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A Crucial Role for the Mitogen-Activated Protein Kinase Pathway in Nicotinic Cholinergic Signaling to Secretory Protein Transcription in Pheochromocytoma Cells

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ABSTRACT

The mitogen-activated protein kinase (MAPK) pathway plays a pivotal role in intracellular signaling, and this cascade may impinge on cAMP response elements (CREs) of target genes. Both the MAPK pathway and chromogranin A expression may be activated by cytosolic calcium influx, and calcium-dependent signals map onto the chromogranin A promoter proximal CRE. We therefore probed the role of the MAPK pathway in chromogranin A biosynthesis after secretory stimulation of PC12 pheochromocytoma cells by the nicotinic cholinergic pathway, the physiological secretory trigger. Chemical inhibition of either MAPK or MAPK kinase blocked the response of a transfected chromogranin A promoter to nicotine or protein kinase C activation [by phorbol-12-myristate-13-acetate (PMA)], although nicotine-evoked catecholamine secretion was unaffected. Activation of the MAP kinase cascade (Ras, Raf, MAPK, or CREB kinase) by cotransfection of pathway components stimulated the chromogranin A promoter. Cotransfection of MAPK pathway dominant negative mutants (for Raf, MAPK, or CREB kinase) blocked nicotinic or PMA activation of chromogranin A, although a dominant negative Ras mutant was without effect. MAPK pathway enzymatic activity was stimulated by both nicotine and PMA. Point mutations of the chromogranin A CRE suggested that this element was necessary in *cis* for stimulation by nicotine, PMA, or chemical activation of the MAPK pathway. Transfer of the CRE to a heterologous promoter conferred inducibility by not only nicotine or cAMP but also MAPK activation. Expression of the CREB antagonist KCREB blocked the response of the chromogranin A promoter to nicotine, cAMP, or MAPK pathway activation by either chemical stimulation or cotransfection of active cascade components. Chromogranin A mRNA responded to MAPK pathway manipulation in a fashion similar to the transfected chromogranin A promoter, in both direction and magnitude. We conclude that the MAPK pathway is a necessary intermediate in signaling from the nicotinic receptor to secretory protein transcription, although not to catecholamine secretion. In *trans*, this response seems to involve the following signal cascade: protein kinase C → Raf → MAPK kinase → MAPK → CREB kinase → CREB. In *cis*, activation by the cascade maps onto the chromogranin A promoter proximal CRE, which is both necessary and sufficient to confer the response.

The MAPK pathway plays an increasingly appreciated role in diverse cellular signaling processes, including signaling of several growth factors and hormones toward gene expression (Kyriakis and Avruch, 1996; Treisman *et al*., 1996; Robinson *et al*., 1997). Growth factor activation of the MAPK pathway may impinge onto the CREs of target genes, with activation of CREB through phosphorylation by CREB kinase, otherwise known as ribosomal protein S6 serine kinase 2 (Rsk2; Xing *et al*., 1996), which is in turn activated through phosphorylation by MAPK (Xing *et al*., 1996).

In previous studies, we probed the mechanism of transcriptional activation of the secretory granule protein chromogranin A by stimulation of chromaffin cells through the physiological nicotinic cholinergic pathway (Tang *et al*., 1996, 1997). The biosynthesis of chromogranin A, the major protein stored and released by exocytosis with catecholamines (Takiyyuddin *et al*., 1990), is activated by nicotinic stimulation (Tang *et al*., 1996), and the response requires the participation of the chromogranin A promoter CRE in *cis* (Tang *et al*., 1996, 1997) and the transcription factor CREB in *trans* (Tang *et al*., 1996, 1997). This response is also absolutely dependent on cytosolic calcium influx and consequent activation of protein kinase C (Tang *et al*., 1997).

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ABBREVIATIONS: CRE, cAMP response element; CREB, cAMP response element binding protein; RskII, ribosomal protein S6 serine kinase II (pp90^{rsk}, CREB kinase); Erk, extracellular signal relay serine/threonine kinase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; bp, base pair(s); MEK, mitogen-activated protein kinase kinase; Raf1, serine/threonine kinase; MEKK, mitogen-activated protein kinase kinase kinase; Ras, guanine nucleotide-binding/exchanging protooncogene product; NGF, nerve growth factor; PMA, phorbol-12-myristate-13-acetate; PAF, platelet-activating factor; TK, thymidine kinase; CAT, chloramphenicol acetyltransferase.

Because the MAPK cascade also can be triggered by protein kinase C activation (Marquardt *et al.*, 1994), we wondered whether the protein kinase C-dependent actions of nicotine might trigger the MAPK pathway in chromaffin cells and whether this sequence of events might be involved in chromaffin cell transcriptional responses to nicotinic stimulation. Our interest was heightened not only because protein kinase C is involved in nicotinic (Tang *et al.*, 1997) and MAPK (Marquardt *et al.*, 1994) signaling but also because both nicotinic (Tang *et al.*, 1997) and MAPK (Xing *et al.*, 1996) transcriptional signals may converge on the protein CREB in *trans* and CREs in *cis*.

We therefore explored the role of the MAPK pathway in the actions of nicotinic cholinergic stimulation to trigger secretory protein transcription in chromaffin cells. Our results suggest that this pathway plays a crucial (indeed indispensable) role in nicotinic signaling to chromogranin A transcription, although not to catecholamine secretion. Furthermore, MAPK responses use the transcription factor CREB in *trans* and the chromogranin A promoter proximal CRE in *cis*.

Materials and Methods

Cell culture and transfections. PC12 (Greene and Tischler, 1976) cells (passages 10–25) were cultured and transfected by lipofection, as described previously (Tang *et al.*, 1996).

Plasmids. In plasmid pXP-1133, a functional mouse chromogranin A promoter drives expression of a luciferase reporter (Wu *et al.*, 1994); the promoter region in this plasmid extends from −1133 bp upstream of the transcription initiation (cap) site to +42 bp downstream of the cap site.

The mouse chromogranin A promoter CRE (CRE box; [−71 bp]5′-TGACGTAA-3′[−64 bp]) occurs at position −71 to −64 bp upstream of the cap site. CRE box mutants M13 and M41 have been described previously (Wu *et al.*, 1995). Mutant M13, in which 6 of 8 CRE bases are changed (to CATCACCA; changes underlined), occurs in a 100-bp promoter/reporter construct (control promoter/reporter plasmid: pXP-100). Mutant M41, a CRE point-gap mutant (to TGA-GTAA) occurs in a 77-bp promoter/reporter (control promoter/reporter plasmid: pXP-77).

The mouse chromogranin A promoter CRE domain (TGACGTAA), a CRE box point mutant (TGA-GTAA), and a consensus CRE (TGACGTCA; Roesler *et al.*, 1988) were positioned just upstream of the heterologous herpes simplex virus TK promoter, in a TK/luciferase promoter/reporter (Wu *et al.*, 1995).

Other eukaryotic expression plasmids used in this research include (1) wild-type human Erk1 (pCMV5Erk1), or its dominant negative mutant K71R (pCMV5 Erk1 K71R), as well as wild-type rat Erk2 (pCMV5Erk2), or its dominant negative mutant K52R (pCMV5 Erk2 K52R), each driven by the CMV promoter (from John K. Westwick, University of North Carolina, Chapel Hill, NC; Westwick *et al.*, 1994; Robbins *et al.*, 1993). (2) Wild-type rat Raf-1 (KSRSPA cRAF), or its dominant negative mutant K375R (KS RSPA cRAF DN), each driven by the RSV-LTR promoter, were from Michael Karin (University of California, San Diego, CA) (Thorburn *et al.*, 1994). (3) Wild-type c-Ha-Ras (pCD-WTras), or its dominant negative mutant pCD-DNras (S17N), each driven by the SV40 early promoter, were from Neil M. Nathanson (University of Washington, Seattle, WA) (Feig and Cooper, 1988; Feig *et al.*, 1986). (4) Wild-type human CREB kinase (also called RSK2, HA-epitope-tagged in pMT2HARSK2), or its K100R dominant negative mutant (pMT2HARSK2KR100), each driven by the adenovirus major late promoter (AML), were from Michael E. Greenberg (Harvard Medical School, Boston, MA) (Xing *et al.*, 1996). (5) The CREB dominant negative (inhibitory) DNA binding domain point mutant KCREB, driven by the RSV-LTR (in pRSV-KCREB), was from Richard H. Goodman (Oregon Health Sciences University, Portland, OR) (Walton *et al.*, 1992). (6) A 71-bp c-Fos promoter, which includes a "calcium response element" at 5′-TGACGTTT-3′ (−62 to −54 bp upstream of the c-*fos* cap site), linked to a CAT reporter ("−71 wt fos CAT"), was from Michael E. Greenberg (Harvard Medical School, Boston, MA) (Sheng *et al.*, 1988).

