciences Clontech, Palo Alto, CA) and a complementary set of 30-bp oligonucleotides containing 2-bp substitutions within the CRE-2 sequence (ggcgtagggcctgcgAcTgctgcagccgc; substituted bases are shown in uppercase letters). The dominant-negative bZIP expression vectors, A-CREB and A-Fos, have been described previously (Olive et al., 1997; Ahn et al., 1998) and were subcloned into either the pRC/CMV500 or pRC/RSV500 plasmids (Invitrogen).

**Determination of Phosphorylated CREB by Immunoblotting.** Immunoblotting assay was performed according to the protocol set forth by Cell Signaling Technology, Inc. (Beverly, MA). Chromaffin cells, plated in 10-cm dishes, were washed with phosphate-buff-

ered saline and scraped off with 300 µl of lysis buffer plus 200 µl of 2× SDS sample buffer. Samples were lysed by sonicating for 15 s. After denaturing at 95 to 100°C for 5 min, cell lysates were centrifuged at 12,000 rpm for 10 min. The supernatant fractions (cell extract) were collected and subjected to SDS-polyacrylamide gel electrophoresis (for 1.5 h at 125 V) on 14% Tris-glycine polyacrylamide gels (NOVEX, San Diego, CA) followed by transfer to polyvinylidene difluoride membranes by electroblotting for 1.5 h at 25 V. Blots were incubated with a 1:1000 dilution of mouse monoclonal antibody specific for Ser-133-phosphorylated CREB (Cell Signaling Technology), followed by peroxidase-labeled anti-mouse second antibody (1:4000 dilution). Immunoreactive bands were detected using an enhanced chemiluminescence Western blotting kit (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK). The optical density of phospho-CREB bands was measured using an Image Station 440 computer-controlled imaging system (Eastman Kodak, Rochester, NY).

## Results

Elevated extracellular potassium increases PENk mRNA and PENk gene transcription in bovine chromaffin cells in culture, and induction is blocked by D600, a pan-specific voltage-sensitive calcium-channel blocker (Siegel et al., 1985; MacArthur et al., 1993). Bovine chromaffin cells were treated with KCl and other agents to induce PENk mRNA 24 h after differential plating onto poly-D-lysine in Dulbecco's modified Eagle's medium with 5% heat-inactivated fetal bovine serum (see *Materials and Methods*). Under these conditions, both up-regulation of PENk mRNA levels by PMA (Kley, 1988), and inhibition of KCl-induced up-regulation of PENk mRNA by PMA (Pruss and Stauderman, 1988; MacArthur et al., 1993) occur, allowing the comparison of calcium-initiated PENk gene regulation in chromaffin cells to induction via stimulation of protein kinases A and C (Hamelink et al., 2002b). PENk mRNA was up-regulated 2.5- to 3-fold by 40 mM KCl, 25 µM forskolin, or 100 nM PMA (Fig. 1). Induction by KCl, forskolin or PMA was not influenced by cotreatment with 25 µM dexamethasone or by the differential plating used to remove adherent nonchromaffin cells before plating and treatment (data not shown).

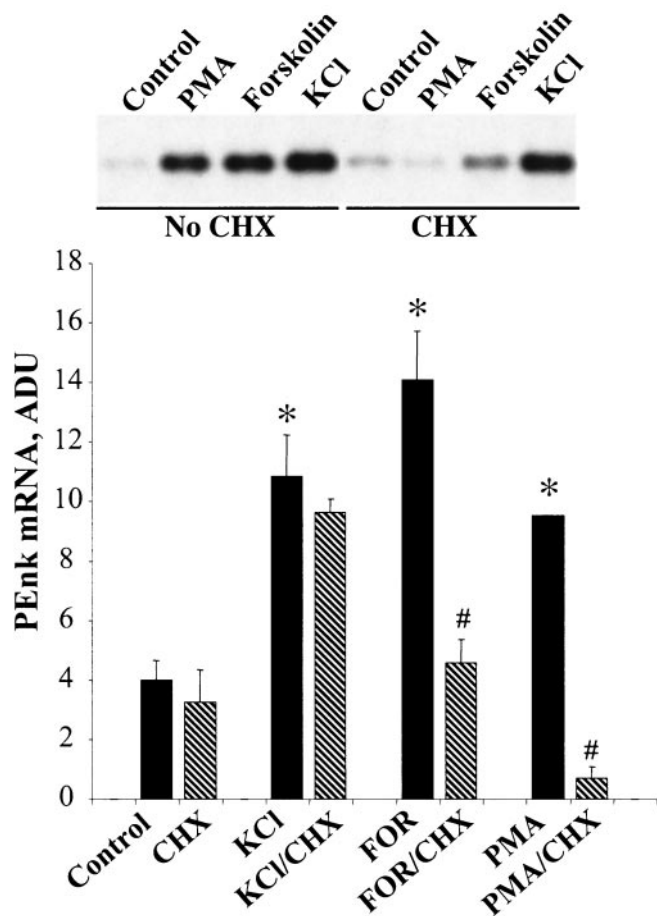
Both cAMP and protein kinase C have been implicated in calcium-initiated signal transduction pathways leading to gene activation in various cell types; however, enkephalin gene transcription stimulated by cell depolarization in chromaffin cells is distinct from that initiated by the elevation of cAMP or activation of protein kinase C in several ways. PENk mRNA up-regulation by forskolin and PMA was sensitive to blockade by the protein synthesis inhibitor cycloheximide (CHX) at 0.5 µg/ml (Fig. 1), a dose at which the induction of new protein synthesis was completely inhibited, with a minimum effect on ambient protein levels (Table 1). The induction of PENk mRNA by KCl, however, was completely insensitive to blockade by CHX (Fig. 1), indicating that under the culture conditions used in this study, calcium signaling to the PENk gene requires only pre-existing protein machinery and is independent of the immediate early gene induction that is reported to occur in chromaffin cells in response to KCl, forskolin, and PMA treatment (Bacher et al., 1996).

