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Secretin Activation of Chromogranin A Gene Transcription

IDENTIFICATION OF THE SIGNALING PATHWAYS *IN CIS* AND *IN TRANS**

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Secretin evokes catecholamine secretion from PC12 pheochromocytoma cells. We tested whether secretin activates transcription of the major vesicular core protein chromogranin A (CgA). Secretin stimulated both endogenous CgA gene transcription (~4–6-fold) as well as transfected CgA promoter activity (~8–10-fold; EC_{50}, ~7 nM) in PC12 cells. Studies on CgA promoter 5′-deletion mutant/luciferase reporter constructs, point mutations of the CgA cAMP response element (CRE), and their transfer to a heterologous promoter implicated CRE *in cis* as both necessary and sufficient for secretin-stimulated CgA gene transcription. Secretin-induced CgA gene transcription was inhibited/abolished by cytosolic Ca^{2+} chelation, chemical blockade of phospholipase C, protein kinase A (PKA), or mitogen-activated protein (MAP) kinase extracellular signal regulated kinase (ERK) 1/2 and the expression of dominant negative mutants of ERK1/2, CRE binding protein (CREB) kinase RSK2, or CREB. Secretin also augmented (~4-fold) phosphorylation of ERK1/2. *Trans*-activation (~21-fold) of GAL4-CREB fusion protein by secretin indicates involvement of CREB in secretin signaling to gene transcription. Electrophoretic mobility shift assays also identified CREB as the mediator of secretin-induced CgA gene transcription, and pCREB supershifts indicated Ser-133 as the active CREB moiety *in vitro*. This conclusion was reinforced *in vivo* by results of chromatin pCREB immunoprecipitation assays. We conclude that secretin signals to CgA gene transcription through the CRE domain in *cis* and through cAMP, Ca^{2+}, PKA, MAP kinase, and the transcription factor CREB in *trans*. Thus, multiple signal transduction pathways seem to subserve the function of stimulus-transcription coupling after this peptidergic stimulus to chromaffin cells.

The gastrointestinal hormone secretin (human amino acid sequence HSDGTFTSELSRLREGARLQRLLQGLV) belongs to the vasoactive intestinal polypeptide (VIP)/pituitary adenylyl cyclase-activating polypeptide (PACAP)[1]/glucagon peptide superfamily (1, 2). Although secretin is mainly produced by the enteroendocrine S cells (3), secretin mRNA is detected in rat hypothalamus, brain stem, and cortex (4), as well as rat cerebellum (5). Besides stimulation of pancreatic and biliary secretion, secretin also increases cardiac output, cardiac muscle adenylate cyclase activity (6–8), and peripheral blood flow (9–11). In the central nervous system, secretin affects turnover and accumulation of dopamine and increases tyrosine hydroxylase (the rate-limiting enzyme in catecholamine biosynthesis) activity in the hypothalamus, superior cervical ganglion, and sympathetic nerve endings (12, 13). Recently, secretin has also been shown to facilitate γ-amino-*n*-butyric acid (GABA) transmission in the rat cerebellum (5).

Because of the expression of secretin receptors in organs outside the gastrointestinal system (2, 14) and the stimulatory effect of secretin on tyrosine hydroxylase activity (13, 15), we looked for effects of secretin on catecholamine secretion and found that it induces up to 26% of cell total catecholamine release from PC12 pheochromocytoma cells (16). Because chromogranin A (CgA) is co-stored and co-released with catecholamines (17), we reasoned that secretin would activate transcription of the CgA gene to replenish the just-secreted CgA protein.

Several physiological implications have, to date, been attributed to CgA. The plasma concentration of CgA is elevated in established human essential hypertension (18). This protein complexes with catecholamines and calcium within secretory vesicles (19) and plays a fundamental role in steering secretory proteins into the regulated pathway (20). Indeed, CgA is now established to be a crucial on/off switch for creation of the regulated secretory pathway in catecholaminergic cells; the subtraction of CgA (by antisense CgA mRNA) deletes the regulated pathway (both functionally and morphologically) from chromaffin cells, whereas re-expression of CgA rescues this phenotype (21). In addition, CgA is proteolytically cleaved to form biologically active peptides such as vasostatin (human CgA_{1-76}), which relaxes vascular smooth muscle (22), catestatin (bovine $CgA_{344-364}$) that inhibits catecholamine release (23, 24), and pancreastatin (porcine $CgA_{240-288}$), which impairs glucose homeostasis (25). There-

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[1] The abbreviations used are: PACAP, pituitary adenylyl cyclase activating polypeptide; AP1, activator protein 1; BAPTA-AM, 1,2-bis(2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl)ester; B-Raf, neuronal Raf isoform; CAT, chloramphenicol acetyltransferase; CgA, chromogranin A; ChIP, chromatin immunoprecipitation; CMV, cytomegalovirus; CRE, cyclic AMP response element; CREB, CRE-binding protein; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; Gs, stimulatory heterotrimeric G-protein; MAP, mitogen-activated protein; MEK, MAP kinase/ERK kinase; PBS, phosphate-buffered saline; pCREB, CREB activated by phosphorylation at Ser-133; PKA, protein kinase A (cAMP-dependent protein kinase); PKI, protein inhibitor of PKA; ΔPKI, inactive mutant of PKI; PMA, phorbol-12-myristate-13-acetate; Rap1,Ras-related small G-protein; RSV, Rous sarcoma virus; RT, room temperature.

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fore, transcriptional regulation of CgA by secretin may modulate various physiological events.

We found that secretin stimulated both endogenous CgA gene transcription as well as transfected CgA promoter activity (EC_{50}, ~7 nM) in PC12 cells. The present communication describes the *cis* and *trans* signaling determinants of CgA gene transcription in response to secretin.

MATERIALS AND METHODS

Cell Culture—Early passage (passage 8–12) rat pheochromocytoma PC12 cells (26) were used in this study. The cells were grown in Dulbecco's modified Eagle's medium/high glucose medium supplemented with 5% heat-inactivated fetal bovine serum, 10% heat-inactivated horse serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin (Invitrogen) at 37 °C, 6% CO_2, in 10-cm or 12-well plates.

Northern Blots for CgA mRNA—PC12 cells were treated with secretin (1 μM) *versus* mock for 1–24 h. Total RNA was isolated by guanidinium thiocyanate extraction (RNAzolB; TelTest, Friendswood, TX). RNAs (10–20 μg) were size-fractionated on denaturing 1% agarose-formaldehyde gels, transferred to nitrocellulose membranes, and fixed with ultraviolet irradiation (StrataLinker; Stratagene, La Jolla, CA). The integrity of the RNA was judged by the appearance of 28 and 18 S rRNA bands on the ethidium bromide-stained gel. The blots were prehybridized, hybridized, and washed as described (27).

Random primer-labeled cDNA probes were a 1.6-kbp rat CgA cDNA (28) and a 381-bp mouse cyclophilin cDNA (29) used as a normalizing probe for a "housekeeping" (constitutively expressed) mRNA. Expression of mRNAs was quantified using the NIH image (version 1.62) software and normalized to cyclophilin gene expression.

