Stimulus Coupling to Transcription Versus Secretion in Pheochromocytoma Cells

Convergent and Divergent Signal Transduction Pathways and the Crucial Roles for Route of Cytosolic Calcium Entry and Protein Kinase C

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Abstract

How do chromaffin cell secretory stimuli program resynthesis of secreted peptides and amines? We previously showed that the physiologic nicotinic cholinergic signal for secretion also activates the biosynthesis of chromogranin A, the major protein released with catecholamines. Here, we examine signal transduction pathways whereby secretory stimuli influence exocytotic secretion versus chromogranin A transcription. Both secretion and transcription depended on initial nicotinic-triggered sodium entry into the cytosol, followed by calcium entry through L-type voltage-gated channels. When calcium entered through L-type channels, activation of secretion paralleled activation of transcription ($r = 0.897$, $P = 0.002$). Calcium entry from intracellular stores or through calcium ionophore channels activated secretion, though not transcription. Nicotinic-stimulated transcription depended upon protein kinase C activation; nicotine caused translocation of protein kinase C to the cell membrane fraction, and inhibition of protein kinase C blocked activation of transcription, while activation of protein kinase C mimicked nicotine effects. Transcriptional responses to both nicotine and protein kinase C mapped principally onto the chromogranin A promoter's cAMP response element (TGACGTAA; CRE box). KCREB, a dominant negative mutant of the CRE-binding protein CREB, blunted activation of chromogranin A transcription by nicotine, phorbol ester, or membrane depolarization. We conclude that activation of chromogranin A transcription by secretory stimulation in chromaffin cells is highly dependent upon precise route of calcium entry into the cytosol; transcription occurred after entry of calcium through L-type channels on the cell surface, and was mediated by protein kinase C activation. The *trans*-acting factor CREB ultimately relays the secretory signal to the chromogranin A promoter's CRE box in *cis*. (*J. Clin. Invest.* 1997. 100:1180–1192.) Key words: catecholamine • acetylcholine • chromaffin • pheochromocytoma • PC12

Introduction

During *trans*-synaptic induction, the response of postsynaptic cells to incoming synaptic signals includes activation of a variety of gene programs, both acute and long-term (1).

When chromaffin cells and sympathetic axons release by exocytosis their complement of cosecreted catecholamines and peptides (2), mechanisms are activated to replenish secretory vesicles with the just-secreted components. The rate-limiting enzyme in catecholamine synthesis, tyrosine hydroxylase (3, 4), is transcriptionally activated by the physiologic nicotinic cholinergic signal to chromaffin cell exocytosis. Induction of tyrosine hydroxylase by chromaffin cell secretory stimuli may require the cAMP response element in the tyrosine hydroxylase promoter, as well as protein kinase A activity in the cell (3, 4). Other catecholamine biosynthetic enzymes, such as phenylethanolamine-*N*-methyltransferase and dopamine β-hydroxylase, also respond to secretory stimuli (5, 6).

The biosynthesis of secreted peptides, such as proenkephalin A (7) and neuropeptide Y (8), is likewise activated by nicotinic stimulation. Chromogranin A is the quantitatively major protein stored and released with catecholamines (2). It is a prohormone, giving rise by proteolytic cleavage to several biologically active peptides (9). We have shown (10) that chromogranin A biosynthesis is activated by nicotinic cholinergic stimulation, that the activation occurs at a transcriptional level, and that discrete chromogranin A promoter domains, including the cAMP response element (CRE box),[1] are involved in the nicotinic response. In those studies, the cAMP response element seemed to be, at least in part, both necessary and sufficient to confer nicotinic induction onto the chromogranin A promoter (10), and nicotinic stimulation resulted in formation of the transcriptionally activated form of the cAMP response element binding protein CREB, phosphorylated at crucial serine residue 133 (10).

Here we examine and contrast signal transduction pathways whereby nicotinic cholinergic stimulation activates catecholamine secretion versus chromogranin A transcription in chromaffin cells. Our results suggest that calcium entry into the cytosol through voltage-gated channels is a necessary intermediary in both responses, but that different calcium-dependent protein kinases are involved in triggering secretion versus transcription; protein kinase A apparently plays no role in these processes. Protein kinase C (PKC) activation seems to be rate-limiting for the transcriptional response; dominant

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1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyltransferase; CREB, cAMP response element-bonding protein; CRE box, cAMP response element; PKC, protein kinase C; RSV, Rous sarcoma virus; TK, thymidine kinase.

negative (inhibitory) mutant studies with the cAMP binding protein CREB suggest that its activation signals transcription through the chromogranin A promoter's proximal cAMP response element. Only entry of calcium into the cytosol from an extracellular source is capable of activating both secretion and transcription; calcium from intracellular stores activates secretion alone.

Methods

Cell culture and transfections. PC12 (11) cells (at passage number 10–25) were cultured and transfected by lipofection, as previously described (10). PC12 derivative A126-1B2, which is deficient in protein kinase A activity (12), was obtained from David Schubert (Salk Institute, La Jolla, CA).

Promoter/reporter plasmids. The plasmid pRSV-CAT was used as a transfection efficiency internal control, as previously described (13).

In plasmid pXP-1133, a functional 1133-bp mouse chromogranin A promoter drives expression of a luciferase reporter (13–15); 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. Construction and transfection of a series of 5′ deletions of the mouse chromogranin A promoter (from ∼ −4800 bp to 0 bp upstream of the cap site), fused to the luciferase reporter, have been described (15).

Mouse chromogranin A promoter cAMP response element (CRE) box (TGACGTAA; at position −71 to −64 bp upstream of the cap site) mutants M13 and M41 have been described previously (15). Mutant M13, which alters six of the eight bases in the cAMP response element (to **CAT**C**ACC**A; mutant residues in bold), was incorporated into a mouse chromogranin A 100-bp promoter (−100 bp to +42 bp)/luciferase reporter construct, while mutant M41 is a cAMP response element single point-gap mutation (TGA-GTAA), incorporated into a mouse chromogranin A 77-bp promoter (−77 bp to +42 bp)/luciferase reporter construct.

Promoter-reporter plasmids in which the rodent chromogranin A cAMP response element (TGACGTAA), a consensus cAMP response element (TGACGT**C**A), or a cAMP response element point-gap mutant (TGA-GTAA) were transferred to a position just 5′ of the heterologous thymidine kinase (TK) promoter, governing expression of the luciferase reporter in the parent vector pTK-luciferase (pTK-LUC), were described previously (15).

A eukaryotic expression plasmid (pRSV-KCREB) in which the Rous sarcoma virus (RSV) long terminal repeat drives expression of the CREB (cAMP response element binding protein) mutant KCREB (dominant negative mutant form of CREB which hetero-dimerizes with and inactivates endogenous CREB) has been described (16, 17), and was the gift of Dr. Richard Goodman and Dr. Roger Cone (Oregon Health Sciences University, Portland, OR).

We obtained eukaryotic expression plasmids for PKC mutants SRα-PKAC (encoding a constitutively activated α-isoform of PKC), and SRα-ΔPKCβ (PKCβΔEE; encoding a constitutively activated β-isoform of PKC) from Dr. Masa-aki Muramatsu (Department of Molecular and Developmental Biology, University of Tokyo, Tokyo, Japan) (18).

A 71-bp *c-fos* promoter/CAT reporter plasmid (−71 wt fos CAT), which contains a calcium response element TGACGT**TT** at position −60 bp upstream of the cap site, was obtained from Dr. Michael Greenberg (Department of Neurology, Harvard Medical School, Boston, MA) (19–21). This element bears 6/8 bp similarity to a consensus cAMP response element, TGACGTCA.

Catecholamine secretion. Catecholamine secretion from PC12 cells preloaded with [^{3}H]L-norepinephrine was accomplished as previously described (22).

To observe catecholamine secretion in the absence of extracellular calcium, [^{3}H]L-norepinephrine-labeled cells were washed twice-with calcium-free secretion buffer (152 mM NaCl, 5 mM KCl, 10 mM Hepes, pH 7.4, and 0.1 mM EGTA), and incubated in this buffer for 90 min. After twice washing again with the same buffer, the cells were incubated in the secretion buffer with or without nicotine.

When protein kinase inhibitors were used in [^{3}H-]L-norepinephrine secretion studies, these inhibitors were added 90 min before study, and maintained in the medium during the 30-min secretory stimulation period.

Pharmacology. The effect of the following agents on chromogranin A transcription and catecholamine secretion was tested at concentrations indicated in a particular experiment's results. Nicotine, EGTA, caffeine, ionomycin, nifedipine, verapamil, $BaCl_2$, dibutyryl cAMP, ATP, and PMA were obtained from Sigma Chemical Company (St. Louis, MO). A23187, ryanodine, BayK-8644, KT-5720, KT-5823, KN-62, SQ-22,536, and MDL-12,330A were obtained from Calbiochem Corp., (La Jolla, CA). The protein kinase inhibitor chelerythrine was from LC Services Corporation (Woburn, MA).