A second feature of calcium-initiated signaling to the PENk gene that is distinct from regulation by cAMP or protein kinase C is its dependence on calcineurin. Ascomycin is a potent and selective inhibitor of KCl-induced PENk mRNA



up-regulation without effect on induction by either PMA or forskolin (Fig. 2). In addition to inhibiting calcineurin as part of a drug-FKBP12 complex, ascomycin and other FK506 derivatives are capable of actions that do not involve calcineurin but may modulate calcium signals, such as interaction with inositol phosphate-3 and ryanodine receptors in

neuroendocrine cells (Steiner et al., 1997). A series of cyclophilin- and immunophilin-binding agents were therefore tested for potency and efficacy in inhibiting KCl-induced up-regulation of enkephalin biosynthesis (Fig. 3). These included ascomycin; the potent FK506 analog L-683590; the mixed calcineurin antagonist L-685818; cyclosporin A, which inhibits calcineurin after binding to the immunophilin cyclophilin rather than to FKBP12; and finally rapamycin, an FKBP12-binding immunosuppressant which lacks calcineurin inhibitory activity in vitro (Bierer, 1994; Dumont et al., 1996). These compounds had a rank order of potency and efficacy in inhibiting the KCl-induced enkephalin biosynthesis expected if the inhibition of calcineurin activation was their primary mechanism of action in inhibiting Penk mRNA induction by KCl (Bierer, 1994). The inhibition of calcineurin, although blocking calcium-dependent Penk biosynthesis,



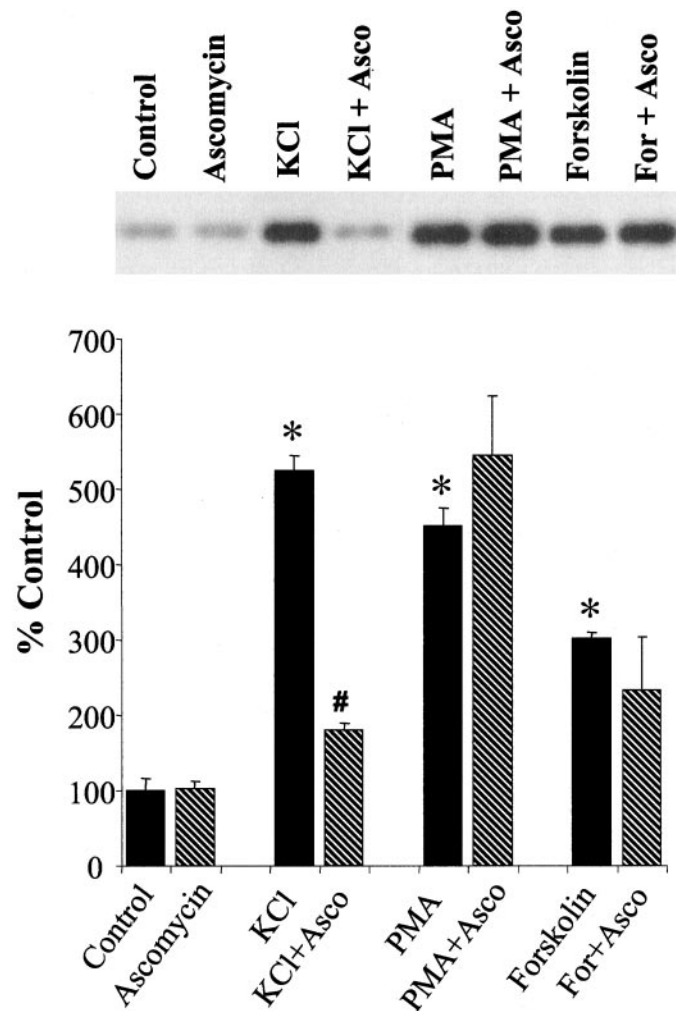
**Fig. 1.** Potassium-induced proenkephalin transcription does not require new protein synthesis. Proenkephalin A mRNA (PENk mRNA) was quantified by densitometric scanning of Northern blots containing RNA extracted from a single well of cultured chromaffin cells per lane 24 h after treatment with 0.1  $\mu$ M PMA, 25  $\mu$ M forskolin (FOR), or 40 mM KCl in the presence or absence of CHX (0.5  $\mu$ g/ml) added 60 min before drug treatment and hybridized with  $^{32}$ P-labeled bovine proenkephalin A cDNA probe. Data shown in the bottom panel are representative of three independent experiments, each performed in triplicate wells. Error bars are the S.E.M. \*, significant difference from control at  $p < 0.01$  (Student's two-tailed  $t$  test); #, significant difference from corresponding treatment in the absence of CHX at  $p < 0.01$  (Student's two-tailed  $t$  test). Top shows result from single wells for each condition representative of  $n = 3$ , shown at bottom.