CgA Promoter-Reporter Constructs and Expression Plasmids—Progressive 5′-deletion mutant constructs of CgA are numbered relative to the transcription initiation (cap) site as +1. For example, pXP1133 contains 1133 bp of the mouse CgA promoter fused to a luciferase reporter in the promoterless luciferase reporter vector pXP1. Generation of deletion as well as site-directed mutant constructs of CgA was described previously (30, 31). pRSV-protein kinase A inhibitor (PKI) is the expression plasmid for the heat-stable inhibitor of cAMP-dependent protein kinase A; pRSV-ΔPKI expresses the inactive PKI mutant (32). Richard A. Maurer (University of Iowa, Iowa City, IA) kindly supplied these plasmids. We obtained the following plasmids: a dominant negative point mutant of human ERK1 (K71R), subcloned into the cytomegalovirus (CMV) promoter-driven pCMV5 expression vector (33) from J. K. Westwick (University of North Carolina, Chapel Hill, NC); a dominant negative point mutant of a hemagglutinin epitope-tagged murine RSK2 (HA-RSK2; K100R), subcloned into the adenovirus major late promoter-driven pMT2 expression vector (34), from Michael E. Greenberg (Harvard Medical School, Boston, MA); and a dominant negative point mutant (KCREB; R287L) of the human cyclic AMP-response element-binding protein (CREB) subcloned into the Rous sarcoma virus (RSV) promoter-driven pRC expression vector (35), from Richard H. Goodman (Vollum Institute, Oregon Health Sciences University, Portland, OR).

Transient Transfection/Co-transfection—Supercoiled plasmid DNA was used in transient transfection/co-transfection studies. Plasmids were purified on columns (Qiagen Inc., Chatsworth, CA) and transfected using the polycationic method (Superfect; Qiagen Inc.) as described previously (27, 36, 37). PathDetect® CREB *trans*-reporting system plasmids, *i.e.* pFR-Luc (reporter plasmid), pFA2-CREB (fusion *trans*-activator plasmid), pFC2-dbd (negative control plasmid), and pFC-PKA (positive control plasmid), were obtained from Stratagene. In case of co-transfections when the reporter or *trans*-activator plasmid did not total the requisite amount of DNA, the balance was composed of supercoiled pBluescript (Stratagene) (27). Four to five hours after transfections, PC12 cells were treated with human secretin (1 μM) either alone or in combination with chemical inhibitors for 16–18 h before harvesting for luciferase assay as described previously (27). To control for transfection efficiency, in some experiments we co-transfected with the neutral promoter/reporter plasmid pRSV-CAT, as described previously (27). In this plasmid, the RSV long terminal repeat drives the expression of the bacterial chloramphenicol acetyltransferase (CAT) reporter. CAT activity was measured by transfer of [^{14}C]acetyl groups from acetyl-CoA to chloramphenicol, as described previously (27).

Quantitative Determination of pERK1/2—To test whether secretin induces MAP kinase (ERK1/2) activity, we quantitatively determined pERK1/2 in the PC12 cell lysate in response to secretin (1 μM) *versus* mock using the pERK1/2 TiterZyme® enzyme immunometric assay (EIA) kit (Assay Designs, Inc., Ann Arbor, MI). In brief, PC12 cells in 12-well tissue culture plates were treated with secretin (1 μM) for 5–160 min followed by washing once with ice-cold PBS and lysis in 300 μl of 10 mM HEPES (pH 7.4), 50 mM β-glycerophosphate, 70 mM NaCl, 2 mM EDTA, and 1% SDS. The cell lysates were diluted 1:100 in the assay buffer, and 100 μl of the diluted lysates were applied to the microtiter plate coated with a monoclonal antibody to phosphorylated ERK and incubated at room temperature (RT) for 1 h with shaking. Then, the microtiter plate was washed four times with wash buffer and incubated with a rabbit polyclonal antibody to pERK (that binds to the captured pERK on the plate) at RT for 1 h with shaking. After the excess antibody was washed out, the plate was incubated with a donkey anti-rabbit IgG conjugated to horseradish peroxidase (that binds to the rabbit polyclonal antibody) for 30 min with shaking followed by washing out the excess conjugate and incubation with the substrate solution containing 3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide for 15 min. The reaction was stopped by the addition of 1N HCl and the optical densities (OD) of the samples were measured at 450 nm. The OD values were converted to pg/ml of pERK by comparing with those for the recombinant pERK standards.

Preparation of Nuclear Extracts—PC12 cells grown on 10-cm tissue culture dishes were treated with secretin (1 μM) *versus* mock for 10 min, and nuclear extracts were prepared by the procedure of Andrews and Faller (38). Briefly, cells were washed once with PBS, scraped off the dishes, collected in 2 ml of ice-cold PBS, and centrifuged at 500 rpm and 4 °C for 3 min. The cell pellet was resuspended in 400 μl of ice-cold buffer (10 mM HEPES-KOH at pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride), incubated on ice for 10 min, and centrifuged at 14,000 rpm (4 °C) for 5 min. The pellet containing nuclei was resuspended in 200 μl of nuclear extraction buffer (20 mM HEPES-KOH at pH 8.0, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) and kept on ice for 30 min. The nuclear extracts were then separated from the debris by centrifugation at 14,000 rpm (4 °C) for 10 min and stored at −70 °C in aliquots until use. Protein concentrations of the nuclear extracts were measured by the Bio-Rad protein assay reagent.

Synthesis and Labeling of Oligonucleotides—Single-stranded mouse CgA CRE (5′-TCCTA**TGACGTAA**TTTCC-3′; CRE sequence in bold) and mutated CgA CRE (5′-TCCTA**TGA-GTAA**TTTCC-3′; deletion of C from the CRE sequence is shown by a hyphen) oligomers and their complementary strands were synthesized and PAGE-purified by Genset (San Diego, CA) at the concentrations of 80 pmol/μl. Oligomers and their complementary strands (2 μl each) were mixed with 60 μl of water and 20 μl of 100 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, and 10 mM dithiothreitol and annealed by heating at 90 °C for 5 min, followed by cooling down to RT such that the final concentration of the oligomer was 2 pmol/μl.

Double-stranded oligomers (2 μl each) were end-labeled with 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol, PerkinElmer Life Sciences) using T4 polynucleotide kinase (Promega, Madison, WI) in a 10-μl reaction mixture. The reaction was terminated by adding 1 μl of 0.5 M EDTA. The radiolabeled oligomers were purified by Nuc Trap probe purification columns (Stratagene) in 100–150 μl of Tris-EDTA buffer.

Electrophoretic Mobility Shift Assay (EMSA)—For EMSAs, 2 μg of nuclear protein extract were incubated in binding buffer (10 mM Tris-HCl at pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.05 μg/μl poly(dI-dC), and 5% glycerol) for 10 min at RT. The mixtures were then incubated with 20 fmol of radiolabeled oligos for 20 min at RT before being applied to 5% non-denaturing polyacrylamide gels that were run at 150 V in 0.5× Tris borate/EDTA (TBE) buffer at 4 °C. The gels were fixed, dried, and exposed to Kodak Biomax MS films with Kodak Biomax Transcreen HE intensifying screen (Eastman Kodak, Rochester, NY) at −70 °C. For competition experiments, the nuclear extracts were incubated with 10-, 30-, or 90-fold molar excess of the cold (unlabeled) oligomers having identical or different sequences than the radiolabeled oligomer for 10 min at RT in the binding buffer prior to addition of the labeled oligo.

The following antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) were used in the supershift assays: anti-CREB-1 (24H4B) (mouse monoclonal antibody that specifically reacts with CREB-1 p43); anti-pCREB-1 (affinity purified goat polyclonal antibody that recognizes serine-133 phosphorylated CREB-1, CREM-1 and ATF-1); and anti-AP2α (affinity purified rabbit polyclonal antibody). Supershift studies were carried out by the addition of the radiolabeled oligomers, followed by the addition of 1 μl of an antibody with the nuclear extract and incubation for 1 h at 4 °C. The reaction mixtures were then elec-

trophoresed, and the gel was dried and autoradiographed as mentioned above.