To deplete PKC, in some experiments cells were pretreated with PMA (10^{-7} M) for 48 h before transfection or secretion studies. PMA (10^{-7} M) was then continued during the 48-h posttransfection period before reporter harvest.

To cause influx of extracellular calcium through L-type voltage-gated cell surface calcium channels independent of nicotinic receptor activation, the following cell membrane-depolarizing (high potassium) medium was added to cells 16 h after transfection, followed by incubation in the same medium for an additional 12 h before cell harvest for reporter assay (100 mM NaCl, 55 mM KCl, 2 mM $CaCl_2$, 10 mM Hepes, pH 7.4, 0.45% glucose, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 10% horse serum, and 5% fetal bovine serum). In some of these experiments, the calcium channel agonist BayK8644 was added.

In some experiments, the secretagogue barium (2 mM $BaCl_2$, in barium medium: 148 mM NaCl, 5 mM KCl, 0 mM $CaCl_2$, 10 mM Hepes, pH 7.4, 0.45% glucose, 1% penicillin/streptomycin, 10% horse serum, and 5% FBS) was added to cells 16 h after the transfection, followed by incubation in the same medium for an additional 24 h before harvest.

Some experiments tested the requirement of extracellular sodium for nicotinic cholinergic-stimulated catecholamine secretion, or for chromogranin A transcription. For secretion, the medium without extracellular sodium was as follows: 150 mM *N*-methyl-D-glucamine, 2.5 mM KCl, 2 mM $CaCl_2$, and 2.5 mM K-Hepes, pH 7.4. For transcription, the cell culture medium without extracellular sodium was as follows: the above medium plus 4,500 mg/liter glucose, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 10% dialyzed heat-inactivated horse serum, and 5% dialyzed heat-inactivated FBS. Sodium-containing medium for transcription was DMEM (110 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 43 mM $NaHCO_3$, pH 7.4), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 10% heat-inactivated horse serum, and 5% heat-inactivated FBS. For sodium dependence of nicotinic-stimulated transcription, cells were first incubated in medium with sodium (without nicotine) for 2 h after transfection. Cells were then switched to medium with or without sodium (see above), and with or without nicotine (10^{-3} M), for 12 h. The medium on all cells was then changed to sodium-containing, without nicotine, for another 10 h. For sodium-dependence of PMA-stimulated transcription, cells were first incubated in medium with sodium (without PMA) for 12 h after transfection. Cells were then switched to medium with or without sodium (see above), and with or without PMA (0.1 μM), for 6 h. The medium on all cells was then changed to sodium-containing, without PMA, for another 6 h. At the end of the stimulation periods (24 h after transfection), cells were harvested for assay of luciferase and protein.

To create a medium free of extracellular calcium in some transcription experiments, cells were washed three times with calcium-free medium (152 mM NaCl, 5 mM KCl, 10 mM Hepes, pH 7.4, 0.1 mM EGTA, 0.45% glucose, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 10% dialyzed heat-inactivated horse serum, and 5% dialyzed heat-inactivated FBS) 16 h after transfection, at which time nicotine

(or mock secretagogue) was added. After 8 h of further culture in this medium with or without nicotine, cells were harvested.

Cytosolic calcium responses of PC12 cells to secretagogues were observed using fluorescence of the calcium-binding dye Fura-2 (23). Cells were preloaded for 20–30 min with Fura-2/AM (acetoxymethylester; Molecular Probes Inc., Eugene, OR), washed twice, and fluorescence was visualized in a fluorometer (Perkin Elmer Corp., Norwalk, CT) with wavelengths of 340 and 380 nm for excitation, and 510 nm for emission.

Secretagogue-activated radiolabeled cation (Ca^{2+} or Na^{+}) fluxes from the extracellular space into PC12 cells were measured over 5 min as previously described (24, 25), using [^{45}Ca]Cl_2 (14.95 mCi/mg; DuPont-NEN®, Boston, MA) or [^{22}Na]Cl (800 mCi/mg, DuPont NEN®).

PKC and protein kinase A assays. PKC activity was measured (SAM^{2}™ Protein Kinase C Assay System; Promega Corp., Madison, WI). After 12 h of nicotinic (10^{-3} M nicotine, versus mock) stimulation, PC12 cells from 10-cm plates were harvested with 0.5 ml PBS (140 mM NaCl, 5 mM KCl, and 15 mM Na-phosphate pH 7.4) and lysed by sonication (40% power, five pulses, 5 s/pulse). Lysed cells were centrifuged at 100,000 *g* for 60 min. To remove endogenous phospholipid activators, the ~ 0.5 ml supernatant (cytosolic fraction) was passed directly though a 1-ml DEAE cellulose column (DEAE Sephacel®; Pharmacia Diagnostics AB, Uppsala, Sweden) equilibrated with ion exchange buffer (25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin, and 1 μg/ml aprotinin), washed with 15 ml of the same buffer, and eluted with 2.5 ml of 0.2 M NaCl in the same buffer. The effluent was used for kinase assay and protein measurement in the cytosol. The pellet (membrane fraction) was dissolved in 0.5 ml ion exchange buffer. After vortexing and centrifugation (10 min, 13,000 *g*), the supernatant was passed though and eluted from a similar DEAE cellulose column. 5 μl of eluted material was assayed for kinase activity in a total volume of 25 μl, with the following final concentrations: 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 0.25 mM EGTA, 0.4 mM $CaCl_2$, 0.1 mM ATP, 0.1 M PKC biotinylated peptide substrate neurogranin$_{28-43}$ (AAKIQASFRGHMARKK), 0.02 μCi/μl γ-[^{32}P]ATP (6,000 Ci/mmol), and with or without PKC activators (230 μg/ml phosphatidylserine in combination with 23 μg/ml diacylglycerol). Mixtures were incubated at 30°C for 20 min, and the reaction was stopped by adding guanidine hydrochloride (final concentration, 2.5 M). 10 μl of terminated reaction mixture was spotted onto a SAM^{2}™ membrane (streptavidin matrix) and washed (according to the protocol) to remove free, unincorporated γ-[^{32}P]ATP, followed by liquid scintillation counting (Ecoscint™; National Diagnostics, Atlanta, GA) for [^{32}P] incorporation into neurogranin. Basal phosphorylation of neurogranin, in the absence of exogenous phospholipid activators, served as the assay blank for PKC activity. Activation of PKC is estimated by translocation of enzymatic activity from cytosolic to membrane fractions.

Protein kinase A activity was measured with a SAM^{2}™ Protein Kinase A Assay System (Promega Corp.). Cells were treated in 6-cm plates as described for the PKC assay, then harvested with 0.2 ml PBS (pH 7.4), and lysed by freezing and thawing in 100 μl cold extraction buffer (25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). After centrifugation (10 min, 13,000 *g*), the supernatant was used directly for protein kinase assay and protein measurement. 5 μl of supernatant was assayed for 5 min at 30°C, in a total volume of 25 μl, with (final concentrations) 40 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$, 0.1 mg/ml BSA, 0.1 M protein kinase A biotinylated peptide substrate Kemptide (LRRASLG), 0.02 μCi/μl γ-[^{32}P]ATP (6,000 Ci/mmol), and 0.1 mM ATP, with or without activation by 5 μM cAMP. Mixtures with exogenous cAMP added measured the total available protein kinase A, while mixtures without exogenous cAMP measured protein kinase A already activated by endogenous cAMP. Reaction termination, membrane spotting and washing, and measurement of [^{32}P] incorporation into substrate were accomplished as in the PKC assay.

RNA extraction and northern blots. Total RNA was prepared from PC12 cells by extraction in guanidinium thiocyanate (RNAzol II; TelTest, Inc., Friendswood, TX). 20 μg of total cellular RNA was fractionated on formaldehyde–agarose gels and transferred to a nylon filter. The integrity of the RNA was judged by the appearance of 28S and 18S rRNA bands on the ethidium bromide–stained gel. Northern blots were done as previously described (26).

Random primer-labeled (27) cDNA probes were a 1.6-kbp rat chromogranin A cDNA (28), and a 381-bp mouse cyclophilin cDNA (29), used as a normalizing probe for a housekeeping (constitutively expressed) mRNA.

Hybridizations and washes were done at 65°C. Between probes, filters were stripped by boiling for 30 min in 0.1 × SSC/0.5% SDS, before rehybridization.

Transcriptional activation (nuclear run-on assay). Nuclear run-on assays, to assess the rate of initiation of new chromogranin A transcripts in PC12 cells after nicotine, PMA, or both, were accomplished as previously described (10).