TABLE 1

Cycloheximide blockade of VIP peptide induction with a minimum effect on basal VIP levels in chromaffin cells

VIP peptide content was measured 24 h after the addition of PMA, preceded by treatment with 0.5  $\mu$ g/ml cycloheximide for 60 min before the addition of PMA. Values are mean  $\pm$  S.E.M. determined from quadruplicate wells. VIP radioimmunoassay was carried out as described by Lee et al. (1999).

	Without Cycloheximide	With Cycloheximide
	pg/well	
Medium alone	95.2 $\pm$ 8.8	71.4 $\pm$ 1.9
100 nM PMA	2,354.0 $\pm$ 175.6	110.6 $\pm$ 12.5



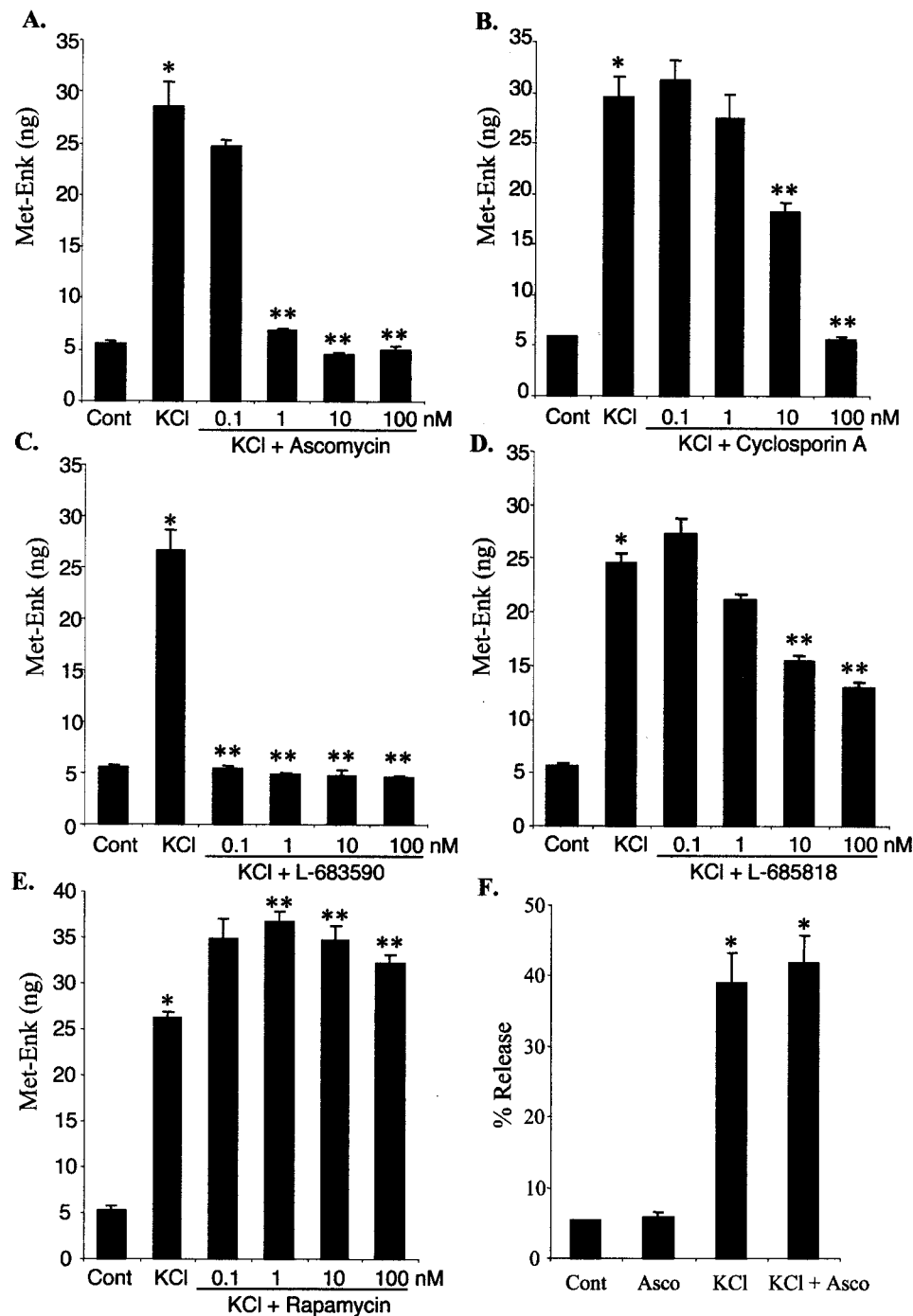
**Fig. 2.** Blockade of KCl induction of PENk mRNA by ascomycin. Chromaffin cells were treated and harvested as described in Fig. 1 and under *Materials and Methods*. Top, representative Northern blot showing induction by 40 mM KCl, 0.1  $\mu$ M PMA, and 25  $\mu$ M forskolin (For), and blockade by 100 nM ascomycin (Asco) of KCl induction but not PMA or forskolin induction. Ascomycin was present 60 min before and during 24-h treatment with KCl, forskolin, or PMA before RNA harvest. Bottom, data shown are representative of three independent experiments, each performed in triplicate, expressed as a percentage of control  $\pm$  S.E.M. \*, significant difference from control at  $p < 0.01$  (Student's two-tailed  $t$  test); #, significant difference from corresponding treatment in the absence of Asco at  $p < 0.01$  (Student's two-tailed  $t$  test).

had no effect on enkephalin release elicited by elevated potassium levels (Fig. 3F), indicating that the steps downstream of calcium influx leading to exocytosis, unlike those leading to enhanced proenkephalin gene transcription, are not calcineurin-dependent.