Chromatin Immunoprecipitation (ChIP) Assay—Immunoprecipitation of transcriptionally active chromatin was performed using the ChIP assay kit from Upstate Biotechnology (Lake Placid, NY). In brief, PC12 cells treated with 1 μM secretin (*versus* mock) for 30 min were then incubated with 1% formaldehyde for 10 min at 37 °C to cross-link proteins to chromatin *in vivo*. The cells were washed with ice-cold PBS containing a protease inhibitor mixture (Sigma P8340; 10 μl/ml) and then lysed in buffer (0.1% TritonX-100, 10 mM KCl, and 10 mM Tris, pH 8); the lysate was passed through a 30-gauge needle four to five times, and chromatin was fragmented between nucleosomes by the 4-base (5′-GATC-3′) cutter *Sau*3AI (0.2 units/μl at 37 °C for 20 min) and then centrifuged at ~15,000 × *g* for 10 min at 4 °C. The supernatant was pre-cleared with a salmon sperm DNA/protein A-agarose 50% slurry to reduce nonspecific background. Immunoprecipitation was carried out on the supernatant using immunoaffinity-purified rabbit anti-phospho-CREB (Upstate Biotechnology), affinity-purified rabbit antibody to CREB-1 (Santa Cruz Biotechnology), or normal rabbit (pre-immune) IgG as a negative control (Santa Cruz Biotechnology) for reaction overnight at 4 °C. Fresh salmon sperm DNA/Protein A-agarose 50% slurry was added, and, after incubation (1 h at 4 °C), the immunocomplex was pelleted, washed 4 times, eluted by 1% SDS/0.1 M $NaHCO_3$, and subjected to reversal of cross-linking by heating in 0.2 M NaCl at 65 °C for 4 h, followed by proteinase K digestion at 45 °C for 1 h. The DNA was subsequently extracted with phenol/chloroform and then chloroform/isoamyl alcohol and precipitated with ethanol in the presence of 20-μg glycogen carriers. The DNA pellet was dissolved in 30 μl of sterile water.

The immunoprecipitated DNA samples were analyzed by PCR using the primers 5′-ACGGAAGGGATGTGAGGCTC-3′ (forward primer at −274/−255 bp), and 5′-TGGTGGCAGTGGCGGTGATG-3′ (backward primer at +38/−19 bp) which encompass the CRE region (at −69/−62bp) of the rat chromogranin A promoter. As a control for nonspecific binding with the antibodies, the immunoprecipitated DNA was also subjected to PCR using primers outside the CRE region, *i.e.* 5′-GGAAACCAACCAGGAAGCAAG-3′ (forward primer at −795/−775 bp), and 5′-AAGTAAGGCAGGTAGCAGTGGGAG-3′ (backward primer at −613/−636 bp). The extracted DNA from the chromatin fragments before antibody precipitation was used as a positive control ("input DNA"). To ensure that the PCR amplification was in the linear range, reactions with different amounts of input DNA samples (both mock- and secretin-treated) were carried out for various (typically 10–40) cycles; a linear range of amplification occurred at ~25 cycles. The PCR products were separated on 1.5% agarose gel, which was stained by ethidium bromide, photographed, and quantified by scanning transmission densitometry (results expressed as (arbitrary) densitometric units) using the NIH-Image (version 1.62) software.

Peptides and Chemicals—Human secretin (HSDGTFTSELSRLREGARLQRLLQGLV) was purified to >97% homogeneity by reverse phase, high performance liquid chromatography (RP-HPLC), and obtained either from Peninsula Laboratories (Belmont, CA) or Calbiochem. H89, ω-conotoxin GVIA, ω-agatoxin, ω-conotoxin MVIIC, bepridil, chelerythrine, Gö6983, BAPTA-AM, U-73122, PD98059, and 5-Iodotubercidin were obtained from Calbiochem, and nifedipine was obtained from Sigma.

Data Presentation and Statistical Analysis—Secretin potency on transcription was estimated as the EC_{50} value (concentration required to give half-maximal effect), using the program KaleidaGraph 228 (Synergy/Abelbeck Software, Reading, PA). Transfection experiments were repeated at least three times with three wells per condition in each experiment. Results are expressed as mean ± S.E. Descriptive and inferential statistics were performed with the program InStat (GraphPad Software, San Diego, CA). Student's *t* tests or analysis of variance (ANOVA) tests followed by Student-Newman-Keuls multiple comparison tests were used, as appropriate. Significance was determined at the $p \leq 0.05$ levels.

RESULTS

Activation of Endogenous CgA Gene Transcription in Response to Secretin—We have already established secretin as an effective chromaffin cell secretagogue (16). Because CgA is co-stored and co-released with catecholamines in response to secretagogues, we tested whether secretin activates transcription of the endogenous CgA gene. Northern blot results revealed that secretin significantly stimulated steady-state CgA mRNA level by ~1.8-fold in 1 h, reaching ~6.2-fold stimulation after 24 h of treatment (Fig. 1).

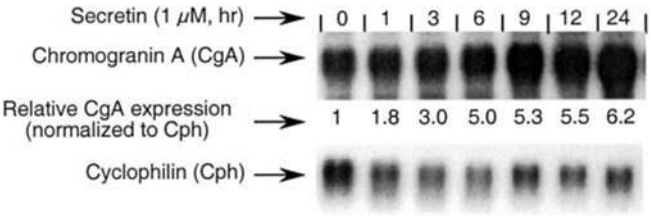

FIG. 1. **Activation of endogenous CgA gene transcription in response to secretin.** PC12 cells were treated with 1 μM secretin for the indicated times. Cells were harvested, and CgA mRNA levels were analyzed by Northern blot. Densitometric values of steady-state mRNAs for CgA were normalized to values obtained from cyclophilin (housekeeping gene) mRNA, and the ratios were recorded.

Transactivation of a Transfected CgA Promoter by Secretin—PC12 cells were transfected with the CgA promoter/luciferase reporter construct pXP1133, which contains 1133 bp of the mouse CgA promoter upstream of the cap (transcriptional start) site, driving a luciferase reporter. Cells were treated with logarithmically ascending doses of secretin (0.0001–1 μM), starting 5 h after transfection, and harvested for luciferase assay 16–18 h after treatment. Secretin dose-dependently increased CgA promoter activity (up to a maximum of 10-fold), with an EC_{50} of ~7 nM (Fig. 2*A*).

Identification of Secretin-responsive domain(s) Within the CgA Promoter—To identify secretin-responsive domains within the CgA promoter, we tested the effects of secretin on a series of 5′-promoter deletion/reporter constructs. Secretin induction of CgA promoter activity dropped dramatically (by ~96%) upon deletion of the −77 to −62 bp domain upstream of the cap site. This region contains a functional cyclic AMP response element (5′-(−71bp)TGACGTAA(−64bp)-3′), indicating that secretin activates CgA gene transcription via the CRE (Fig. 2*B*). To confirm involvement of the CRE, we transfected PC12 cells with several site-directed mutant constructs within the CRE region and treated them with secretin (1 μM), starting 5 h after transfections and continuing for 16–18 h. Mutations within the CRE region (M12, M13, and M41) profoundly (by ~94–96%) diminished the secretin effect (Fig. 2*C*). By contrast, point-conversion of the mouse CgA CRE (TGACGTAA) to a classic consensus CRE (TGACGTCA; mutant M14) preserved the response to secretin (Fig. 2*C*). To control for transfection efficiency, we co-transfected PC12 cells with the neutral promoter/reporter plasmid pRSV-CAT, as described under "Materials and Methods." Transfer of the CgA CRE (TGACGTAA) or a consensus CRE (TGACGTCA) onto a previously unresponsive heterologous thymidine kinase (TK) promoter, followed by secretin (1 μM) treatment, resulted in a secretin stimulatory effect on CgA gene transcription (Fig. 2*D*). Of note, this stimulatory effect of secretin was abolished when CgA CRE-mutant (TGA-GTAA) was transferred to the thymidine kinase promoter (Fig. 2*D*).