Statistics. Results are reported as the mean value±one SEM. Data were analyzed by either *t* test (two groups) or ANOVA (three or more groups), using the software packages Statworks or Cricketgraph (Cricket Software, Malvern, PA), or StatView (Abacus Concepts Inc., Berkeley, CA), each for the Macintosh microcomputer. Differences were considered significant if $P < 0.05$.

Results

Effects of secretagogues on cation (sodium or calcium) fluxes from the extracellular space into PC12 cells. Dependence of nicotinic cholinergic-stimulated catecholamine secretion and chromogranin A transcription on extracellular sodium influx into cells. Nicotine (60 μM) triggered uptake of extracellular $^{22}Na^{+}$ into PC12 cells (from 0±9.8 [after mock secretagogue]–699± 3.0 [after 60 μM nicotine] net dpm/well in 5 min, $P < 0.001$).

Nicotine (60 μM) also triggered extracellular $^{45}Ca^{2+}$ net uptake into PC12 cells (to 6,107±188 dpm/well; $P < 0.02$); this stimulated uptake was decreased by 84% (to 960±1.4 dpm/well; $P < 0.001$) by the nicotinic cholinergic antagonist hexamethonium (10 μM), and completely abolished (to < 0±177 dpm/well; $P < 0.001$) by the L-type calcium channel antagonist nifedipine (10 μM). By contrast, membrane depolarization with 55 mM KCl also caused cellular uptake of $^{45}Ca^{2+}$ (to 6,357±457 dpm/well; $P < 0.02$), but such stimulated uptake could be blocked by nifedipine (to 86±505 dpm/well; $P < 0.02$), though not by hexamethonium (to 5,706±674 dpm/well).

Removal of sodium from the extracellular space prevented both catecholamine secretory and chromogranin A transcriptional responses to nicotinic cholinergic stimulation (Table I), where transcription was evaluated by expression of a transfected 1,133-bp mouse chromogranin A promoter/luciferase reporter plasmid.

Effects of nicotinic cholinergic stimulation and/or PKC activation, alone or combined, on catecholamine secretion and chromogranin A biosynthesis. Both nicotinic cholinergic stimulation (by nicotine, 10^{-3} M) and PKC activation (by PMA, 10^{-7} M) augmented the level of the endogenous chromogranin A mRNA (Fig. 1).

In a nuclear runoff experiment (Fig. 2) after 1 h of exposure of PC12 cells to either nicotine or PMA, the effects of each agent on chromogranin A gene expression seemed to occur at the level of transcriptional activation (that is, an increase in rate of initiation of new chromogranin A transcripts).

Table I. Extracellular Na^+ Dependence of Nicotine- or Phorbol Ester–stimulated Catecholamine Secretion or Chromogranin A Transcription (Transfected 1133-bp Mouse Chromogranin A Promoter/Luciferase Reporter) in PC12 Cells

	Assay			
	Catecholamine secretion		Chromogranin A transcription	
Extracellular [Na^+]	150 mM Na^+	0 mM Na^+	150 mM Na^+	0 mM Na^+
	%			
Stimulus				
Mock 1	5.23±0.18	4.91±0.20	268.5±10.4	178.4±6.7
Nicotine	14.6±0.65*	5.64±0.33	543.6±23.5*	182.8±9.1
Mock 2	5.41±0.25	5.13±0.19	233.6±9.7	218.5±8.9
PMA	11.2±0.57*	9.81±0.41*	557.1±19.8*	530.2±20.4*

Each secretion period was 30 min. In the first transcription experiment (mock 1 versus nicotine), the stimulation period was 12 h; in the second transcription experiment (mock 2 versus PMA), the stimulation period was 6 h. Secretion data are expressed as percent of cell total of [^{3}H]L-norepinephrine released, mean±SEM (n = 3). Transcription results are luciferase activity/mg protein in each well, mean±SEM (n = 4 plates/condition). PMA concentration was 0.1 μM for both secretion and transcription. Nicotine concentrations were 60 μM for secretion, and 1 mM for transcription. *$P < 0.05$, comparing secretagogue to the corresponding mock stimulation (same column).

Nicotine or PMA, given separately, each activated catecholamine secretion, as well as a transfected 1,133-bp mouse chromogranin A promoter/luciferase reporter plasmid (Table II). cAMP activated chromogranin A transcription, though not catecholamine secretion.

While removal of extracellular sodium from the medium impairs both the catecholamine secretory and the chromogranin A transcriptional responses to nicotine, both of these responses to PMA were preserved (Table I).

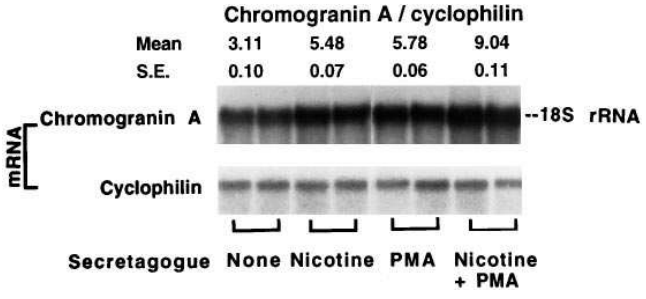

Figure 1. Northern blot quantitation of chromogranin A mRNA signals in PC12 cells after nicotine (10^{-3} M, 16 h), PMA (10^{-7} M, 6 h) or nicotine (16 h) plus PMA (6 h) stimulation. Two lanes (representing two separate plates of PC12 cells) are shown for each stimulus. The cyclophilin probe recognizes 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. The ratios (integrated density/integrated density) of chromogranin A/cyclophilin mRNAs were as follows: mock, 3.11±0.10; nicotine, 5.48±0.07; PMA, 5.78±0.06; and nicotine plus PMA, 9.04±0.11.

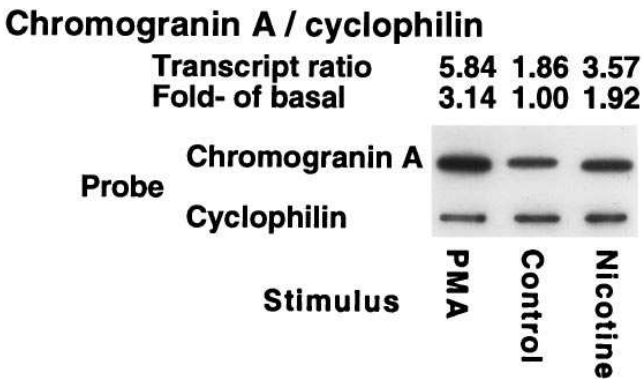

Figure 2. Nuclear runoff: chromogranin A transcriptional response to nicotine or PMA (phorbol ester). 1 h after exposure of PC12 cells to either nicotine (10^{-3} M) or PMA (10^{-7} M), nuclei were harvested for measurement of the rate of initiation of new chromogranin A transcripts. Newly initiated transcripts were labeled with α-[^{32}P]rUTP, and then hybridized with nitrocellulose strips to which a 3.3-kbp rat chromogranin A genomic DNA fragment, or a rodent cyclophilin cDNA, had been previously affixed. Radioactivity (cpm) was quantitated by band excision (guided by fluorography) and liquid scintillation counting. The ratios (cpm/cpm) of chromogranin A/cyclophilin transcripts were as follows: PMA, 5.84:1; control, 1.86:1; and nicotine, 3.57:1.

Signal transduction during activation of exocytotic catecholamine secretion by nicotinic cholinergic stimulation: protein kinases and calcium entry. The dependence of nicotinic-stimulated catecholamine secretion (Table III) on protein kinase classes and calcium entry was somewhat different from the pattern for chromogranin A transcription (Table IV).

Catecholamine secretion (Table III) in response to nicotine was partially blocked by inhibition of either PKC (with either acute chelerythrine, or chronic PMA pretreatment) or calcium–calmodulin-dependent PKC; combined inhibition of PKC plus

Table II. Coeffects of Nicotine with Phorbol Ester on Catecholamine Secretion or Transfected Chromogranin A Promoter Activity

Stimulus	Norepinephrine secretion	Chromogranin A promoter activity
	%	*luciferase/CAT*
Control	5.1±0.44*	6.2±0.56*
Nicotine (10^{-3} M)	14.9±1.02‡	19.2±1.14‡
PMA (10^{-7} M)	10.0±0.77§	16.4±0.94‡
Nicotine + PMA	14.5±0.47‡	58.6±3.87§
cAMP (10^{-3} M)	4.9±0.17*	25.2±1.14‡
Nicotine + cAMP	15.0±1.02‡	50.9±2.56§

Stimulation times were 48 h for promoter activity and 30 min for secretion. Secretion units are percent of total cellular norepinephrine secreted. Transcription units are luciferase activity (from the transfected mouse chromogranin A 1133-bp promoter/luciferase reporter construct) normalized to CAT activity (from the transfection efficiency control, cotransfected pRSV-CAT). *, ‡ or § indicate groups within each column significantly ($P < 0.05$) different from each other; for example, values labeled * are similar to each other within a column, while * versus ‡ differ from each other. Results are mean±SEM (n = 4). cAMP, dibutyryl cAMP.