Despite their very different mechanisms of action, both forskolin (data not shown) and KCl caused an increase in CREB phosphorylation in chromaffin cells (Fig. 4). As an indication that CREB phosphorylation is probably involved in depolarization-induced signaling to the PENk gene, increased CREB phosphorylation after KCl treatment was blocked by both D600 and ascomycin, at concentrations that

inhibit KCl-induced up-regulation of enkephalin-peptide biosynthesis and PENk mRNA levels in chromaffin cells (Fig. 4).

A human PENk minimal promoter (pENK12-Luc) containing the cAMP- and calcium-responsive *cis*-regulatory elements ENKCRE-1, ENKCRE-2, and AP-2 was transfected into bovine chromaffin cells to demonstrate the mechanism of KCl-induced proenkephalin biosynthesis at the level of transcriptional activation of the proenkephalin A gene. Stimulation of reporter-gene activity was examined after exposure to 40 mM KCl. Exposure to KCl elicited a 2- to 3-fold increase in reporter-gene activity that was similar to the KCl-induced increase in endogenous PENk mRNA. Depolarization-in-



**Fig. 3.** Inhibition of KCl-induced up-regulation of met-enkephalin (Met-Enk) biosynthesis, but not KCl-induced secretion, by immunophilin-binding calcineurin inhibitors. Chromaffin cells were pretreated with increasing doses of immunophilin-binding drugs (A, ascomycin; B, cyclosporin A; C, L-683590; D, L-683818; and E, rapamycin) or vehicle alone 30 min before treating with 40 mM KCl as described under *Materials and Methods*. Total met-enkephalin peptide (met-enkephalin extracted from cell homogenate plus met-enkephalin secreted into medium) was measured 72 h after the addition of KCl. F, release of enkephalin from chromaffin cells treated with vehicle, 0.1  $\mu$ M ascomycin, 40 mM KCl, or 0.1  $\mu$ M ascomycin plus KCl was measured as the percentage of total met-enkephalin peptide (cell plus medium) secreted into the medium. Values for all experiments are mean  $\pm$  S.E.M. determined from triplicate determinations, and the results represent experiments repeated at least two times with similar results. \*, values significantly different from control at  $p < 0.01$  (Student's two-tailed  $t$  test); \*\*, values significantly different from KCl treatment in the absence of inhibitor at  $p < 0.01$  (Student's two-tailed  $t$  test).

duced up-regulation of pENK12-Luc reporter-gene activity was blocked by both ascomycin and D600, indicating that the minimal promoter was able to mimic the regulated behavior of the endogenous gene (Fig. 5). Calcium responsiveness was dependent on the ENKCRE-2 element, because its mutation to a nonconsensus sequence resulted in abrogation of stimulation of the transfected minimal promoter by KCl (Fig. 6). This hypothesis was confirmed by EMSA analysis of protein complexes formed between the ENKCRE-2 and chromaffin cell nuclear extracts. A gel-shift band present in both stimulated and unstimulated chromaffin cells was supershifted with antibodies directed against CREB (Fig. 7).

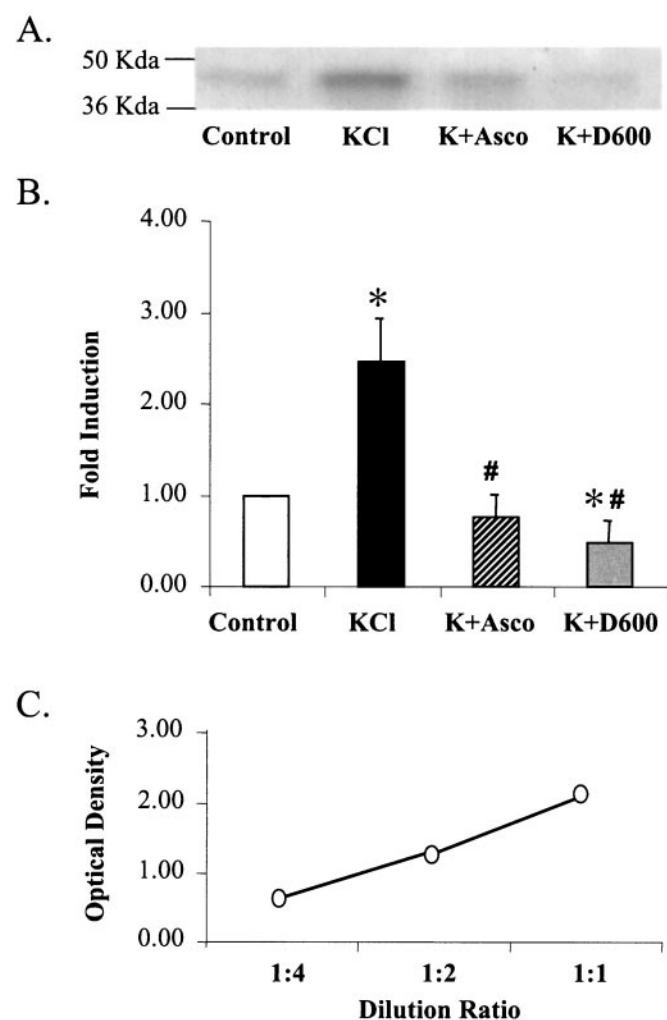
To confirm that KCl induction of pENK12-Luc reporter gene in chromaffin cells is mediated by CREB but not by