Assays and catecholamine secretion. Luciferase reporter activity was measured in cell extracts by luminometry (Dewet *et al.*, 1986). In the c-*fos* promoter/CAT reporter study, CAT activity was measured by incorporation of [^{14}C]acetyl groups into chloramphenicol (Gorman *et al.*, 1982). Catecholamine secretion from PC12 cells preloaded with [^{3}H]-L-norepinephrine was accomplished as described previously (Parmer *et al.*, 1993). Protein was measured by the Coomassie blue dye binding assay (Bradford, 1976).

MAPK pathway activity was measured with an MAP Kinase Assay Kit (Stratagene, La Jolla, CA) (Boulton *et al.*, 1991; Davis, 1993). PC12 cells (in 10-cm plates) under different experimental conditions were harvested with PBS and then lysed with 1 ml of lysis buffer (2.5 mM HEPES, pH 7.5, 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 2 mM Na vanadate). After pelleting cell lysates, supernate protein concentrations were measured, and an anti-MAPK antibody (Stratagene) was added to supernates at 1 μl/mg protein. After a 1-hr incubation at 4°, 20 μl of protein A beads (Sigma Chemical, St. Louis, MO) was added in lysis buffer, followed by continued incubation for 30 min. The beads were washed three times with lysis buffer and dissolved into a final volume of 24 μl of lysis buffer, subsequently used for MAPK assay. Samples from the above immunoprecipitation were mixed with [γ-^{32}P]ATP in reaction buffer, with the MAPK substrate PHAS-I (phosphorylated heat- and acid-stable protein regulated by insulin). Mixtures were incubated at 30° for 20 min and then electrophoresed on Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis gradient gels (10–27%; NOVEX, La Jolla, CA), followed by exposure to Kodak X-ray film at −70°. After fluorographic exposure, ^{32}P-labeled protein bands were excised from gels based on autoradiography, and the dpm of each band was obtained by liquid scintillation counting.

Pharmacology. The effect of the following agents on transcription or secretion was tested at concentrations and times indicated for a particular experiment. Nicotine was from Sigma Chemical. Doses of nicotine for activation of chromogranin A transcription (maximal at 1 mM nicotine) versus catecholamine secretion (maximal at 60–100 μM nicotine) were optimized by previous dose-response analyses (Tang *et al.*, 1996). PMA, the MAPK inhibitor apigenin (Kuo and Yang, 1995; Sato *et al.*, 1994), the MAPK kinase inhibitor PD-98059 (Alessi *et al.*, 1995), and the MAPK pathway activators ATA (Okada and Koizumi, 1995), PAF (C_{16}; Honda *et al.*, 1994), and *N*-hexanoyl-D-*erythro*-sphingosine (C_6-ceramide; Jayadev *et al.*, 1995) were obtained from Calbiochem/Novabiochem (La Jolla, CA).

Statistics. Results are reported as the mean ± 1 standard error value. Data were analyzed by either *t* test (two groups) or analysis of variance (three or more groups) using the software packages Statworks or Cricketgraph (Cricket Software, Malvern, PA) or StatView (Abacus Concepts, Berkeley, CA), each for the Macintosh microcomputer. Differences were considered significant at $p < 0.05$.

Results and Discussion

Involvement of the MAPK pathway in nicotinic cholinergic signaling to chromogranin A transcription, although not catecholamine secretion: Effects of MAPK pathway chemical inhibitors. We used the MAPK pathway enzymatic activity inhibitors apigenin (MAPK inhibitor; Sato *et al.*, 1994) or PD98059 (MAPK kinase inhibitor; Alessi *et al.*, 1995) to probe that pathway's potential involvement in nicotinic cholinergic signaling in PC12 cells. Both nicotine and the protein kinase C activator PMA stimulated the transfected chromogranin A promoter, and in each

case the stimulation was substantially blunted by inhibition of either MAPK or MEK (Table 1). The inhibitors alone (without the activators [nicotine or PMA]) had only a modest effect on basal expression of the transfected promoter.

Nicotine also activated catecholamine secretion, but neither basal nor nicotine-stimulated catecholamine release was affected by inhibition of either MAPK or MEK (Table 1), despite preincubation of PC12 cells with each inhibitor for 1 hr before nicotinic secretory stimulation. Thus, the MAPK pathway seems to be involved in the chromogranin A tran-

TABLE 1

Effects of MAPK pathway chemical inhibitors on nicotine-stimulated catecholamine secretion or chromogranin A transcription in pheochromocytoma cells

Chromogranin A transcription: After transfection of the 1133-bp mouse chromogranin A promoter/luciferase reporter, apigenin (MAP kinase inhibitor, 12.5 μM) or PD98059 (MAP kinase kinase inhibitor, 20 μM) was added and incubated for 48 hr. Nicotine (1 mM) was added at the same time as the inhibitors, whereas PMA (0.1 μM) was added 6 hr before cell harvest. Data shown are luciferase activity/mg of protein in mean ± standard error for four replicates/condition.

Inhibitor		Chromogranin A transcription (luciferase/protein)					Catecholamine secretion		
Target	Compound	Basal	Nicotine (1 mM)	Ratio (nicotine/basal)	PMA (0.1 μM)	Ratio (PMA/basal)	Basal	Nicotine (60 μM)	Ratio (nicotine/basal)
								%	
None	Mock	233 ± 14.4	619 ± 28.4[b]	2.66	513 ± 22.7[b]	2.20	4.3 ± 0.25	13.1 ± 0.58[b]	3.0
MAPK	Apigenin (12.5 μM)	189 ± 10.8[a]	172 ± 7.54[a]	0.91	237 ± 12.2[ab]	1.20	4.7 ± 0.31	13.8 ± 0.64[b]	2.9
MEK	PD98059 (20 μM)	170 ± 5.34[a]	198 ± 7.18[a]	1.16	195 ± 2.03[a]	1.10	4.0 ± 0.22	12.9 ± 0.41[b]	3.2

[a] Inhibitor treatment group significantly ($p < 0.05$) different from mock group for that stimulation (same column).

[b] Stimulated group significantly ($p < 0.05$) different from unstimulated group for that treatment (same row). Activation of this plasmid by nicotine or phorbol ester has been described previously (Tang *et al.*, 1996, 1997).

Catecholamine secretion: Each group was preincubated with inhibitors for 1 hr. During the 30-min secretion period (with or without 60 μM nicotine), inhibitors also were maintained in each group. Percent norepinephrine secretion was calculated as [secreted dpm/(secreted dpm + cellular dpm)][100]. Ratio data are stimulated secretion/mock-stimulated secretion for each treatment. Results are mean ± standard error for three replicates/condition. [ab], See above.