Table III. Influence of Protein Kinase Type-specific Inhibition, or L-type Calcium Channel Blockade, on Norepinephrine Secretion (30-min stimulation) in Response to Nicotine or PMA in PC12 Cells

Category	% Norepinephrine secretion		
	Mock	Nicotine (10^{-3} M)	PMA (10^{-7} M)
Control	4.8±0.07	15.4±0.45*	12.7±0.39*
Protein kinase C inhibition			
Chelerythrine (10^{-5} M)	5.2±0.11	8.7±0.21*‡	5.4±0.20‡
PMA preincubation (10^{-7} M, 48 h)	4.2±0.27	11.9±0.16*‡	5.3±0.32‡
Protein kinase A inhibition			
KT-5720 (5×10^{-8} M)	5.3±0.18	16.0±1.05*	13.1±0.84*
cGMP-dependent protein kinase inhibition			
KT-5823 (5×10^{-7} M)	4.7±0.16	14.6±0.42*	13.0±0.38‡
Ca^{2+}-calmodulin-dependent protein kinase inhibition			
KN-62 (10^{-6} M)	5.2±0.36	7.5±0.17‡	11.6±0.75*
Coeffect of two inhibitors			
Chelerythrine + KN-62	5.0±0.19	7.7±0.42‡	6.3±0.32‡
PMA preincub. + KN-62	4.0±0.28	6.2±0.48‡	5.8±0.49‡
L-type voltage-gated Ca^{2+} channel antagonist			
Nifedipine (10^{-5} M)	4.2±0.39	4.1±0.27‡	6.2±0.42‡

Units of secretion are as defined in Table I. Preincub., preincubation. *Significantly ($P < 0.05$) different from the mock value for that condition (same row). ‡Significantly ($P < 0.05$) different from the control (no inhibitor) value for nicotine or PMA stimulation (same column). Results are mean value±one standard error ($n = 4$ determinations/condition).

calcium–calmodulin-dependent protein kinase virtually abolished nicotinic-stimulated secretion. Blockade of calcium entry through L-type voltage-gated channels also prevented nicotinic-stimulated secretion. Inhibition of other classes of protein kinases (including protein kinase A) had no effect on secretion.

Activation of PKC by acute PMA (10^{-7} M) also stimulated catecholamine secretion, and the effect was selectively blocked by PKC inhibition, though not by inhibition of other protein kinases; in particular, inhibition of calcium–calmodulin-dependent protein kinase did not affect PMA-stimulated secretion.

Table IV. Influence of Protein Kinase Type-specific Inhibition, or L-type Calcium Channel Blockade, on the Transfected Chromogranin A Promoter (Transcriptional) Response to Nicotine or PMA

Category	Chromogranin A promoter activity (luciferase/CAT)		
	Mock	Nicotine (10^{-3} M)	PMA (10^{-7} M)
Control	3.52±0.121	11.64±0.716*	9.33±0.549*
Protein kinase C inhibition			
Chelerythrine (10^{-5} M)	3.15±0.112	6.94±0.176*‡	4.02±0.289‡
PMA preincubation (10^{-7} M, 48 h)	4.12±0.127	8.63±0.171*‡	5.75±0.392‡
Protein kinase A inhibition			
KT-5720 (5×10^{-8} M)	4.24±0.174	12.42±0.392*	10.06±0.434*
cGMP-dependent protein kinase inhibition			
KT-5823 (5×10^{-7} M)	3.61±0.183	12.24±0.557*	8.94±0.388*
Ca^{2+}-calmodulin-dependent protein kinase inhibition			
KN-62 (10^{-6} M)	3.30±0.210	11.1±0.467*	9.62±0.429*
L-type voltage-gated Ca^{2+} channel antagonist			
Nifedipine (10^{-5} M)	4.67±0.154	7.18±0.388*‡	8.54±0.296*
Coeffect of two inhibitors			
Chelerythrine + nifedipine	3.78±0.234	4.12±0.396‡	4.53±0.378‡
PMA preincub. + nifedipine	3.98±0.376	4.57±0.674‡	5.04±0.377‡

The transfected 1133-bp mouse chromogranin A promoter drives expression of a luciferase reporter. The nicotine stimulation time was 48 h, while the PMA stimulation time was 6 h before cell harvest. Units of promoter activity are as defined in Table I. Preincub., preincubation. *Significantly ($P < 0.05$) different from the mock value for that condition (same row). ‡Significantly ($P < 0.05$) different from the control value for nicotine or PMA stimulation (same column). Results are mean value±one standard error ($n = 4$ determinations/condition).

Table V. Activation of PKC or Protein Kinase A by Nicotine (1 mM, 12 h) in PC12 Cells

	PKC activity				Protein kinase A activity		
Treatment	In cytosol	On membrane	Total	% of total on membrane	Already activated (before cAMP)	Total (after cAMP)	% of total activated
Mock	10435±624	2783±174	13218±675	21.1±0.96	5348±265	24055±1187	22.2±1.54
Nicotine (1 mM)	4612±238*	17865±878*	22477±987*	79.5±4.78*	5014±267	23107±785	21.7±0.97

Data are recorded as mean values±SEM ($n = 4$ replicates). *$P < 0.05$ (nicotine versus mock treatment [same column]). PKC activity was measured with the biotinylated peptide substrate neurogranin$_{28–43}$ (AAKIQASFRGHMARKK), while protein kinase A activity was measured with the biotinylated peptide substrate Kemptide (LRRASLG). Kinase activities are expressed as enzyme-specific activity, in pmol ATP incorporated into substrate/min. Mixtures with exogenous cAMP (5 μM) added measured the total available protein kinase A (*Total*), while mixtures without exogenous cAMP measured protein kinase A already activated by endogenous cAMP (*Already activated*).

Signal transduction during activation of chromogranin A transcription by nicotinic cholinergic stimulation:protein kinases and calcium entry. Since nicotinic cholinergic secretory responses in chromaffin cells are associated with influx of calcium through L-type voltage-gated calcium channels (30), we tested the involvement of extracellular calcium influx, as well as several classes of protein kinases, in the chromogranin A transcriptional response to nicotinic stimulation (Table IV).

Nicotinic stimulation augmented by 3.3-fold ($P < 0.05$), reporter expression directed by the transfected chromogranin A promoter in PC12 chromaffin cells. The response was substantially ($P < 0.05$) blunted (though not completely abolished) during PKC inhibition, either by the specific inhibitor chelerythrine, or by PKC inactivation by prior chronic PMA. By contrast, inhibitors of protein kinase A, cGMP-dependent protein kinase, or calcium-calmodulin dependent protein kinase did not affect the nicotinic response of the chromogranin A promoter.

Blunting ($P < 0.05$, Table IV) of the chromogranin A promoter response to nicotine by nifedipine suggested a requirement for extracellular calcium influx in the transcriptional response. The combination of PKC inhibition plus calcium channel blockade virtually abolished nicotinic activation of the chromogranin A promoter (Table IV). Sequestration of cytosolic free calcium by EGTA/AM also blunted the transcriptional response of the transfected chromogranin A promoter to nicotine.

Activation of PKC by acute PMA (10^{-7} M) also stimulated the chromogranin A promoter, and the effects of acute PMA were blocked by PKC inhibition by either chelerythrine or chronic PMA pretreatment, though not by blockade of calcium entry by nifedipine.

After nicotinic stimulation, total cellular PKC activity increased by ~ 70% (Table V, from 13,218±675 to 22,477±987 pmol ATP incorporated into substrate/min, $P < 0.05$), and there was a marked shift of PKC activity distribution within the cell, from the cytosol to the membrane fraction (21.1± 0.96–79.5±4.78%, Table V, $P < 0.05$). Thus, not only was existing PKC activated (translocated to membranes) by exposure of PC12 cells to nicotine, but its biosynthesis was also likely stimulated. By contrast, after nicotine there was no change in either total cellular protein kinase A activity or that fraction activated by endogenous cAMP (Table V).

Expression of constitutively activated mutants of either α- or β-isoforms of PKC each activated the chromogranin A promoter (Table VI), and each synergized activation of the chromogranin A promoter by nicotine.

In a PC12 variant lacking protein kinase A activity (line A126–1B2), both the chromogranin A transcriptional and the catecholamine secretory responses to nicotine were maintained (Table VII).