Effect of Chemical Blockade of Protein Kinase A (PKA), Overexpression of a PKA Inhibitor Plasmid, or Inhibition of Protein Kinase C (PKC) on Secretin-induced Transcription of the CgA Gene—To investigate secretin signaling through the PKA pathway, PC12 cells were transfected with a CgA promoter/luciferase reporter construct (pXP1133), and treated with a chemical inhibitor of PKA (H-89;10–20 μM) either alone or in combination with secretin (1 μM) for 18 h. PKA inhibition dose-dependently diminished the secretin response of CgA gene transcription (Fig. 3*A*). Furthermore, co-transfection of an expression plasmid for the PKA inhibitor PKI (pRSV-PKI) with the CgA promoter/luciferase reporter construct dose-dependently blocked (up to 72%) secretin-induced transcription of the CgA

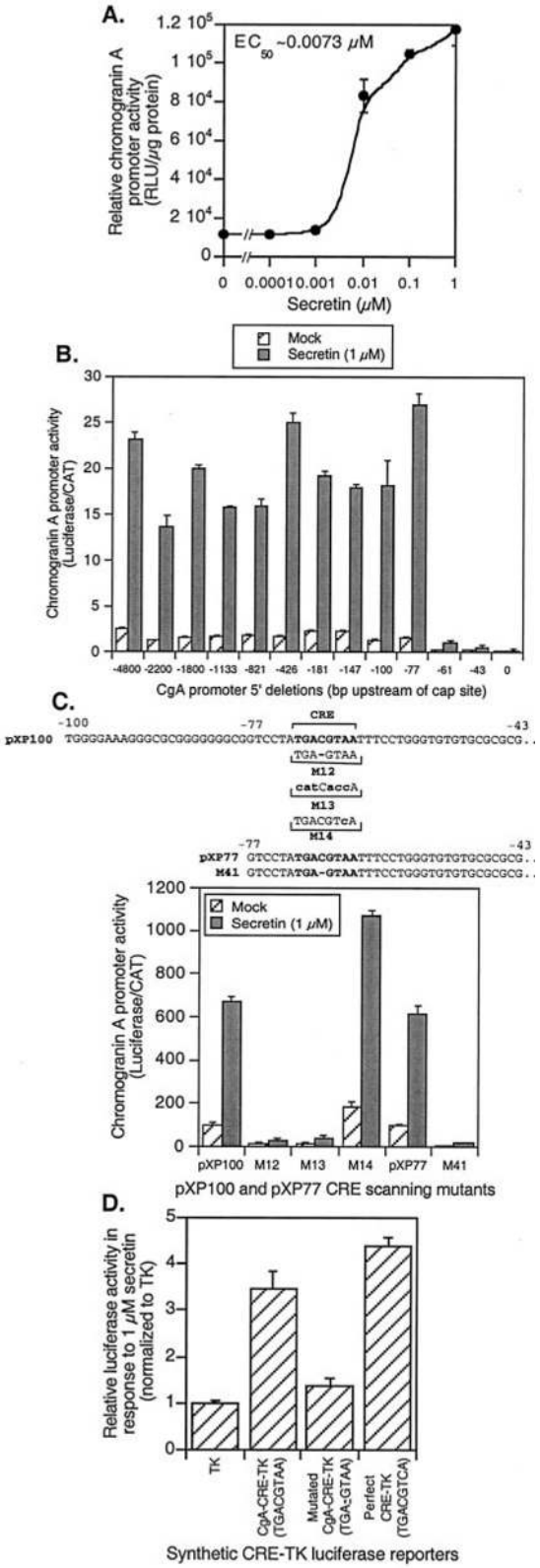

FIG. 2. **Transactivation of a transfected CgA promoter by secretin.** PC12 cells were transfected either with a CgA promoter/luciferase reporter construct (pXP1133) or a series of progressive 5′-deletion mutant constructs treated with secretin (0.0001–1 μM) 5 h after transfections. Cells were harvested 16–18 h after treatment for luciferase assay. *A*, dose-response study. *RLU*, relative light unit. *B*, identification of secretin-responsive domain(s) within the CgA promoter. The CRE in this promoter lies at (−71 bp)5′-TGACGTAA-3′(-64 bp). *C*, involvement of CRE in secretin response to CgA gene transcription. *M12* and *M41*, single base deletion mutations within CRE; *M13*, six base changes within CRE; *M14*, single base change in CgA CRE to a perfect CRE. *D*, transfer of CRE and its mutants to a heterologous thymidine kinase (*TK*) promoter. To control for transfection efficiency, in some experiments we co-transfected PC12 cells with the neutral promoter/reporter plasmid pRSV-CAT and measured ratios of luciferase/CAT activities (see "Materials and Methods").

gene (Fig. 3*B*). By contrast, overexpression of the protein kinase inhibitor inactive mutant ΔPKI dose-dependently reversed PKI-induced inhibition of secretin-activated CgA gene transcription (Fig. 3*C*). These data clearly suggest an involvement of PKA in secretin signaling to CgA gene transcription. In contrast, chemical inhibition of PKC (10 μM chelerythrine, 10 μM Gö6983, or pretreatment with 200 nM phorbol-12-myristate-13-acetate (PMA) for 16 h failed to modulate secretin-induced CgA gene transcription (data not shown).

Role of Ca^{2+} in Secretin-induced CgA Gene Transcription—It is now well established that Ca^{2+} plays a pivotal role in secretagogue-induced neuronal gene transcription (39–41). Therefore, we tested the role of extracellular as well as intracellular Ca^{2+} in secretin-induced CgA gene transcription. Chemical inhibition of L (nifedipine, 10 μM), N (ω-conotoxin GVIA, 1 μM), P (ω-agatoxin, 0.5 μM), Q (ω-conotoxin MVIIC, 0.5 μM), or T (bepridil, 10 μM) types of calcium channels produced no significant blockade of transcription (data not shown). However, secretin-induced CgA gene transcription was distinctly inhibited (~61%) by a blockade of nonspecific plasma membrane Ca^{2+} channels (by $ZnCl_2$, 200 μM) (Fig. 4*A*).

Because secretin-induced catecholamine secretion relies upon Ca^{2+} release from intracellular stores,[2] we tested CgA gene transcription in the presence of the cytosolic Ca^{2+} chelator BAPTA-AM (50 μM) (Fig. 4*B*) and found a marked (~50%) inhibition of secretin-stimulated CgA gene transcription. The inhibition of phospholipase C (PLC) by 20 μM U73122 also resulted in ~62% inhibition of secretin-induced CgA gene transcription, further implicating Ca^{2+} release from intracellular stores (Fig. 4*C*).

Involvement of the Mitogen-activated Protein (MAP) Kinase Pathway in Secretin Signaling to Transcription of the CgA Gene—MAP kinase pathways are known to mediate peptidergic signaling to gene transcription (42). PC12 cells were transfected with a CgA promoter/luciferase reporter construct (pXP1133) and treated with chemical inhibitors of MEK (43) (PD98059, 2–50 μM) or ERK (44) (5-iodotubercidin, 0.1–2 μM) either alone or in combination with secretin (1 μM) for 18 h. Secretin response to transcription was dose-dependently inhibited (up to ~61%) by PD98059 (Fig. 5*A*). Chemical blockade of ERK by 5-iodotubercidin caused almost complete abolition (~87%) of secretin-induced transcription of the CgA gene (Fig. 5*B*).

Profound (~72–85%) inhibition of secretin-induced transcription of the CgA gene after over-expression of dominant negative mutants of ERK1 or ERK2 (Fig. 5*C*) also implicates the MAP kinase pathway in secretin signaling to CgA gene transcription. In addition, suppression of secretin-induced CgA gene transcription following over-expression of a dominant negative mutant of CREB kinase (RSK2) indicates RSK2 as the downstream signaling molecule to ERK (Fig. 5*C*).