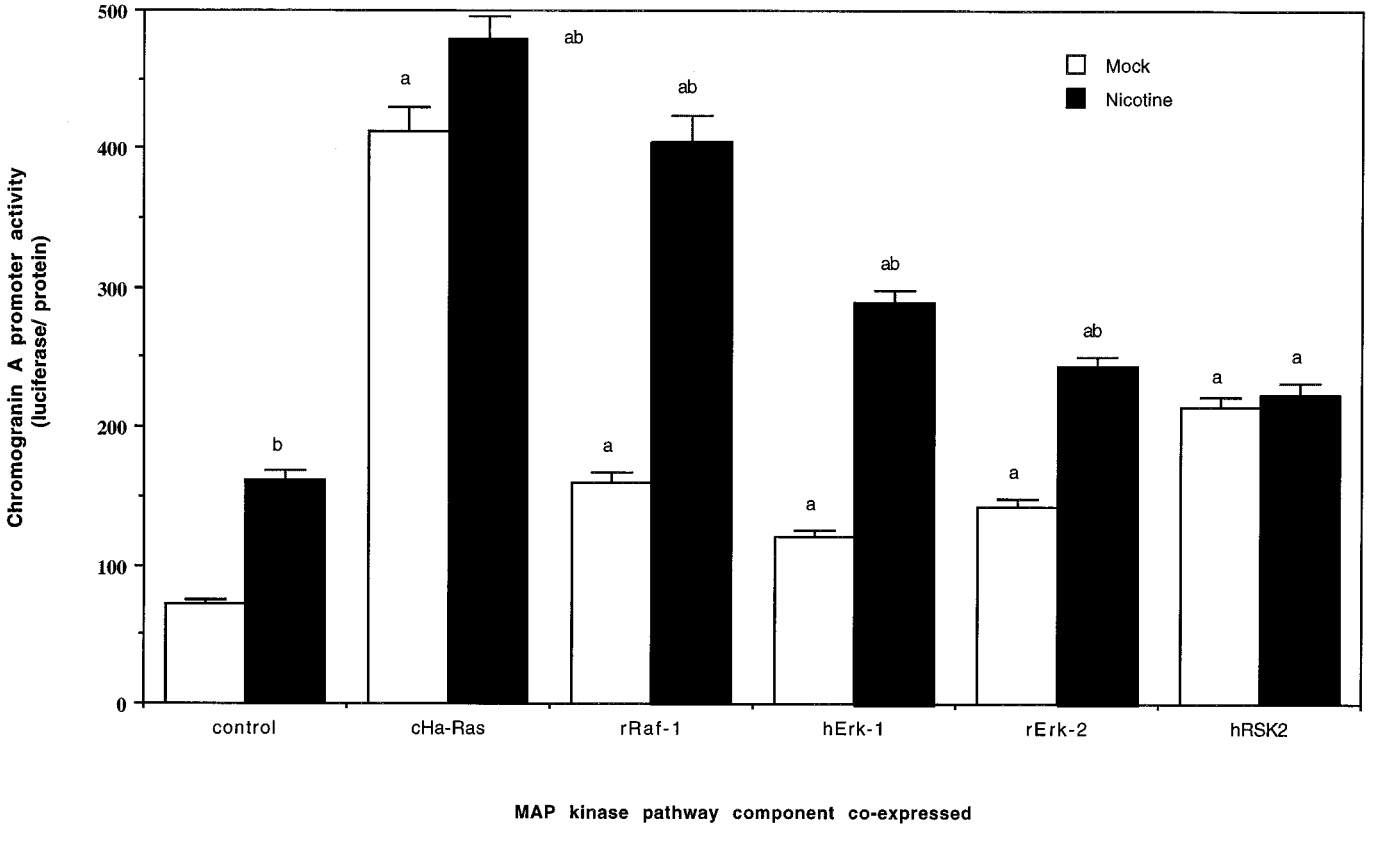

Fig. 1. Effects of cotransfection of MAPK pathway expression plasmids on nicotinic cholinergic control of chromogranin A promoter expression in pheochromocytoma cells. The 1133-bp mouse chromogranin A promoter/luciferase reporter (3 μg/ml) was cotransfected along with the indicated MAPK pathway expression plasmid (3 μg/well), at 1 ml/well, in 12-well plates of PC12 cells. Stimulation occurred over 48 hr, with nicotine (10^{-3} M) versus vehicle (mock). Results shown are luciferase activity/mg sample protein [four replicates/condition; mean ± standard error; a, $p < 0.05$, compared with the pBluescript (control) cotransfection group for each stimulation (same color)]. b, $p < 0.05$ compared with the unstimulated group for each cotransfection (same activator gene coexpressed). *cHa-Ras*, wild-type c-Ha-Ras expression driven by the SV40 promoter, in the plaasmid pCD-WTras; *rRaf-1*, wild-type rat Raf-1 expression driven by the RSV LTR (Rous sarcoma virus long terminal repeat) promoter, in the plasmid KSRSPA cRAF, *hErk-1*, wild-type human Erk1 expression driven by the CMV (cytomegalovirus) promoter, in the plasmid pCMV5Erk1; *rErk-2*, wild-type rat Erk2 expression driven by the CMV promoter, in the plasmid pCMV5Erk2; *hRSK2*, wild-type human RSK2 (ribosomal serine kinase 2; CREB kinase) expression driven by the AML (adenovirus major late promoter) in the plasmid pMT2HARASK2.

scriptional (although not the catecholamine secretory) response to nicotine or PMA.

Activation of the MAPK pathway by cotransfection: Effect on chromogranin A transcription. During growth factor stimulation (Ginty *et al*., 1994), the MAPK pathway may relay signals to the transcription factor CREB by the action of MAPK (Erk) on CREB kinase (Rsk2), which in turn activates CREB by phosphorylation on CREB Ser133 (Xing *et al*., 1996).

We overexpressed several components of the MAPK pathway (Ras, Raf, Erk, or RSK2; Okada and Koizumi, 1995; Pang *et al*., 1995; Johnson and Nathanson, 1994) in PC12 cells by cotransfection with an 1133-bp mouse chromogranin A promoter/luciferase reporter plasmid. Each of these components *trans*-activated the transfected chromogranin A promoter (by 1.7–5.7-fold; Fig. 1) and in some cases (e.g., Raf-1 or Erk-1) substantially augmented the chromogranin A transcriptional response to nicotine. Thus, overexpression of MAPK pathway components also can activate the chromogranin A promoter.

Inhibition of the MAPK pathway by cotransfection: Effect on chromogranin A transcription. ATP binding domain, dominant negative (inhibitory) point mutants have been developed for several components of the MAPK cascade: Ras (Feig *et al*., 1986; Feig and Cooper, 1988), Raf (Thorburn *et al*., 1994), Erk (Robbins *et al*., 1993; Westwick *et al*., 1994), and CREB kinase (Xing *et al*., 1996).

The chromogranin A transcriptional response to nicotine was almost completely inhibited by expression of dominant negative mutants (Fig. 2) of three of these MAPK pathway components (Raf, Erk, or CREB kinase; Kyriakis and Avruch, 1996; Treisman *et al*. 1996; Robinson *et al*., 1997), although not by a dominant negative Ras mutant. Likewise, the chromogranin A transcriptional response to protein kinase C activation (by PMA) was at least partially blocked by dominant negative mutants of Raf, Erk, or CREB kinase, although not by the Ras mutant (Fig. 2).

As a positive control, the effects of NGF on chromogranin A transcription also were evaluated in the same experiment. NGF signaling to chromogranin A transcription was substantially blocked by each of the dominant negative mutants (for Ras, Raf, Erk, or CREB kinase). Thus, the Ras dominant negative mutant was functional, and its lack of inhibition of nicotine or PMA effects, suggests that Ras does not signal for nicotine or PMA. Thus, specific mutant disruption of the MAPK pathway inhibits the chromogranin A transcriptional response to nicotine or PMA but only at the Raf, Erk, or CREB kinase steps (although not at the level of Ras).