Table VI. Effects of Constitutively Activated PKC Mutants on the Chromogranin A Promoter and its Response to Nicotine

Cotransfected PKC plasmid		Stimulation	
Name	Function	Mock	Nicotine, 1 mM
pBluescript	Control	239±18.8	502±23.5*
SRα-PKAC	Constitutively activated α-isoform of PKC	607±10.7‡	1718±119.1*‡
SRα-ΔPKCβ (PKCβΔEE)	Constitutively activated β-isoform of PKC	716±39.6‡	1795±258.0*‡

Stimulation time was 48 h. The mouse chromogranin A 1133-bp promoter/luciferase reporter plasmid (pXP-1133) was cotransfected with PKC mutants SRα-PKAC or SRα-ΔPKCβ (PKCβΔEE), or a control plasmid (pBluescript). Units are luciferase activity normalized to mg protein in each sample. *Nicotine treatment group significantly ($P < 0.05$) different from unstimulated group in the same row. ‡SRα-PKAC or SRα-ΔPKCβ group significantly ($P < 0.05$) different from the pBluescript group in the same column. Results are mean value±SEM; $n = 4$ replicates/condition.

Table VII. Nicotine Effects on Catecholamine Secretion or Transfected Chromogranin A Promoter Activity in Protein Kinase A-deficient (A126-1B2) PC12 Cells

PC12 cell type	Treatment	Catecholamine secretion	Chromogranin A transcription
		%	*luciferase/CAT*
Wild-type	Mock	6.5±0.23	44.8±1.88
	Nicotine	14.2±0.78*	84.6±3.67*
A126-1B2 (PKA-deficient)	Mock	11.1±0.69	30.5±2.02
	Nicotine	22.0±1.66*	76.3±2.52*

The transfected 1133-bp mouse chromogranin A promoter drove expression of a luciferase reporter. Concentrations of nicotine were 60 μM for secretion (30 min) and 1 mM for transcription (48 h). Units of secretion and promoter activity are as defined in Tables II and III. *$P < 0.05$ versus each mock (no secretagogue) condition. Results are shown as mean values±SEM ($n = 4$). PKA, cAMP-dependent protein kinase A.

Neither of two adenylyl cyclase inhibitors (SQ-22,536 [10^{-3} M] or MDL-12,330A [10^{-4} M]) affected either basal or nicotine (10^{-3} M)-stimulated expression of the transfected mouse chromogranin A promoter in PC12 cells (data not shown).

Transcriptional and secretory effects of agents that selectively influence cytosolic calcium. Since blockade of L-type voltage-gated cell surface calcium channels (by nifedipine) blunted or abolished the effects of nicotinic stimulation on both catecholamine secretion and chromogranin A promoter activity (Tables III and IV), we examined the effects of several agents that influence entry of calcium into the cytosol from extracellular or intracellular sources.

After cytosolic calcium was chelated by intracellular EGTA, nicotinic stimulation of chromogranin A transcription was substantially blunted.

Agents whose actions affect extracellular calcium entry into the cytosol through cell surface L-type voltage-gated calcium channels (such as nicotine, membrane depolarization with 55 mM KCl, the L-type channel agonist BayK 8644, or the combination of KCl plus BayK 8644) coactivated both catecholamine secretion and chromogranin A transcription (Table VIII). Calcium entry through ATP-gated P_{2x} cell surface calcium channels (31) also activated both secretion and transcription. By contrast, the calcium ionophores A23187 or ionomycin, which allow extracellular calcium entry by creating artificial calcium pores in the cell membrane, triggered catecholamine secretion but not chromogranin A transcription; indeed, chromogranin A transcription fell by $\sim$ 50% after the calcium ionophores, even as catecholamine secretion rose markedly (by 2.8- to 12.8-fold).

In the absence of extracellular calcium (Table VIII), the effects of both nicotine and PMA on catecholamine secretion were abolished; the effects of nicotine on transcription were blunted, while those of PMA were abolished.

There was a general correspondence (Fig. 3; $r = 0.897$, $P = 0.002$) between activation of secretion and activation of the chromogranin A promoter by maneuvers that influence calcium entry into the cytosol through L-type calcium channels, although prevention of calcium entry from the extracellular space (by either calcium removal or calcium channel blockade) more effectively inhibited secretion than transcription during nicotinic stimulation.

Agents that modulate calcium release from intracellular stores (Table VIII) had no effect on chromogranin A promoter activity; among these agents, caffeine stimulated catecholamine secretion. Barium, whose complex actions on chro-

Table VIII. Importance of Route of CA^{2+} Entry Into the Cytosol for Secretion Versus Transcription in PC12 Cells

Category	Norepinephrine secretion	Chromogranin A promoter activity
	%	*luciferase/CAT*
Mock	5.0±0.289	4.68±0.274
Agents affecting Ca^{2+} entry from the extracellular space		
Nicotine (10^{-3} M)	14.85±1.021*	12.13±0.712*
L-type channel opening		
KCl (55 mM)	34.3±0.315*	10.15±0.814*
BayK-8644 (10^{-6} M)	16.3±0.480*	7.27±0.189*
KCl + BayK-8644	53.3±1.612*	17.79±1.021*
Ca^{2+} ionophores		
A23187 (10^{-6} M)	13.8±0.071*	2.56±0.091*
Ionomycin (10^{-5} M)	63.9±1.923*	2.12±0.055*
Ca^{2+}-free medium		
Mock	4.9±0.311	3.92±0.401
Nicotine (10^{-3} M)	4.8±0.216	4.41±0.571
PMA (10^{-7} M)	6.3±0.488	9.59±0.261*
P_{2x} ATP-gated Ca^{2+} channel		
ATP (10^{-4} M)	13.2±0.688*	9.85±0.412*
Agents affecting Ca^{2+} entry from intracellular stores		
Agents causing release from stores		
Caffeine (10 mM)	10.1±0.375*	4.71±0.317
$BaCl_2$ (2 mM in Ca^{2+}-free medium)	77.0±0.388*	5.01±0.462
Agent inhibiting release from stores		
Ryanodine (10^{-5} M)	5.3±0.086	3.98±0.356

Response of norepinephrine secretion or transfected chromogranin A promoter activity to agents that influence Ca^{2+} entry from the extracellular space versus internal depots. The transfected 1133-bp mouse chromogranin A promoter drives expression of a luciferase reporter. Stimulation times were 12 h for KCl, and 48 h for other stimuli to promoter/reporter expression, and 30 min for secretion. Units of secretion and promoter activity are as defined in Tables II and III. *Significantly ($P < 0.05$) different from the mock (no agent added) value for each experiment (same column). Results are mean±SEM ($n = 4$).

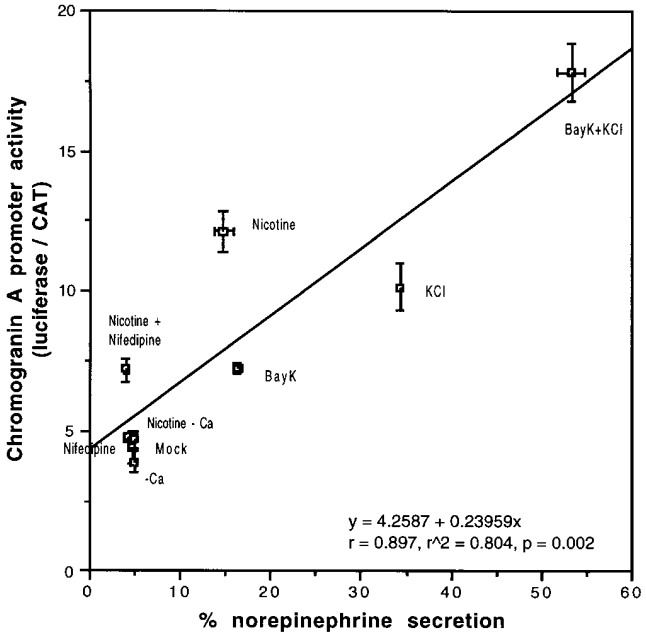

Figure 3. Importance of route of Ca^{2+} entry for its mediation of secretion versus transcription in PC12 cells. Effects on catecholamine secretion and chromogranin A promoter activity of agents that influence calcium entry through L-type voltage-gated Ca^{2+} channels. The transfected 1,133-bp mouse chromogranin A promoter drives expression of a luciferase reporter. Nicotine minus Ca indicates the administration of nicotine in the absence of extracellular Ca^{2+}. Bay K-8644 (BayK) is a dihydropyridine agonist at L-type channels, while nifedipine is an L-type channel dihydropyridine antagonist. Standard error bars are shown for each agent. Units of secretion and promoter activity are as defined in Tables II and III. The doses used were as follows: nicotine, 10^{-3} M; KCl, 55 mM; Bay K-8644, 10^{-6} M; or nifedipine, 10^{-5} M. Mock, no additions; −Ca, no extracellular Ca^{2+}.