To further support the involvement of MAP kinase, we quantitatively estimated the concentration of phosphorylated ERK1/2 in response to secretin. Secretin augmented the phosphorylation of ERK1/2 by ~2-fold at 5 min, and the phosphorylation reaches a maximum of 4-fold at 10 min (Fig. 6). The secretin-induced phosphorylation of ERK1/2 was sustained at least up to 160 min of treatment (Fig. 6).

[2] N. R. Mahapatra, M. Mahata, D. T. O'Connor, and S. K. Mahata, unpublished observations.

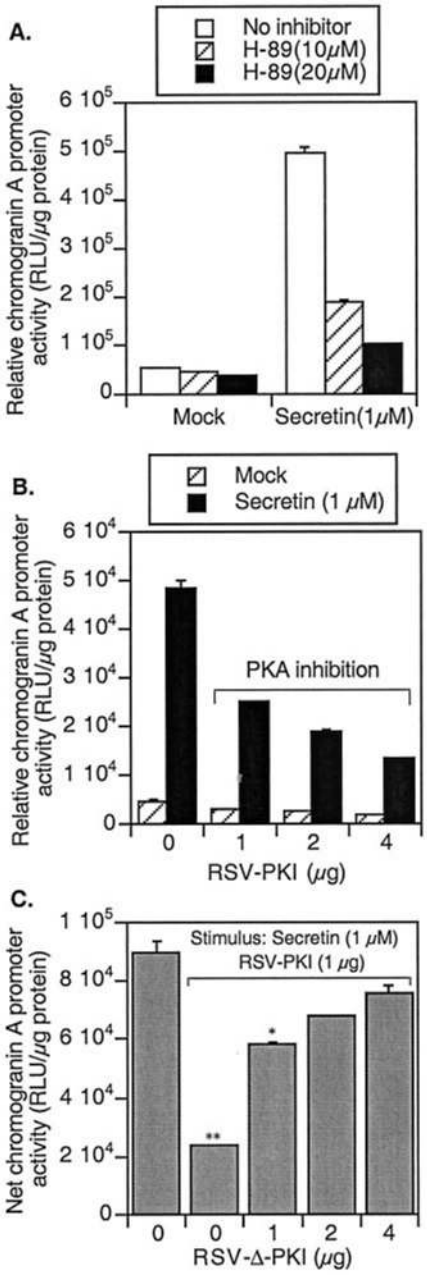

FIG. 3. **Effects of the chemical blockade of PKA and overexpression of a PKA inhibitor plasmid on secretin-induced transcription of the CgA gene.** PC12 cells were transfected either with a 1133-bp CgA promoter/luciferase reporter construct or co-transfected with the PKA inhibitor plasmid PKI. Cells were also co-transfected with a mutant PKI, ΔPKI. Transfected cells were treated with chemical inhibitors of PKA (H-89, 10–20 μM) either alone or in combination with secretin (1 μM) for 18 h before harvesting for luciferase assay. Co-transfected cells were treated with secretin (1 μM) *versus* mock for 18 h before harvesting for luciferase assay. *A*, chemical blockade of PKA. *B*, overexpression of the PKA inhibitor plasmid PKI. *C*, overexpression of the PKI inhibitor plasmid ΔPKI (1 to 4 μg) in presence of PKI (1 μg). Net CgA promoter activity is secretin-induced activity minus mock; *, $p < 0.001$, **, $p < 0.0001$.

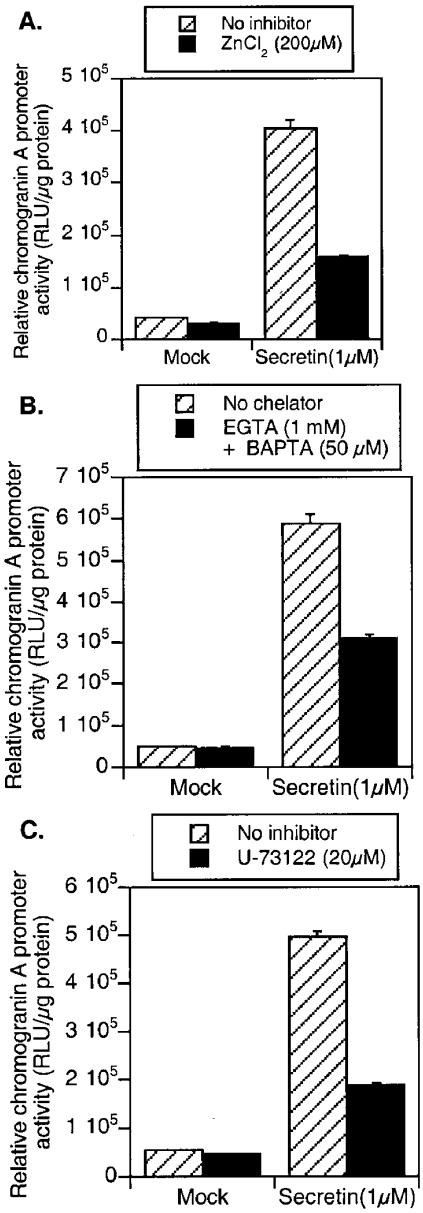

FIG. 4. **Role of Ca^{2+} in secretin-induced CgA gene transcription.** PC12 cells were transfected with CgA promoter/luciferase reporter construct (pXP1133), pre-treated with 200 μM $ZnCl_2$ (*A*), 1 mM EGTA plus 50 μM BAPTA-AM in Ca^{2+}-free medium (*B*), or pre-treated with U-73122 (20 μM) for 30 min before treatment with 1 μM secretin (*C*). Cells were harvested 16–18 h after treatment for luciferase assay. *RLU*, relative light unit

Participation of CREB in Secretin Signaling to Gene Transcription—PC12 cells were transfected with a fusion *trans*-activator plasmid pFA2-CREB (where the human CMV immediate-early promoter drives constitutive expression of CREB), either alone or in combination with a positive control plasmid, pFC-PKA (where the human CMV immediate early promoter drives constitutive expression of the catalytic subunit of PKA). PC12 cells were also transfected with a negative control plasmid pFC2-dbd (where the GAL4-DNA binding domain is under the control of the human CMV promoter). A luciferase reporter plasmid, pFR-Luc (where 5× GAL4 binding elements are fused to the TATA box and the luciferase reporter), was co-transfected in accordance with the manufacturer's protocol. Transfected cells were treated with secretin (1 μM) and harvested 18 h after treatment for luciferase assay. Secretin caused dramatic *trans*-activation (~21-fold) of the GAL4-CREB fusion protein (Fig. 7*A*), indicating involvement of CREB in secretin signaling to gene transcription.

In another set of experiments, the CgA1133 plasmid was co-transfected with dominant negative mutants of CREB. Secretin-induced transcription of the CgA gene was substantially diminished following expression of dominant negative mutant of CREB (90%) (Fig. 7*B*).