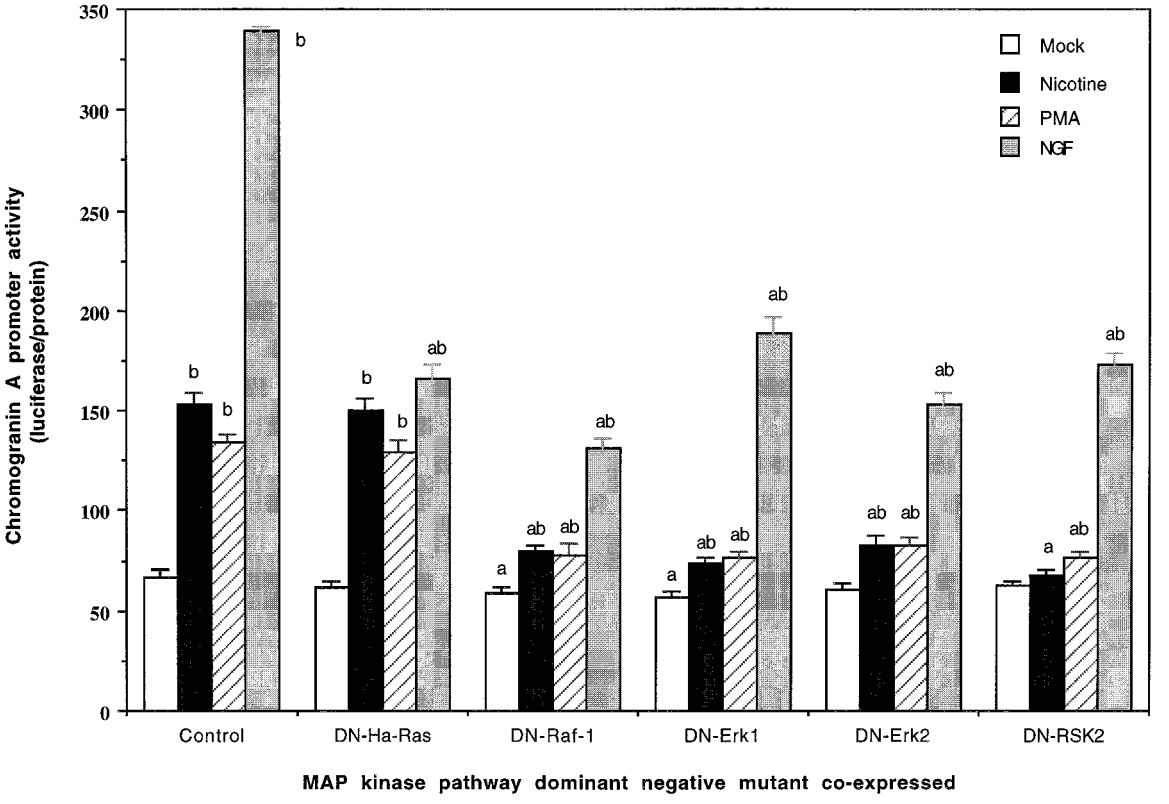

Fig. 2. Effects of cotransfected MAPK pathway dominant negative (inhibitory) mutants on the response of the chromogranin A promoter to stimulation by nicotine, phorbol ester, or NGF in pheochromocytoma cells. The 1133-bp mouse chromogranin A promoter/luciferase reporter (3 μg/ml) was cotransfected along with the indicated (*left*) *trans*-inhibitor expression plasmid (3 μg/ml). Stimulations were nicotine, 1 mM, 48 hr; PMA (phorbol ester), 0.1 μM, 6 hr; NGF (positive control), 0.1 μg/ml, 48 hr; or vehicle (mock). Results shown are luciferase activity/mg protein [four replicates/condition, mean ± standard error; a, $p < 0.05$, compared with the control (pBluescript) cotransfection group for each stimulation (same color)]. b, $p < 0.05$, compared with the unstimulated group for each cotransfection (same mutant expressed). Control: cotransfection of pBluescript. *DN-Ha-Ras*, dominant negative c-Ha-Ras (S17N) expression driven by the SV40 promoter, in the plasmid pCD-DNras; *DN-Raf-1*, dominant negative rat Raf-1 (K375R) expression driven by the RSV LTR (Rous sarcoma virus long terminal repeat) promoter, in the plasmid KS RSPA cRAF DN; *DN-Erk1*, dominant negative human Erk1 (K71R) expression driven by the CMV (cytomegalovirus) promoter, in the plasmid pCMV5 Erk1 K71R; *DN-Erk2*, dominant negative human Erk2 mutant (K52R) expression driven by the CMV promoter, in the plasmid pCMV5 Erk2 K52R; *DN-RSK2*, dominant negative human RSK2 (ribosomal serine kinase 2; CREB kinase) mutant (K100R) expression driven by the AML (adenovirus major late promoter), in the plasmid pMT2HARSK2(KR100).

Nicotine or PMA effects on MAPK pathway enzymatic activity in PC12 cells. In an MAPK activity assay (Fig. 3), nicotine augmented phosphorylation of an MAPK substrate by 1.8-fold at 30 min, 4.6-fold at 1 hr, and 2.2-fold at 12 hr. PMA increased activity by 3.2-fold at 30 min. Thus, MAPK pathway enzymatic activity is triggered by nicotine or PMA in PC12 cells.

MAPK pathway signaling to the chromogranin A promoter: Localization of the activation to the CRE. Because nicotinic cholinergic stimulation of chromogranin A transcription maps substantially onto the chromogranin A promoter proximal CRE, [−71 bp]5′-TGACGTAA-3′[−64 bp] (Tang *et al*., 1996), we tested the effects of not only nicotine but also several MAPK pathway activators on transfected proximal chromogranin A promoter/reporter constructs: the MAPK activator ATA (Okada *et al*., 1995), the MAPK and MEK activator PAF (C_{16}) (Honda *et al*., 1994), or the MAPK activator N-hexanoyl D-*erythro*-sphingosine (C_6-ceramide) (Jayadev *et al*., 1995).

Not only nicotine and PMA but also each of the MAPK pathway activators (ATA, PAF, or C_6-ceramide), *trans*-activated the transfected chromogranin A promoter in PC12 cells (Table 2). Both a 100-bp promoter (in plasmid pXP-100) and a 77-bp promoter (in plasmid pXP-77) were activated by each stimulus.

The degree of nicotinic activation of the 100-bp (2.28-fold; pXP-100; Table 2), 77-bp (1.91-fold; pXP-77; Table 2), or 1133-bp (2.66-fold; pXP-1133; Table 1) transfected chromogranin A promoters was similar. Likewise, the degree of PMA activation of the 100-bp (2.01-fold; pXP-100; Table 2), and 77-bp (1.84-fold; pXP-77; Table 2) transfected chromogranin A promoters was similar.

MAPK pathway activators stimulated the transfected 100-bp chromogranin A promoter (pXP-100) to an extent (2.32–2.65-fold) similar to nicotinic (2.28-fold) or PMA (2.01-fold) stimulation (Table 2). Likewise, MAPK pathway activators stimulated the transfected 77-bp chromogranin A promoter (pXP-77) to an extent (1.86–2.36-fold) similar to nicotinic (1.91-fold) or PMA (1.84-fold) induction (Table 2).

Site-directed mutation of the chromogranin A promoter CRE (TGACGTAA; Table 2), either a 6/8-bp change (to CATCACCA) in a 100-bp promoter (mutant M13) or a single base gap (to TGA-GTAA) in a 77-bp promoter (mutant M41), virtually abolished the chromogranin A promoter response to not only nicotine or PMA but also the three MAPK pathway activators: ATA, PAF, and C_6-ceramide. Thus, the chromogranin A promoter proximal CRE, [−71 bp]5′-TGACGTAA-3′[−64 bp], seemed to be necessary (indeed, indispensable) in *cis* for activation of transcription in response to nicotinic stimulation, protein kinase C activation, or MAPK pathway activation.

Role of the chromogranin A CRE as a sufficient domain to transmit MAPK pathway activation signals. To test whether the mouse chromogranin A promoter proximal CRE, [−71 bp]5′-TGACGTAA-3′[−64 bp], was sufficient to account for the response to MAPK pathway activation, we fused the chromogranin A CRE (TGACGTAA) to a previously unresponsive, heterologous (TK) promoter, which was in turn fused to a luciferase reporter (pTK-LUC; Wu *et al*., 1994, 1995), yielding the plasmid mCgA-CRE-TK. We also fused a mutant CRE (TGA-GTAA) to the TK promoter, yielding the plasmid Mutant-CRE-TK (Wu *et al*., 1995); and a consensus CRE (TGACGTCA) to the TK promoter, yielding the plasmid Perfect-CRE-TK (Roesler *et al*., 1988; Wu *et al*., 1995).

The MAPK pathway activators ATA, PAF, and C_6-ceramide each stimulated the isolated CRE box (Table 3), and the degree of stimulation (2.27–3.16-fold) was similar to that seen for the intact 1133-bp chromogranin A promoter (pXP-1133; 2.94–4.31-fold). By contrast, the three MAPK pathway activators did not stimulate the control heterologous TK promoter without a CRE (in pTK-LUC), nor was this minimal promoter stimulated by nicotine or cAMP. The chromogranin A CRE (in mCgA-CRE-TK) was stimulated to a similar degree by direct MAPK activators (2.27–3.16-fold), nicotine (4.06-fold), or cAMP itself (3.92-fold). A similar plasmid with a consensus CRE (TGACGTCA; Perfect-CRE-TK) also was stimulated to similar extents by MAPK activators (3.87–4.16-fold), nicotine (5.03-fold), or cAMP (8.11-fold). Thus, the proximal CRE seems to be a sufficient element in *cis* to account for activation of the chromogranin A promoter by MAPK pathway stimulation.