AP-1, cotransfection experiments were performed using dominant-negative expression vectors for CREB and AP-1 (Olive et al., 1997; Ahn et al., 1998). Coexpression of pENK12-Luc with the dominant-negative CREB expression vector A-CREB blocked KCl-stimulated pENK12-Luc transcription, whereas coexpression with dominant-negative Fos expression vector A-Fos had no effect. This result suggests that binding of CREB, rather than AP-1, to the ENKCRE-2 mediates calcium-initiated transcriptional activation of the proenkephalin gene in chromaffin cells (Fig. 8).

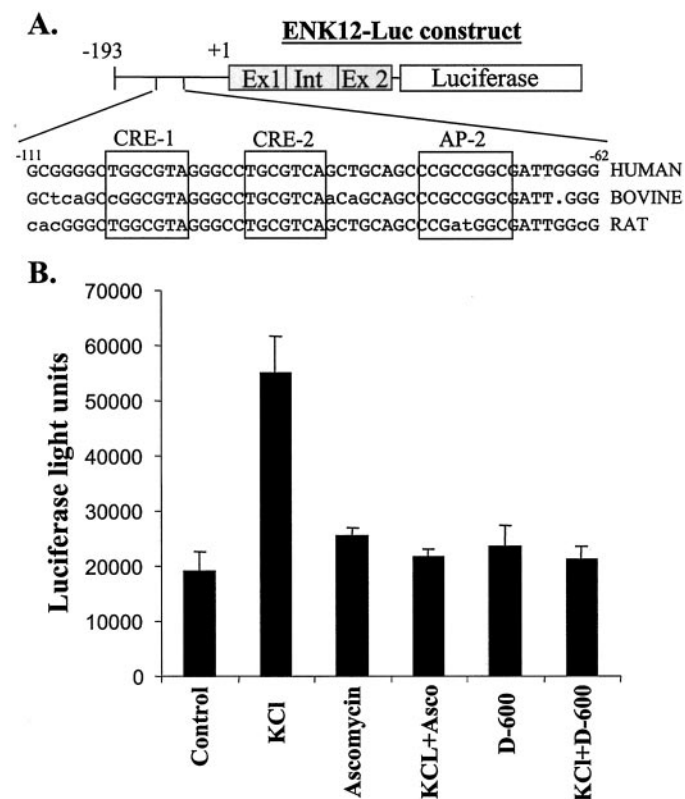
Induction of PENk mRNA by forskolin or pituitary adenylyl cyclase-activating protein is not blocked by 10  $\mu$ M H89 (Fig. 9), confirming the previously reported cAMP-dependent/protein kinase A-independent pathway for activation of CRE-containing target genes in chromaffin cells (Hamelink et al., 2002a). Induction of PENk mRNA by 40 mM KCl, however, is selectively blocked by this serine/threonine protein kinase inhibitor (Fig. 9), implicating two potential CREB kinases, protein kinase A and mitogen- and stress-activated protein kinase isoform 1 (MSK1) (Davies et al., 2000), in regulation of CREB phosphorylation after the activation of calcineurin.

## Discussion

In this report, we demonstrated that endogenous PENk mRNA and the transcription of a 406 base-pair promoter-



**Fig. 4.** Stimulation of CREB phosphorylation by KCl, and effects of pretreatment with ascomycin and D600. Phospho-CREB ( $n = 4$ ) was detected by immunoblotting in extracts from chromaffin cells cultured in 10-cm dishes using an antibody specific for the Ser-133 phosphorylated CREB. Cells were pretreated with 100 nM Ascomycin (Asco), 30  $\mu$ M D600, or vehicle alone for 30 min before treating with 40 mM KCl (K) and harvested 1 h after KCl treatment. A representative blot is shown in A, and B shows quantitative results obtained by densitometric scanning of the blots expressed as fold induction from control (mean  $\pm$  S.E.M. determined from quadruplicates). C, a calibration curve validating the densitometric intensity measurements, obtained by scanning phospho-CREB bands produced from serial dilutions of KCl-treated chromaffin cell extracts. \*, values significantly different from control at  $p < 0.01$  (Student's two-tailed  $t$  test); #, values significantly different from KCl treatment in the absence of inhibitor at  $p < 0.01$  (Student's two-tailed  $t$  test).



**Fig. 5.** Stimulation of transcription from the human proenkephalin A gene minimal promoter by elevated KCl in chromaffin cells is sensitive to D600 and ascomycin. A, the human proenkephalin gene proximal promoter containing conserved *cis*-regulatory elements ENKCRE-1 and -2 and the adjacent AP-2 site and fused to the luciferase reporter gene was transfected into chromaffin cells by calcium phosphate precipitation as described under *Materials and Methods*. B, stimulation of transcription by 40 mM KCl and inhibition by both ascomycin and D600 are shown as mean  $\pm$  range determined in duplicate. The experiment was repeated once with similar results.