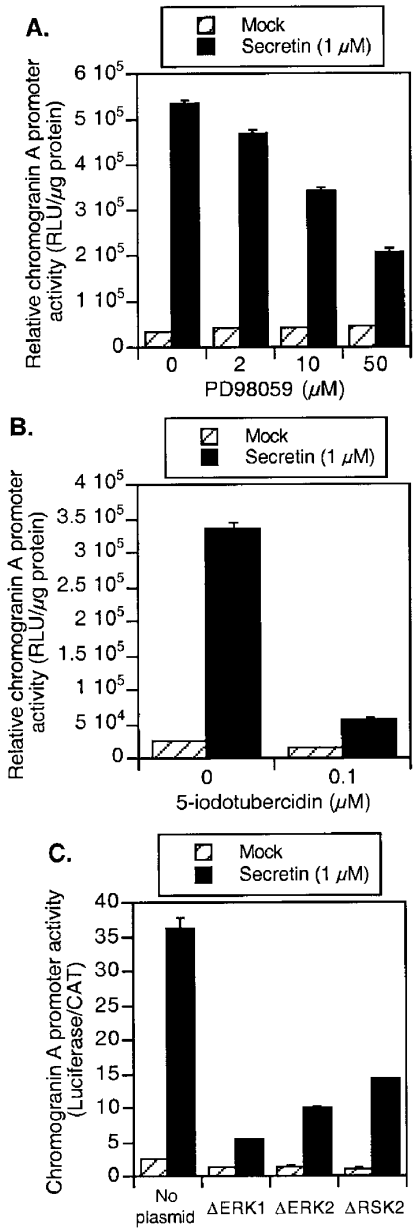

FIG. 5. **Involvement of the MAP kinase pathway in secretin signaling to transcription of the CgA gene.** PC12 cells were transfected with CgA promoter/luciferase reporter construct (pXP1133) and treated with chemical inhibitors of MEK (PD98059; 2–50 μM) (*A*) or ERK (5-iodotubercidin; 0.1 μM) (*B*) either alone or in combination with secretin for 18 h. *C*, PC12 cells were transfected with CgA promoter/luciferase reporter construct (pXP1133), co-transfected either with ΔERK1, ΔERK2, or ΔRSK2 and treated with secretin (1 μM) *versus* mock. Cells were harvested 16–18 h after treatment for luciferase assay. *RLU*, relative light unit

Interaction of PC12 Nuclear Proteins with the CRE Binding Site in the CgA Promoter—Although nuclear proteins extracted from PC12 cells formed a major complex with the radiolabeled CgA-CRE oligonucleotide (Fig. 8*A*, *lane 1*), the radiolabeled mutated CgA-CRE oligonucleotide did not form that DNA-protein complex (Fig. 8*A*, *lane 2*). This protein-DNA interaction was specific, because there was a dose-dependent reduction in the intensity of the complex when an increasing amount of the unlabeled competitor probe (10-, 30-, and 90-fold molar excess; Fig. 8*A*, *lanes 3–5*) was added to the binding reaction. In contrast, the mutated CgA-CRE oligonucleotide failed to abolish the major complex formation even at 90-fold molar excess (Fig. 8*A*, *lanes 6–8*).

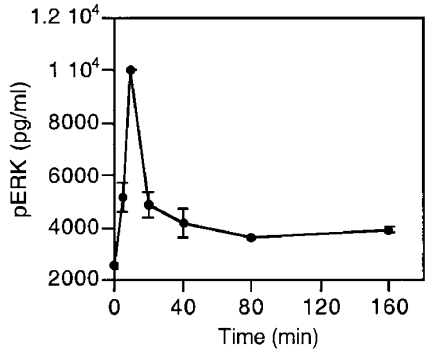

FIG. 6. **Secretin-induced phosphorylation of ERK1/2.** PC12 cells were treated with secretin (1 μM) for 5–160 min and phosphorylated ERK1/2 was determined using the pERK1/2 TiterZyme® enzyme immunometric assay (EIA) kit.

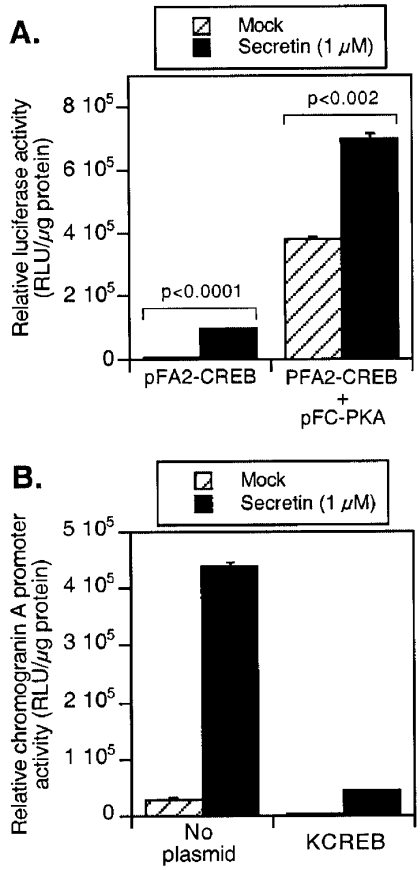

FIG. 7. **Participation of CREB in secretin signaling to gene transcription.** *A*, PC12 cells were transfected with a fusion *trans*-activator plasmid (pFA2-CREB) either alone or in combination with a positive control plasmid (pFC-PKA). A luciferase reporter plasmid (pFR-Luc) was co-transfected in each transfection in accordance with the manufacturer's protocol. *B*, PC12 cells were transfected with CgA promoter/luciferase reporter construct (pXP1133) and co-transfected with KCREB *versus* mock. Transfected cells were treated either with mock or secretin (1 μM) and harvested 18 h after treatment for luciferase assay. *RLU*, relative light unit

Secretin Augments the Binding Activity of CREB to CgA-CRE and CREB Phosphorylation—Secretin (1 μM; 10 min) augmented binding of PC12 nuclear proteins to the CgA-CRE oligomer, suggesting involvement of the CREB/ATF transcription factor family (Fig. 8*B*, *lanes 1* and *5*). To confirm the binding of a CREB/ATF protein to the CRE region, we performed supershift assays using an antibody that specifically recognizes CREB-1 and does not cross-react with other ATF/CREB proteins (Fig. 8*B*, *lanes 2* and *6*) The higher binding

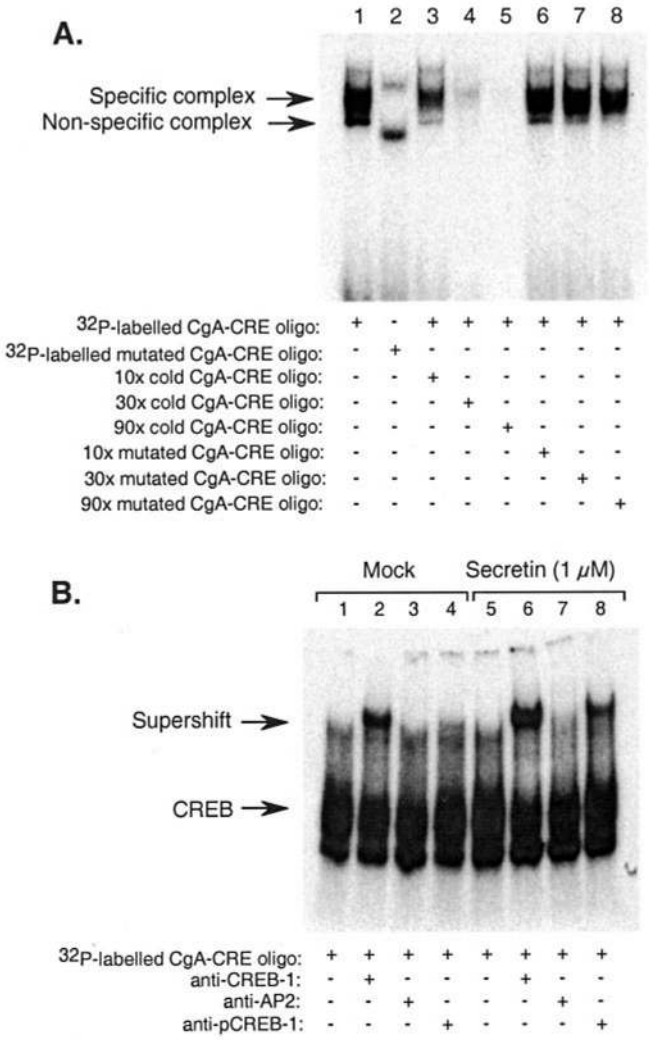

FIG. 8. **EMSA on PC12 nuclear proteins in the presence or absence of secretin.** *A*, competition experiment with the CgA-CRE and mutated CgA-CRE. Nuclear proteins extracted from PC12 cells were incubated with either the CgA-CRE or mutated CgA-CRE. Competition experiments were carried out in the presence of 10-, 30-, or 90-fold molar excess of the cold CgA-CRE or mutated CgA-CRE. *B*, EMSA supershift assay. Nuclear proteins were extracted from mock *versus* secretin-treated (1 μM) PC12 cells, which were incubated with the labeled double-stranded CgA-CRE oligonucleotide probe either alone or in the presence of anti-CREB-1, anti-AP2α, or anti-pCREB-1.

activity of CREB-1 in the presence of secretin is shown by supershift of the major complex to a greater extent as compared with the untreated sample (Fig. 8*B*, *lanes 2* and *6*). As a negative control, anti-AP2α failed to shift the DNA-protein complex (Fig. 8*B*, *lanes 3* and *7*). Because CRE-dependent *trans*-activation requires phosphorylation of CREB at serine 133, we also performed supershift assays using an antibody directed against the serine 133-phosphorylated CREB. Although secretin caused shifting of the phosphorylated CREB-CRE complex, the mock treatment resulted in a very little shifting of the protein-DNA complex (Fig. 8*B*, *lanes 4* and *8*).