A "calcium response element," similar in sequence (TGACGTTT) to a CRE (TGACGT[C/A]A), also can be activated by nicotinic cholinergic stimulation, through an MAPK-dependent pathway. Greenberg *et al*. (Ginty *et al*., 1994) characterized a "calcium response element" in the c-Fos promoter that responds to cytosolic calcium-dependent signals. A c-Fos promoter/reporter plasmid

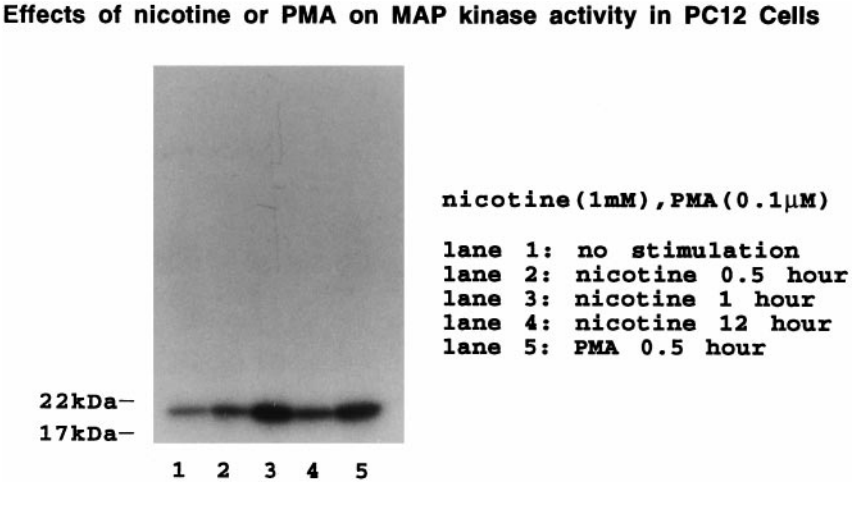

Fig. 3. Stimulation of MAPK pathway enzymatic activity by nicotine or protein kinase C activation in pheochromocytoma cells. After stimulation of PC12 cells by secretatogue (nicotine, 10^{-3} M, or PMA, 10^{-6} M) or vehicle, MAPK was immunoprecipitated from cell extracts and then incubated with [γ-^{32}P]ATP and the MAPK substrate PHAS-I ("phosphorylated heat- and acid-stable protein regulated by insulin"). Electrophoresis was performed in a Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. The dpm (disintegrations/minute) in the labeled bands were *lane 1*, 7,220; *lane 2*, 13,200; *lane 3*, 33,300; *lane 4*, 16,200, and *lane 5*, 22,900.

(−71 wt *fos* CAT) containing the calcium response element also was activated ($p < 0.05$) by either nicotine (1.8-fold) or PMA (1.4-fold), and each of these activations was almost entirely blocked ($p < 0.05$) by inhibition of the MAPK pathway, either at MAPK (by apigenin, 12.5 μM) or at MEK (by PD98059, 40 μM). Thus, the calcium response element (TGACGTTT) responds to nicotine and PMA stimulation in much the same MAPK pathway-dependent fashion as the chromogranin A CRE (TGACGTAA).

Role of the transcription factor CREB in relaying signals from the MAPK pathway: studies with chemical activators of MAPK. Goodman *et al.* (Walton *et al.*, 1992) developed a CREB point mutant in the CREB DNA-binding domain, the expression of which acts as in dominant

TABLE 2

Necessity of the CRE box for MAPK pathway-mediated signaling to chromogranin A gene expression in pheochromocytoma cells

Mouse chromogranin A promoter/reporter plasmids were transfected into PC12 cells. Cells were harvested for reporter assay after the times noted. Mutant bases in the CRE box are designated in bold type. In the mouse chromogranin A promoter, the functional wild-type CRE box (TGACGTAA) is at position −71 to −64 bp upstream of the cap (transcription initiation) site. Data are shown as mean ± standard error values (n = 4) for luciferase/mg protein ratios.

Plasmid name CgA promoter length (bp 5′ of cap site) CRE box (mutations in bold type)	pXP-100 100 TGACGTAA (wild-type CRE)	M13 100 **CAT**C**ACC**A (6/8 bp **replaced**)	pXP-77 77 TGACGTAA (wild-type CRE)	M41 77 TGA-GTAA (1 bp point-gap)
Pathway				
Stimulus				
None				
Mock	2,510 ± 98.6	944 ± 37.7[a]	6930 ± 435	3,270 ± 230[a]
Nicotinic cholinergic				
Nicotine (1 mM, 48 hr)	5,730 ± 187[b]	1,160 ± 55.2[a]	13,200 ± 621[b]	3,590 ± 109[a]
Ratio (nicotine/mock)	2.28	1.22	1.91	1.10
Protein kinase C activator				
PMA (0.1 μM, 6 hr)	5,040 ± 145[b]	1,220 ± 64.1[a]	11,400 ± 745[b]	3,490 ± 211[a]
Ratio (PMA/mock)	2.01	1.29	1.84	1.07
MAPK pathway activators				
ATA (100 μM, 6 hr)	6,630 ± 267[b]	987 ± 37.9[a]	16,300 ± 475[b]	2,990 ± 88.0[a]
Ratio (ATA/mock)	2.65	1.04	2.36	0.91
PAF (C_{16}) (100 nM, 6 hr)	5,820 ± 491[b]	885 ± 69.1[a]	12,900 ± 801[b]	2,730 ± 236[a]
Ratio (PAF/mock)	2.32	0.94	1.86	0.84
C_6 ceramide (10 μM, 6 hr)	6,000 ± 196[b]	947 ± 59.3[a]	13,600 ± 1,010[b]	2,610 ± 163[a]
Ratio (C_6/mock)	2.39	1.00	1.96	0.80

[a] Stimulated group significantly ($p < 0.05$) different from mock for the same plasmid (same row).

[b] Significantly ($p < 0.05$) different from the corresponding wild-type group in the same stimulation condition (same column). CgA, chromogranin A; CRE, cAMP response element; cap site, transcription initiation site; pXP-100 or pXP-77, 100- or 77-bp mouse chromogranin A promoter (wild-type) fused to a luciferase reporter; M13, cAMP response element mutant (**CAT**C**ACC**A) of pXP-100; M41, cAMP response element mutant (TGA-GTAA) of pXP-77; ATA, aurintricarboxylic acid, stimulator of MAP kinase phosphorylation; PAF (C_{16}), platelet activating factor-16, activator of MAPK and MEK; C_6 ceramide; *N*-hexanoyl D-erythro-sphingosine, activator of MAP kinase.

The effect of nicotine or phorbol ester on gene expression by these mutants has been reported previously (Tang *et al.*, 1996, 1997).

TABLE 3

Sufficiency of the CRE box for MAPK pathway-mediated signaling to chromogranin A gene expression in pheochromocytoma cells

PC12 cells were transfected with the indicated promoter/luciferase reporter plasmid (top row) and in the presence or absence of secretagogues (left column), and cells were harvested for reporter assay after the times noted. In the TK (thymidine kinase) plasmids, the herpes simplex virus 110-bp TK promoter drives expression of a luciferase reporter; isolated CRE (cAMP response element) boxes have been fused just 5′ (upstream) of the TK promoter. Both the mouse chromogranin A (mCgA) promoter and the herpes simplex virus TK promoter contain TATA boxes. Units are luciferase activity/mg protein. In the wild-type mouse Cg A promoter, the functional CRE box (TGACGTAA) is located at position −71 to −64 bp upstream of the cap site.

	Plasmid				
	pXP-1133	mCgA-CRE-TK	Perfect-CRE-TK	Mutant-CRE-TK	pTK-LUC (vector)
Promoter CRE box	1133 bp mCgA TGACGTAA	TK (110 bp) TGACGTAA	TK (110 bp) TGACGTCA	TK (110 bp) TGA-GTAA	TK (110 bp) None
Pathway					
Stimulus					
None					
Mock	318 ± 12.4	407 ± 17.7	513 ± 20.1	154 ± 10.0	128 ± 11.5
Nicotinic cholinergic					
Nicotine (1 mM, 48 hr)	948 ± 47.6[a]	1650 ± 67.8[a]	2580 ± 98.7[a]	208 ± 12.1	157 ± 9.23
Ratio (nicotine/mock)	2.98	4.06	5.03	1.35	1.23
cAMP					
cAMP (0.1 mM, 48 hr)	1130 ± 52.8[a]	1600 ± 74.5[a]	4160 ± 251[a]	172 ± 10.2	135 ± 8.67
Ratio (cAMP/mock)	3.55	3.92	8.11	1.11	1.05
MAPK pathway activators					
ATA (100 μM, 6 hr)	1370 ± 65.5[a]	1290 ± 60.2[a]	2130 ± 77.8[a]	194 ± 11.5	127 ± 8.56
Ratio (ATA/mock)	4.31	3.16	4.16	1.26	0.99
PAF (C_{16}) (100 nM, 6 hr)	935 ± 60.2[a]	924 ± 52.8[a]	2020 ± 124[a]	186 ± 9.78	163 ± 7.79
Ratio (PAF/mock)	2.94	2.27	3.93	1.21	1.27
C_6 ceramide (10 μM, 6 hr)	1080 ± 40.3[a]	1140 ± 43.9[a]	1990 ± 111[a]	241 ± 12.3[a]	148 ± 6.62
Ratio (C_6/mock)	3.39	2.79	3.87	1.56	1.16

[a] Results in a treatment (secretagogue) group significantly ($p < 0.05$) different from the mock group in the same column. Results shown are mean ± standard error for four replicates/condition. mCgA-CRE, form in which the functional CRE box occurs in the mouse chromogranin A promoter (TGACGTAA); Perfect-CRE, consensus 8 bp CRE motif (TGACGTCA), originally described in the somatostatin promoter; Mutant-CRE, single point-gap mutation in the mCgA CRE box (TGA-GTAA). The effect of nicotine or cAMP on gene expression of these plasmids has been reported previously (Tang *et al.*, 1996; Wu *et al.*, 1995).

negative (inhibitory) fashion, perhaps by heterodimerizing with and thereby inactivating wild-type CREB. When we coexpressed KCREB along with the 1133-bp mouse chromogranin A a promoter/reporter plasmid in PC12 cells (Fig. 4), the chromogranin A promoter response to nicotine was diminished by 73% ($p < 0.05$), whereas the response to three MAPK pathway chemical activators (ATA, PAF, or C_6-ceramide) was virtually abolished. As a positive control, the effects of cAMP on chromogranin A transcription also were blocked by KCREB expression (Fig. 4). Thus, CREB seemed to be a necessary downstream element in signaling by MAPK pathway chemical activation.