Table IX. Effect of Secretagogues on Cytosolic [Ca^{2+}] Concentration in PC12 Chromaffin Cells. Stratification by Route of Calcium Entry

Route of Ca^{2+} entry to cytosol	Secretagogue	Modulator	*n*	Peak increment in cytosolic Ca^{2+} concentration
				nM
From extracellular space	Nicotine (60 μM)		5	300±63
	Nicotine	Verapamil (1 μM)	3	27±17
	Nicotine	ω-conotoxin (0.5 μM)	2	179±11
	KCl (55 mM)		8	965±120
	KCl	Verapamil	5	622±134
	KCl	ω-conotoxin	2	130
	A-23187 (5 μM)		1	130
From intracellular stores	Caffeine (10 mM)		5	167±29
	Caffeine	EGTA (3 mM)	6	119±23
	Caffeine	No extracellular Ca^{2+}	1	115
	Caffeine	Verapamil	2	203±14
	Caffeine	ω-conotoxin	1	179
	$BaCl_2$ (2 mM)		2	2015±994
	$BaCl_2$	Verapamil	2	1233±897
	$BaCl_2$	EGTA	2	1200±423

PC12 cells in stirred suspension within a cuvette were loaded with Fura-2/AM, washed, resuspended, and recorded in a fluorometer. The peak response of cytosolic calcium concentration to various secretagogues (grouped by effects on Ca^{2+} entry from extracellular versus intracellular sources) and modulators is listed. The concentration of each secretagogue or modulator is given only at the first listing in the column. Unless otherwise indicated, the extracellular fluid (bath) contained 2 mM $CaCl_2$. The average basal calcium concentration was 339 nM. $BaCl_2$ is administered in the absence of extracellular calcium in the bath.

maffin cells may involve barium entry through L-type calcium channels and subsequent release of calcium from intracellular stores (32, 33), triggered secretion, but not chromogranin A transcription.

Were the transcriptional effects of long-term membrane depolarization with 55 mM KCl (see Table XI) specifically medicated by opening of L-type voltage-gated calcium channels? 100 μM verapamil had no effect on basal expression of the transfected mouse chromogranin A promoter/luciferase reporter in PC12 cells, but blocked KCl-induced activation of expression by 79% (data not shown), arguing for such specificity.

Effect of secretagogues on cytosolic calcium concentration in PC12 chromaffin cells. Using PC12 cells loaded with the calcium-fluorescent dye Fura-2 (Table IX), we studied the effects on cytosolic calcium of those agents (Table VIII) that provoked catecholamine secretion or chromogranin A promoter activation.

The increments of cytosolic calcium after nicotine or membrane depolarization by KCl were substantially blocked by the L-type channel antagonist verapamil, but largely unaffected by the *N*-type channel antagonist ω-conotoxin; thus, calcium entry after either nicotine or KCl seemed to involve L-type channels.

The more modest increments in cytosolic calcium after bradykinin, caffeine, or thapsigargin were largely unaffected by the absence (or chelation by EGTA) of extracellular calcium, or by blockade of cell surface L-type or *N*-type calcium channels; these results are compatible with calcium release from intracellular (endosomal) stores. The effects of barium on cytosolic calcium were blunted by verapamil, consistent with a requirement for entry of barium into the cell through L-type channels (32, 33); the lack of effect of extracellular calcium chelation by EGTA indicates that the cytosolic calcium increment after barium was at least partly derived from intracellular stores.

Role of the chromogranin A promoter's proximal CRE box in nicotinic activation. When progressive 5′ deletions of the mouse chromogranin A promoter (Fig. 4) were exposed to nicotine or phorbol ester, much of the promoter activation by each compound mapped to the region between −77 bp to −61 bp upstream of the cap site; this is the region of the promoter's functional (15) cAMP response element (TGACGTAA; −71 to −64 bp). There were, however, some differences in response to nicotine or PMA in other promoter regions. Deletion from −425 bp to −328 bp, a region that includes a partial consensus match for an AP-1 site ([−418 bp] 5′-TGAG**C-G**AG-3′ [−425 bp], partial [6/8-bp match] homology with an AP1 consensus TGAGTCAG [34]), diminished the promoter response to nicotine, though not to PMA. A 43-bp promoter retained a minimal response to PMA, though no response to nicotine (in this promoter [13], the TATA box [here: TATATAAAA] is at position −25 to −17 bp).

We then evaluated two site-directed mutants of the chromogranin A promoter's cAMP response element (Table X), in mouse chromogranin A promoter/reporter plasmids. Both a 6/8-bp change in the cAMP response element (from TGACG-TAA to **CAT**C**ACC**A; changes in bold) and a more subtle 1-bp point–gap mutation (from TGACGTAA to TGA-GTAA) substantially ($P < 0.05$) blunted the promoter's responses to both nicotine and PMA.

Is a cAMP response element sufficient to mediate promoter responses to a variety of calcium-dependent stimuli? Several cAMP response element variations were transferred to a neutral, heterologous promoter TK/luciferase reporter

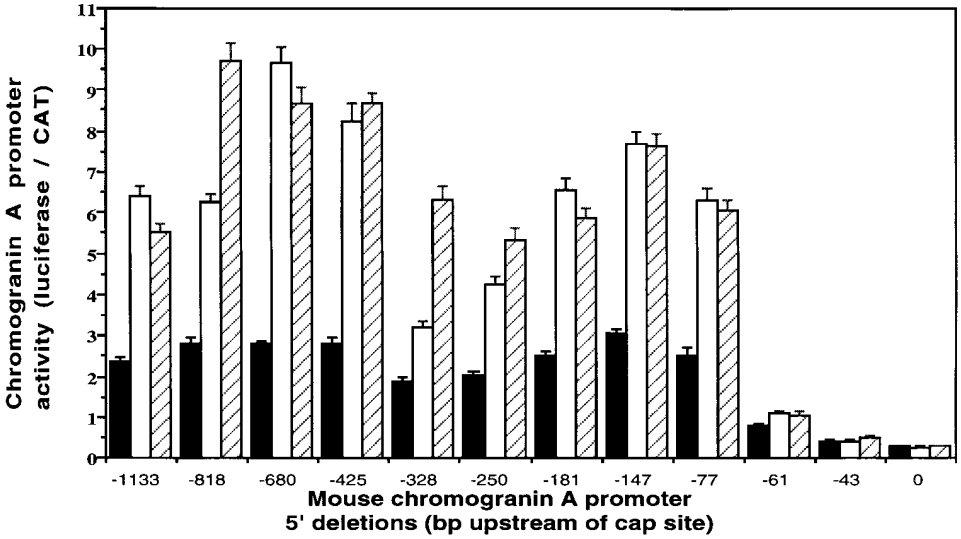

Figure 4. Stimulation of progressive 5′ promoter deletions, in mouse chromogranin A promoter/luciferase reporter plasmids, after nicotine (1 mM), PMA (0.1 μM), or vehicle (mock). The stimulation time was 48 h. The response of each promoter fragment was calculated as the ratio of nicotine/mock (vehicle) values of luciferase/CAT. 0 bp of promoter length indicates the promoterless luciferase reporter vector, pXP1. Results are mean values±one standard error ($n = 4$ transfections at each deletion point). Data on regional chromogranin A promoter effects of nicotine alone have been presented previously (10). *Black bars*, mock; *white bars*, nicotine; *striped bars*, PMA.

(parent plasmid, pTK-LUC), then transfected and stimulated with nicotine, KCl, cAMP, or PMA (Table XI). The chromogranin A cAMP response element (TGACGTAA) as well as a consensus cAMP response element (TGACGT**C**A) increased their expression in response to all four kinds of stimulation, while a point–gap mutant cAMP response element (TGA-GTAA) responded partially to nicotine, KCl, or PMA, but not to cAMP.

Greenberg et al. (35) identified an 8-bp motif (TGA-CGT**TT**), at position −60 bp in the *c-fos* promoter, which confers a promoter response to cytosolic calcium increments, and which is similar in sequence (6/8 bp match) to a cAMP response element consensus (TGACGTCA); this sequence has been referred to as a calcium response element (36). When a 71-bp *c-fos* promoter/CAT reporter plasmid containing this calcium response element at −60 bp (36) was transfected into PC12 cells, its basal CAT reporter expression (48 h after transfection) of 3,016±123 cpm/mg protein, was increased ($P <$ 0.05) to 5,433±187 cpm/mg protein after nicotinic stimulation (1 mM, 48 h). After 48 h of 0.1 μM phorbol myristate acetate, CAT reporter activity was also stimulated ($P < 0.05$) to 4,223±154 cpm/mg protein.

To test the role of the cAMP response element binding protein CREB in relaying the nicotinic signal to the chromogranin A promoter's cAMP response element, we cotransfected a plasmid expressing the CREB dominant negative mutant (i.e., CREB antagonist) KCREB (Table XII). KCREB expression diminished the chromogranin A promoter's response to cAMP by ∼ 97%, to nicotine by ∼ 70%, to KCl by ∼ 63%, and to PMA by ∼ 64%.