Secretin Up-regulates the in Vivo Binding of pCREB to the Endogenous CgA Promoter—To examine whether CREB binding to the endogenous CgA promoter is altered by secretin treatment, we utilized ChIP assays. Formaldehyde cross-linked, fragmented chromatins were isolated from secretin- or mock-treated PC12 cells and then immunoprecipitated with anti-CREB-1 (raised against a peptide mapping near the carboxyl terminus of CREB-1) or an antibody specific for the serine 133-phosphorylated version of CREB (pCREB). DNA

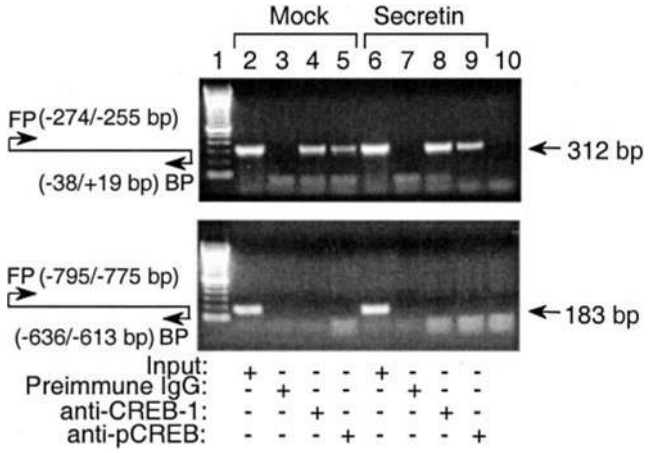

FIG. 9. **ChIP assay shows up-regulation of CREB binding to rat CgA promoter in the context of chromatin by secretin.** Chromatin from PC12 cells treated with mock (*lanes 2–5*) *versus* 1 μM secretin (*lanes 6–9*) was formaldehyde cross-linked, and the *Sau*3AI-fragmented chromatin was immunoprecipitated with anti-phospho-CREB (pCREB; Ser(P)-133) (*lanes 5* and *9*), anti-CREB-1 (*lanes 4* and *8*), or preimmune rabbit IgG as a negative control (*Lanes 3* and *7*). After de-crosslinking, the purified DNA fragments were subjected to PCR using primers to amplify a 312-bp segment spanning the CRE region of the promoter (*top panel*) or a 183-bp segment outside the CRE region of the promoter (*bottom panel*). *Input*, DNA (used as a positive control) from fragmented chromatin prior to immunoprecipitation (*both panels*, *lanes 2* and *6*). *Lane 10*, *both panels*, as a negative control for PCR, a reaction without any DNA was carried out. *Lane 1*, DNA size standards (100-bp DNA ladder; Invitrogen). *FP*, forward primer; *BP*, backward primer. Secretin treatment led to a ~1.77–2.35-fold increase in anti-pCREB- or anti-CREB-1-immunoprecipitated CRE target (*top panel*, *lanes 8* and *9 versus lanes 4* and *5*). PCR primer positions in the CgA promoter are shown on the *left*.

was extracted from anti-CREB-1 or anti-pCREB immuno-isolated chromatin and subjected to PCR amplification by primers flanking the CRE region (Fig. 9, *top panel*).

Secretin treatment, followed by anti-pCREB immunoprecipitation, significantly enriched the CRE target in the complexes (Fig. 9, *top panel*, *lane 9 versus lane 5*). In $n = 3$ separate experiments, the values for mock treatment were 0.46 ± 0.02 densitometric units, climbing to 1.08 ± 0.08 units after secretin (2.35-fold enrichment, $p < 0.002$). Thus, secretin treatment increased the amount of endogenous pCREB bound to the CgA promoter *in vivo*.

Secretin apparently also increased the amount of CREB-1 bound to the CgA promoter CRE, as judged by ChIP using an antibody recognizing CREB-1 regardless of phosphorylation state (Fig. 9, *top panel*, *lane 8 versus lane 4*). In $n = 3$ experiments, the mock treatment values were 1.12 ± 0.11 units, rising to 1.99 ± 0.17 after secretin (1.77-fold enrichment, $p < 0.02$). This increase is compatible with cAMP induction of the biosynthesis of CREB itself as documented by Meyer *et al.* (45), who found three functional CREs in the CREB promoter.

To ensure specificity of the immunoprecipitation, control rabbit IgG was added instead of the CREB-1 antibody or pCREB antibody to the chromatin fragment solution in some experiments, but it failed to precipitate the CRE sequence in either mock or secretin-treated samples (Fig. 9, *top panel*, *lanes 3* and *7*). As another negative control, immunoprecipitation without the addition of any antibody was carried out, which also did not precipitate the CRE fragment in either the mock or secretin-treated samples (data not shown).

To confirm the specificity of the immunocomplex, PCR amplification was carried out using primers outside the CRE region, which failed to generate any product (Fig. 9, *bottom panel*, *lanes 3–5* and *7–9*). Furthermore, no PCR product was obtained when the formaldehyde cross-linking step was omit-

ted (data not shown) or when no DNA was added to the PCR mixture (Fig. 9, both *top* and *bottom panels*, *lane 10*). As positive controls for the PCR reactions, DNA extracted from the chromatin fragments prior to immunoprecipitation (input DNA) did amplify on PCR (Fig. 9, both the *top* and *bottom panels*, *lanes 2* and *6*). Thus, secretin seems to specifically increase the amounts of both pCREB and CREB-1 bound to the CgA CRE sequence in the context of chromatin *in vivo*.

DISCUSSION

We recently found secretin to be an effective chromaffin cell secretagogue, inducing 26% of cell total catecholamine release (16). Secretin-induced catecholamine release appears to depend solely upon Ca^{2+} release from intracellular stores. Because CgA, the major soluble protein in chromaffin vesicles, is co-stored and co-released with catecholamines (17), we tested whether secretin induces resynthesis of just-secreted protein by activating transcription of the CgA gene. We found that secretin augmented transcription of the endogenous CgA gene (~6.2-fold) as well as *trans*-activation of the CgA promoter/luciferase reporter construct (8–10-fold). Among peptidergic secretagogues, secretin appears to be the most potent (EC_{50}, ~7 nM; Fig. 2*A*) and effective in stimulating CgA gene transcription (46).