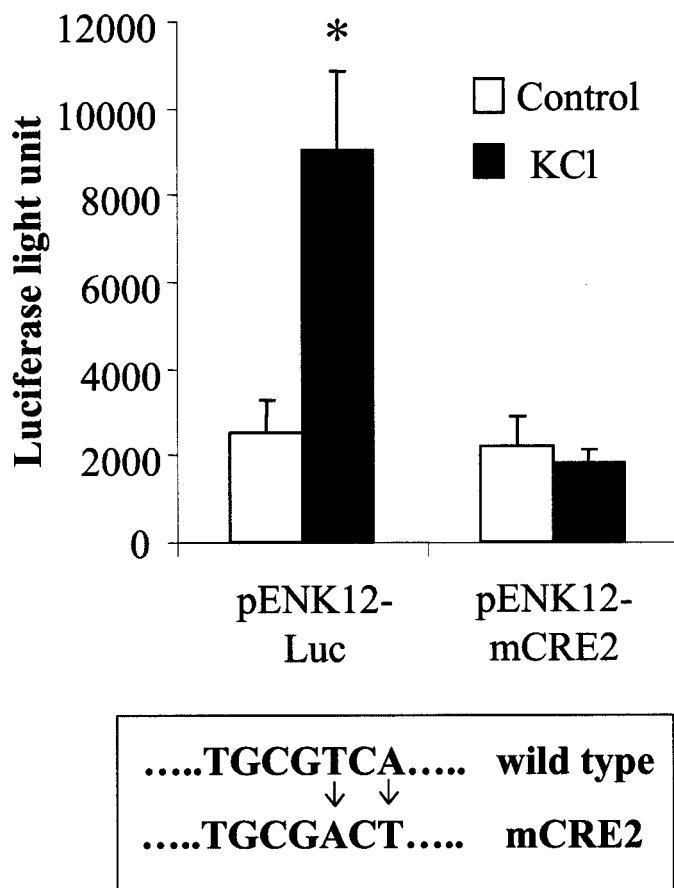


proximal PEnk reporter gene are similarly regulated by depolarization in bovine chromaffin cells. This has allowed in turn the application of reporter gene behavior to hypotheses about depolarization-induced regulation of the cognate endogenous neuropeptide gene. Under these conditions, nuclear extracts of bovine chromaffin cells contain a CREB-immunoreactive protein that binds to the ENKCRE-2, and depolarization induces D600- and ascomycin-sensitive CREB phosphorylation, revealing a direct link between depolarization-stimulated calcium influx and CREB-dependent PEnk gene transcription via the calcium-activated protein phosphatase calcineurin. These observations provide a definitive picture of depolarization-induced regulation of neuropeptide gene expression in neuroendocrine cells and a novel mechanism for CREB-dependent calcium signaling to the nucleus.

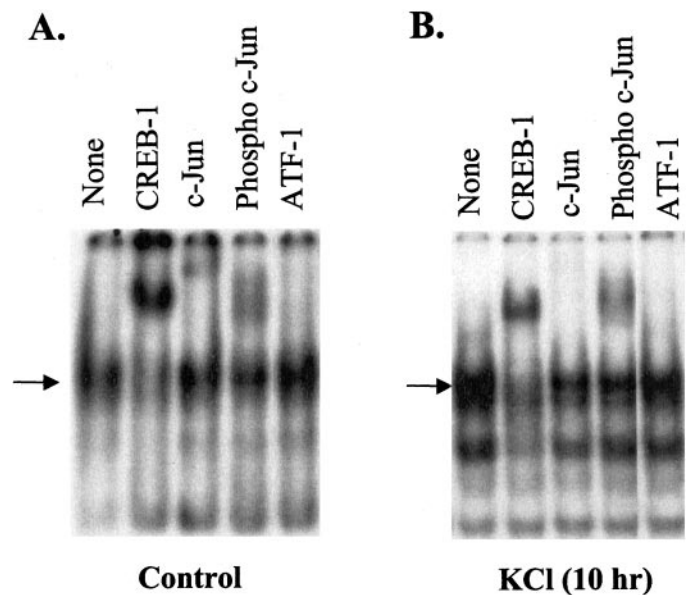
Previous reports on the regulation of proenkephalin biosynthesis regulation by depolarization in chromaffin cells have emphasized the potential role of IEGs in transcriptional regulation. Thus, depolarization has been reported previously to increase the abundance of mRNAs encoding IEGs, including c-Jun and c-Fos in bovine chromaffin cells, and increased PEnk mRNA abundance elicited by potassium depolarization has been reported to be blocked by the inhibition

of new protein synthesis (Bacher et al., 1996). However, such experiments do not distinguish between the action of IEGs per se, and nonspecific effects of cycloheximide in inhibiting the production of rapidly turning over but constitutively expressed proteins permissive for enkephalin gene transcription. Furthermore, increased gene transcription upon depolarization with nicotine or histamine is not blocked by c-Fos antisense oligonucleotide treatment, suggesting that newly synthesized c-Fos is not involved in this signaling pathway (Farin et al., 1990). Finally, third and fourth messengers acting downstream of calcium influx to mediate the calcium responsiveness of the PEnk gene in chromaffin cells have not yet been characterized. Thus, the signaling pathway lying between calcium influx and *cis*-activation through the ENKCRE-2 in chromaffin cells remains largely uncharacterized.