Discussion

Although much is known about how nicotinic cholinergic stimulation triggers exocytotic corelease of catecholamines and peptides from the chromaffin cell (31, 37–39), the mechanisms whereby the cell resynthesizes the secreted peptides are not yet completely understood (10, 40). For this reason, we analyzed signal transduction during the responses of both catecholamine secretion and chromogranin A promoter activity to

Table X. Necessity of the CRE box for Calcium-mediated Signaling to Chromogranin A Gene Expression in PC12 Cells

Mouse CgA promoter/reporter plasmid			Stimulation				
CgA promoter length (bp 5′ of cap site)	CRE box	Plasmid	Mock	Nicotine (1 mM)	Nicotine/ mock ratio	PMA (0.1 μM)	PMA/ mock ratio
100	TGACGTAA (wild-type)	pXP-100	28.5±1.64	62.4±2.58‡	2.19	77.3±3.17‡	2.71
100	**CATCACC**A (6/8 bp replaced)	M13	18.2±1.02*	27.5±0.94*‡	1.51	30.1±1.54*‡	1.65
77	TGACGTAA (wild-type)	pXP-77	32.0±1.13	69.8±2.78‡	2.18	64.2±2.67‡	2.00
77	TGA-GTAA (1 bp point-gap)	M41	22.6±0.89*	29.1±1.26*	1.29	35.3±1.58*‡	1.56

Effects of nicotine (1 mM, 48 h) or phorbol ester (PMA, 0.1 μM, 6 h) on mouse chromogranin A (CgA) promoter/luciferase reporter plasmids with cAMP response element mutations. 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 values±SEM ($n = 4$) for luciferase/protein ratios. *Significantly ($P < 0.05$) different from the corresponding wild-type group in the same stimulation condition (same column). ‡Stimulated group was significantly ($P < 0.05$) different from mock in the same plasmid (same row). All (nicotine- or PMA-) stimulated group ratios were significantly ($P < 0.05$) higher than the corresponding mock group (same row). CgA, chromogranin A. CRE, cAMP response element. Cap site, transcription initiation site. pXP-100, 100-bp mouse chromogranin A promoter (wild-type) fused to luciferase reporter. pXP-77, 77-bp mouse chromogranin A promoter (wild-type) fused to luciferase reporter. M13, cAMP response element mutant (**CAT**C**ACC**A) of pXP-100. M41, cAMP response element mutant (TGA-GTAA) of pXP-77.

Table XI. Sufficiency of the CRE box for Ca^{2+}-mediated Signaling to Chromogranin A Gene Expression in PC12 Cells

Promoter/luciferase reporter plasmid			Stimulus				
Plasmid	Promoter	CRE box	Mock	Nicotine (1 mM)	KCl (55 mM)	cAMP (100 μM)	PMA (0.1 μM)
pXP-1133	mCgA (1133 bp)	TGACGTAA	1203±67.8	3014±124*	2275±107*	3126±154*	2851±112*
mCgA-CRE-TK	TK (110 bp)	TGACGTAA	283±13.5	892±34.5*	645±28.9*	852±41.6*	721±25.5*
Perfect-CRE-TK	TK	TGACGT**C**A	564±10.9	1582±54.7*	1016±33.5*	1458±67.7*	1013±43.2*
Mutant-CRE-TK	TK	TGA-GTAA	206±10.5	328±14.6*	285±11.9*	223±9.37	293±10.1*
pTK-LUC (vector)	TK (alone)	None	123±6.52	153±7.14	138±8.25	145±10.4	141±9.4

Effects of stimulation by nicotine (1 mM), membrane depolarization (KCl, 55 mM), cAMP (dibutyryl cAMP, 0.1 mM) or PMA (0.1 μM) on transfected promoter/luciferase reporter plasmids, where the promoter driving luciferase expression is a heterologous TK promoter (from Herpes simplex virus), to which an isolated cAMP response element has been fused (just 5′ of the TK promoter). Both the mouse chromogranin A promoter and the Herpes simplex virus TK promoter contain TATA boxes. Stimulation times were 48 h for nicotine, or 8 h for cAMP or KCl. Units are luciferase activity (from the transfected mouse chromogranin A 1133-bp promoter/luciferase reporter construct), normalized to mg protein of each sample. In the wild-type mouse chromogranin A promoter, the functional CRE box (TGACGTAA) is located at position −71 to −64 bp upstream of the cap site. CRE box mutations are shown in bold type. *Results in a treatment group significantly ($P < 0.05$) different from the unstimulated group in the same row. Results shown are mean±SEM; $n = 4$ replicates/condition. cAMP, dibutyryl cAMP; TK, thymidine kinase (Herpes simplex virus) 110-bp promoter; mCgA, mouse chromogranin A. CRE, cAMP response element; perfect-CRE, consensus 8-bp CRE motif (TGACGT**C**A), originally described in the somatostatin promoter; Mutant-CRE, single point-gap mutation in the mCgA CRE box (TGA-GTAA).

physiologic (nicotinic cholinergic) stimulation of chromaffin cells, as well as to stimuli that provoke calcium entry and enzymatic (protein kinase) responses to calcium into such cells.

Initial results on cation fluxes indicated that nicotine triggers both Na^+ and Ca^{2+} entry into PC12 cells (Table I), however, membrane depolarization–stimulated Ca^{2+} entry could be blocked by inhibition of L-type calcium channels, though not by nicotinic cholinergic antagonism with hexamethonium. Hence, entry of Ca^{2+} through L-type channels seems to be a step distal to initial Na^+ entry through the nicotinic cholinergic cation pore, which, in neuronal nicotinic receptors, is generally most permeable to Na^+ (41). Indeed, removal of Na^+ from the extracellular space virtually abolished both the catecholamine secretory and the chromogranin A transcriptional responses to nicotinic cholinergic stimulation (Table I), consistent with the role of the nicotinic cholinergic receptor as an extracellular ligand-gated cation pore permeable principally to sodium (41), and as the initial point of signal transduction for all processes responding to nicotinic cholinergic stimulation (41).

We found that nicotinic cholinergic stimulation augmented both catecholamine release and chromogranin A promoter activity in PC12 cells (Tables III and IV). The protein kinase dependencies of these two responses to nicotinic stimulation were different: chromogranin A transcription was largely mediated by PKC activation, while secretion was, in part, mediated by both PKC and Ca^{2+}-calmodulin–dependent protein kinase (Tables III and IV). Activation of endogenous PKC by nicotine was confirmed enzymatically (Table V): a marked shift in PKC enzymatic activity from the cytosol to the membrane fraction confirmed typical posttranslational activation of the enzyme, while an increase in total cellular PKC activity (cytosolic plus membrane forms; Table V) also suggested an increase in biosynthesis of the enzyme after nicotine. Pharmacologic activation of PKC by acute phorbol ester exposure augmented both secretion and chromogranin A transcription (Tables III and IV).

Involvement of protein kinases in chromaffin cell catecholamine secretion has been documented previously by Burgoyne and Norman (42, 43), and Holz and co-workers (44, 45) presented evidence for involvement of both PKC and calcium–calmodulin-dependent protein kinase (10). Involvement of PKC in the chromogranin A biosynthetic response to nicotinic stimulation in chromaffin cells was suggested by Simon et al. (46), who found that PKC inhibition (by either sphingosine, or

Table XII. Role of the Transcription Factor CREB in Relaying Ca^{2+}-mediated Signals to the Chromogranin A Promoter in PC12 Cells. Effects of a CREB Dominant Negative Mutant (KCREB) on Response of the Transfected Mouse Chromogranin A Promoter to Secretory Stimuli

	Stimulus				
Cotransfection	Mock	Nicotine (1 mM)	KCl (55 mM)	cAMP (100 μM)	PMA (0.1 μM)
pBluescript (control)	2271±112	5154±278*	4537±203*	4736±211*	4896±215*
pRSV-KCREB	2059±103	2919±126*‡	2907±119*‡	2137±104‡	3015±174*‡

The mouse chromogranin A 1133-bp promoter/luciferase reporter plasmid (pXP-1133) was cotransfected with either the KCREB expression vector (pRSV-KCREB, in which KCREB expression is driven by the strong RSV long terminal repeat), or an irrelevant control plasmid (pBluescript). Transfection amounts were as follows: pXP-1133, 3 μg/ml, plus either pRSV-KCREB or pBluescript, each 3 μg/ml; there were 0.4 ml/well in 12-well plates. Units are luciferase activity normalized to mg protein of each sample. Stimulation times were 48 h for nicotine or cAMP, 6 h for PMA, and 8 h for KCl. *Treatment group significantly ($P < 0.05$) different from mock (unstimulated) group in the same row. ‡KCREB group significantly ($P < 0.05$) different from pBluescript group in the same column. Results are mean value±SEM, $n = 4$ replicates/condition. cAMP, dibutyryl cAMP.

phorbol ester pretreatment) inhibited or abolished incorporation of [^{35}S]methionine into newly biosynthesized chromogranin A protein.