Studies with a series of 5′-progressive deletion mutants of the CgA promoter revealed a dramatic (~96%) drop in secretin-mediated CgA promoter activity upon deletion from −77 to −62 bp (Fig. 2*B*), a region encompassing the CRE box, at 5′-(−71 bp)TGACGTAA(−64 bp)-3′ (31). The crucial role of the CRE in secretin response was further verified by several point mutations within the CRE itself (Fig. 2*C*) and upon their transfer to a heterologous thymidine kinase promoter (Fig. 2*D*). In addition, we have shown in our competition experiments that PC12 nuclear proteins formed specific complexes with CgA-CRE oligonucleotides, because 10- to 90-fold molar excesses of the unlabeled CgA-CRE dose-dependently abolished the CgA-CRE-nuclear protein complex (Fig. 8*A*). These findings indicate that the CRE *in cis* is both necessary and sufficient for mediation of secretin-induced CgA gene transcription. Of note, other secretory stimuli such as nicotine or PACAP also mediate their effects on CgA gene transcription through the CRE *in cis* (27, 46–48).

Diminution of secretin-induced CgA gene transcription after chemical blockade of PKA (Fig. 3*A*) or after overexpression of the PKA inhibitor protein PKI indicate involvement of the cAMP-PKA pathway in secretin signaling to CgA gene transcription (Fig. 3, *B* and *C*). In addition, the present findings of general chemical blockade of plasma membrane Ca^{2+} channels by $ZnCl_2$ (Fig. 4*A*), cytosolic Ca^{2+} chelation by BAPTA-AM (Fig. 4*B*), or chemical inhibition of phospholipase C (Fig. 4*C*) point to a crucial role of Ca^{2+} (influx from extracellular medium and release from intracellular stores) that contributes 50–62% of secretin signaling to CgA gene transcription.

How might PKA and Ca^{2+} cross-talk to mediate secretin signaling to CgA gene transcription? The existing literature indicates that the ultimate target of both PKA and Ca^{2+} is the transcription factor CREB (49). It is already well established that, following increases in intracellular cAMP levels and activation of PKA, the catalytic subunit of PKA translocates into the nucleus and phosphorylates CREB, leading to stimulation of gene transcription (50). Subsequent studies have identified additional CREB kinases, including members of the calcium/calmodulin-dependent kinase family (51, 52) and the ERK-stimulated RSK kinases (34, 53). Previous studies have demonstrated that a variety of kinase-signaling pathways can differentially contribute to Ca^{2+}-mediated phosphorylation of CREB in PC12 cells (49). It has also been reported that calcium-mediated phosphorylation of CREB in PC12 cells occurred via ERK-dependent phosphorylation of the CREB kinase, RSK2 (54). Using the PC12 cell model system, it has been shown that both calcium and cAMP stimulated CREB-dependent transcription via a Rap1 (Ras-related small G-protein)-B-Raf (neuronal Raf isoform)-ERK pathway (55, 56). Consistent with these findings, we found almost complete abolition of secretin-induced CgA gene transcription after a chemical blockade of MEK (Fig. 5*A*) or ERK (Fig. 5*B*) or overexpression of dominant negative mutants of ERK1, ERK2, or RSK2 (Fig. 5*C*). Involvement of ERK is further confirmed by the finding of augmented phosphorylation of ERK1/2 in response to secretin (Fig. 6). Of note, nicotine also transactivates CgA gene transcription through the MAP kinase pathway (57).

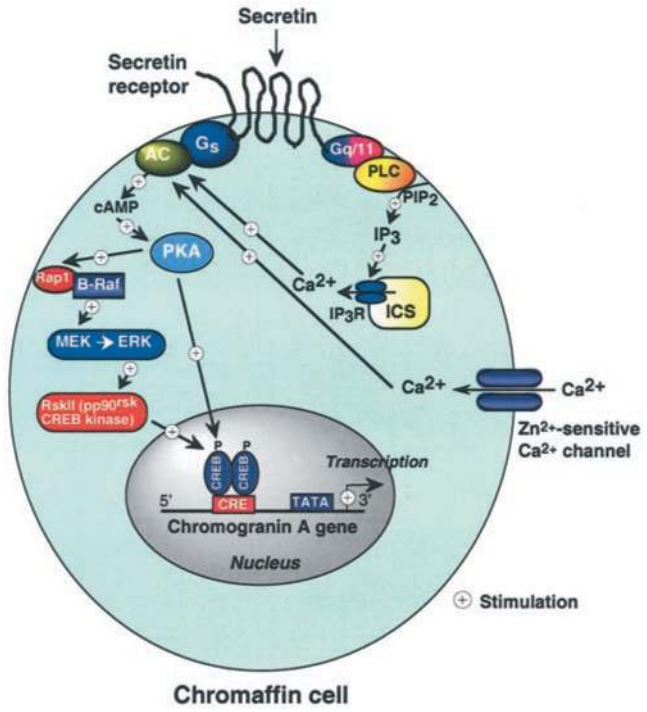

FIG. 10. **Proposed signal transduction scheme for activation of chromogranin A gene transcription by secretin with results suggested by the current experiments.** *AC*, adenylyl cyclase; *CRE*, (−71 bp5′-TGACGTAA-3′−64 bp); *Gs*, stimulatory heterotrimeric G-protein; *Gq/11*, heterotrimeric G-protein of the Gq/11 family; *ICS*, intracellular Ca^{2+} store; IP_3, inositol 1,4,5-trisphosphate; IP_3R, receptor for IP_3; *B-Raf*, neuronal Raf (MAP kinase kinase kinase) isoform; PIP_2, phosphatidylinositol-4-biphosphate.

The involvement of CREB in secretin signaling to CgA gene transcription is further substantiated by the following findings: (i) substantial *trans*-activation (~21-fold) of a GAL4-CREB *trans*-activator fusion protein in response to secretin (Fig. 7*A*); (ii) complete abolition of secretin-induced CgA gene transcription after overexpression of the dominant negative mutant of CREB (KCREB) (Fig. 7*B*); and (iii) secretin-induced binding of CREB to the CgA CRE as shown by an EMSA gel shift experiment with CREB-1 anti-serum (Fig. 8*B*, *lanes 2* and *6*). Stimulation of CREB-dependent transcriptional activity is generally achieved by phosphorylation of CREB at serine 133 (50). In line with this, we found that secretin stimulated phosphorylation of CREB using an antibody that specifically recognizes the CREB Ser-133 epitope in its phosphorylated state (Fig. 8*B*, *lanes 4* and *8*). Consistent with the *in vitro* EMSA gel shift assays (Fig. 8), the ChIP assay confirms that secretin significantly up-regulated the binding of pCREB and CREB-1 to the endogenous CgA CRE *in vivo* (Fig. 9, *top panel*, *lanes 4*, *5*, *8*, and *9*). Based on our findings, we thus propose that secretin

stimulates PKA through the following two pathways: (i) Gsα → AC → cAMP → PKA (Fig. 3); and (ii) Ca^{2+} → AC → cAMP → PKA (Fig. 4*B*) (where Gsα is stimulatory heterotrimeric G-protein α and AC is adenylyl cyclase). Activated PKA phosphorylates CREB either directly or through the Rap1 → B-Raf → MEK → ERK → CREB kinase (RSK2) pathway, ultimately resulting in activation of CgA gene transcription (Fig. 10).

It seems appropriate to point out that peptidergic signaling pathways to chromogranin/secretogranin gene transcription appear to be heterogeneous. Although PACAP signals to CgA gene transcription through the calcium-independent PKA → CREB pathway (50), PACAP signals to secretogranin II gene transcription through a calcium-dependent MAP kinase → AP1 pathway (58). In contrast, secretin signals to CgA gene transcription through both calcium-dependent and calcium-independent pathways, impinging upon the PKA → MAP kinase → CREB kinase → CREB signaling pathway (Fig. 10). In each case, PACAP or secretin signals through the CRE *in cis*.

We conclude that secretin signals to CgA gene transcription through the CRE domain *in cis* and through cAMP, Ca^{2+}, PKA, the MAP kinase, and the transcription factor CREB *in trans*. Thus, multiple signal transduction pathways seem to subserve the function of stimulus-transcription coupling after this peptidergic stimulus to chromaffin cells.

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