To appropriately dissect the signal transduction pathway for stimulus-secretion synthesis coupling, it is critical to be able to compare it with other signal transduction pathways present in chromaffin cells, both to provide specificity controls for pharmacological inhibitors and exogenous signal transduction proteins and to determine whether other pathways converge on the calcium-transduction pathway. Here, we demonstrate that PEnk gene transcription in short-term chromaffin cell cultures is responsive to KCl, PMA, and forskolin, allowing the pathways that stimulate PEnk gene transcription and initiated by these agents to be directly compared. It is clear from both the analysis of protein-synthesis dependence and inhibition by immunosuppressive agents that the potassium depolarization/calcium influx-regulated pathway is distinct from both PMA- and cAMP-initiated signaling pathways that converge on the enkephalin gene. Specifically, KCl signaling to the endogenous PEnk gene is sensitive to inhibition by calcineurin antagonists, as



**Fig. 6.** Mutation of pENK12-Luc within the ENKCRE-2 attenuates transcriptional activation by KCl. Chromaffin cells plated at  $1 \times 10^6$  cells/well in 12-well plates and transfected with pENK12-Luc (wild type) or pENK12-mCRE2 construct containing two bp substitutions within the conserved ENKCRE-2 sequence (shown in box) were assayed for luciferase activity after treatment with 40 mM KCl or medium alone. Mean  $\pm$  S.E.M. of triplicate values are shown; the experiment was repeated with qualitatively similar results. \*, significant difference from untreated control at  $p < 0.01$ .



**Fig. 7.** ENKCRE-2 sequence is predominantly bound by CREB-1 in chromaffin cell nuclear extracts both unstimulated and stimulated by KCl. EMSA was performed using  $^{32}$ P-labeled ENKCRE-2 probe (gggct-gcgtaacagcag) and nuclear extracts prepared from chromaffin cells either untreated (A) or treated with KCl for 10 h (B). For the supershift assays, antibodies raised against each of the members of CREB and AP-1 family were added in the reaction (see *Materials and Methods*). Arrow indicates band shifted by chromaffin nuclear proteins, and additional unmarked supershifted bands are also shown.

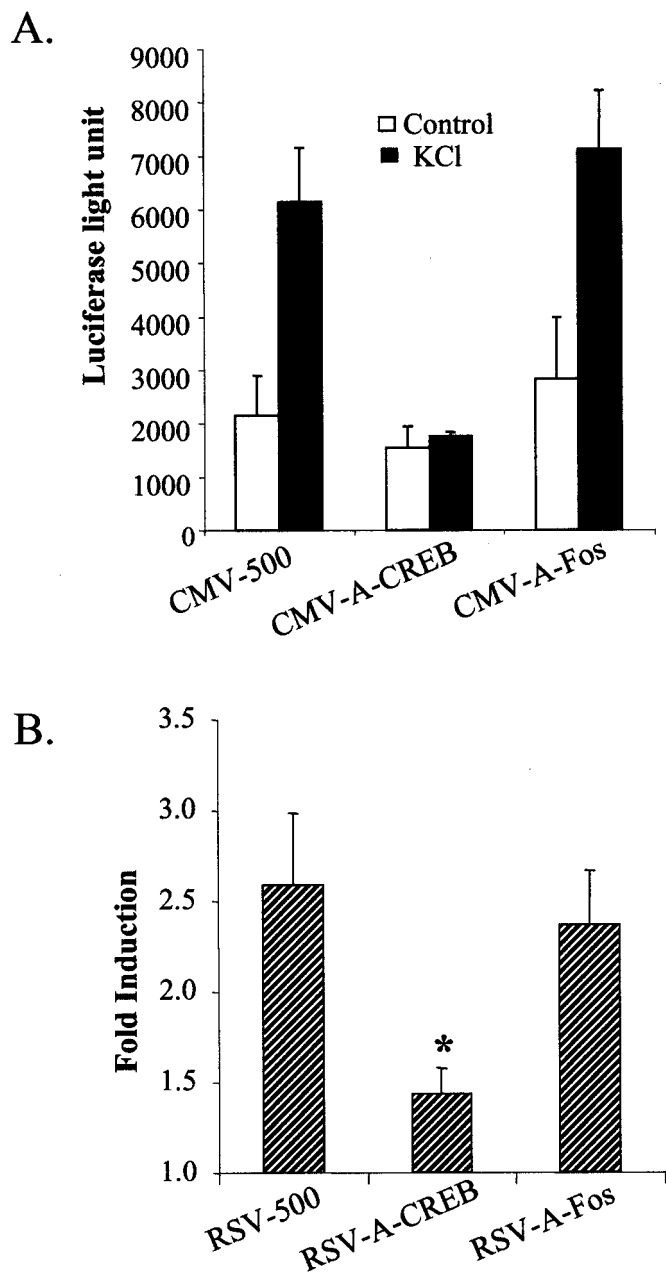
reported for calcium signaling to CRE-containing reporter genes in pancreatic and lymphoid cell lines (Schwaninger et al., 1993; Krüger et al., 1997).