Role of the transcription factor CREB in relaying signals from the MAPK pathway: studies with transfected overexpression of MAPK pathway components. Overexpression by cotransfection of Ras, Raf, Erk, or CREB kinase each activated the transfected chromogranin A promoter in PC12 cells, by 1.8- to 3.8-fold (Fig. 5). Coexpression of the CREB antagonist KCREB blunted the effects of not only nicotine (Fig. 4) but also Ras, Raf, Erk, or CREB kinase (Fig. 5); blockade of Raf, Erk, or CREB kinase effects was virtually complete. Thus, CREB seemed to be a necessary downstream element in signaling by each of these MAPK pathway components (Ras, Raf, Erk, and CREB kinase).

Response of the endogenous (chromosomal) chromogranin A gene in PC12 cells to the MAPK pathway. The studies reported above relied on activation of a transfected chromogranin A promoter. Do the same observations apply to the chromogranin A gene as it exists in its usual chromosomal location? For this question, we turned to mRNA blots, evaluating the steady state levels of the chromogranin A message (Fig. 6). The chromogranin A mRNA was augmented 2.78-fold by nicotine, and this induction was entirely blocked by either the MAPK inhibitor apigenin or the MEK inhibitor PD98059.

Two MAPK activators each increased the chromogranin A mRNA: 2.51-fold for ATA, and 3.02-fold for PAF. When coadministered, nicotine plus the MAPK activators had slightly less than additive effects: 4.64-fold for nicotine plus ATA and 3.88-fold for nicotine plus PAF. Thus, the endogenous chromogranin A gene responds to MAPK pathway activation in a manner similar in direction (Table 1) and magnitude (Tables 2 and 3; Fig. 4) to the responses of the transfected chromogranin A promoter.

Conclusions. Our results can be summarized and interpreted most parsimoniously by the following model (see Fig. 7). Nicotinic cholinergic stimulation triggers an initial cytosolic influx of sodium (Tang *et al.*, 1997), creating membrane depolarization that then admits calcium to the cytosol through voltage-gated calcium channels (Tang *et al.*, 1996, 1997). Calcium activates protein kinase C, thereby triggering the MAPK cascade, initially by Serine/Threonine phosphorylating and thereby activating Raf (Kyriakis and Avruch, 1996; Treisman *et al.*, 1996; Robinson *et al.*, 1997), which in

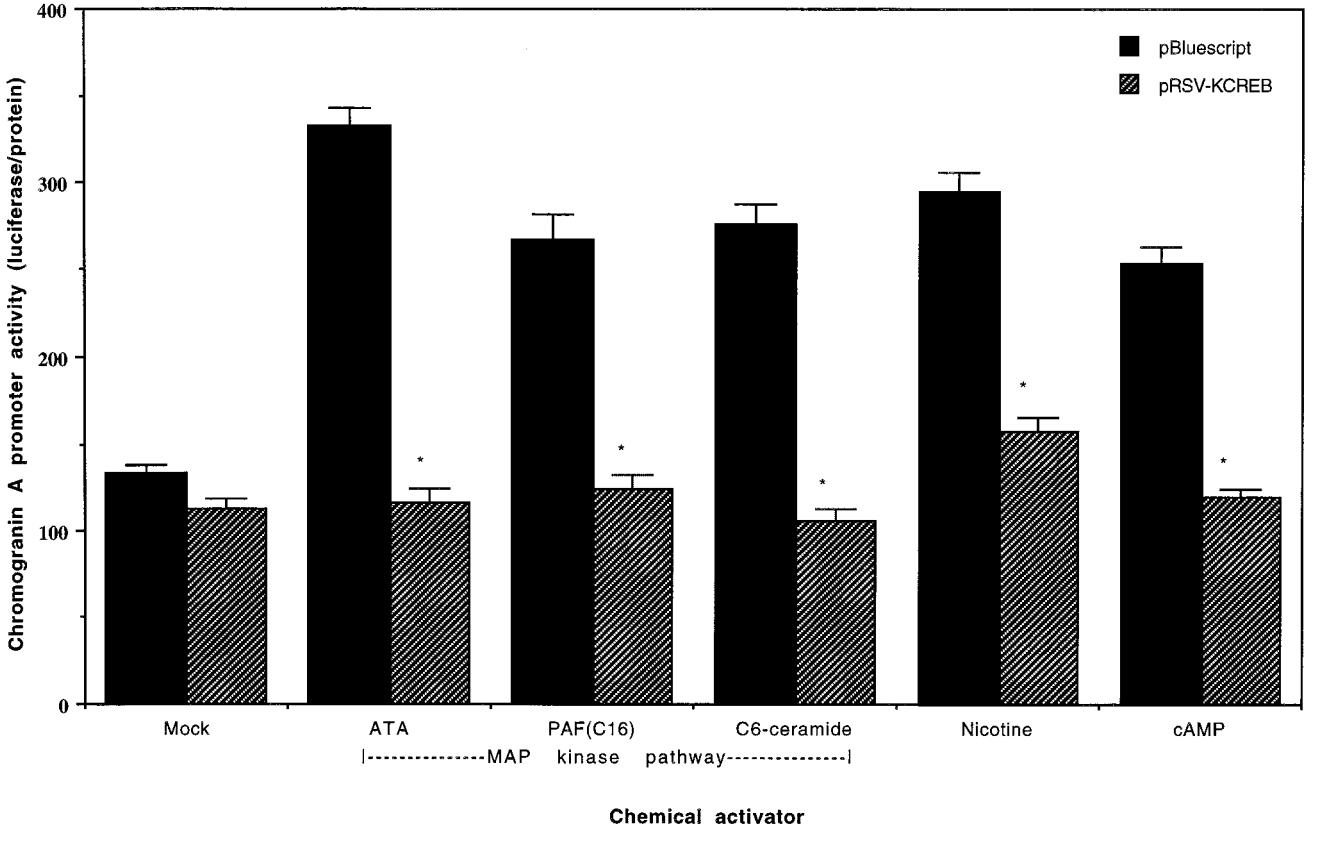

Fig. 4. Effect of the CREB antagonist KCREB on response of the chromogranin A promoter to stimulation by nicotine or MAPK pathway activators in pheochromocytoma cells. The 1133-bp mouse chromogranin A promoter/luciferase reporter plasmid (3 μg) was cotransfected into PC12 cells, along with either pRSV-KCREB (4 μg), or a DNA mass control (pBluescript, 4 μg), in a 1-ml transfection volume. Exposure (treatment) times were nicotine or cAMP, 48 hr; ATA, PAF (C_{16}), or C_6-ceramide, 6 hr. Data shown are luciferase activity/mg cell protein (mean ± standard error, four replicates/condition). *, $p < 0.05$, comparing KCREB with its control (pBluescript). ATA, stimulator of MAPK phosphorylation; PAF (C_{16}), activator of MAPK and MEK; C_6-ceramide, activator of MAPK. *cAMP*, dibutyryl cAMP.

turn causes the sequential phosphorylation and activation of MEK, MAPK, CREB kinase, and CREB. CREB, activated by phosphorylation on Ser133 (Tang *et al.*, 1996), finally relays the transcriptional signal to the CRE ([−71 bp]5′-TGACGTAA-3′[−64 bp]) of the chromogranin A proximal promoter.

Although the protooncogene Ras activated chromogranin A transcription (Figs. 1 and 5) and a dominant negative Ras mutant (S17N) blocked chromogranin A promoter activation by NGF (Fig. 2), the dominant negative Ras mutant did not affect nicotinic signaling to chromogranin A (Fig. 2). Thus, although Ras is a potent early activator of the MAPK pathway (Figs. 1 and 5), nicotinic or protein kinase C signaling into the MAPK pathway seems to proceed through Raf-1 without involving Ras (Fig. 7).