Is there a role for cAMP-dependent protein kinase A in the transcriptional response of chromogranin A to nicotinic cholinergic stimulation? Involvement of protein kinase A might be suggested by the involvement of the chromogranin A promoter's CRE box in *cis* and the transcription factor CREB in *trans.* The nicotinic transcriptional response mapped onto the chromogranin A promoter's cAMP response element (Tables X–XII; Fig. 4), and was impaired by selective CRE box mutations (Table X). Furthermore, dominant negative KCREB mutant studies (Table XII) established a role in *trans* for the transcription factor CREB in relaying the nicotinic signal to the cAMP response element in *cis.* Nicotinic stimulation of PC12 cells also phosphorylates CREB on serine 133 (10). Protein kinase A, however, was not activated by nicotine (Table V), and the transcriptional response of chromogranin A to nicotine was unimpaired by protein kinase A chemical (KT-5720) inhibition (Table IV) or absence (variant A126-1B2; Table IX) in PC12 cells. By contrast, nicotinic transcriptional activation of chromogranin A was blocked by several types of PKC inhibition (Table IV), and augmented by PKC overexpression (Table VI). Of note, activation of CREB through phosphorylation at serine 133 by calcium-dependent protein kinases, including PKC, has been established in PC12 cells and other systems (20, 35, 47–49). Thus, our data are most consistent with a key role for CREB in nicotinic activation of chromogranin A biosynthesis, but with CREB activation through phosphorylation by PKC.

By contrast, Sabban et al. (4) have established a role for both protein kinase A and the promoter's cAMP response element in the transcriptional response of tyrosine hydroxylase to nicotinic secretory stimulation. In that system (4), a nicotinic-triggered influx of calcium into the cytosol may activate an adenylyl cyclase, but here adenylyl cyclase chemical inhibition did not impair the transcriptional response of chromogranin A to nicotine. We did however, uncover a subtle interaction between cAMP and nicotine in activation of chromogranin A transcription: when nicotine and cAMP were combined, their effects on chromogranin A transcription were synergistic (Table II), suggesting that their signal transduction towards chromogranin A transcription is not entirely via independent pathways; indeed, both nicotine and cAMP signal to chromogranin A gene expression through the same *cis* and *trans* elements, the cAMP response element (Tables X and XI) and the transcription factor CREB (Table XII).

Blockade of calcium entry through L-type cell surface calcium channels inhibited nicotinic activation of both secretion and chromogranin A transcription (Tables III and IV). We therefore examined the secretory and transcriptional effects of a variety of agents that selectively perturb entry of calcium into the cytosol by several routes (Table VIII). Agents that influenced calcium entry through L-type voltage-gated calcium channels exerted parallel effects on both secretion and chromogranin A transcription (Fig. 3), although prevention of calcium entry into the cytosol during nicotinic cholinergic stimulation (by calcium channel blockade or removal of calcium from the extracellular fluid) more effectively prevented secretion than chromogranin A transcription. ATP, which allows extracellular calcium to enter the cytosol through P_{2x} ligand-gated cell surface calcium channels in PC12 cells (31) also triggered both secretion and transcription (Table IX).

By contrast, agents that allowed calcium entry into the cytosol from artificial cell surface calcium pores (such as the calcium ionophores A23187 or ionomycin), or agents that provoked calcium efflux from intracellular stores (such as caffeine or barium) increased secretion, but did not augment chromogranin A transcription (Table VIII).

Livett et al. (32, 33) have previously reported a lack of effect of the powerful secretory stimulus barium on chromaffin cell gene expression for the secreted peptide proenkephalin A and the catecholamine biosynthetic enzyme phenylethanolamine-*N*-methyltransferase. The effects of the secretagogue barium in chromaffin cells are complex and incompletely understood (29, 32, 33, 50–52); barium may gain entry to the cell by the L-type voltage-gated cell surface calcium channel (Table XI) (50), and then induce release of calcium from internal stores (Table VIII) (50). PKC may also mediate barium's actions on secretion (52).

What is the significance of route of calcium entry into the cytosol (Tables VIII and IX) in triggering transcription? Gallin and Greenberg (53) and Ginty (54) have also noted very different neuronal gene expression responses to extracellular calcium influx through L-type voltage-gated calcium channels versus ligand-gated calcium channels (e.g., NMDA receptors), though they have not investigated the effects of intracellular calcium store release on gene expression. Different routes of calcium entry may also differ in intrinsic permeability to calcium as well as kinetics of calcium flux (55). Other evidence (56) suggests that an increase in calcium concentration within the nucleus itself is crucial to activation of CREB-mediated gene expression; whether influx of calcium to the cytosol from the extracellular space results in greater increments in nuclear calcium than does influx from intracellular stores is not known.

Nicotinic stimulus transcription coupling in chromaffin cells has also been examined for responsible promoter regions in the catecholamine biosynthetic enzymes tyrosine hydroxylase (3, 4, 57, 58), dopamine β-hydroxylase (59), and phenylethanolamine *N*-methyltransferase (60), as well as the secretory peptide proenkephalin A (61). Such previous studies suggest that two promoter domains may be crucial in nicotinic responses: the cAMP response element (3, 4) and the AP1 site (62). Likewise, the cAMP response element in the chromogranin A promoter is crucial (Fig. 4; Tables X–XII).

In a previous report (10), we optimized dose-response relationships for nicotine (from 10^{-8} M to 10^{-2} M) on both catecholamine secretion and chromogranin A biosynthesis in PC12 cells. Catecholamine secretion was maximal at 10^{-4} M nicotine, while chromogranin A transcription was maximal at 10^{-3} M nicotine; both responses declined at higher doses of nicotine, suggesting desensitization at the highest agonist dose (63). While a maximal secretory response to 10^{-4} M nicotine is typical for chromaffin cells (64), the response of a transfected promoter takes place over a much longer time course (48 h versus 30 min), perhaps allowing for further time-dependent desensitization. The dissociation constant (K_d) values for acetylcholine binding to various nicotinic cholinergic receptor species range from 1 to 158 μM (41).

The emerging picture of nicotinic stimulus coupling to secretion versus chromogranin A transcription in chromaffin cells, based on these studies, is outlined schematically in Fig. 5. Nicotinic cholinergic stimulation results initially in entry of sodium from the extracellular space through the nicotinic cation pore (41), which depolarizes the cell membrane, thereby open-

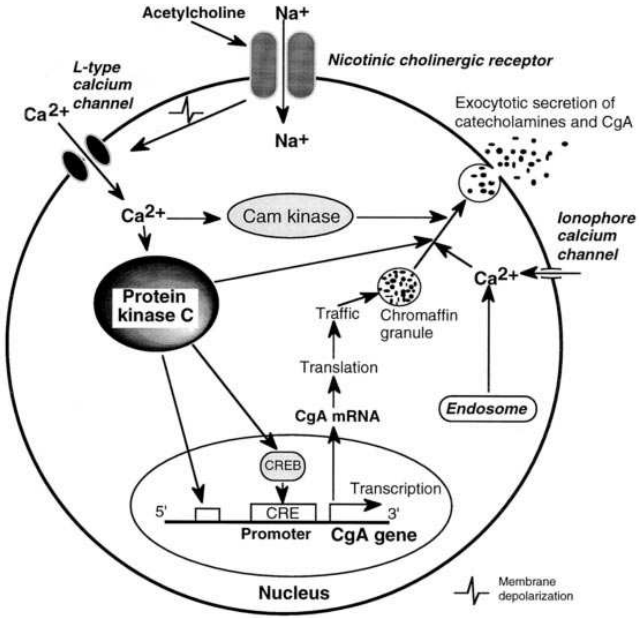

Figure 5. Outline of proposed signal transduction pathways for activation of catecholamine secretion versus chromogranin A (CgA) transcription by nicotinic cholinergic stimulation in PC12 chromaffin cells. CRE, cAMP response element; Cam kinase, calcium-calmodulin–dependent protein kinase. The upstream promoter signaling arrow for PKC refers to an upstream (−425 to −328 bp upstream of the cap site; see Fig. 4) domain, suggesting a positive response to nicotine.

ing cell surface L-type voltage-gated calcium channels. Once in the cytosol, this pool of calcium activates PKC to trigger both the chromogranin A promoter and catecholamine secretion; the same calcium pool also activates calcium–calmodulin-dependent protein kinase, contributing to secretion but not transcription. Agents that result in increments of cytosolic calcium through other routes of entry, such as opening artificial calcium channels with calcium ionophores, or releasing calcium from internal (endosomal) stores, may result in secretion, but no activation of the chromogranin A promoter (Table VIII).

Thus, the chromogranin A promoter responds (through the crucial intermediary of protein kinase C activation) to receptor-triggered increments in cytosolic calcium entering from the extracellular space. Unlike the response of catecholamine secretion, however, the chromogranin A transcriptional response is highly selective in recognition of the precise route of calcium entry into the cytosol.

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