The calcineurin inhibitors used in this study blocked KCl-induced PENk mRNA elevation and peptide synthesis without affecting secretion, demonstrating involvement of calmodulin-dependent phosphatase activity in mediating stimulus-synthesis coupling in chromaffin cells. Blockade of

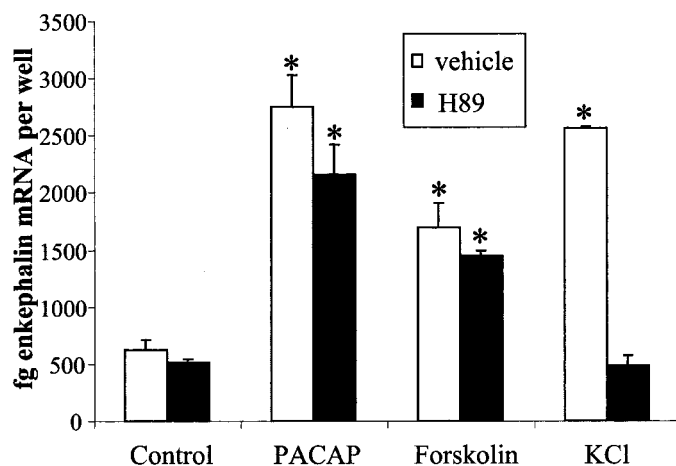
depolarization-induced PENk gene up-regulation by ascomycin and cyclosporin A, but not by rapamycin, firmly implicates calcineurin as a calmodulin-dependent downstream component of this calcium-initiated signal transduction pathway. Calcium-initiated secretion, on the other hand, seems to be independent of calcineurin activation in chromaffin cells.

The signal transduction pathway leading to enhanced transcription of the proenkephalin gene under depolarizing conditions established here can be summarized as the following:  $\text{Ca}^{2+}$  influx  $\rightarrow$  calmodulin/calcineurin activation  $\rightarrow$  CREB activation  $\rightarrow$  PENk transcriptional up-regulation via ENKCRE2. This signal transduction pathway relies only on pre-existing rather than newly induced IEG products. Other workers have observed that the elevation of proenkephalin A mRNA levels by potassium depolarization, like that evoked after treatment with forskolin or PMA, requires new protein synthesis; i.e., it is blocked by cycloheximide (Bacher et al., 1996). Cycloheximide sensitivity has also been reported for the induction of enkephalin mRNA by nicotine (Farin et al., 1990), which, like potassium depolarization, is dependent on calcium influx (Eiden et al., 1984) and is blocked by L-type calcium-channel blockers. The IEG-independent transcriptional effects of depolarization reported here may have been overlooked in previous reports in which cycloheximide concentrations were not titrated to the minimal dose required to inhibit de novo protein synthesis, avoiding potential effects on resting protein levels over a 24- to 72-h period.

Clear demonstration of a role for both calcineurin and CREB in coupling depolarization-induced calcium influx to enkephalin gene transcription in chromaffin cells represents an important advance in delineating the cellular components of activity-dependent signaling to the nucleus in homeostatic regulation of the secretory products of neuroendocrine cells. Steps downstream of calcineurin linking it to the phosphorylation and activation of CREB and the generality of this



**Fig. 8.** KCl-induced transcription of pENK12-Luc is blocked by cotransfection of A-CREB but not A-Fos. Chromaffin cells were cotransfected with pENK12-Luc reporter plasmid and one of the dominant-negative expression vectors for A-CREB or A-Fos cloned in either pRc/CMV500 (A) or pRc/RSV500 (B), or the empty vector, before treating with 40 mM KCl or medium alone. Cells were harvested 48 h after treatment for luciferase activity. Results are shown as a representative experiment done in duplicate expressed as mean  $\pm$  range (A) and as a fold induction over control after KCl treatment determined from four separate experiments and shown as mean  $\pm$  S.E.M. \*, significant difference from empty vector (RSV500) cotransfection at  $p < 0.05$  (Student's two-tailed  $t$  test).



**Fig. 9.** Selective inhibition of KCl-stimulated PENk mRNA elevation by H89. Chromaffin cells were treated with vehicle or 10  $\mu$ M H89 and 30 min later, they were exposed to 40 mM KCl, 100 nM pituitary adenyl cyclase-activating protein (PACAP), or 25  $\mu$ M forskolin in the continued absence ( $\square$ ) or continued presence ( $\blacksquare$ ) of H89. Twenty-four hours later, RNA was harvested from cells and reverse-transcribed, and PENk mRNA was quantified as described under *Materials and Methods*. Confirmation of equivalency of input RNA was determined by polymerase chain reaction quantification of glyceraldehyde-3-phosphate dehydrogenase mRNA in each sample. Values represent the mean  $\pm$  S.E.M. of three individual determinations (wells). \*, significant difference from control (vehicle or H89 alone) at  $p < 0.05$  (Student's two-tailed  $t$  test).



pathway to stimulus-secretion synthesis coupling in other neuronal systems can now be established. Inhibition of calcineurin/CREB-dependent calcium signaling to the proenkephalin A gene by the serine/threonine kinase inhibitor H89 suggests that one of the H89-sensitive CREB kinases, such as protein kinase A or MSK1 (Arthur and Cohen, 2000; Davies et al., 2000), is also a component in this pathway. There is as yet no evidence for calcineurin-dependent activation of either of these CREB kinases; however, one well-established pathway for MSK1 activation is via the extracellular signal-regulated kinase (ERK) cascade (Deak et al., 1998). In myocytes, the activation of ERK by  $\beta$ -agonists is reported to require calcineurin (Zou et al., 2001). Whether this pathway is accessed by calcineurin upon calcium influx after depolarization in neuroendocrine cells is a subject for future investigation; however, the observation that ERK1/2 phosphorylation is stimulated by elevated KCl in bovine chromaffin cells (Chen and Eiden, unpublished data) supports this possibility.

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