Nicotinic cholinergic stimulation stimulates the enzymatic activity of the MAPK pathway (Fig. 3), and disruption of the MAPK pathway, by either chemical inhibition (Table 1) or dominant negative mutants of pathway components (Fig. 2), abolishes nicotinic signaling toward secretory protein transcription. Thus, the MAPK pathway is necessary in *trans* for nicotinic signaling to the chromogranin A promoter. The CRE (TGACGTAA) seems to be both necessary (Table 2) and sufficient (Table 3) in *cis* for transmission of signals initiated by either nicotine or the MAPK pathway.

MAPK pathway chemical activation triggered increases in chromogranin A promoter activity similar in direction and magnitude to that obtained after nicotine (Tables 2 and 3); similarly, MAPK pathway chemical inhibition diminished the chromogranin A promoter response to nicotine (Table 1). While we measured MAPK pathway enzymatic activity (phosphorylation of the MAPK substrate PHAS-I) under only a few circumstances (nicotine or phorbol ester; Fig. 3), inhibition of the nicotine response by both chemical (Table 1) and genetic (Fig. 2) blockade of the MAPK pathway argues for a crucial role of this pathway in mediating the nicotinic response of chromogranin A. Although MAPK pathway activation seems to be crucial for transcriptional responses to nicotinic cholinergic stimulation (Tables 1, Fig. 2), this pathway apparently does not impinge on catecholamine secretion (Table 1) triggered by the same nicotinic stimulus.

Xing *et al.* (1996) first identified Rsk2 as CREB kinase, and they pointed out its central role in MAPK cascade signaling to c-Fos gene expression at the CRE in *cis*. Our studies extend the spectrum of CREB/cAMP targets for the MAPK

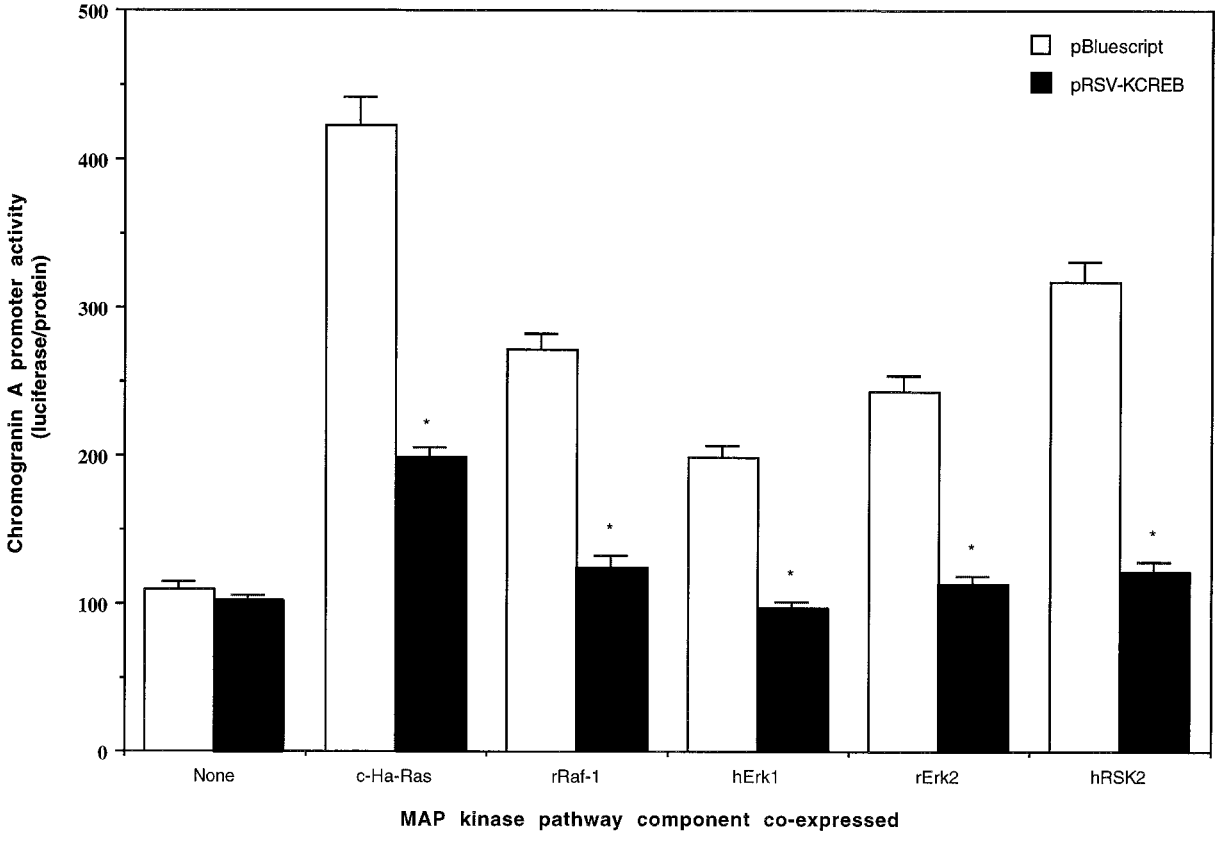

Fig. 5. Role of CREB in relaying MAPK pathway activation signals to the chromogranin A promoter in pheochromocytoma cells. A 1133-bp mouse chromogranin A promoter/luciferase reporter was cotransfected into PC12 cells with the indicated MAPK pathway activator plasmid (*left*), in the presence or absence of an expression plasmid for the CREB antagonist KCREB. After 48 hr, the cells were harvested for reporter assay. Transfection amounts (in 1 ml each) were 3 μg of chromogranin A promoter/luciferase reporter, 4 μg of pRSV-KCREB, and 2 μg of MAPK pathway expression plasmid. pBluescript was cotransfected as a DNA mass control, in the absence of activator or inhibitor plasmid or plasmids. Data shown are luciferase activity/mg protein (mean ± standard error, four replicates/condition). *, $p < 0.05$, comparing KCREB with its control (pBluescript). *KCREB*, dominant negative CREB antagonist; *RSV*, Rous sarcoma virus long terminal repeat.); *cHa-Ras*, wild-type c-Ha-Ras expression driven by the SV40 promoter, in the plaasmid pCD-WTras; *rRaf-1*, wild-type rat Raf-1 expression driven by the RSV LTR (Rous sarcoma virus long terminal repeat) promoter, in the plasmid KSRSPA cRAF; *hErk1*, wild-type human Erk1 expression driven by the CMV (cytomegalovirus) promoter, in the plasmid pCMV5Erk1; *rErk2*, wild-type rat Erk2 expression driven by the CMV promoter, in the plasmid pCMV5Erk2; *hRSK2*, wild-type human RSK2 (ribosomal serine kinase 2; CREB kinase) expression driven by the AML (adenovirus major late promoter) in the plasmid pMT2HARASK2.

pathway to secretory proteins (such as chromogranin A) and extend the range of MAPK pathway activators to the nicotinic cholinergic system (Tang *et al*., 1996, 1997), the physiological mediator of neurotransmission in autonomic ganglia (both sympathetic and parasympathetic), chromaffin cells, and a diverse population of central neurons (Wonnacott, 1997).

The CREB antagonist KCREB virtually abolished the response of the chromogranin A promoter to chemical activation of the MAPK pathway or to cAMP (Fig. 4), but the response to nicotine was incompletely (only 73%) disrupted by KCREB. Because both nicotinic and MAPK pathway activation of chromogranin A map onto the promoter CRE (Tables 2 and 3), perhaps a *trans*-activating factor other than CREB may signal in a quantitatively minor fashion to the promoter CRE, accounting for a portion of the nicotinic response. For example, other members of the CREB/activating transcription factor family may bind to CRE elements, as may members of the AP-1/Jun-Fos/Fra family (Habener, 1990).

Xia *et al*. (1996) described MAPK cascade activation by cytosolic calcium influx after glutamate/*N*-methyl-D-aspartate receptor activation in primary neurons. Because glutamate/*N*-methyl-D-aspartate receptors and nicotinic cholinergic receptors are both members of the same superfamily of extracellular ligand-gated heteropentameric cation channels, our results perhaps are analogous, although involving different receptor family members (Conley, 1996).

Rosen and Greenberg (1996) also found MAPK stimulation after voltage-sensitive calcium channel activation. Although our dominant negative mutant results seemed to exclude a role for Ras (Fig. 2), and our inhibitor results (Tang *et al*., 1997) instead suggest a necessary role for protein kinase C, Rosen and Greenberg (1996) and Rosen *et al*. (1994) found that some calcium-influx signals to the MAPK cascade were quite Ras dependent. Different results in particular signaling pathways may reflect the very different targets we studied. For example, Rosen and Greenberg (1996) and Rosen *et al*. (1994) found that the Ras-dependent pathway also was activated by release of calcium from intracellular stores, while we found that only extracellular calcium influx could trigger transcription of chromogranin A (Tang *et al*., 1996, 1997).

In conclusion, our results suggest that the MAPK pathway plays a crucial role in nicotinic cholinergic signaling in chromaffin cells to secretory protein transcription, although not to catecholamine secretion. In *trans*, our results suggest this signal transduction cascade: protein kinase C → Raf → MEK → MAPK → CREB kinase → CREB. In *cis*, the chromogranin A promoter CRE is both necessary and sufficient to confer both nicotinic and MAPK responses.

Acknowledgments

Gifts of expression plasmids included activator/inhibitor Ras (N. M. Nathanson, University of Washington, Seattle, WA), activator/inhibitor Raf (M. Karin, University of California, San Diego, CA), activator/inhibitor Erk1 and Erk2 (J. K. Westwick, University of North Carolina, Chapel Hill, NC),

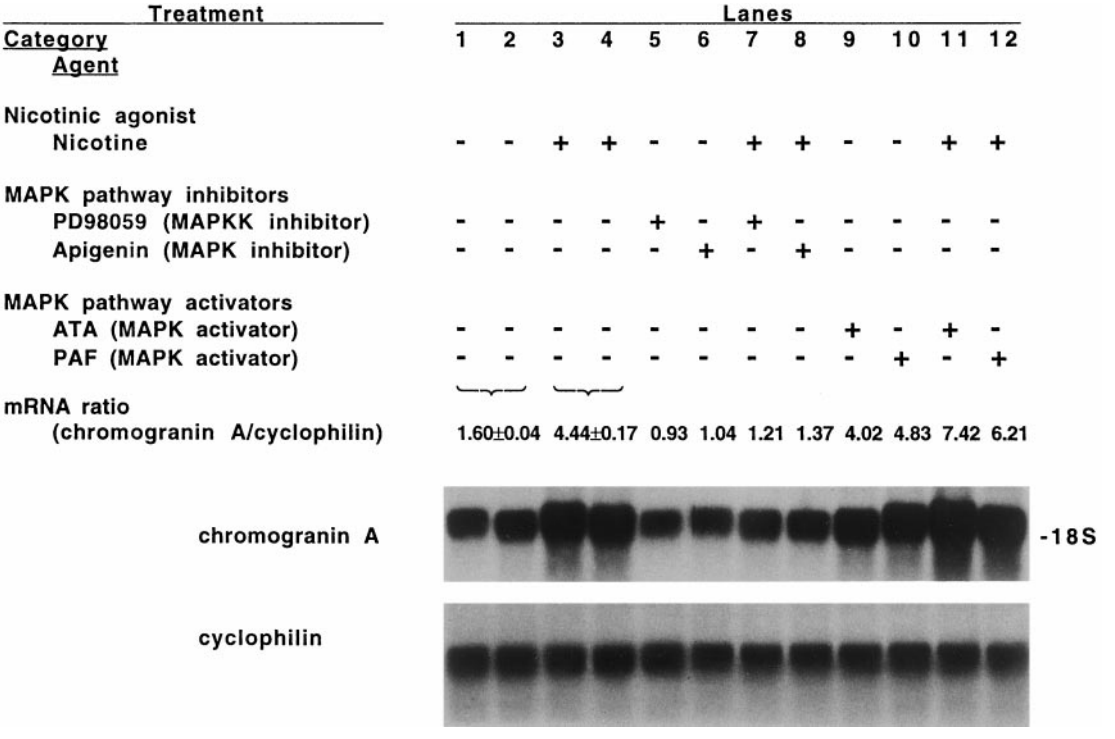

Fig. 6. Chromogranin A mRNA response to nicotine in pheochromocytoma cells showing the effect of activators or inhibitors of the MAPK pathway. Northern blot quantification of MAPK pathway inhibitor or activator effects on the chromogranin A mRNA (endogenous gene) response to nicotine (10^{-3} M, 16 hr). Two *lanes* (representing two separate culture plates of PC12 cells) are shown at certain time points. The cyclophilin probe recognizes a constitutive or "housekeeping" mRNA, at the same time points. *18S rRNA*, position of the 18S form of ribosomal RNA on the ethidium bromide-stained gel. PD98059 (MEK inhibitor, 20 μM) or apigenin (MAPK inhibitor, 12.5 μM) was added at the same time as nicotine. ATA (MAPK activator, 100 μM) or PAF (C_{16}) (MAPK activator, 100 nM) was added 6 hr before cells were harvested. *Lanes 1 and 2*, control. *Lanes 3 and 4*, nicotine alone. *Lane 5*, PD98059 alone. *Lane 6*, apigenin alone. *Lane 7*, PD98059 plus nicotine. *Lane 8*, apigenin plus nicotine. *Lane 9*, ATA alone. *Lane 10*, PAF alone. *Lane 11*, ATA plus nicotine. *Lane 12*, PAF plus nicotine.

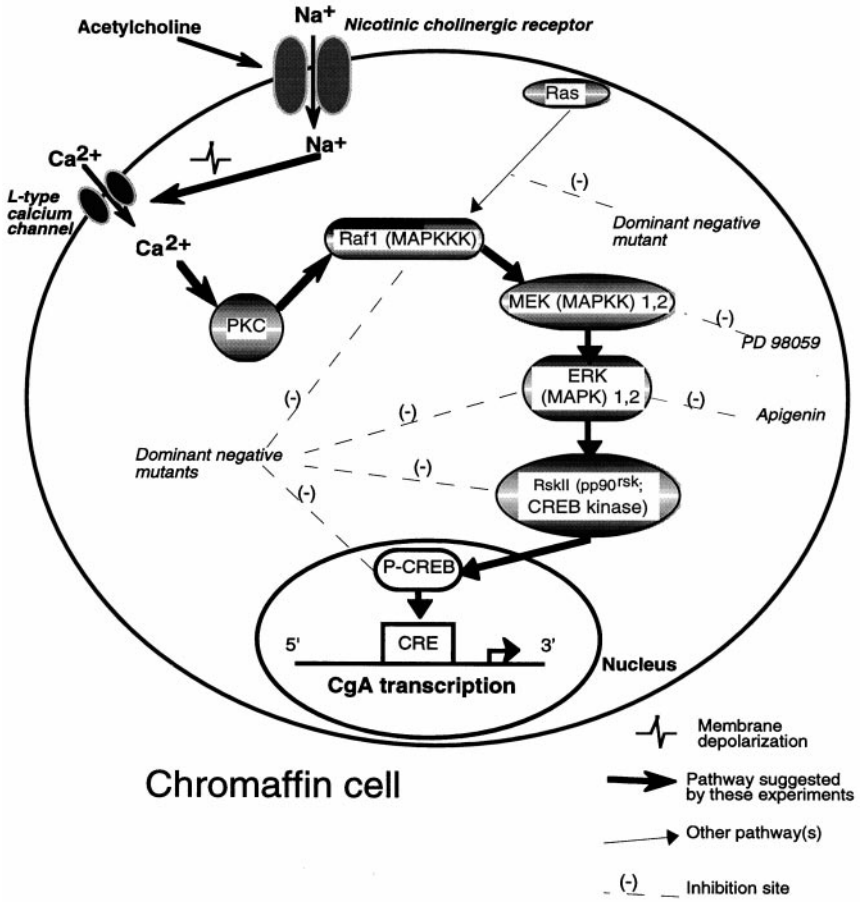

Fig. 7. Outline of proposed signal transduction pathway for activation of chromogranin A transcription by nicotinic cholinergic stimulation in pheochromocytoma cells. *CgA*, chromogranin A; *P-CREB*, Ser133-phosphorylated (activated) CREB; *PKC*, protein kinase C; *MAPKK*, MEK; *MAPKKK*, MEK kinase. *Dashed lines with brackets*, inhibition (or an inhibitor). *Broad arrows*, signaling pathways suggested or confirmed by the current experiments. *Thin arrow*, a pathway (Ras → Raf-1) excluded by the current experiments from participation in nicotinic cholinergic signaling.

activator/inhibitor CREB kinase and 71-bp c-*fos*/CAT promoter/reporter (M. E. Greenberg, Harvard Medical School, Boston, MA), and KCREB (R. H. Goodman, Oregon Health Sciences University, Portland, OR).

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